

Genetic diversity and structure analysis of *Linum glaucum* populations

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Abstract

Linum glaucum is a member of the section *Linum* of the genus *Linum*. This species is distributed in the western regions of Iran. In the present study, the inter-simple sequence repeat (ISSR) technique was used to estimate infraspecific variation in different populations of *L. glaucum*. Samples from nine populations were collected from different regions of the country during spring 2011. For ISSR investigations, populations were divided into three geographical regions. Detrended correspondence analysis showed that ISSR loci were distributed in different parts of the genome and showed no clustering with close association. Gst analysis of ISSR loci showed that some of these loci have good discriminating power. STRUCTURE analysis showed that except for population No. 9, the other populations contained almost similar allelic composition, but differed in the frequency of these alleles using ISSR data. The studied populations were separated in principal coordinate analysis plots and in unweighted paired group cluster analysis. Based on the observed variation, populations were distinguished as ecotypes in the studied populations.

Key words: genetic structure, infraspecific variations, ISSR, *Linum glaucum*, population.

Abbreviations: CCA, canonical correspondence analysis; DCA, detrended correspondence analysis; ISSR, inter-simple sequence repeat; NJ, neighbor joining; PCO, principal coordinate analysis; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; UPGMA, unweighted paired group using average method.

Introduction

Genetic diversity, one of the basic levels of biodiversity, determines the evolutionary potential of a taxon or population to adapt to variable environmental conditions (Musche 2008). Genetic diversity arises from different factors such as mutation or gene flow, while events, for example, genetic drift and directional selection, can decrease the diversity. Frankham et al. (2002) suggested that the relative impact of these mentioned factors depends on population size and varies among traits, coding as well as non-coding DNA, and protein polymorphism and its quantitative features. Human-induced habitat fragmentation, which divides large and continuous populations into smaller and more isolated remnants, has a major impact on the strength of genetic drift and the magnitude of gene flow (Musche 2008).

Inter simple sequence repeat (ISSR) technique is a polymerase chain reaction (PCR) based approach. The technique involves amplification of DNA segments that exist at amplifiable distance between two identical microsatellite repeat regions oriented in opposite directions.

Genetic variation patterns have been successfully assessed in population studies using ISSR markers (Raina et al. 2001). ISSR have been shown to be more reliable, in comparison with other fingerprinting techniques such as random amplified polymorphic DNA (RAPD), which may be due to longer primers that anneal at higher temperatures (Qian et al. 2001). Each ISSR fragment of differing length is treated as a unique locus and the amplified PCR fragment represents a diallelic marker (Wolfe, Randle 2001). This technique has been successfully used to estimate the extent of genetic variation at inter- and intra-specific levels in a wide range of taxa such as *Brassica napus* (Charters et al. 1996), *Triticum* spp. (Nagaoka, Ogihara 1997), *Zea mays* (Kantety et al. 1995), *Vigna* spp. (Ajibade et al. 2000), *Ipomoea* spp. (Huang, Sun 2000) and *Plantago* spp. (Wolff, Morgan-Richards 1998).

Linum glaucum Boiss. & Nöe is a member of the section *Linum* of the genus *Linum* L. This species is an herbaceous perennial, stems divergent, 30 to 50 cm, bearing a many-flowered panicle of monochasial cymes. Leaves are alternate, glaucous, linear or linear-oblong, acute, thick, 15 to 20 × 1.5 to 5 mm, smooth or minutely

scaly (rarely papillose). Pedicels are deflexed or recurved in fruit. Flowers are hermaphrodite and heterostyled, sepals prominently 3 to 5-nerved, 5 to 6 mm. Petals are blue with length 15 to 20 mm. Stigmas are oblong-capitate, capsule 7 to 8 mm, slightly longer than broad (Davis 1967).

L. glaucum is regarded as a medicinal plant, since justicidin B occurs in callus and plantlet cultures of this species. Justicidin B is an aryl-naphthalene lignan that has strong cytotoxicity on chronic myeloid and chronic lymphoid leukemia cell lines (Mohagheghzadeh 2009).

This species naturally grows in Iran (Rechinger 1974; Sharifnia, Assadi 2001), but in comparison to other species of this genus such as *Linum austriacum* L. and *Linum album* Ky. ex Boiss, *L. glaucum* is mainly restricted to western regions of the country.

In the present study, the ISSR technique was used to estimate infraspecific genetic variation and structure in different populations of *L. glaucum* in Iran. We found no previously published studies on the genetic structure of different populations of this species.

Materials and methods

Plant material

Nine populations of *L. glaucum* were randomly collected from western regions of Iran during spring 2011 (Table 1). Samples were identified based on descriptions provided in Flora Iranica (Rechinger 1974) and Flora of Iran (Sharifnia, Assadi 2001). Voucher specimens were deposited in the herbarium of Shahid Beheshti University (HSBU). For each population five samples (in total 45 flowering stems) were selected randomly and were pooled for genetic analysis.

ISSR assay

Total genomic DNA was extracted from fresh leaves using the CTAB method (Murray, Thompson 1980) with the modification described by De la Rosa et al. (2002). Six commercial (University of British Columbia) ISSR primers were used: UBC823, UBC811, UBC834, UBC807, CA7AT and CA7AC. PCR reactions were performed in a 25 μ L volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM

KCl; 1.5 mM MgCl₂; 0.2 mM of each dNTP; 0.2 μ M of a single primer; 20 ng genomic DNA and 3 units of Taq DNA polymerase (Bioron, Germany). Amplification reactions were performed in a Techne thermocycler (Germany) with the following programme: 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 with a final extension step of 7 min at 72 °C. Amplification products were visualized by electrophoresis on a 2% agarose gel, followed by ethidium bromide staining. Fragment size was estimated by using a 1 kb base pair (bp) molecular size ladder (Fermentas, Germany).

Amplified ISSR fragments were treated as binary characters and coded accordingly (presence = 1, absence = 0). To assess genetic diversity, different genetic parameters including the number of common alleles and their frequency, number of specific alleles, Shannon's information index, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity and fixation index (Weising et al. 2005; Freeland et al. 2011) were determined for each population and for combined all studied populations (Podani 2000).

Bayesian model-based clustering was also used to determine the genetic structure among populations using STRUCTURE v. 2.3 (Pritchard et al. 2000). This program implements a model-based clustering method for inferring population structure using genotype data consisting of unlinked markers. It is assumed that within populations, the loci are at Hardy-Weinberg equilibrium and linkage equilibrium. We utilized an admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K for 10⁶ iterations after a burn-in period of 105. All other parameters were set at their default values. The proportional membership of each cluster was estimated for each individual and each population. The choice of the most likely number of clusters (K) was carried out comparing log probabilities of data [Pr (X|K)] for each value of K (Pritchard et al. 2000), as well as by calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as described by Evanno et al. (2005).

Reticulation was performed by T-REX (Tree and Reticulogram Reconstruction) ver. 3. (2000), which infers a reticulogram from distance matrix. For reticulation, we first built a supporting phylogenetic tree using the neighbor joining (NJ) method, followed by a reticulation branch that minimized the least-squares at each step of the algorithm (Legendre, Makarenkov 2002). Two statistical criteria, Q1 and Q2, were used to measure the gain in fit provided by each reticulation branch. The numerator of these functions is the square root of the sum (or the sum itself) of the quadratic differences between the values of the given evolutionary distance δ and the corresponding reticulogram estimates d , n is the number of taxa in the considered set X, and N is

Table 1. Location and herbarium accession numbers of the studied populations of *L. glaucum* collected by Talebi in Kurdistan

No.	Location	Herbarium No.
1	Sanandaj to Hasan Abad, 1684 m	HSBU2011153
2	Sanandaj, Darbandeh village, 1559 m	HSBU2011154
3	Sanandaj, Kani moshkan, 1678 m	HSBU2011155
4	Sanandaj, Kilaneh, 1471 m	HSBU2011156
5	Sanandaj, 1585 m	HSBU2011158
6	Baneh to Saqqez (20 km), 1546 m	HSBU2011160
7	Baneh, 1623 m	HSBU2011161
8	Saqqez, 1570 m	HSBU2011162
9	Saqqez to Divandareh, 1617 m	HSBU2011163

the number of branches in the reticulogram, i.e. total of the phylogenetic tree branches and reticulation branches. The minimum of Q1 and Q2 can define a stopping rule for the addition of reticulation branches (Legendre, Makarenkov 2002; Makarenkov, Lapointe 2004). The multivariate method canonical correspondence analysis (CCA), was used to test the relationship between the studied populations and environmental factors such as altitude, longitude, latitude and mean annual maximum and minimum temperature. The statistical analyses were performed by using NTSY ver. 2 (1998), SPSS ver. 9 (1998) GENEALX ver. 6 (2006) and STRUCTURE ver. 2.2 (2009).

Results

In total, 76 ISSR fragments/loci were amplified. In this study nine populations of *L. glaucum* were selected from western regions of Iran. CCA plot showed the relationships between the sampled populations and their environmental factors (Fig. 1). The populations were divided into three geographical regions: (1) Sanandaj (populations No. 1 to 5), (2) Baneh (populations No. 6 and 7), and (3) Saqqez (populations No. 8 and 9). The habitats of the sampled populations are mountainous, and populations of the Sanandaj region are separated from the other regions by mountains. The habitat in the Sanandaj region is not flat relief and the populations surrounded by mountains are relatively small. Pairwise geographical distances between the studied populations were given in Table 2.

The DCA analysis showed that ISSR loci were spread throughout the plot without clustering and close association, indicating they are located in different parts of the genome (Fig. 2). The G_{st} analysis of ISSR loci showed that some of these loci had good discriminating power ($G_{st} > 0.60$), they were loci No. 2, 12, 37, 38, 41, 61, 62, 67 and 73.

The highest number of bands were observed in samples from geographical region 1 (Sanandaj, 63 bands), and the lowest number in samples from region 3 (Saqqez). Specific bands were identified in samples from regions 1 and 2 (Fig. 3). Some of the ISSR bands were specific for a single population, for example band No. 14 (2500 bp) of primer CA7AC and band 2 (750 bp) of primer 811 occurred only

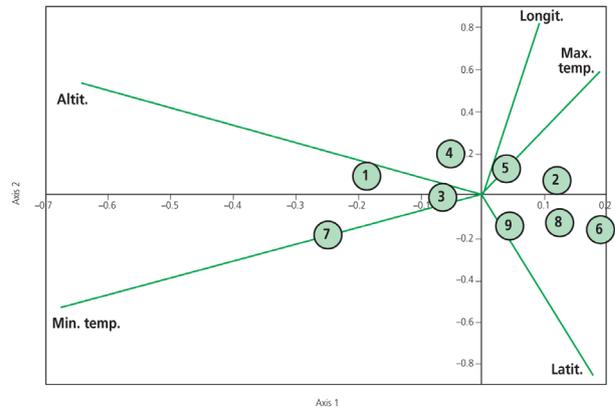


Fig. 1. CCA plot of the relationships between genetic diversity variables and ecological factors of populations habitat. Min. temp., mean annual minimum temperature; Max. temp., mean annual maximum temperature; Latit., latitude; Altit. altitude; Longit., longitude (numbers 1 to 9 refer to populations given in Table 1).

in population No. 8. Similarly, band No. 4 (820 bp) of ISSR primer 811 was present only in population No. 2, while ISSR band No. 9 (2000 bp) of this primer occurred only in population No. 5.

Genetic diversity parameters were determined in the three geographical regions (Table 3). The highest number of effective alleles (1.41), Shannon information index (0.37), expected heterozygosity (0.24) and polymorphism percentage (72.37), occurred in region 1 (Sanandaj). The lowest number of effective alleles (1.27), expected heterozygosity (0.15), Shannon information index (0.23) and polymorphism percentage (38.16) occurred in Saqqez region. The AMOVA test with 999 permutations showed no significant molecular difference among the three geographical regions, but the PCoA plot showed that Sanandaj with its five populations differed genetically from the Baneh and Saqqez regions (Fig. 4). Nei's genetic identity determined among the three geographical regions showed a higher similarity between regions 1 and 3 (0.86), followed by regions 1 and 2 (0.83). The lowest genetic identity (largest difference) occurred between regions 2 and 3 (0.77).

Fig. 5 presents results of STRUCTURE analysis and shows the estimated membership coefficients for each

Table 2. Pairwise geographical distances between the studied populations of *L. glaucum* (all values in km)

Populations	1	2	3	4	5	6	7	8	9
1	0								
2	18	0							
3	21	16	0						
4	23	65	16	0					
5	23	27	30	33	0				
6	205	235	225	245	235	0			
7	265	269	271	275	257	43	0		
8	203	209	212	215	197	33	71	0	
9	110	114	117	120	105	100	160	100	0

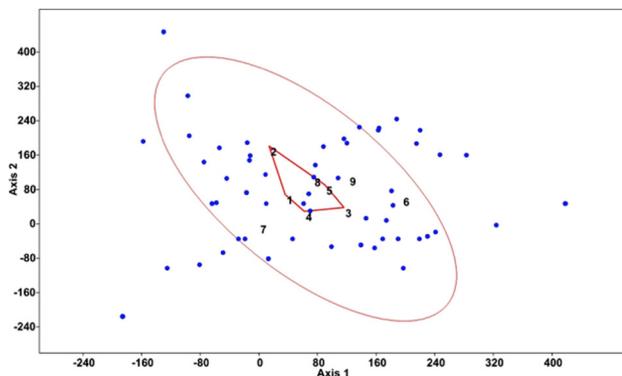


Fig. 2. DCA plot of ISSR loci of *L. glaucum* with convex hulls and 95% ellipses (numbers 1 to 9 refer to populations given in Table 1).

population to a pre-defined number of groups. Population No. 1 showed lack of a red coloured segment that was present in the other four populations of Sanandaj region (populations No. 2 to 4). Similarly, among two populations of Baneh region, population No. 7 did not have a red coloured segment while population No. 6 had this segment. Population No. 9 of the Saqqez region differed completely from population No. 8 of the same region and also differed greatly from all other populations. The presented results indicated that there was some degree of population differentiation between the populations, with the exception of population 9, which was very different. These genetic differences indicate the occurrence of extensive genetic rearrangements in the genome of *L. glaucum* during population diversification and perhaps due to local adaptation.

Similarities in genetic structure of the studied populations may be due to gene exchange among them. The obtained reticulogram (Fig. 6) also showed some degree of gene exchange among these populations, supporting results obtained using STRUCTURE.

The studied populations were separated in the NJ and UPGMA trees (Fig. 7, 8) as well as the PCO plot (Fig. 9). Populations from the Sanandaj region were placed in two different branches, populations No. 1, 4 and 5 were clustered closely together in the same branch, and populations No. 2 and 3 clustered separately from other populations of this region. Separation of populations was also found in other

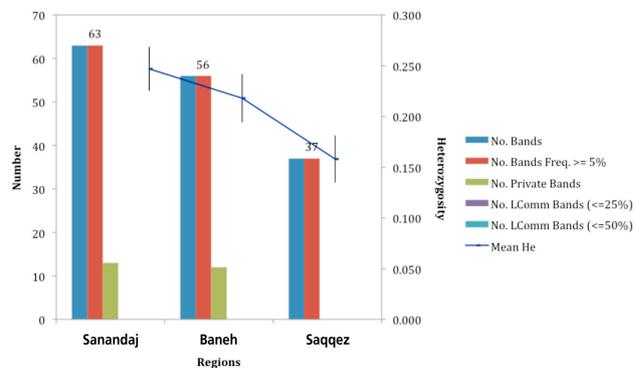


Fig. 3. ISSR band characteristics in *L. glaucum* populations in the studied geographical regions. Sanandaj, populations 1 to 5; Baneh, 6 and 7; Saqqez, 8 and 9.

regions. Populations from the Baneh and Saqqez regions clustered separately from each other. The arrangement of populations in plot and trees confirmed degrees of genetical similarity between populations.

Discussion

Populations of *L. glaucum* are found naturally in Iran, but they have restricted distribution and occur only in the western region of the country on slopes of the Zagros mountain chain, from Sanandaj to south of West Azerbaijan province. In the present study, infraspecific variation in nine populations of this species was determined. Genetic diversity was studied in the populations using a DNA molecular marker technique.

Previous studies (e.g. Divakara et al. 2010; Ashkani et al. 2012) have shown that DNA molecular markers can provide information on polymorphism. The genetic composition is unique for each taxon and is not influenced by physiological conditions or age. The ISSR-PCR marker technique, which has been used successfully for genetic investigation of populations within a species, was also used in this study to reveal the infraspecific variation in *L. glaucum* populations. This technique is efficient for genetic characterization even at the varietal level of a species. For example, Charters et al. (1996) distinguished 20 cultivars of *Brassica napus* using ISSR markers. This marker technique has been used also for assessment of genetic diversity in *Theobroma*

Table 3. *L. glaucum* genetic diversity parameters in studied geographical regions. Na, average number of different alleles; Ne, average number of effective alleles = $1 / (p^2 + q^2)$; I, Shannon's information index = $-1 \times [p \times \ln(p) + q \times \ln(q)]$; He, expected heterozygosity = $2 \times p \times q$; UHe, unbiased expected heterozygosity = $[2N / (2N - 1)] \times He$; %P, polymorphism percentage. Sanandaj, populations 1 to 5; Baneh, 6 and 7; Saqqez, 8 and 9.

Region	N	Na	Ne	I	He	UHe	%P
Sanandaj	5.000	1.553	1.418	0.373	0.247	0.274	72.37
Baneh	2.000	1.263	1.372	0.318	0.218	0.291	52.63
Saqqez	2.000	0.868	1.270	0.231	0.158	0.211	38.16
Total	3.000	1.228	1.353	0.307	0.208	0.259	54.39

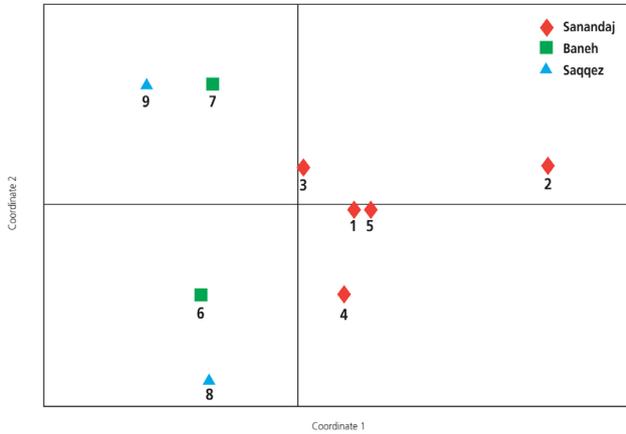


Fig. 4. PCoA plot of the ISSR data of the studied *L. glaucum* populations. Numbers 1 to 9 refer to populations given in Table 1.

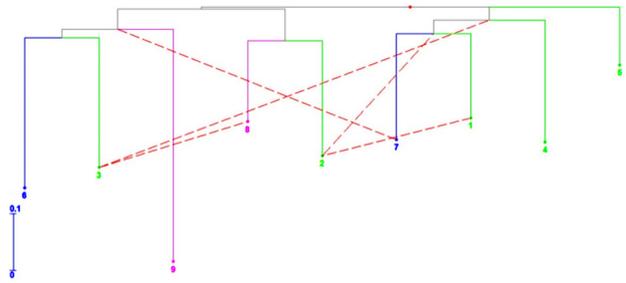


Fig. 6. Reticulogram of *L. glaucum* populations. Dashed lines indicate gene exchange. Numbers 1 to 9 refer to populations given in Table 1.

cacao (Charters, Wilkinson 2000), gymnosperms such as *Pseudotsuga menziesii* and *Cryptomeria japonica* (Tsumura et al. 1996) and even fungi (Hantula et al. 1996).

The studied populations were separated in the UPGMA hierarchical tree, and PCO analysis confirmed the UPGMA clustering. Not only populations from different regions were separated from others, but also populations from the same region clustered separately. In addition, population No. 9 was separated from the other populations. On the basis of Nei's genetic identity, highest similarity in genomic structure occurred between populations of regions 1 and 3, while highest genetic difference was seen between regions 2 and 3. In addition, largest and smallest values of expected heterozygosity and also polymorphism percentage were recorded in regions 1 and 3, respectively. Thus, we expect to find highest genetical difference between populations of regions 1, and in contrast highest genetical similarity among populations of region 3. The ISSR technique has been successfully applied to determine genetic diversity among and within *Jatropha curcas* populations and to group plants according to genetic similarity. Tanya et al. (2011) reported that the average Nei's total genetic diversity of *Jatropha* populations (HT) was 0.355 and that the intra-population genetic diversity (HS) was 0.068, while the inter-population genetic differentiation coefficient (GST) was 0.807 due to a low level of genetic variation among the populations,

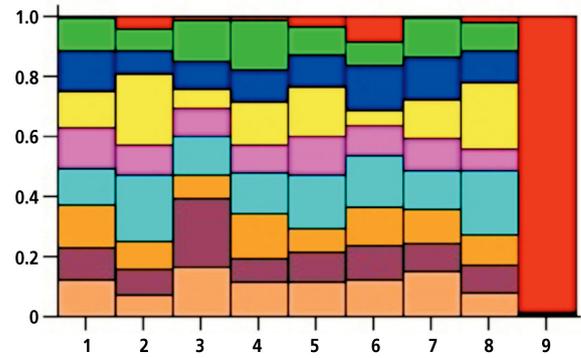


Fig. 5. STRUCTURE analysis of the studied *L. glaucum* populations. Numbers 1 to 9 refer to populations given in Table 1.

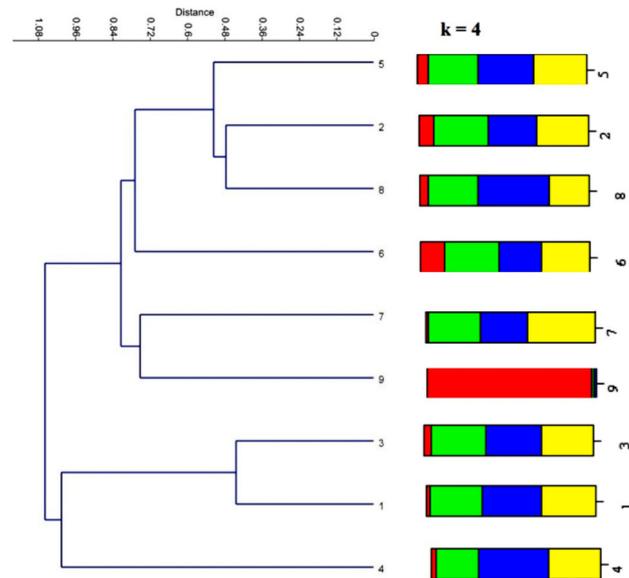


Fig. 7. NJ tree of the studied populations of *L. glaucum* based on ISSR data. Numbers 1 to 9 refer to populations given in Table 1.

as determined with ISSR markers. Genetic diversity in different accessions of *J. curcas* from Brazil was investigated by Grativol et al. (2010). In that study use of ISSR markers showed a high level of genetic diversity among these accessions. Khurana-Kaul et al (2012) showed low levels of variation in *J. curcas* from India, which was attributed to the small number of introductions and vegetative propagation.

Two regions of the studied (Saqqez and Baneh) were geographically close together, while Sanandaj region was separated. The localities of the Sanandaj region populations were close to each other. Due to the geographical proximity between populations in this area, it was expected that these populations would be genetically similar, but the populations did not form a clear cluster in the analyses, especially regarding populations No. 3 and 2, which were placed far from other populations. This was also true in the case of Baneh and Saqqez regions. As these two areas

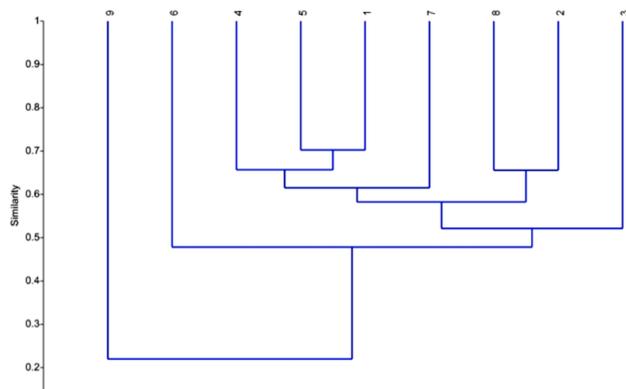


Fig. 8. UPGMA tree of the studied populations of *L. glaucum* based on ISSR data. Numbers 1 to 9 refer to populations given in Table 1.

were located close to each other, it was expected that their populations would be genetically similar, but this was not confirmed.

The observed genetic variation between the studied populations can be the outcome of several factors, such as mutation rate, breeding size of the population, breeding strategy, migration, selection (Ramel 1998), gene flow, genome size (Talebi et al. 2012) and chromosome number. In flow cytometry investigations, previous study showed that variation of about 1.19 times occurred between the maximum and minimum mean genome sizes in populations of *L. glaucum* (Talebi et al. 2015). The breeding system in the genus *Linum* has been widely discussed. Different studies have shown that heterostyly (distyly) is widespread in this genus and two forms of flowers, i.e. long-styled as well as short-styled flowers, are found in many species of the genus *Linum* (for example, Ockendon 1968; Dulberger 1973; Rogers 1979; Talebi et al. 2012; Güvensen et al. 2013). This phenomenon can affect various plant characteristics. For example Talebi et al. (2012) found that morphological and palynological traits as well as nuclear genome size differed between long-styled and short-styled populations in *L. glaucum*. At the chromosomal level, variation in B chromosomes are well known: B chromosomes are additional dispensable chromosomes that are present in some individuals from some populations in some species, which have probably arisen from A chromosomes, but follow their own evolutionary pathway (Beukeboom 1994). However, B chromosomes have been identified in some species of the genus *Linum* (Muravenko et al 2010). Thus, no evidence of the presence of this type of chromosome has been provided in *L. glaucum*.

Frequencies of B chromosomes vary between different populations of *Crepis capillaries*, being higher in intermediate altitudes populations, which suggest that these chromosomes are excluded in populations growing under suboptimal conditions (Parker et al. 1991). However, the explanation for this is still unclear.

The absence or presence of gene exchange might be

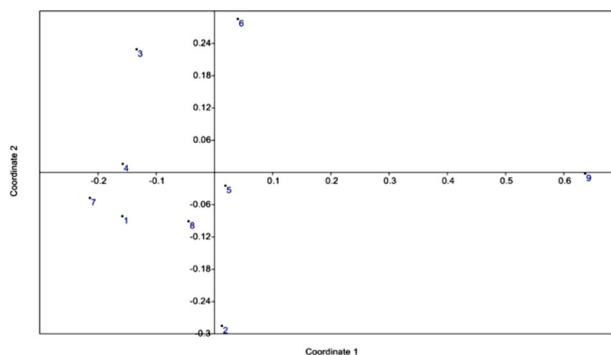


Fig. 9. PCO plot of ISSR data of the studied populations of *L. glaucum*. Numbers 1 to 9 refer to populations given in Table 1.

one of the reasons for the observed variation/similarities between the populations. Based on the obtained reticulogram, the studied populations, with the exception of populations No. 4, 5, 6 and 9, had gene exchange between each other. This resulted in greater genetic similarity, shown by population clusters in analyses. In contrast, because of lack of gene exchange, populations No. 4, 5, 6 and 9 were genetically differentiated from the others. Biabani et al. (2013) showed that genetic structure is an important trait that reflects different events such as gene flow, mating systems in a population and the extent of diversity within populations.

The habitats of the studied populations are mountainous, separating the populations of Sanandaj region from the others. These mountains act as barriers and in some cases, can prevent gene flow between populations. These conditions have led to the separation of populations and genetic differentiation between the studied populations. Gene flow may compensate for the loss of genetic variations between populations. Its magnitude may be influenced by different environmental factors such as the spatial separation of populations (Wright 1943) as well as presence of physical barriers (Keller, Largiadère 2003), or by different characteristics of species as dispersal abilities and breeding system (Loveless, Hamrick 1984).

Genetic diversity is essential for both short-term adaptation to environmental change and long-term impact on species and communities. However the foundation for biodiversity and evolution is genetic variation within species. This intraspecific variation provides material for long-term evolutionary adaptation and short-term adaptation to seasonal and rapid fluctuations in environmental factors (Ramel 1998).

An ecotype is a distinct set of genotypes (or populations) within a species, resulting from adaptation to local environmental conditions, but which are capable of interbreeding with other ecotypes of the same species (Hufford, Mazer 2003). Ecotypes are populations adapted to local conditions, at a variety of spatial and temporal

scales. Creation of an ecotype is very important because this type of variation has adaptive value for each species and increases biodiversity in the ecosystems. Matching ecotypes to local conditions increases restoration success. A practical value of recognizing ecotypic variation in grasses is in identifying the most suitable ecotypes for conservation, restoration, renovation, landscaping, and bioremediation (Gibson 2009).

Based on the above definition, some ecotypes were distinguished within the studied populations. Some of these populations had distinct morphological traits and also nuclear DNA amount, such as populations No. 4 and 6 (Talebi et al. 2015), and others were genetically differentiated, such as population No. 9.

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