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Genetic and morphological diversity in *Stachys lavandulifolia* (Lamiaceae) populations

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Stachys lavandulifolia Vahl. (Lamiaceae) is an important medicinal plant that grows in different parts of Iran and forms many geographical populations. We have no information on its population genetic structure, genetic diversity, and morphological variability in Iran. Therefore, we planned a genetic and morphological investigation in *St. lavandulifolia* geographical populations in Iran. The obtained data are important for conservation and germplasm management of this medicinal plant species. Seventy-four plants were randomly collected from 14 geographical populations and studied for genetic diversity (ISSR molecular markers) and morphological variability. The highest value for gene diversity occurred in populations 1 and 4 (0.133 and 0.129, respectively). The latitude and altitude were positively correlated with gene diversity and genetic polymorphism while longitude was negatively correlated with them. The Mantel test showed correlation between genetic distance and geographical distance. AMOVA revealed a significant genetic difference among populations and showed that 58% of total genetic variation was due to within-population diversity. The STRUCTURE analysis and K-Means clustering identified two gene pools for *St. lavandulifolia*. The consensus tree of both molecular and morphological data identified divergent populations.

Key words: genetic admixture, genetic fragmentation, gene flow, *Stachys*

INTRODUCTION

The plant species that are distributed in various geographical regions face different ecological conditions and variable altitudes and therefore are subjected to different selection pressures and sometimes suffer from population fragmentation. In these situations, plants reveal morphological and genetic divergence among geographical populations (Azizi et al., 2014; Sheidai et al., 2012, 2013, 2014; Minaeifar et al., 2015), while medicinal plants may also reveal variability in their phytochemical compositions and their trichome types (Rezakhanlo, Talebi, 2010; Khadivi-Khub et al., 2014).

Population genetics analyses produce important data on the levels of genetic variation, the partitioning of genetic variability within/between populations, gene flow, inbreeding, self-pollination versus outcrossing, effective population size

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and population bottleneck. The obtained information can help in developing effective management strategies for endangered and/or invasive species (Chen, 2000; Ellis, Burke, 2007).

The family Lamiaceae contains about 200 genera and 3500 species. The woundwort (*Stachys* Vahl.) is one of the largest genera in Lamiaceae with about 300 species. These species grow everywhere in the world with the exception of Australia, New Zealand, and the Arctic regions. The number of species is particularly high in the Mediterranean region, Eastern Europe, Cape Province, and Chile. *Stachys* species grow mainly in the mild regions of the Mediterranean and in southwest Asia (Potoğlu Erkara, Koyuncu, 2007).

Stachys is a genus of shrubs and annual or perennial herbs. The stems vary from 50–300 cm in height with simple, opposite, triangular leaves with serrate margins. In most species, the leaves are softly hairy. The flowers are 1 to 2 cm long, clustered in the axils of the leaves on the upper part of the stem. The corolla is 5-lobed with the top lobe forming a 'hood', varying from white to pink, purple, red or pale yellow (Tutin et al., 1972; Simon, 1992).

Many species of *Stachys* are of medicinal value. For example, *St. officinalis* is used against cough due to its expectorant and mucolytic effect (Vogl et al., 2013). The essential oil of *St. plumose* has antimicrobial activity (Petrovic et al., 2006), and extracts of *St. lavandulifolia* has anxiolytic effects (Rabbani et al., 2003). Many other *Stachys* species have medicinal properties and are used as sage and in popular medicines to treat genital tumors, sclerosis of the spleen, inflammatory tumors, coughs and ulcers. Teas prepared from the whole plant or leaves are used in phytotherapy, possessing sedative, antispasmodic, diuretic and emmenagogue activities (Potoğlu Erkara, Koyuncu, 2007).

St. lavandulifolia Vahl. (Family Lamiaceae) is an important medicinal plant of Iran that is distributed in different regions of the country and forms several local populations. We have no report on genetic variability, population genetic structure and gene flow or population fragmentation of this valuable species in the country. Therefore, we planned a genetic and morphological investigation in *St. lavandulifolia* geographical populations in Iran. The obtained data are important for conservation and germplasm evaluation of this medicinal plant in Iran.

Molecular markers were used to study the species relationship in the genus *Stachys* in Iran (Kharazian et al., 2015), but no reports exists on population genetic structure, gene flow and the degree of genetic variability within these species. Different molecular markers have been used in population genetic studies. We used ISSR (inter-simple sequence repeats) since these markers are reproducible, cheap, and easy to work and are known to be efficient in population genetic diversity studies (Azizi et al., 2014; Sheidai et al., 2012; 2013; 2014; Minaeifar et al., 2015).

MATERIALS AND METHODS

Plant material

Seventy-four plant specimens of *St. lavandulifolia* were randomly collected from 14 geographical populations in Iran (Table 1, Fig. 1). Samples were identified on the bases of descriptions provided in Flora Iranica (Rechinger, 1982) and flora of Iran (Jamzad, 2012). The voucher specimens were deposited in the herbarium of Shahid Beheshti University (HSBU).

Morphological study

The studied morphological characters are presented in Table 2. Since we could not get flowers for population 14, the analysis was done on 13 populations only. In total, twenty nine quantitative morphological characteristics of both vegetative and reproductive organs were examined. From each population 5–6 flowering stem were collected, and for each trait 5–6 replications were measured.

DNA extraction and ISSR assay

Fresh leaves were collected randomly in each of the studied populations and dried in silica gel powder. Genomic DNA was extracted using CTAB with activated charcoal protocol

Table 1. *St. lavandulifolia* populations, their locality and voucher number

Population	Province	Locality	Altitude (m)	Longitude	Latitude	Voucher No.
1	Semnan	Chashm village	2263	53° 15’	35° 53’	HSBU73
2	Zanjan	Zanjan	1942	48° 23’	36° 30’	HSBU74
3	East Azerbaijan	Abbas Abad	1950	46° 49’	38° 53’	HSBU75
4	Kurdistan	Marivan	2900	46° 10’	35° 31’	HSBU76
5	Semnan	Shahroud	1870	55° 03’	36° 42’	HSBU77
6	Mazandaran	Shahrestanak, Chalus Road	2500	51° 21’	35° 58’	HSBU78
7	Mazandaran	Lasem	2623	52° 13’	35° 48’	HSBU79
8	Tehran	Fasham	2170	51° 31’	35° 56’	HSBU80
9	West Azerbaijan	Sero	2200	44° 44’	37° 30’	HSBU81
10	Markazi	Sangak	2335	49° 44’	35° 15’	HSBU82
11	Markazi	Sefidkhani	2400	49° 34’	33° 58’	HSBU83
12	Kohgiluyeh Va Boyer Ahmad	Babakan village	2003	51° 15’	30° 40’	HSBU84
13	Kohgiluyeh Va Boyer Ahmad	Sisakht	2340	51° 29’	30° 51’	HSBU85
14	Qazvin	Gharmak	1967	50° 19’	36° 33’	HSBU86

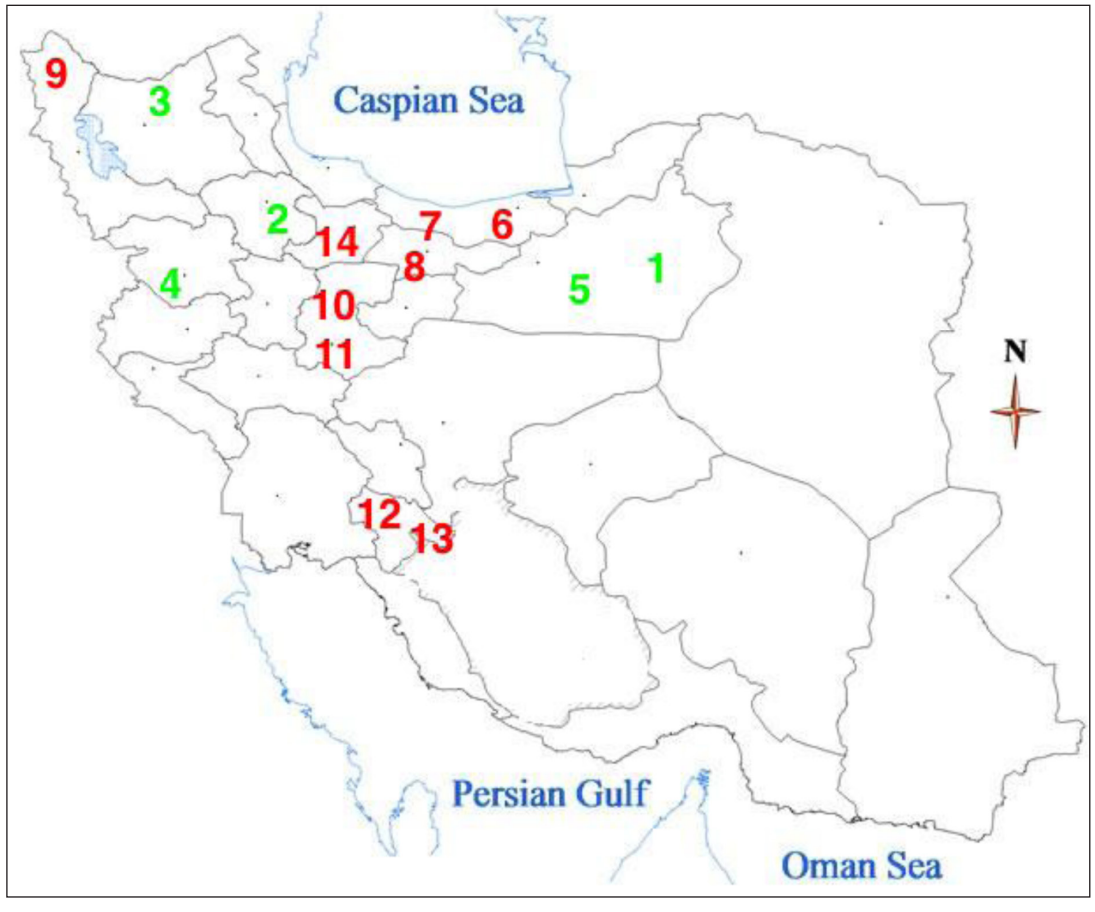

Fig. 1. Distribution map of the studied *St. lavandulifolia* populations (population numbers 1–14 are according to Table 1)

Table 2. Morphological characters studied in *St. lavandulifolia* populations

Pop	Plant height (cm)	Branch no	Basal leaf length (mm)	Basal leaf width (mm)	Basal leaf length/ width ratio	Floral leaf length (mm)	Floral leaf width (mm)	Floral leaf length/ idth ratio	Inflorescence length (cm)
	1	2	3	4	5	6	7	8	9
1	10.3	1.6	15.82	3.7	4.53	13.82	6.17	2.22	3.66
2	15.9	1	18.26	2.4	7.58	12.87	5.6	2.32	8.5
3	15.5	1	27.33	3.16	8.56	18.7	6.2	2.86	7.5
4	7.5	1.66	15.33	2.58	5.93	15.5	4.66	3.5	20.66
5	12.6	1.4	16.82	3.42	5.162	18.06	5.45	3.292	6.75
6	17.7	3.6	18.21	3.34	5.682	17.37	6.12	2.88	6.86
7	14.2	1.8	22.33	4.22	5.57	18.5	6.11	2.954	5.92
8	20.8	1	19.94	3.72	5.534	14.7	5.3	2.806	12.85
9	23.12	2.25	21.9	4.56	4.81	13.07	5.962	2.22	14.38
10	9.1	1.8	20.22	6.2	3.456	14.76	4.71	3.598	5.69
11	14	2	20.2	4.8	4.65	15.47	6.16	2.556	7.4
12	16.7	4.8	21.49	3.97	5.38	12.93	5.29	2.39	7.59
13	15.7	4.8	21.35	4.77	4.466	13.2	5.17	2.526	7.134

Stem/ inflorescence Length ratio	Calyx length (mm)	Calyx width (mm)	Calyx length/width ratio	Calyx teeth length (mm)	Calyx dent width (mm)	Calyx teeth length/ width ratio	Corolla length (mm)
10	11	12	13	14	15	16	17
2.38	17.52	3.76	4.61	11.86	1.20	10.04	14.60
0.87	17.06	3.65	4.76	10.20	1.12	9.58	13.96
1.08	20.53	3.75	5.48	13.66	1.16	12.33	14.06
2.88	16.00	3.25	4.86	10.16	0.80	13.90	8.00
0.92	18.7	3.61	5.26	12.82	1.17	11.01	15.46
1.38	22.74	3.74	6.06	16.12	1.21	13.58	16.10
0.88	19.48	4.26	4.62	12.30	1.05	12.32	16.62
0.71	23.32	4.26	5.46	14.40	1.20	12.62	17.64
0.90	20.95	3.362	6.24	13.12	1.00	13.00	14.77
0.78	17.82	3.7	4.89	11.90	1.15	10.54	13.72
4.35	20.46	4.45	4.58	15.26	1.05	14.64	17.28
0.93	19.27	3.17	6.06	13.36	1.34	9.94	13.46
1.04	18.20	4.66	3.89	11.75	1.25	9.58	17.66

Corolla width (mm)	Corolla length/ width ratio	Maximum upper lobe width (mm)	Min upper lope width (mm)	Lower-right lope width (mm)	Lower-left lope width (mm)	Stamen length large (mm)	Short stamen length (mm)	Style length (mm)	Calyx/ corolla length ratio	Pedicle length (mm)	Floral no per cycle
18	19	20	21	22	23	24	25	26	27	28	29
3.40	4.46	6.80	3.80	2.56	2.51	2.9	3.7	10.9	1.356	1	6
2.96	4.86	5.20	3.68	2.15	2.15	2.45	3.35	9.55	1.23	1.94	6
3.16	4.36	5.50	3.91	2.58	2.41	2.25	2.91	9.66	1.48	1.5	6
2.75	2.86	3.50	2.41	1.66	1.66	1.83	2.41	5.58	2.1	1.11	5.66
2.85	5.48	6.92	4.60	2.65	2.65	3.3	4.3	8.96	1.19	1.2	5.8
3.07	5.35	7.10	4.75	2.95	2.95	3.46	4.38	11.05	1.38	1.48	6
3.30	5.00	7.62	4.80	2.97	2.97	3.9	4.8	12	1.16	1.51	6
3.12	5.76	6.41	4.15	2.75	2.65	4.17	3.97	12.82	1.302	2.3	6
2.85	5.18	6.25	4.56	2.81	2.81	2.68	3.66	12.35	1.41	1.43	6
2.85	4.63	7.15	4.15	2.75	2.75	2.45	3.25	10.6	2.126	1.55	6
3.25	5.30	7.10	5.10	3.27	3.27	3.7	4	11.26	1.17	1.55	6
2.75	4.88	5.70	4.07	2.60	2.6	2.07	2.65	10.15	1.42	1.36	6
3.07	5.89	8.98	5.99	3.70	3.7	3.8	3.4	11.16	1	1.26	6

(Sheidai et al., 2013). The quality of extracted DNA was examined by running on 0.8% agarose gel. Ten ISSR primers: $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9C$, UBC807, UBC811, $(GA)_9A$, 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).

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 the 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 fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

DATA ANALYSIS

Morphological analysis

Morphological characters were coded accordingly. Euclidean and Gower distances were used for multivariate statistical analyses (Podani, 2000). The canonical correspondence analysis (CVA) was used to reveal morphological differences in the studied populations. The principal components analysis (PCA) was used to identify the most variable morphological characters. The coefficient of correlation was determined to show correlation between environmental features (altitude, longitude, and latitude) with morphological characters.

Genetic diversity and population structure

The obtained ISSR bands were coded as binary characters (presence = 1, absence = 0). The genetic diversity parameters like allele diversity (Weising et al., 2005), Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Freeland et al., 2011) were determined for each population.

Nei's genetic distance was used for clustering (Weising et al., 2005; Freeland et al., 2011). Neighbour Joining (NJ) clustering and Neighbour-Net method of networking were used for grouping after 100 times bootstrapping/permutations (Freeland et al., 2011). The Mantel test was performed to check correlation between geographical and genetic distances of the studied populations (Podani, 2000). PAST ver. 2.17 (Hamer et al., 2012) and DARwin ver. 5 (2012) programs were used for these analyses. The Pearson coefficient of correlation was determined between geographical features (altitude, longitude, and latitude) and genetic diversity parameters.

AMOVA (analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall, Smouse, 2006) and Nei's Gst analysis of GenoDive ver. 2 (2013) (Meirmans, Van Tienderen, 2004) were used to reveal significant genetic differences among the studied populations (Sheidai et al., 2014).

The population genetic differentiation was studied by G'st_est = standardized measure of genetic differentiation (Hedrick, 2005) and D_est = Jost measure of differentiation (Jost, 2008). 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) (Tero et al., 2003).

Bayesian model-based STRUCTURE analysis (Pritchard et al., 2000) and maximum likelihood-based method of K-Means clustering were used to study the genetic structure of populations (Sheidai et al., 2014). For STRUCTURE analysis, data were scored as dominant markers (Falush et al., 2007). The Evanno test (Evanno et al., 2005) and K-Means clustering were used to identify optimum k genetic groups (Sheidai et al., 2014). Two summary statistics: (1) pseudo-F and (2) Bayesian Information Criterion (BIC), provide the best fit for *k* (Meirmans, 2012).

Gene flow

Gene flow was determined by different approaches: (1) calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 – Gst)/Gst. This approach considers equal amount of gene flow among all populations; (2) reticulation analysis that is based on the least square method; and (3) population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2 (2013).

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 (longitude, latitude, and altitude) of the studied populations. The analysis was done by LFMM program ver. 1.2 (2013).

RESULTS

Genetic diversity

The determined parameters of genetic diversity in the studied populations are presented in Table 3. The highest value for gene diversity occurred in populations 1 and 4 (0.133 and 0.129, respectively). The highest level of genetic polymorphism (15.79) occurred in population 2, while the lowest value of the same occurred in population 4 (35.62).

The latitude was positively correlated with Nei gene diversity and genetic polymorphism ($r = 0.5.31$, $P = 0.05$) and ($r = 0.59$, $P = 0.05$), respectively. Similarly, the altitude showed weak positive correlation with genetic polymorphism ($r = 0.10$, $P > 0.05$) and gene diversity ($r = 0.12$, $P > 0.05$). However, the longitude was negatively correlated with gene diversity as well as genetic polymorphism ($r = –0.39$, $P > 0.05$) and ($r = –0.41$, $P > 0.05$), respectively. These results revealed a complex interaction between geographical features and genetic diversity in *St. lavandulifolia* geographical populations. The Mantel test produced significant correlation ($r = 0.21$, $P = 0.01$) between geographical distance and genetic distance of the studied populations. Therefore, isolation by distance occurred in *St. lavandulifolia* populations.

Table 3. Genetic diversity parameters in the studied populations of *St. lavandulifolia* (population numbers are according to Table 1)

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	5.000	0.440	1.090	0.086	0.056	0.062	17.33
Pop2	4.000	0.280	1.055	0.051	0.034	0.039	9.33
Pop3	4.000	0.387	1.098	0.090	0.060	0.068	17.33
Pop4	5.000	0.453	1.087	0.086	0.055	0.061	18.67
Pop5	7.000	0.853	1.184	0.184	0.116	0.125	42.67
Pop6	7.000	0.667	1.181	0.164	0.108	0.116	33.33
Pop7	7.000	0.747	1.193	0.176	0.115	0.124	37.33
Pop8	7.000	0.587	1.157	0.138	0.091	0.098	28.00
Pop9	3.000	0.320	1.105	0.086	0.059	0.071	14.67
Pop10	5.000	0.453	1.119	0.107	0.071	0.079	20.00
Pop11	5.000	0.547	1.189	0.154	0.105	0.117	26.67
Pop12	5.000	0.613	1.145	0.141	0.092	0.102	16.00
Pop13	5.000	0.373	1.097	0.083	0.056	0.062	28.00
Pop14	4.000	0.333	1.108	0.084	0.059	0.067	13.33

N – number of populations, 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.

Population genetic structure

AMOVA produced a significant genetic difference (PhiPT = 0.42, $P = 0.001$) among the studied populations. It also revealed that 58% of total genetic variation was due to diversity within population and 42% was due to genetic differentiation among populations. Pairwise AMOVA produced a significant difference among these populations. The Hickory test also produced high Theta B value (0.50) supporting AMOVA.

Gst (0.42, $P = 0.001$), Hedrick' standardized fixation index (G'st = 0.47, $P = 0.001$), and Jost' differentiation index (D-est = 0.10, $P = 0.001$) revealed that the studied populations were genetically differentiated.

The Neighbour-Net diagram is presented in Fig. 2. It produced four major clusters. Most of the plants in each population were placed close to each other, but some were placed intermixed with other populations.

Plants of populations 1–3 formed the first cluster, while plants of populations 4 and 5 comprised the second cluster. Plants of populations 6 and 7 were placed in the third cluster, and plants of 8–14 populations formed the forth cluster. The side bars formed between branches in the Neighbour-Net diagram indicate genetic

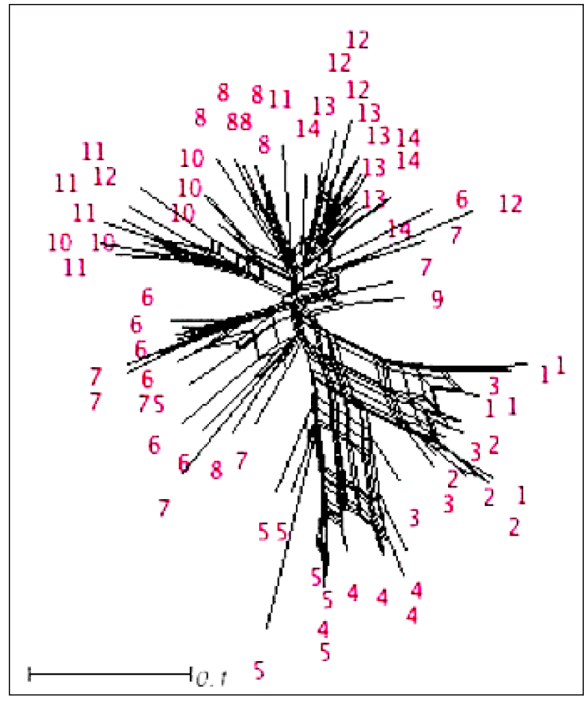

Fig. 2. Neighbour-Net diagram of the studied *St. lavandulifolia* populations (population numbers are according to Table 1)

differences of the studied individuals. Therefore, the plants of populations 1–3, 4, and 5 differed genetically to some extent.

The NJ tree based on the mean genetic distance of the studied populations produced a better picture on population genetic affinity (Fig. 3). The population 14 and populations 1–3 were placed far from other populations and formed separate clusters. The populations 5–14 formed the third major cluster. A higher genetic similarity was observed between populations 5–7, then between 9–11, and finally between populations 12–14. The population 8 had a position between 5–7 and 9–11.

The Evanno test performed on STRUCTURE analysis and pseudo-F index of K-Means clustering produced the optimum number of $k = 2$. These results indicated that we had 2 genetic groups in the studied populations. The STRUCTURE plot (Fig. 4) based on $k = 2$ identified these two genetic groups (gene pools).

The obtained STRUCTURE plot based on admixture model and Bayesian approach revealed that populations 1–5 were genetically

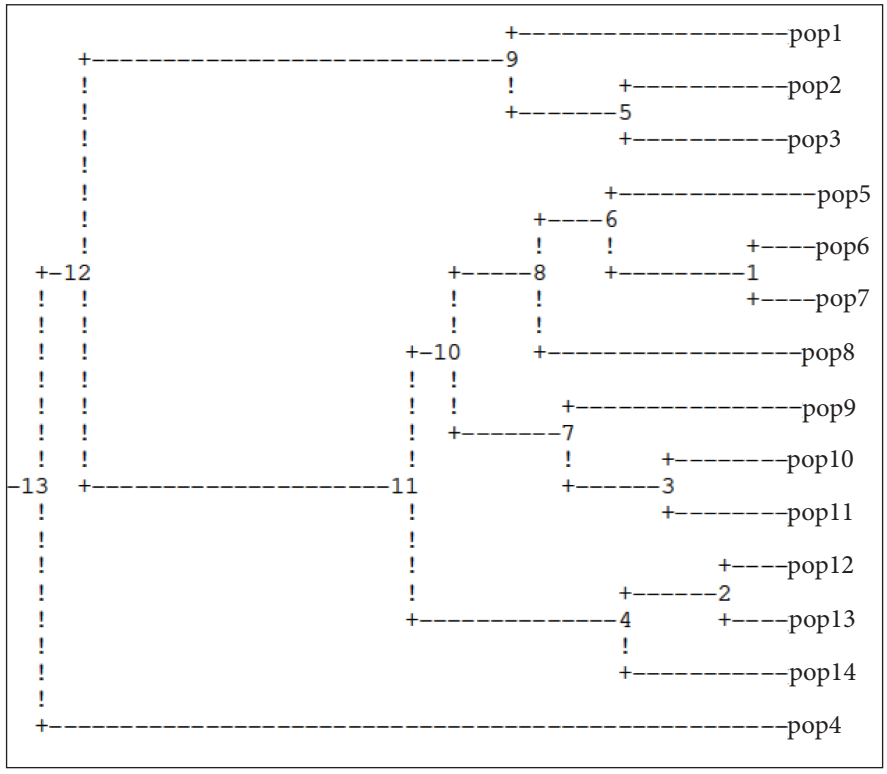

Fig. 3. NJ tree of the studied *St. lavandulifolia* populations (population numbers 1–14 are according to Table 1)

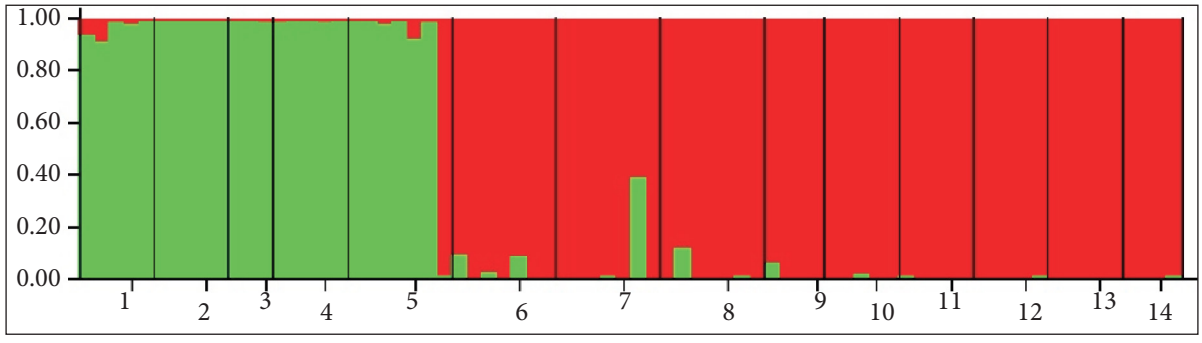

Fig. 4. STRUCTURE plot of *St. lavandulifolia* populations (population numbers are according to Table 1)

more alike and comprised the first gene pool, while populations 6–14 formed the second gene pool. This plot also revealed a low degree of genetic admixture between the two gene pools and indicated strong genetic differentiation between populations.

Gene flow

We used different indirect approaches to study the level of gene flow or ancestral shared alleles in *St. lavandulifolia* populations. These approaches were based on different assumptions and mathematical models. The Nm approach assumes equal rate of gene flow among populations that might not be true always. The NM analysis produced the mean Nm = 0.47 which indicates a low degree of gene flow among populations. This result was in agreement with the STRUCTURE result presented before. Out of 75 ISSR loci studied, few had a high Nm value (1.00–6.00) (for example, ISSR loci 1, 5, 6, 13, 14, 21–26, 29, and 30). These loci were exchanged more frequently among the studied populations, while other loci were private loci and were confined to one or more local populations.

The reticulation method of networking is based on the least square method and identified similar/shared alleles between populations. The obtained reticulogram (Fig. 5) revealed some degree of gene flow or ancestral shared alleles between populations 1–5, 4 and 12, 1 and 10. This approach mostly identified gene flow between the populations of the first gene pool recognized by STRUCTURE plot (populations 1–5). It also showed a limited gene flow between some of the populations in the two gene pools (population 1 and 10, 4 and 12).

More detailed information was obtained by the population assignment test (Table 4). In this approach, the assignment of the current plant to the inferred population is determined by the maximum likelihood method (Meirmans, Van Tienderen, 2004).

The assignment test revealed that some plants in population 1 were closer to population 2, and vice versa. The same holds true for plants of populations 10 and 11, as well as 12 and 13. Therefore, the population assignment test supported the reticulograms (occurrence of gene flow among populations of the first gene pool) and also revealed some degree of gene flow among populations of the second gene pool.

The absence of gene flow between populations 3 and 6 that are located in east and west Azerbaijan is interesting. Though they are growing in two neighbouring provinces, they belong to two different gene pools. They are growing at 400 km distance from each other in the mountainous regions. Several mountains and also Lake Uremia separate these two populations and may act as a barrier against gene flow between them.

Mountains also separate population 2 (Zanjan) from population 14 (Qazvin) that grow in the neighbouring provinces. The populations 1 and 5 of the Semnan province are separated from populations 6–8 by the same barriers. Populations 1 and 5 are surrounded by Zagros Mountain reaching up to 3000 m in height.

LFMM analysis revealed that out of 75 ISSR loci, 14 were adaptive (Table 5). Some of these ISSR loci had a low Nm value (<1) and were private alleles, for example, ISSR loci 47, 57, 58, 68, and 69. Other loci had a high Nm value (>1.0) and were more frequently shared by the studied populations.

Morphological variability

The CVA plot of morphological characters separated *St. lavandulifolia* populations from each other indicating their morphological difference (Fig. 6). PCA analysis revealed that the first three components comprised about 75% of total variation. It revealed that characters 9, 22, 10, and 27 separated population 4 from others. Characters 1, 4, and 23 separated populations 12 and 13, characters 5 and 6 separated populations 2, 3, 8.

The correlation coefficient determined for morphological characters and environmental features revealed that altitude was positively correlated with the length of inflorescence (r = 0.26, P = 0.05) and negatively correlated with the length/width of calyx (r = –0.30,

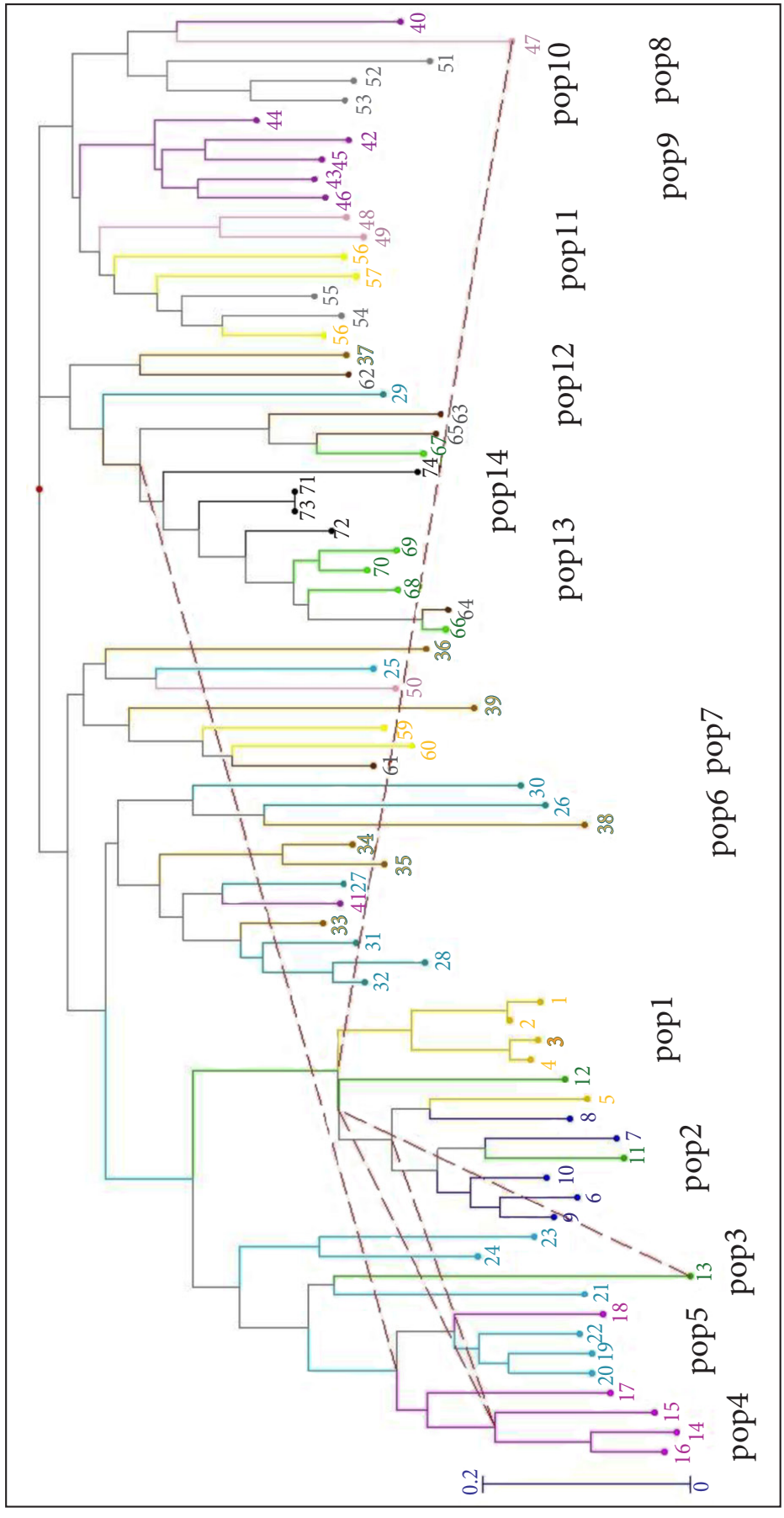

Fig. 5. Reticulogram of the studied *St. lavandulifolia* populations (dashed lines = gene flow). Population numbers are according to Table 1

Table 4. Population assignment test results showing plants inferred to be from other populations

Individual	Current	Inferred	Lik_max	Lik_home	Lik_ratio
5	Pop001	Pop005	–41.52	–41.846	0.651
6	Pop002	Pop001	–23.554	–	–
7	Pop002	Pop001	–27.461	–	–
8	Pop002	Pop001	–20.782	–	–
9	Pop002	Pop001	–16.875	–	–
10	Pop003	Pop001	–14.102	–	–
11	Pop003	Pop001	–15.489	–	–
12	Pop003	Pop001	–24.689	–	–
13	Pop003	Pop005	–29.607	–	–
17	Pop004	Pop005	–12.81	–14.144	2.667
18	Pop004	Pop005	–11.553	–24.165	25.224
25	Pop005	Pop006	–20.302	–60.71	80.815
26	Pop006	Pop005	–29.471	–35.093	11.244
29	Pop006	Pop007	–35.061	–47.116	24.11
33	Pop007	Pop006	–11.725	–18.972	14.493
39	Pop007	Pop006	–21.3	–32.787	22.974
40	Pop008	Pop007	–19.275	–47.465	56.379
48	Pop009	Pop011	–23.983	–	–
49	Pop009	Pop010	–23.298	–	–
50	Pop009	Pop007	–21.52	–	–
54	Pop010	Pop011	–15.511	–19.92	8.816
55	Pop010	Pop011	–22.021	–22.851	1.659
56	Pop011	Pop010	–29.32	–37.91	17.18
60	Pop011	Pop010	–28.591	–31.513	5.844
61	Pop012	Pop011	–30.257	–32.566	4.617
64	Pop012	Pop013	–8.539	–13.375	9.672
65	Pop012	Pop013	–23.86	–24.782	1.846
66	Pop013	Pop012	–10.201	–13.173	5.945
67	Pop013	Pop012	–12.398	–26.819	28.842

Table 5. Manhatan table of LFMM analysis showing adaptive ISSR loci

Name	Zscore	-log10 (p-value)	p-value
ISSR1	10.8072	26.4975	3.1807E-27
ISSR30	3.51392	3.35502	0.000441547
ISSR32	6.47345	10.0187	9.57931E-11
ISSR33	2.42011	1.80923	0.0155158
ISSR36	5.29885	6.93355	1.16533E-07
ISSR47	2.34904	1.72534	0.0188217
ISSR48	2.58746	2.01464	0.00966863
ISSR57	5.12813	6.53368	2.9263E-07
ISSR58	2.41069	1.79799	0.0159223
ISSR59	4.64536	5.46917	3.39491E-06
ISSR63	4.8781	5.97016	1.07113E-06
ISSR66	2.73189	2.20084	0.00629731
ISSR68	2.7494	2.22399	0.00597045
ISSR69	2.19948	1.55527	0.0278437

$P < 0.05$). The longitude was positively orrelated with the ratio of length/width of the basal leaf ($r = 0.28$, $P < 0.05$) and the length of inflorescence leaf ($r = 0.33$, $P = 0.01$). The latitude was negatively correlated with the length of inflorescence ($r = –0.4811$, $P < 0.01$), but was positively correlated with the size of corolla ($r = 0.378$, $P < 0.01$).

The consensus tree of both molecular and morphological data is presented in Fig. 7. The plants of populations 1, 2, and 4 formed separate clusters. They differed from the other studied populations in both genetic and morphological features. Some plants of populations 6 and 10 also differed from the other populations and were placed in separate clusters. A detailed study revealed that population 4 had the highest value of characters 9 and 27 and

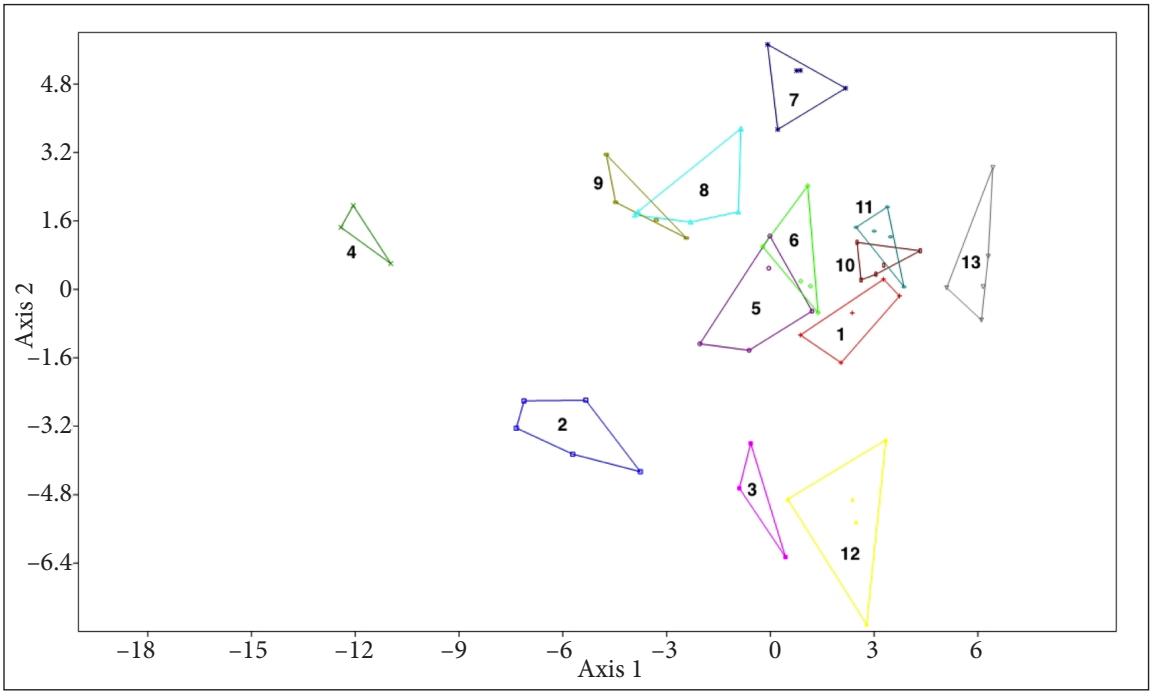

Fig. 6. CVA plot of morphological characters in *St. lavandulifolia* populations (population numbers are according to Table 1)

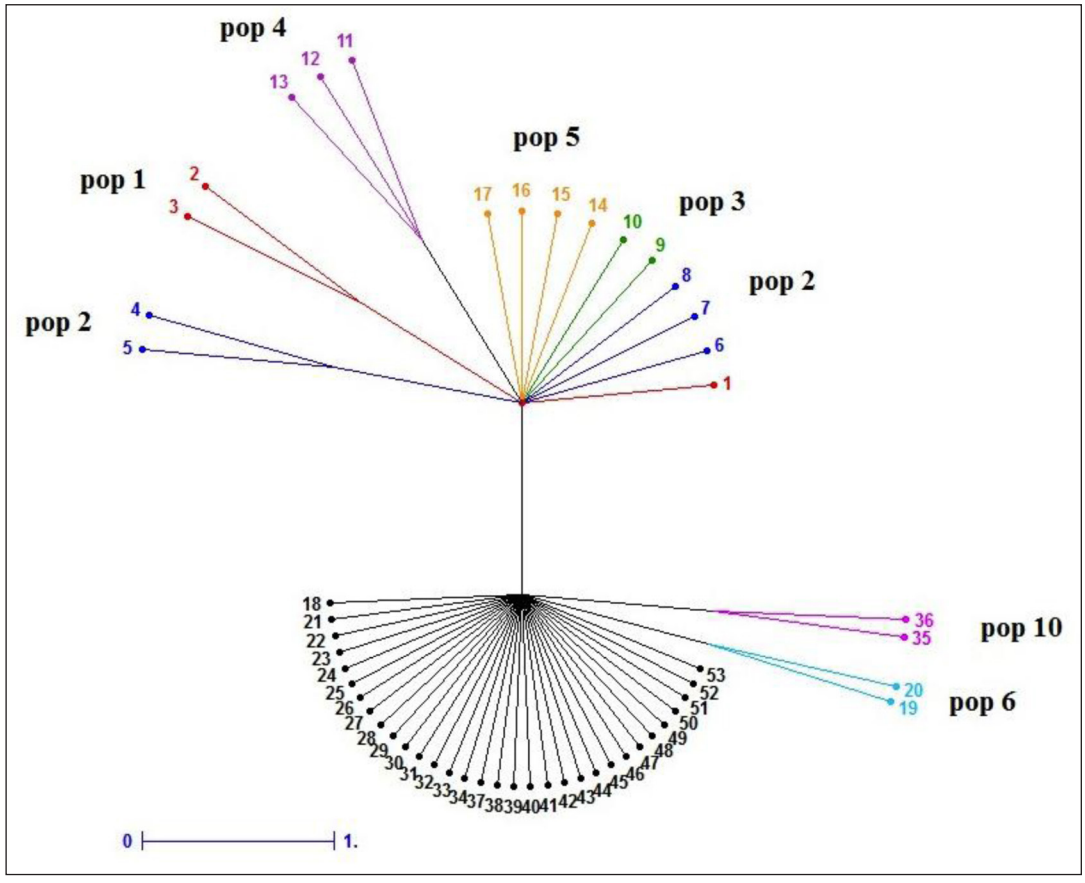

Fig. 7. Consensus tree of morphological and molecular data in *St. lavandulifolia* populations

the lowest value for character 28, compared to the other populations. Similarly, population 3 had the highest value for characters 5, 6.

DISCUSSION

St. lavandulifolia, like other medicinal plants, is consumed by locals and therefore is subjected to elimination from the natural habitat. Therefore, it is important to monitor the size of its natural populations and study their level of genetic variability as well as population genetic fragmentation. The disappearance and fragmentation of natural populations in general reduce the rate of gene flow and increase the genetic differentiation of populations. In such case, the genetic drift becomes active and reduces the within-population genetic variability (Setsuko et al., 2007; Hou, Lou, 2011).

The result of AMOVA and used genetic differentiation parameters revealed that *St. lavandulifolia* populations were genetically differentiated but contained some good level of within-population genetic variability (58% of their total genetic variation). This should be related to the outcrossing nature of this species. High genetic diversity is an essential factor in continuity of the plant species and adaptation to fluctuating environmental conditions (Çalişkan, 2012).

The moderate within-population genetic diversity observed may be due to the ongoing gene flow, too. Gene flow via introducing new genes into the local populations increases their genetic variability (Hou, Lou, 2011; Sheidai et al., 2014). The present study revealed some degrees of gene flow among *St. lavandulifolia* populations by reticulation and population assignment tests.

Studying the patterns of molecular genetic variation were useful in identifying and prioritizing the remaining within-species biodiversity for conservation actions and provided insight about the potential for adaptive differences to have developed between plants from different regions (Waples, 1998). The Mantel test revealed a pattern of isolation-by distance across the distribution range of the studied *St. lavandulifolia* populations. It suggested that the dispersal of these populations might be constrained by distance, and gene flow is most likely to occur between neighbouring populations. As a result, more closely situated populations tend to be more genetically similar to one another (Slatkin, 1993; Hutchison, Templeton, 1999; Medrano, Herrera, 2008). However, as we presented before, populations 3 and 9 (located in east and west Azerbaijan, respectively) that were growing in the neighbouring provinces differed in their genetic structure and were placed in two different gene pools. The local environmental and ecological features, such as local mountains and Lake Uremia, acted as a block against gene flow between these populations.

St. lavandulifolia populations showed a general trend of positive association between genetic variability with altitude and latitude, while genetic variability was negatively related to longitude. Therefore, a complex interaction existed between genetic diversity and environmental features in *St. lavandulifolia*.

Several studies suggested altitude as an important factor for population genetic differentiation (Ohsawa, Ide, 2008; Noormohammadi et al., 2015). Populations not only are differentiated on mountains along vertical axes, but genetic changes can also occur along horizontal axes. For instance, ridges may provide geographical barriers to gene flow between populations on their opposite sides, so genetic differentiation may occur across ridges (Taberlet et al., 1998). However, some studies also reported the absence of differentiation between populations at low and high altitudes (Ohsawa et al., 2007) due to the overlap of flowering phenology in populations at different altitudes, species' extensive pollen flow, and long-distance seed dispersal between different altitudes by animals, particularly birds.

Identification of gene pools is essential for planning pant conservation and hybridization programs. STRUCTURE analysis and K-Means clustering identified two gene pools in *St. lavandulifolia* in Iran. The balance between

drift and gene flow is the primary determinant of what fraction of a species' genetic variability is available in local gene pools, but the local system of mating then takes the gene pool variation available at the gametic level and transforms it into genotypic variation at the individual level (Templeton, 2006).

The studied populations differed morphologically from each other, and the consensus tree separated a few populations that differed from the others in their genetic and morphological features. Variations in genetic, cytogenetic, morphological, trichomes, and essential oil composition of *Stachys* species were reported in different geographical populations (Rezakhanlo, Talebi, 2010; Zakaria, Zare, 2013; Khadivi-Khub et al., 2014). In conclusion, the present study indicates that genetic and morphological divergence, limited gene flow, and local adaptation have played role in diversification of *St. lavandulifolia* populations in Iran. These findings may be of use in conservation of this medicinal plant in the country.

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Stachys lavandulifolia (LAMIACEAE) POPULIACIJOS GENETINĖ IR MORFOLOGINĖ ĮVAIROVĖ

Santrauka

Stachys lavandulifolia Vahl. (Lamiaceae) yra svarbus vaistinis augalas, augantis įvairiose Irano vietovėse ir formuojantis daugelį geografinių populiacijų. Mes ištyrėme šį augalą, kadangi Irane nėra informacijos apie jo genetinę struktūrą, genetinę įvairovę ir morfologinį kintamumą. Gauti duomenys yra svarbūs šio vaistinio augalo išsaugojimui ir genofondui. Genetinės (ISSR molekuliniai žymenys) ir morfologinės įvairovės tyrimui buvo surinkti 74 augalai iš 14 geografinių populiacijų. Didžiausia genų įvairovė aptikta 1 ir 4 populiacijose (0,133 ir 0,129 atitinkamai). Geografinė platuma ir aukštuma teigiamai koreliuoja su genų įvairove ir genetiniu polimorfizmu, o ilguma – neigiamai. Mantel testas atskleidė koreliaciją tarp genetinio ir geografinio atstumo. Remiantis AMOVA analize, nustatytas reikšmingas genetinis skirtumas tarp populiacijų ir tai, kad 58 % viso genetinio kintamumo lėmė vidinė populiacijos įvairovė. Struktūrinė ir klasterinė analizės leido išskirti du *St. lavandulifolia* genų fondus. Molekulinių ir morfologinių duomenų medis rodo išsiskiriančias populiacijas.

Raktažodžiai: genetinė priemaiša, genetinė fragmentacija, genų srautas, *Stachys lavandulifolia*