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RESEARCH ARTICLE

Infraspecific morphological and molecular variation of *Linum nervosum* (Linaceae) in Iran

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Abstract

Linum nervosum is among species that can hybridize with *L. usitatissimum* and produce fertile offsprings. Genetic diversity analysis of this wild relative of flax is important from conservation and breeding points of view. In the present study, 55 randomly selected plants of six different populations of *L. nervosum* varieties, including var. *nervosum* and var. *bungei*, were studied for morphological and genetic variability as well as population structure. Analysis of variance (ANOVA) did not show significant morphological difference between populations. PCA as well as PCA biplot confirmed that some morphological traits have taxonomic value. UPGMA clustering separated the populations of varieties in two distinct clusters, indicating degrees of morphological differentiation between them. Furthermore, UPGMA confirmed the variability in morphological characters within populations. Neighbor Joining tree and Neighbor-Net analysis of ISSR data revealed inter- and intrapopulation genetic variability. STRUCTURE plot revealed allelic difference of these varieties and some degree of intervarietal gene flow. K-means clustering showed the fragmentation of populations in support of AMOVA test, which revealed significant genetic difference among them. In general, obtained results confirmed the alternation of taxonomic level of *L. bungei* to the variety of *L. nervosum*.

Keywords: *Linum nervosum*, *Linum bungei*, genetic structure, morphology, variety

Introduction

Linum L. is a type genus of Linaceae (DC.) Dumort, and it is traditionally subdivided into five sections (Rechinger 1974). Flaxes are widespread and comprises about 200 species (Hickey 1988). Several *Linum* species are represeted by shrubs distributed in tropical areas, while perennial and annual taxa occur in temperate areas of the world (Muir & Westcott 2003).

Linum nervosum Waldst & Kit. from the section *Linum* is a heterostylous diploid species ($2n=2x=18$, Samadi et al. 2007). This species is distributed in mountains and upland steppes (Dzybov 2013; Zolotukhin et al. 2014). It has also been introduced to flower gardens as an ornamental plant (Catlow 2012). *L. nervosum* along with some other *Linum* species can hybridize with *L. usitatissimum* L. and transfers genes via such hybridization (Cullis 2011; Jhala et al. 2008). Therefore analysis of genetic diversity of *L. nervosum* is not only important from biodiversity and conservation points of view, but also is useful for boarding the available gene pool for hybridization and breeding programs in flax (Cullis 2011).

There is protracted discussion about infraspecific classification of *L. nervosum*, and different taxonomical ranks have been proposed. For example, recently Sharifnia & Assadi (2001) changed taxonomical rank of *L. bungei* Boiss. and made new combination of it departing to the level of variety of *L. nervosum*, therefore two variety of this species can be considered for Iran – *L. nervosum* var. *nervosum* Waldst & Kit. and *L. nervosum* var. *bungei* (Boiss.) Sharifnia. In addition, one new combination in this species, *L. nervosum* subsp. *jailicola* (Juz.) Egor., was also introduced by Egorova (2000) for Caucasian flora.

The present study was performed with two main objectives: (1) to analyse genetic diversity and population structure of *L. nervosum* varieties, which can produce important data for genetic conservation of these taxa; and (2) to evaluate the similarity between *L. nervosum* var. *nervosum* and *L. nervosum* var. *bungei* in different geographical populations and with different population structure. These results may improve our insight about intraspecific variation in this species.

Material and methods

Plant material

Collection of material in the field was undertaken during 2010–2014 throughout the Iran and six geographical populations were identified for *L. nervosum* varieties (Tab. 1). From each of populations four to five individuals were selected and identified following Flora Iranica (Rechinger 1974) and Flora of Iran (Sharifnia & Assadi 2001). The vouchers were deposited in the herbarium of Shahid Beheshti University (HSBU).

Morphological analysis

In total 25, including 18 quantitative and 7 qualitative, morphological traits of both reproductive and vegetative organs of *L. nervosum* var. *nervosum* and *L. nervosum* var. *bungei* from different populations were investigated. These traits were: the stem height and its diameter, number of veins in the basal leaf, the basal and floral leaf shape, the width and length of the basal and floral leaves, the length/width ratio of the basal and floral leaves, the shape of floral and basal leaf apex and margin, the sepal width, length and its length/width ratio, the calyx width and its length/width ratio, the corolla length, width and its color, the style

Table 1. Sampled material.

Nr	Taxon	Locality	Voucher
1	*L. nervosum* var. *nervosum*	Alborz, Marzanabad, 2000 m a.s.l.	HSBU2011223
2	*L. nervosum* var. *nervosum*	Mazandaran, Chalous, 5 km Siahbisheh, 2193 m a.s.l.	HSBU2011130
3	*L. nervosum* var. *bungei*	Mazandaran, Chalous, 5 km Siahbisheh, 2193 m a.s.l.	HSBU2011129
4	*L. nervosum* var. *bungei*	Alborz, Marzanabad, 2000 m a.s.l.	HSBU2011220
5	*L. nervosum* var. *nervosum*	Mazandaran, Pole Zangooleh, 2325 m a.s.l.	HSBU2011221
6	*L. nervosum* var. *bungei*	Semnan, Shahroud, Abr Jungle, 2033 m a.s.l.	HSBU2011222

and anther length (Fig. 1). Two replications were made for each character per each flowering stem. All studied variables were statistically processed. The mean values and standard deviations of some quantitative characters are presented in the Tab. 2. Three populations were examined per each variety.

DNA extraction and ISSR assay

For molecular studies, fresh leaves were collected randomly from four to five randomly selected plants in each population. Nuclear DNA was extracted using CTAB activated charcoal protocol (Križman et al. 2006). These procedure is based on the activated charcoal and polyvenyl pyrrolidone (PVP) for binding of polyphenolics components during extraction. The mild extraction and precipitation conditions promoted the high-molecular weight DNA isolation without interfering contaminants. The quality of obtained DNA was examined by running on 0.8 % agarose gel.

The applied ISSR primers were: $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9C$, UBC807, UBC811, $(GA)_9A$ and $(GT)_7CA$ commercialized by UBC (University of British Columbia). PCR reactions were performed in 25 µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94 °C, 30 s at 94 °C; 1 min at 50 °C and 1 min at 72 °C. The reaction was completed by final extension step of 7 min at 72 °C.

The amplification products were visualized by running on 2 % agarose gel, followed by the ethidium bromide staining. The fragments size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analysis

The analysis of variance (ANOVA) was performed for quantitative morphological characters to indicate the significant difference between the studied populations. Principal coordinate analysis (PCoA) and Canonical Variant Analysis (CVA) were performed to group the samples on the basis of the standardized (mean = 0, variance = 1) morphological traits. NTSYS ver. 2 and SPSS ver. 9 softwares were used for statistical analyses.

ISSR bands were treated as binary characters and coded accordingly (presence = 1, absence = 0). Parameters of genetic variation were determined in each population. Percentage of allelic polymorphism, allele diversity, Nei's gene

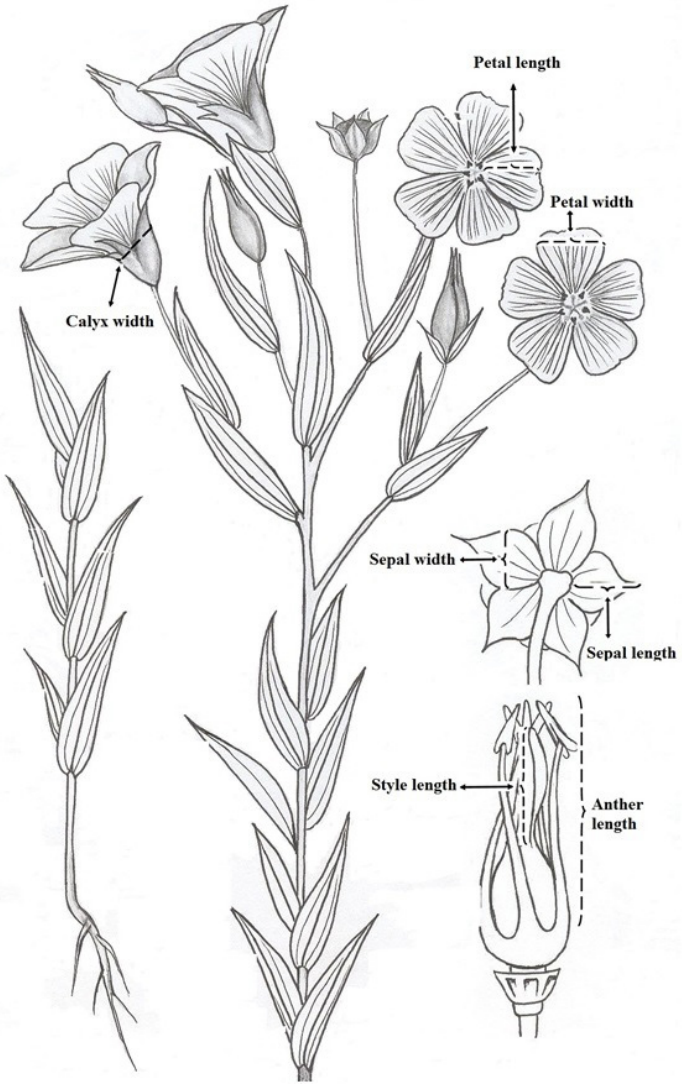

Figure 1. General view of *Linum nervosum* with demonstration of some measured features.

diversity (H), Shannon information index (I), the number of effective alleles and percentage of polymorphism were obtained by using of PAST ver. 2.17 and DARwin ver. 5 softwares (Weising 2005 et al.; Freeland et al. 2011; Hamer et al. 2012).

Nei's genetic distance (Weising et al. 2005; Freeland et al. 2011) was determined between the studied plant specimens and used for Neighbor Joining (NJ) clustering after 100 times bootstrapping (Freeland et al. 2011) in PAST ver. 2.17 (Hamer et al. 2012) and DARwin ver. 5 softwares too.

Genetic affinity of the populations was determined by distance-based Neighbor-Net as implemented in SplitsTree4 (Huson & Bryant 2006). The Mantel test was performed to check correlation among geographical and genetic distances of the studied populations (Podani 2000).

Table 2. Some important morphological characteristics studied in the populations (all values are provided in cm).

Population		Stem length	Stem diameter	Vein number	Basal leaf length	Basal leaf width	basal leaf length / wide ratio	Floral leaf length	Floral leaf width	Calyx width	calyx length / wide ratio	Sepal length	Sepal width	Petal length	Petal width	Anther length	Style length
var. *nervosum*, Marzanabad	Mean	34.75	0.12	3.00	1.72	0.32	5.25	1.12	0.22	0.28	1.93	0.55	0.14	1.44	0.77	0.71	0.73
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	7.80	0.02	0.00	0.45	0.06	0.64	0.25	0.05	0.02	0.35	0.12	0.04	0.51	0.39	0.10	0.12
var. *nervosum*, Siahbisheh	Mean	31.75	0.11	3.00	1.37	0.28	4.92	1.10	0.15	0.30	1.91	0.57	0.18	1.37	0.69	0.90	0.92
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	1.12	0.04	0.00	0.35	0.05	0.83	0.25	0.06	0.01	0.27	0.05	0.02	0.73	0.81	0.21	0.20
var. *bungei*, Siahbisheh	Mean	31.75	0.10	1.00	1.40	0.23	6.11	0.85	0.09	0.27	1.74	0.47	0.19	1.40	0.65	0.85	0.86
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	7.97	0.02	0.00	0.35	0.06	1.61	0.129	0.01	0.05	0.17	0.05	0.01	0.62	0.36	0.15	0.19
var. *bungei*, Marzanabad	Mean	31.00	0.12	1.00	1.60	0.26	6.17	0.72	0.08	0.27	1.83	0.47	0.17	1.37	0.58	0.80	0.82
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	6.48	0.03	0.00	0.21	0.05	1.48	0.09	0.02	0.02	0.19	0.05	0.09	0.84	0.77	0.20	0.24
var. *nervosum*, Pole Zangooleh	Mean	24.75	0.08	3.00	1.45	0.33	4.36	0.82	0.11	0.28	1.94	0.68	0.16	1.32	0.79	0.95	0.98
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	4.64	0.019	0.00	0.10	0.04	0.73	0.22	0.02	0.03	0.29	0.06	0.04	0.52	0.45	0.13	0.18
var. *nervosum*, Pole Zangooleh	Mean	24.75	0.08	3.00	1.45	0.33	4.36	0.82	0.11	0.28	1.94	0.68	0.16	1.46	0.74	0.67	0.74
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	4.64	0.019	0.00	0.10	0.04	0.73	0.22	0.02	0.03	0.29	0.06	0.04	0.48	0.79	0.19	0.16
var. *bungei*, Abr Jungle	Mean	28.25	.52	1.00	1.57	0.28	5.74	1.00	0.16	0.32	1.82	0.55	0.24	1.49	0.70	0.99	1.03
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	SD	6.39	0.84	0.00	0.49	0.10	1.52	0.36	0.06	0.05	0.29	0.05	0.04	1.11	1.36	0.18	0.15

In order to study significant genetic difference among the populations, different methods were applied: (1) AMOVA (Analysis of molecular variance) test with 1000 permutations was performed in GenAlex 6.4 (Peakall & Smouse 2006), and (2) Nei's G_{ST} analysis in GenoDive ver. 2, which was originally developed by Meirmans & Van Tienderen (2004). Moreover, other parameters of genetic differentiation such as G_{ST} and D_{EST} were determined (Hedrick 2005; Jost 2008):

$$G_{ST} = (Ht - Hs) / Ht \quad \text{and}$$

$$D_{EST} = n / (n - 1) \cdot (Ht - Hs) / (1 - Hs), \quad \text{where}$$

n – the number of sampled populations;
Ht – heterozygosity over all populations;
Hs – mean heterozygosity within the populations.

Furthermore, in order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program Hickory ver. 1.0 (Holsinger & Lewis 2003) was used to estimate parameters related to genetic structure (Theta B value). Three runs were performed with default sampling parameters (burn-in = 50,000, sample = 250,000, thin = 50) to ensure consistency of results (Tero et al. 2003).

The genetic continuity versus population stratification was checked by two methods. First, we carried out the structure analysis (Pritchard et al. 2000). For this, data were scored as dominant markers and analysis followed the method suggested by Falush et al. (2007). Second, we performed K-means clustering in GenoDive ver. 2.

In the K-means clustering, the optimal clustering is the one with the smallest amount of difference within clusters, which is calculated using the within-clusters sum of squares. The minimization of the within-groups sum of squares that is used in K-means clustering is, in the context of a hierarchical AMOVA, equivalent to minimizing the among-populations-within-groups sum of squares, SSDAP/WG. The hierarchical population structure in the AMOVA then consists of different hierarchical levels: individuals, populations, and clusters of populations. Different F-statistics can be calculated on the basis of the variance components for the different hierarchical levels. In terms of F-statistics, the minimization of SSDAP/WG comes down to a maximization of FCT, the variance among clusters (C) relative to the total variance (T) (Meirmans & Van Tienderen 2004).

We used two summary statistics to present K-means clustering: (1) pseudo-F (Caliński & Harabasz 1974) and (2) Bayesian Information Criterion (BIC, Schwarz 1978). Pseudo-F relates r^2, the fraction of the total variance that is explained by the clustering, to the number of clusters k and the number of population's n:

$$F_k = r^2 / (1 - r^2) \cdot (n - k), \quad \text{where}$$

$$r^2 = (SSDT - SSDAP / WG) / (SSDT - SSDWP)$$

The clustering with the highest value for pseudo-F is regarded to provide the best fit. The BIC is calculated as:

$$BIC_k = n \cdot \ln (SSE) + k \cdot \ln (n)$$

In order to identify ISSR loci, which were more frequently involved in gene flow among populations, the Nm analysis of POPGENE ver. 2 was performed according to the following formulae:

$Nm = 0.5 (1 - G_{ST}) / G_{ST}$, where
Nm – estimate of gene flow from G_{ST}.

Recently Frichot et al. (2013) introduced the statistical model called Latent Factor Mixed Models (LFMM) that tests correlations between environmental and genetic variation while estimating the effects of hidden factors that represent background residual levels of population structure. We used this method to check if ISSR markers show correlation with environmental features of the studied populations. The analysis was done in LFMM ver. 1.2.

Results

Morphometric analysis

In the populations Siahbisheh and Marzanabad both varieties were presented, while in the rest populations the only variety was found. Both varieties had blue petals. In the studied samples of these verieties, basal and floral leaves were linear and lanceolate, respectively. Leaves were entire and the shape of the apex and the base of leaves were stable between verities and their populations. *L. nervosum* var. *nervosum* had three-veined basal leaves, while *L. nervosum* var. *bungei* had one-veined leaves.

The ANOVA test performed on the quantitative morphological characters did not show significant difference ($p < 0.05$) among the studied populations with the exceptions of the sepal length and floral leaf width (Tab. 3). The CVA plot (Fig. 2) revealed the morphological similarity between some populations, for example, individuals of both varieties from Siahbisheh were placed close together. Samples from the Marzanabad populations of these varities were also clustered near each other, and close to *L. nervosum* var. *nervosum* from the Pole Zangooleh population. Samples of the Jungle Abr population (Shahroud) of *L. nervosum* var. *bungei* were located farthermost from other studied populations.

UPGMA dendrogram based on the morphological characters separated two studied varieties in two nearly distinct clusters (Fig. 3).

PCA analysis revealed that the first three PCA axes comprised about 75% of total morphological variability (Fig. 4). In the first axis with almost 46% of total variation, such parameters like number of leaf veins ($r = 0.98$), ratio of length/width of calyx ($r = 0.67$) and sepal length/width ratio ($r = 0.52$) were the most variable. While in the second axis that comprised about 25% of total morphological variability, the most variable parameters were length of stem leaf ($r = 0.78$) and floral leaf length/ width ratio($r = 0.85$).

PCA biplot (Fig. 5) revealed that each variety had distinct morphological features. For example, sepal length was a distinct character of *L. nervosum* var. *bungei*, and the basal leaf length was the prominent trait for *L. nervosum* var. *nervosum*.

Analysis of genetic diversity

Genetic diversity parameters are presented in Tab. 4. The highest value of the Shanon information index ($I = 0.288$), gene diversity ($He = 0.189$) and percentage of polymorphism (59.09) occurred in the Siahbisheh population of *L. nervosum* var. *nervosum*, while the Marzanabad population of *L. nervosum* var. *bungei* contained the lowest values for the same parameters (0.167, 0.111, and 31.82, respectively).

The AMOVA test revealed significant ($p = 0.01$) genetic difference among the studied populations. Moreover, it revealed

Table 3. Results of ANOVA test on the quantitative morphological features.

Characters		Sum of Squares	Df	Mean Square	F	Sig.
Stem length	Between groups	237.875	5	47.575	0.802	0.563
	Within groups	1067.750	18	59.319		
	Total	1305.625	23			
Stem diameter	Between groups	0.586	5	0.117	0.970	0.462
	Within groups	2.175	18	0.121		
	Total	2.761	23			
Basal leaf length	Between groups	0.367	5	0.073	0.582	0.714
	Within groups	2.272	18	0.126		
	Total	2.640	23			
Basal leaf width	Between groups	0.027	5	0.005	1.182	0.356
	Within groups	0.083	18	0.005		
	Total	0.111	23			
Floral leaf length	Between groups	0.524	5	0.105	1.862	0.151
	Within groups	1.012	18	0.056		
	Total	1.536	23			
Floral lead width	Between groups	0.054	5	0.011	5.607	0.003
	Within groups	0.035	18	0.002		
	Total	0.089	23			
Floral leaf length / wide ratio	Between groups	40.928	5	8.186	2.471	0.072
	Within groups	59.623	18	3.312		
	Total	100.552	23			
Petal length	Between groups	0.498	5	0.099	1.862	0.090
	Within groups	0.963	18	0.053		
	Total	1.461	23			
Petal width	Between groups	0.378	5	0.075	1.862	0.076
	Within groups	0.896	18	0.049		
	Total	1.276	23			
Calyx length	Between groups	0.035	5	0.007	1.094	0.398
	Within groups	0.114	18	0.006		
	Total	0.149	23			
Calyx width	Between groups	0.007	5	0.001	0.990	0.451
	Within groups	0.024	18	0.001		
	Total	0.031	23			
Sepal length	Between groups	0.123	5	0.025	4.693	0.006
	Within groups	0.094	18	0.005		
	Total	0.217	23			
Sepal width	Between groups	0.023	5	0.005	1.856	0.153
	Within groups	0.046	18	0.003		
	Total	0.069	23			
Anther length	Between groups	0.130	5	0.026	4.705	0.12
	Within groups	0.099	18	0.005		
	Total	0.229	23			
Style length	Between groups	0.137	5	0.027	4.717	0.13
	Within groups	0.094	18	0.005		
	Total	0.235	23			

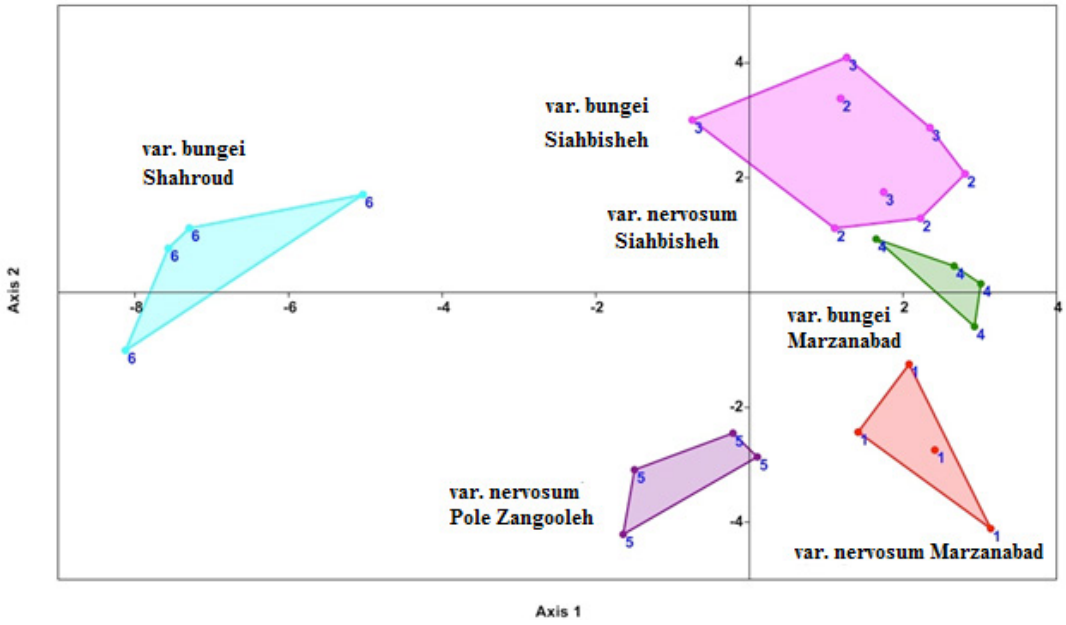

Figure 2. CVA plot of varieties and populations of *Linum nervosum* based on the morphological characters.

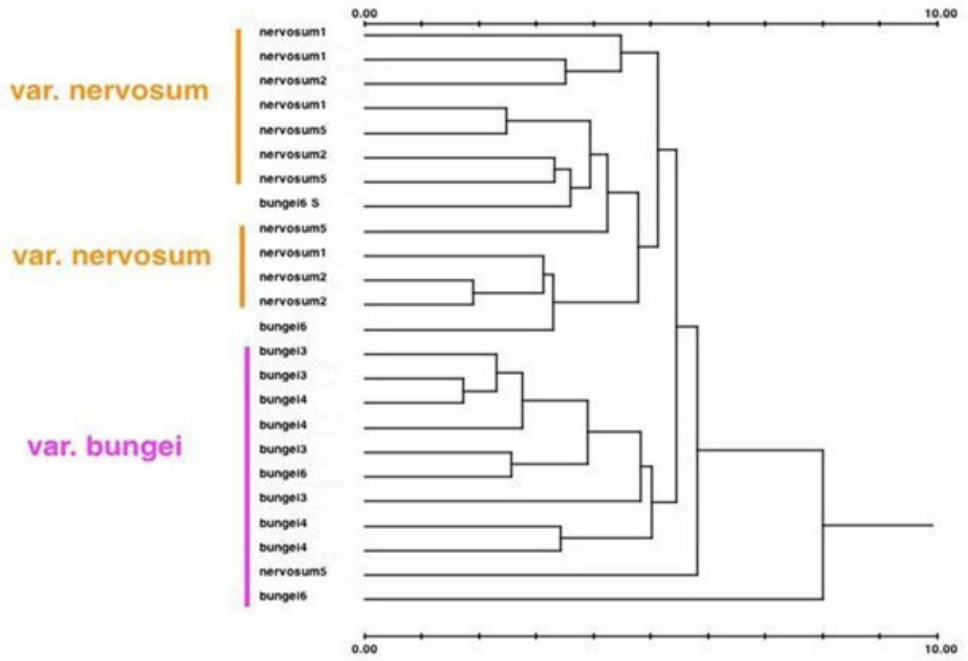

Figure 3. UPGMA tree of *Linum nervosum* varieties based on the morphological characters. Numbers correspond to the populations from the Tab. 1.

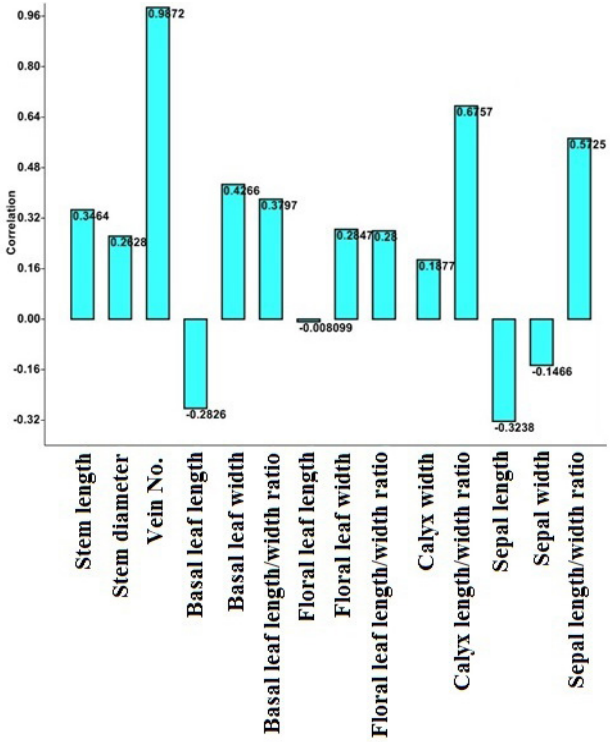

Figure 4. PCA analysis of some morphological features of *Linum nervosum*.

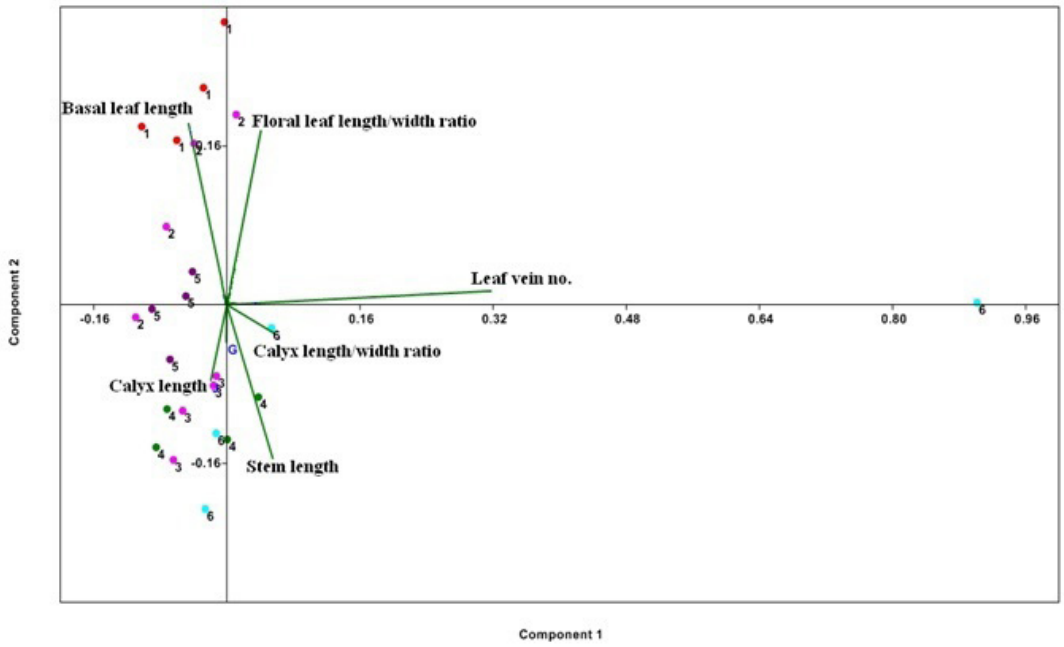

Figure 5. PCA biplot of morphological characters with populations. Numbers correspond to the populations from the Tab. 1.

Table 4. Genetic diversity parameters in the studied populations. **Ne** – number of effective alles; **He** – gene diversity; **% P** – percentage of polymorphism.

Population	Ne	I	He	% P
var. *nervosum*, Marzanabad	1.212	0.220	0.138	53.64%
var. *nervosum*, Siahbisheh	1.310	0.288	0.189	59.09%
var. *bungei*, Siahbisheh	1.221	0.212	0.137	46.36%
var. *bungei*, Marzanabad	1.190	0.167	0.111	31.82%
var. *nervosum*, Pole Zangooleh	1.161	0.193	0.114	54.55%
var. *bungei*, Abr Jungle	1.213	0.219	0.138	50.00%

that 70% of total variability is due to infrapopulation variability, while 30% is a result of diversity between populations.

The values of G_{ST} (Nei) = 0.27 (p = 0.01) and D_{EST} = 0.11 (p = 0.01) along with Hickory test (Theta B = 0.40), supported AMOVA test results and revealed genetic differentiation of the studied populations.

On the NJ tree the populations of *L. nervosum* var. *bungei* from Siahbisheh and Jungle Abr were placed closer to each other due to their higher degree of genetic affinity, while the Marzanabad population of this variety stood far from others (Fig. 6). Mantel test showed significant correlation (p = 0.01) between genetic distance and geographical distance of the studied populations.

The Neighbor-Net diagram supported grouping of NJ tree and revealed more details about genetic affinity of the studied populations and intrapopulation diversity. This diagram showed the presence of three major splits, separating the Marzanabad populations of *L. nervosum* var. *bungei* and and *L. nervosum* var. *nervosum* from the rest of accessions (Fig. 6).

Genetic structure of populations

Genetic structure and possible genetic fragmentation was studied with Bayesian STRUCTURE analysis and K-Means clustering method applied. The STRUCTURE plot is presented on the Fig. 7. Different colors in this plot indicate allelic forms that present in the populations. Plants of the Marzanabad population of *L. nervosum* var. *nervosum* has blue-colored segment as the main allelic form, which is different from the other studied populations. The same holds true for the Siahbisheh population of the same variety with red-colored segment, and Marzanabad population of *L. nervosum* var. *bungei* with yellow colored segment as the main allelic form. However, the Pole Zangooleh population of *L. nervosum* var. *nervosum together* with the Jungle Abr (Shahroud) and Siahbisheh populations of *L. nervosum* var. *bungei* demonstrated a mixture of differently colored segments (different allelic forms), possibly due to the gene flow.

The best clustering of populations according to the Caliński and Harabasz' pseudo-F value was k = 2, while the best

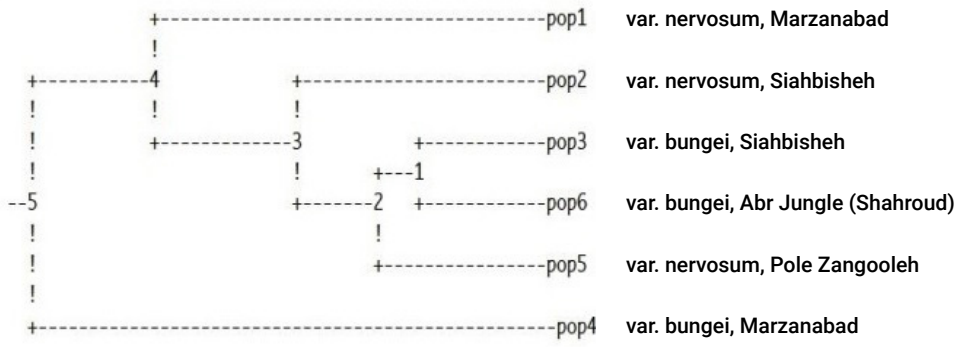

Figure 6. NJ clustering after 100 times bootstrapping based on Nei's genetic distances.

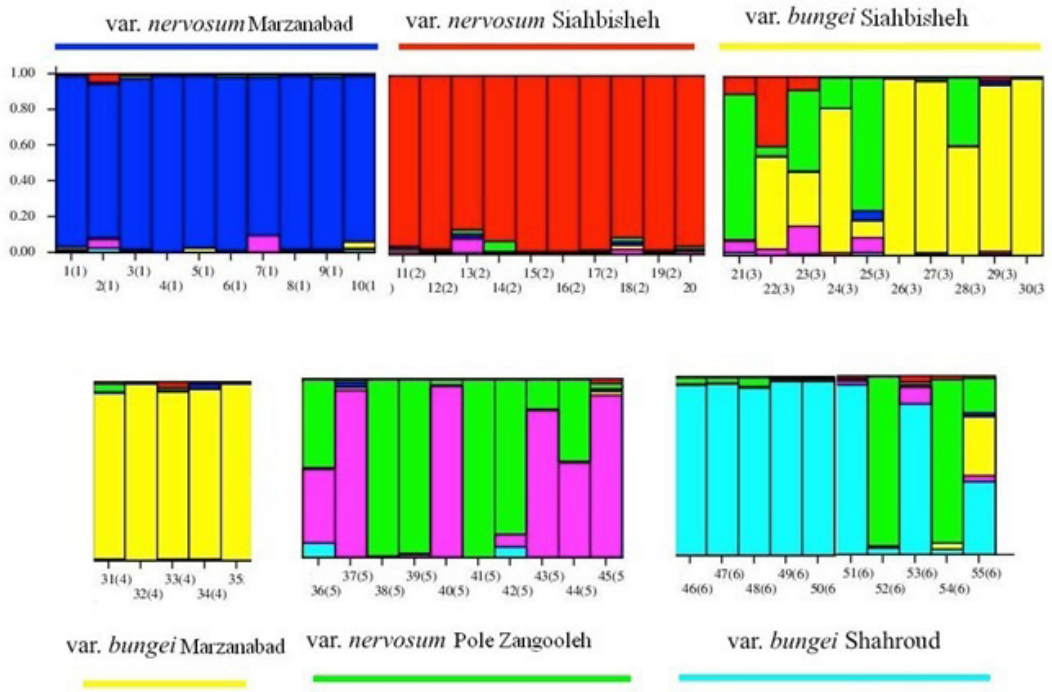

Figure 7. STRUCTURE plot of the studied plants based on ISSR data.

clustering according to the BIC index was k=5 (Tab. 5).

POPGENE analysis of ISSR data showed that most of the studied loci had high Nm value (Nm is an estimator of migration from G_{ST}) ranging from 0.02 to 4.1 with mean value of 2.06, which is a high value of the gene flow. This high Nm value probably corresponds to the breeding system and distyly of this species. ISSR loci with highest Nm values are those loci that are exchanged more frequently among plants within populations and between populations.

Table 5. K-means clustering results of the studied populations of *Linum nervosum*. * – best clustering according to Calinski & Harabasz's pseudo-F (k = 2); ** – best clustering according to Bayesian Information Criterion (k = 5).

k	SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	BIC
1	817.127	0	0	0	0	372.826
2*	817.127	85.442	731.686	0.105	6.189	370.759
3	817.127	147.307	669.82	0.18	5.718	369.908
4	817.127	207.907	609.22	0.254	5.802	368.699
5**	817.127	252.976	564.151	0.31	5.605	368.479
6	817.127	286.559	530.568	0.351	5.293	369.111

We applied latent factor mixed model (LFMM) to our data using dominant method provided in the program to screen genomes for signatures of local adaptation. LFMM showed that ISSR loci 17 (Nm = 0.45), 18 (Nm = 0.33), 50 (Nm = 0.85), and 54 (Nm = 3.40) have $-\log_{10}$ (p-value) of 1.00 to 3.68 and significantly correlate with the studied environmental parameters (p = 0.05). These results are summarized in the Manhattan plot (Fig. 8).

Discussion

The genus *Linum* comprised of about 200 species and different taxonomical patterns have been proposed for it, but most of them were challenged (Talebi et al. 2012). Infraspecific variation plays a key role and aggravates the situation.

The studied varieties of *L. nervosum* are morphologically similar and ANOVA test, as well as CVA plot confirm this. The main morphological differences between the varieties are related to the leaf venation, as well leaf and sepal size. PCA analyses confirmed that the number of veins in the leaf is among the most variable features. *L. nervosum* var. *bungei* has one-veined leaves, while *L. nervosum* var. *nervosum* distinguishes by three veins. Hence, this trait was used for the identification key in the Flora of Iran (Sharifnia & Assadi 2001). In addition, *L. nervosum* var. *bungei* has smaller leaf rather than the other variety; it was mainly related to basal leaf width that significantly differs between these varieties.

Ockendon (1971) showed that the leaf width has taxonomic value and is useful for identification of different taxa in the section *Linum*. For example, this trait is the main feature applied to distinguish *L. perenne* L. subsp. *extraaxillare* (Kit.) Nyman from *L. perenne* L. subsp. *alpinum* (Jacq.) Stoj. & Stef., as well as is applicable in delimitation of *L. trinervium* B. Heyne ex Roth and *L. austriacum* L. subsp. *austriacum* from the rest taxa in section *Linum*. Although, different studies showed that the leaf width has some degrees of phenotypic plasticity. For example, plants of one topodeme of *L. perenne* subsp. *alpinum* growing in partial shade had the leaf widths of 2.5–4.5 mm in comparison with 1.5–4.0 mm measured for the rest of the population. In cultivation, several subspecies showed greater leaf width than those found in the wild. Perhaps, this is a question of the greater vigor of plants in cultivation (Ockendon 1971).

Obtained results showed that sepal length is another important characteristic for delimitation of the varieties. Before it was applied as a diagnostic feature for

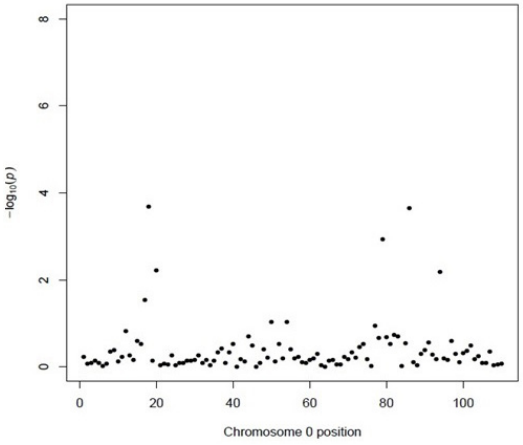

Figure 8. Manhatan plot of ISSR data.

identification of *L. perenne* subspecies and segregation of *L. austriacum* subsp. *austriacum* from the rest of taxa in *L. perenne* group from the section *Linum* (Ockendon 1971).

The length of the branches in Neighbor-Net diagram and presence of horizontal lines between plant specimens indicate genetic differences among the studied populations. Different infraspecific studies on the various species in the genus produce similar results. For example, Sheidai et al. (2014a) investigated genetic diversity and genome size variability in 16 populations of *L. austriacum* in Iran. Different analyses such as AMOVA test, G_{ST} value and Hickory test showed the presence of genetic variability both among and within the studied populations. Nuclear genome size differ significantly in the populations. Similarly, investigation of 12 populations of *L. album* Kotschy ex Boiss. revealed that they differ in many quantitative and some qualitative morphological traits, as well as in molecular characteristics and genome size (Sheidai et al. 2014b).

The result of STRUCTURE plot confirmed genetic differences between the Siahbisheh plants of both studied varieties. These varieties are genetically differentiated from each other, although they are growing in sympatry. However, a small segment that is red-colored occurred in the populations of both varieties possibly due to limited inter-varietal gene flow confirming incomplete isolation of reproductive systems of the varieties. Sympatric speciation is the source of a separating mechanism with the evolution of a delimiter to gene flow (Futuyma & Mayer 1980). Gavrilets (2004) agreed with Futuyma & Mayer (1980) and stated that the coexistence of sexually reproducing taxa needs substantial isolation of reproductive system. The evolution of this phenomenon is generally interpreted as the prominent and intolerable aspect of divergence. The STRUCTURE plot showed genetic difference of the studied varieties in general, as their populations differed greatly in their allelic forms. This result is in agreement with UPGMA tree of morphological characters.

The intrapopulation variation was realized in both morphological characters and genetic structure. For example, plants of *L. nervosum* var. *bungei* from the Siahbisheh population demonstrated a high level of intrapopulation variability and were scattered among the plants of *L. nervosum* var. *nervosum* from the Pole Zangooleh population and plants of *L. nervosum* var. *bungei* from the Jungle Abr population.

It seems that heterostyly predicts high diversity within populations of the genus *Linum*. Talebi et al. (2012) showed that morphological and palynological features, as well as nuclear genome size vary between heterostyled plants within populations of *L. austriacum*, *L. album* and *L. glaucum* Boiss. & Noë. Furthermore, heterostyled samples of four subspecies of *L. mucronatum*

Bertol. also differed significantly (Talebi et al. 2014; Sheidai et al. 2015).

Adaptation to local environment often occurs through natural selection acting on a large number of loci, each having a weak phenotypic effect (Frichot et al. 2013). One way to detect these loci is to identify genetic polymorphisms that exhibit high correlation with environmental variables and used as proxies for ecological pressures. Frichot et al. (2013) proposed new algorithms based on the population genetics, ecological modeling and statistical learning techniques to screen genomes for signatures of local adaptations.

Our study revealed that some of examined loci have significant correlation ($p=0.05$) with ecological factors. However, Manhattan plot showed that only one of them have a high Nm value, while other three loci have low to medium Nm values. It seems that adaptive nature of these loci is not related to their migration nature. Therefore, we may suggest that a combination of genetic drift, limited gene flow and local adaptation may have influence on genetic divergence of *L. nervosum*.

Morphological studies showed that the varieties growing closer to each other and in the same ecological conditions become similar. It means that ecological conditions have strong influence on the features of *L. nervosum*, what is clearly seen in Jungle Abr population. Although molecular analyses confirmed significant genetic difference between the studied taxa, but in some cases the populations were placed close to each other. This confirms general molecular similarity between studied varieties, and therefore we support suggestion of Sharifnia & Assadi (2001) that *L. bungei* should be considered as a variety of *L. nervosum*.

Conclusions

Our morphological and molecular studies revealed a high similarity between *L. nervosum* and *L. bungei*. Because in some habitats these two taxa grow together, it is thought that sympatric speciation could happen. Sharifnia & Assadi (2001) in Flora of Iran altered the taxonomic rank of *L. bungei* to the level of *L. nervosum* var. *bungei*, and our study confirms that opinion.

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