BIODIVERSITAS
Volume 16, Number 2, October 2015
Pages: 145-150

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d160207

Within and among-genetic variation in Asian flax *Linum austriacum* (Linaceae) in response to latitude changes: Cytogenetic and molecular analyses

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Manuscript received: 31 May 2015. Revision accepted: 24 June 2015.

Abstract. *Noormohammadi Z, Shafaf T, Farahani F, Sheidai, Talebi SM, Hasheminejad-Ahangarani-Farahani Y. 2015. Within and among-genetic variation in Asian flax Linum austriacum (Linaceae) in response to latitude changes: Cytogenetic and molecular analyses. Biodiversitas 16: 145-150. Linum austriacum* L. (Linaceae) which is known as Asian flax is an herbaceous medicinal plant species that grow in Iran in different latitudes and forms some local populations particularly in the West and North-West of the country. Within-and between-genetic diversity in response to altitude changes was investigated in geographical populations of *L. austriacum* by using cytogenetic and ISSR molecular markers. These populations were diploid with 2n = 18 and differed significantly ($P<0.05$) in their mean chiasma frequency and chromosome pairing. Significant positive correlation ($r >0.90$, $P < 0.05$) occurred between latitude and the mean number of quadrivalents. The highest level of genetic diversity parameters as Shanon Information Index and gene diversity occurred in Salavat-abad population. Moreover, the STRUCTURE analysis also identified this population as the most varied population. This population had medium altitude distribution. Mantel test performed between genetic distance and geographical distance showed no significant correlation ($R^2 = 0.09$, $p = 0.39$). Pearson coefficient of correlation determined between genetic diversity parameters and altitude, produced a significant negative correlation ($r =-0.85$, $P<0.01$) with the number of effective alleles. The present study revealed that *Linum austriacum* populations do have some cytogenetic and molecular variation in response to altitude.

Key words: Chromosome pairing; Fst; genetic variability; ISSR; wild flax.

INTRODUCTION

The genus *Linum* is the type genus for the flax family, Linaceae DC. (Dumort) comprising 22 genera and about 300 species distributed worldwide. *Linum* is the largest genus (about 200 species) within the family and is found both in the Mediterranean region and the Americas. It includes both horticultural plants with various flower colors and one field crop (*Linum usitatissimum* L.), and have been used as a source of fiber (*L. usitatissimum*), seed oil, fodder, medicine and as ornamentals (Muir and Westcott 2003). Many species are cross-pollinated due to heterostyly. Distyly is widespread and very common in the genus *Linum* (about 40 % of the *Linum* species are distylous) (Sheidai et al. 2015). It occurs in *Linum pubescens*, *L. grandiflorum* and *L. mucronatum*, *L. perenne*, *L. grandiflorum*, *L. alpinum*, *L. aretioides*, *L. austriacum*, *L. album*, and *L. glaucum* (Talebi et al. 2012).

In recent years researchers has been extensively studied to conserve and explore germplasm of crop wild relatives. Crop wild relatives are species closely related to crops, including their progenitors, which may contain beneficial traits such as pest or disease resistance and yield improvement (Sheidai et al. 2014). Genetic diversity analysis and population structure have been the subject of several studied in cultivated and wild flax species (see for example, Fu 2006, Fu and Allaby 2010, Abou El-Nasr and Mahfouze 2013, Sheidai et al. 2014).

Linum austriacum L. is an herbaceous medicinal plant containing important lignans such as arylnaphthalene lignan, 3,4-dimethoxy-3',4'-methylenedioxy-2,7'-cycloligna-7,7'-dieno-9,9'-lactone (1,0.03-0.73% dry wt), together with justicidin B (2, 0.18-1.69% dry wt) (Mohagheghzadeh et al. 2002). Because elevation is a complex factor, altitudinal gradients comprise an assemblage of environmental variables that influence the distribution of population genetic variation of plant species. Along altitudinal gradients, the genetic differentiation between populations of some plant species results in rapid, elevation-related changes in environmental conditions. However, in other taxa, only a little or no differentiation with respect to altitude occurs (Di et al. 2014).

Species have three options that may allow them to survive rapidly changing environments: dispersal (expansion), phenotypic plasticity, or adaptation (Heather and Freeland 2011). The plant species expansion of the core population is associated with reduced within-population genetic diversity (Austerlitz et al. 2000), and reduced levels of phenotypic variation (Pujol and Pannell 2008).

The present study considers cytological and the genetic diversity analysis of some *Linum* populations distributed in different altitudes and investigates degree of within-and among-population genetic variability and tries to study genetic variability response to altitude changes. For molecular study, we used ISSR markers that are powerful molecular tools to differentiate the species populations and reveal their genetic diversity (see, for example, Sheidai et al. 2012, 2013).

MATERIAL AND METHODS

Plant materials

For cytological studies, suitable flower buds have been obtained in five populations of *L. austriacum* (Table 1). Different flower buds were selected randomly from at least ten plants in each population and used for cytological preparations. For ISSR studies, 70 plants were randomly selected from four populations including, Saleh-abad and Hamekasi Village, from Hamedan Province, Iran, and Salavat-abad and Abidar-Sanandaj, from Kurdistan Province (Table 1). The voucher specimens were deposited in the Herbarium of Shahid Beheshti University (HSBU), Tehran, Iran.

Cytological study

The young flower buds collected were fixed and used for cytological investigation by the squash method according to our previous report (Sheidai et al. 2012). Meiotic characters including polyploidy level, chiasma frequency and distribution, as well as chromosome pairing and segregation were observed in plants collected.

ISSR assay

The genomic DNA was extracted from silica gel dried leaves by using CTAB activated charcoal protocol (Krizman et al. 2006). The quality and quantity of extracted DNA were assessed by running on 0.8% agarose gel and NanoDrop® spectrometer respectively. Ten ISSR primers: (AGC)5GT, (CA)7GT, (AGC)5GG, UBC 810, (CA)7AT, (GA)9C, UBC 807, UBC 811, (GA)9A and (GT)7CA commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 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). Amplification 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 a final extension step of 7 min at 72°C. Amplification products as well as No DNA (control sample) were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany). The experiment was replicated three times and constant ISSR bands were used for further analyses.

Data analyses

ANOVA (Analysis of variance) was performed to reveal cytogenetic difference among the studied populations. Pearson correlation determined among geographical features (longitude and latitude) and meiotic characters. PCA (Principal Components Analysis) biplot was used to group populations based on cytogenetic similarity.

ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The Dentrented correspondence analysis plot was used to check distribution of ISSR loci and their effects on genotypes grouping. The role of each locus in discriminating plant genotypes was checked by Gst analysis performed by POPGENE program ver. 2. (1999). Genetic diversity parameters were determined in each population. These parameters were percentage of allelic polymorphism, allele diversity (Weising et al. 2005), Nei's gene diversity (H), Shannon information index (I), the number of effective alleles and percentage of polymorphism and polymorphic information content (PIC) (Weising et al. 2005; Freeland et al. 2011).

Jaccard similarity index and Nei's genetic distance (Freeland et al. 2011), were determined among plants and used for the grouping of the genotypes. Neighbor Joining (NJ) tree followed by 100 times bootstrapping, and Principal Coordinate Analysis biplot (PCoA) (Freeland et al. 2011, Podani 2000) were used for this purpose. DARwin ver. 5 (2012), was used for these analyses.

Genetic differences among the studied populations were determined by: 1-AMOVA (Analysis of molecular variance) test (with 1000 permutations), and 2-Nei's Gst analysis of GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004). Moreover, to avoid possible problem caused by the dominant nature of ISSR markers, Hickory test (Hickory program ver. 1.0) that is a Bayesian approach was used to reveal populations' genetic differentiation (Holsinger et al. 2003).

The genetic structure of geographical populations was studied by Bayesian based model STRUCTURE analysis (Pritchard et al. 2000). Data were scored as dominant markers (Falush et al. 2007).

RESULTS AND DISCUSSION

Cytology

Linum austriacum populations studied showed n = 9 (2n = 2x = 18) chromosome number (Table 1, Figure 1, A-E). The mean value for total chiasmata varied from 16.36 in Tehran-Lashkarak population to 20.38 in Tehran population. These populations had the lowest and highest values of terminal chiasmata too (15.27 and 19.21 respectively). The lowest value of intercalary chiasmata (0.28) occurred in Salavat-abad population, while the highest value of the same parameter occurred in Tehran population (1.17). Although these populations are diploid and mostly formed bivalents (Table 1), they formed few univalent and quadrivalents in metaphase I cells (Figure 1). The chromosomes mostly showed normal segregation during anaphase and telophase stages, but laggard

chromosomes and micronuclei were formed in some cases (Figure 1).

ANOVA test showed significant difference ($p<0.05$) for chiasma frequency and chromosome pairing among populations studied. Pearson correlation determined among ecological and meiotic characters showed significant positive correlation between longitude and the mean number of quadrivalents as well as between latitude and mean number of quadrivalents. Significant negative correlation was observed between longitude and the mean number of univalents.

PCA biplot of meiotic characters (Figure 2), separated the populations studied in distinct groups, indicating cytogenetic differences of the populations studied, also supported by ANOVA test. It also showed that, Tehran population was differentiated from the other populations due to its intercalary chiasmata and number of quadrivalents, while Salavat-abad population was differentiated from the others due to mean number of total chiasmata, ring bivalents and terminal chiasmata. The number of rod bivalents differentiated Tabriz-Ahar population, while No. of univalents separated Roodbar population from the other populations studied.

Within-population genetic diversity

Genetic diversity parameters determined among 4 populations studied are presented in Table 2. The highest level of genetic polymorphism occurred in Hamekasi Village population of Hamedan Province (Pop. 2) (42.42%), while the lowest value of the same parameter occurred in Saleh-abad, Hamedan Province (Pop. 1) and Salavat-abad population, Kurdistan Province (Pop. 3) (40.40%). The highest value for effective No. of alleles (1.326), I (0.258) and gene diversity (0.179) occurred in Abidar population of Kurdistan Province (Pop. 4). Within-population genetic diversity was investigated by using Jaccard similarity index. In Saleh-abad population of Hamedan (Pop. 1), Jaccard similarity among plants of this population ranged from 0.26-0.94.

Similarly, in Hamekasi Village population of Kordestan (Pop. 2), Jaccard index ranged from 0.43-0.81. In Salavat-abad population (Pop. 3), the range of Jaccard index was 0.34-1.00, while the same value in Abidar population of Sanandaj (pop 4) was 0.38-0.90. STRUCTURE analysis (Figure 3) that is based on Bayesian approach also revealed the occurrence of a higher degree of within-population genetic variability in Salavat-abad population (Pop. 3).

Pearson coefficient of correlation determined between genetic diversity parameters and altitude, produced a significant negative correlation ($r =-0.85$, $P<0.01$) with the number of effective alleles only (Table 3). Longitude did not show correlation with genetic diversity parameters, but latitude had a significant negative correlation with Shanon Information Index ($r =-0.90$, $P<0.01$), mean gene diversity ($r =-0.88$, $P<0.01$) and mean unbiased gene diversity ($r =-0.86$, $P<0.01$ $P<0.01$; Table 3).

Table 2. Genetic diversity parameters in populations studied

Pop	N	Na	Ne	I	He	UHe	%P
1	15.000	0.899	1.256	0.210	0.142	0.147	40.40%
2	19.000	0.990	1.264	0.227	0.153	0.157	42.42%
3	20.000	0.859	1.292	0.242	0.166	0.170	40.40%
4	16.000	0.899	1.326	0.258	0.179	0.185	41.41%

Abbreviations: Na = No. of different Alleles, Ne = No. of Effective alleles, I = Shannon's Information Index, He = Gene diversity, UHe = Unbiased gene diversity, and % P = Percentage of polymorphism. Populations 1-4 are: Saleh-abad and Hamekasi Village from Hamedan Province and Salavat-abad and Abidar-Sanandaj from Kurdistan Province respectively.

Table 1. Cytogenetic features of *L. austriacum* populations studied.

Population	Province	Longitude	Latitude	Altitude (ft)	n	TOX	IX	TX	ROD	RB	I	IV	Voucher No.
Tehran-Lashkarak	Tehran	35°15′.50″	51°34′.00″	2100	9	11.23	1.00	10.23	6.70	1.87	0.10	0.20	-
Roodbar	Gilan	36°50′.00″	49°25′.00″	544	9	10.65	0.15	10.5	7.04	1.77	0.19	0.04	2011126
Salavat-abad	Hamedan	35°08′.00″	47°52′.00″	1900	9	12.04	0.82	11.23	5.91	2.91	0.14	0.04	2011108
Tabriz-Ahar	East Azarbayejan	38°09′.00″	46°39′.00″	3809	9	10.67	0.75	9.92	7.33	1.58	0.17	0.00	2011136
Abidar-Sanandaj	Kurdistan	35°19′.00″	46°57′.00″	1645	9	10.5	0.64	9.86	7.36	1.5	0.28	0.00	2011112

Abbreviations: TOX = Total chiasmata, TX = Terminal chiasmata, IX = Intercalary chiasmata, RB = Ring bivalents, ROD = Rod bivalents, I = Univalents, IV = Quadrivalents.

Table 3. Correlation between geographical features and genetic diversity parameters

	N	Na	Ne	I	He	UHe	%P	Altitude	Longitude	Latitude
N	0									
Na	0.09214	0								
Ne	-0.01326	-0.41076	0							
I	0.22846	-0.28382	**0.96518**	0						
He	0.18355	-0.32895	**0.97856**	**0.99813**	0					
UHe	0.13186	-0.33415	**0.98731**	**0.99446**	**0.99863**	0				
%P	0.21938	**0.90224**	0.005495	0.15685	0.10866	0.10047	0			
altitude	0.5045	0.23777	**-0.8529**	-0.72148	-0.74822	-0.78166	-0.0634	0		
longitude	0.37928	-0.76166	-0.09971	-0.09805	-0.08002	-0.1033	-0.81555	0.429	0	
latitude	-0.61235	0.22796	-0.78123	**-0.90863**	**-0.88952**	**-0.86438**	-0.18283	0.36649	-0.11801	0

Abbreviations: Na = No. of different Alleles, Ne = No. of Effective alleles, I = Shannon's Information Index, He = Gene diversity, UHe = Unbiased gene diversity, and % P = Percentage of polymorphism.

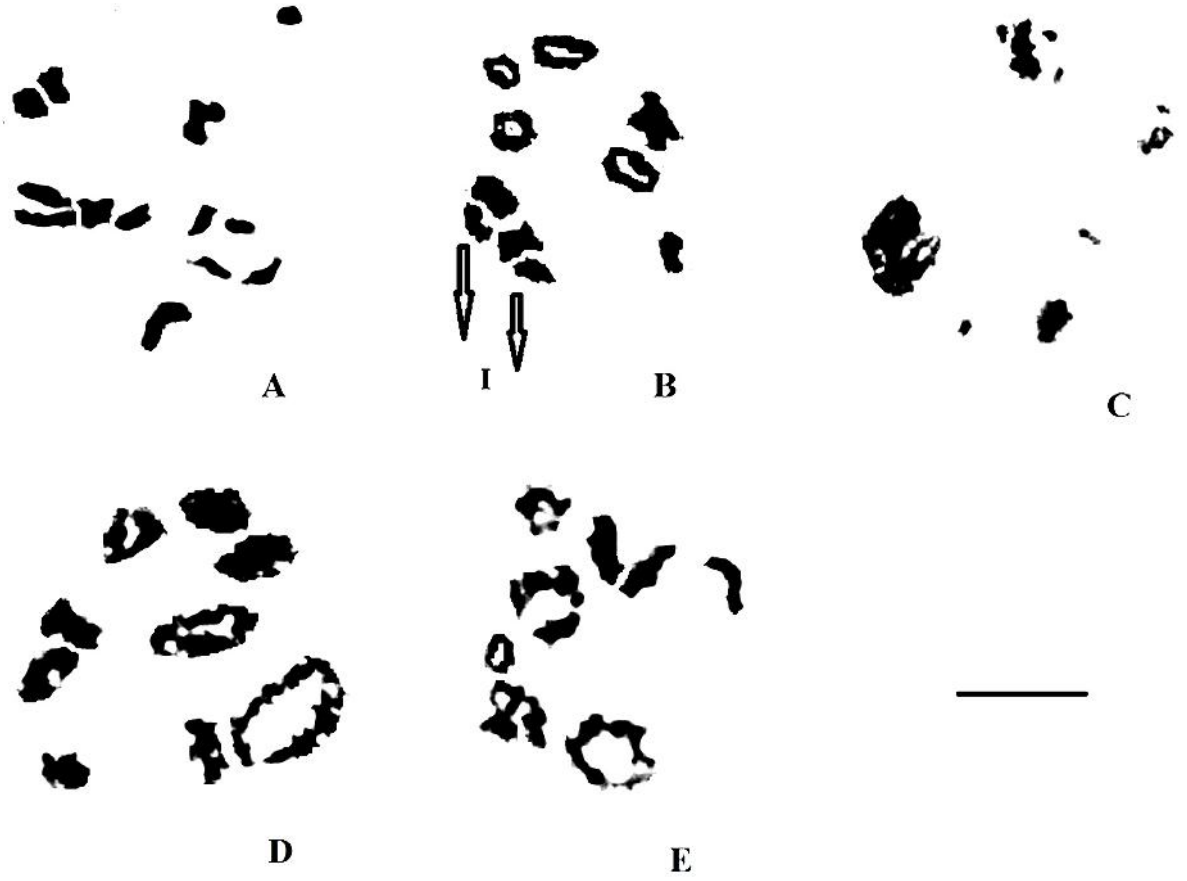

Figure 1. Representative meiotic cells in *L. austriacum* populations studied. A-E = A: metaphase I cell, B: univalent (arrows), C: laggard chromosome, D and E: metaphase I cells

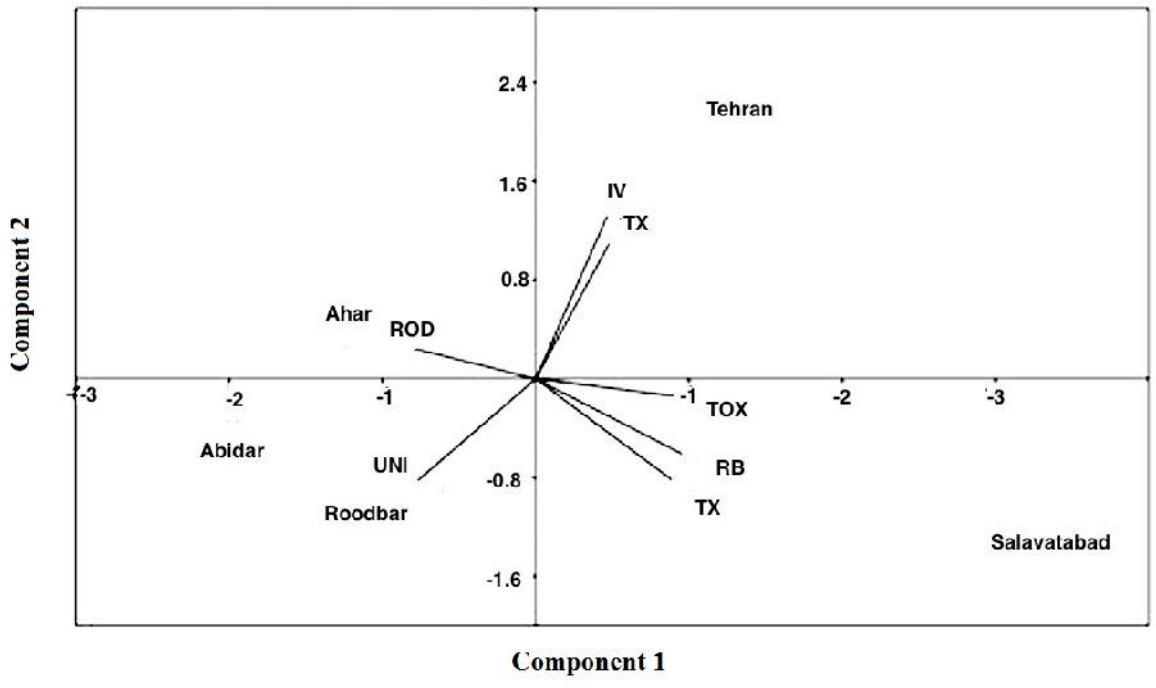

Figure 2. PCA biplot of meiotic characters. Abbreviations: TOX = Total chiasmata, TX = Terminal chiasmata, IX = Intercalary chiasmata, RB = Ring bivalents, ROD = Rod bivalents, I = Univalents, IV = Quadrivalents.

Figure 3. STRUCTURE plot showing higher degree of withi-population genetic diversity in population. Populations 1-4 are Saleh-abad, Hamekasi village, Salavat-abad and Abidar, respectively

Genetic differentiation of populations

AMOVA test showed significant differences among the studied populations ($p<0.01$). It showed that 63% of total variation is due to, among populations and 37% due to within populations. Irrespective of the good genetic variability observed within each population, Neighbor Joining tree of all 70 plants separated the studied populations from each other (data not shown). The plant specimens of each population were placed close to each other and formed a separate group. Moreover, high values of θB (>0.52) showed great genetic difference among pairs of populations which support AMOVA result. These results indicated genetic distinctness of the studied populations.

Mantel test performed between genetic distance and geographical distance showed no significant correlation ($R^2 = 0.09$, $p = 0.39$). However, Fst values of populations showed negative significant correlation with the Eastern distribution ($r = -.92$, $p = 0.05$), and high negative (but not significant) correlation with altitude of populations ($r = -0.81$). Fst values showed high (but not significant) positive correlation with Western distribution and minimum temperature.

Nm values obtained for all ISSR loci in the studied populations ranged from 0.0 to 1.0, with the mean Nm value of 0.44. This is a moderate value and indicates a moderate degree of gene flow among the studied populations.

Discussion

Linum austriacum populations studied showed n = 9 (2n = 2x = 18) chromosome number, supporting Öztürk et al. (2009) report. Although these populations are diploid and mostly formed bivalents, they formed a few quadrivalents in metaphase I cells. Quadrivalent were formed due to the occurrence of the heterozygote translocations, which may have adaptive value (Sheidai et al. 2012). Cytogenetic studies performed by Gill and Yermanos (1967) in six 18-chromosome *Linum* taxa and nine of their interspecific hybrids, also indicated the role of translocations in *Linum* species diversification. They showed that *L. altaicum* differs from *L. alpinum, L. austriacum, L. julicum, L. narbonense*, and *L. perenne* by one reciprocal translocation. *L. austriacum* and *L. narbonense*, and *L. julicum* and *L. narbonense* also differed by one translocation, whereas *L. perenne* and *L. narbonense* differed by two translocations.

Variation in chiasma frequency and localization is genetically controlled and has been reported in several plant species (Quicke 1993; Sheidai et al. 1999). Such a variation in species or populations with the same chromosome number is considered a means for generating new forms of recombination influencing the variability within natural populations in an adaptive way (Rees and Dale 1974).

Therefore, significant difference observed for chiasma frequency and chromosome pairing among *Linum austriacum* populations indicate a change in genetic control of chromosome pairing during population diversification. Significant positive correlation between longitude and the mean number of quadrivalents, as well as between latitude and mean number of quadrivalents, indicate that heterozygote translocations may have played a role in response to these ecological parameters.

ISSR analysis of the studied populations revealed almost high degree of within population genetic variability and significant genetic differentiation among populations (significant AMOVA result). However, in spite of significant genetic difference among populations, we did not observe isolation by distance (IBD) (Mantel test showed no correlation between genetic distance and geographical distance of the studied populations). This result suggested some degree of gene flow among populations that are supported by the fact that Fst values of populations decreased towards Eastern distribution (possibly due to limited occurrence of gene flow). However, the studied populations become genetically more differentiated towards the western part of the country where the minimum temperature is lower.

Genetic variability of these populations was reduced in response to an increase in altitude (Pearson coefficient of correlation produced a significant negative correlation between altitude and the number of effective alleles). These results reveal complex interaction between genetic diversity distribution of *Linum austriacum* populations and environmental features.

In the present study, irrespective of the genetic variability observed within each population, NJ tree of all 70 plants separated the studied populations from each other. The plant specimens of each population were placed close to each other and formed a separate group. This clearly reveals that each local population has its own specific genetic contents along the altitude its plants grow.

This is particularly true for Salavat-abad population of Kurdistan Province (Pop. 3) that formed a distinct cluster and was placed far from the other studied populations.

Evaluation of within and among-population genetic variations have been considered to prioritize populations for conservation efforts (Petit et al. 1998), high within-population variation. These populations may have increased likelihood of persistence over less variable population and hence the ability of a population to contribute demographically to the species through time, and have increased adaptability in the face of future environmental change.

Genetic diversity within populations can vary along altitudinal gradients. In many cases the populations at intermediate altitudes have greater diversity than populations at lower and higher altitudes (see for example, Gapare et al. 2005; Ohsawa and Ide 2008; Di et al. 2014).

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