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Species delimitation and genetic diversity analysis in *Salvia* with the use of ISSR molecular markers

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Abstract – Thirty-nine plant specimens of six *Salvia* species were collected from different localities of the Alborz mountain region in Iran and studied for morphological and genetic variability and species relationship. Inter simple sequence repeats (ISSR) molecular markers showed a high degree of within-species and interspecific genetic variability in *Salvia*. Analysis of molecular variance and Hickory tests showed significant molecular difference among the studied populations. A principal coordinate analysis plot of morphological characters grouped the species into two distinct groups, supporting their taxonomic treatment. This was partly supported by ISSR networking. The Mantel test did not show a correlation between genetic distance and the geographical distance of the studied populations. STRUCTURE and reticulation analyses revealed some degree of gene flow among the species. The present study showed that ISSR molecular markers could be used in *Salvia* species delimitation along with morphological study.

Key words: ISSR, network, population structure, *Salvia*

Introduction

The genus *Salvia* L. is the largest genus of plants in the mint family Lamiaceae, which contains about 900 species distributed throughout the Old and New World growing in temperate and subtropical areas (Standley and Williams 1973, Özdemir and Senel 1999). Within the Lamiaceae, *Salvia* is member of the tribe Mentheae within the subfamily Nepetoideae. It is one of several genera commonly referred to as sage. Western Asia and Mediterranean regions have been considered as the original centers of the distribution of *Salvia* (Wu and Li 1982). *Salvia* has undergone marked species radiations in three regions of the world: Central and South America (500 spp.), central Asia/Mediterranean (250 spp.), and eastern Asia (90 spp.) (Walker et al. 2004).

Salvia species are herbaceous, suffruticose or shrubby perennials, rarely biennial or annual, often strongly aromatic. These species are of horticultural, commercial and medicinal value. They contain monoterpene with antiseptic characteristics (Özdemir and Senel 1999) and the compounds obtained from these species decrease DNA synthesis in the cell, an important feature in the diagnosis and treatment of cancer. Many species of the Lamiaceae are aromatic and are often used as herbs, spices, folk medicines and fragrances. In addition, *Salvia* species are grown in parks and gardens as ornamental plants (Özdemir and Senel 1999). For example, *S. nemorosa* L., which is also included in the present study, is a popular herbaceous perennial species with both seed and vegetative propagated cultivars. *S. spinosa* L., another taxon discussed in the present work, contains essential oil in its aerial parts with antibacterial properties (Salehi-Sormaghi et al. 2006).

There have been about 70 *Salvia* species reported from Flora Iranica (Hedge 1982) with 40% endemism. These species are distributed in subtropical, temperate, sub-arctic and arctic areas as well as in the tropical regions of Iran (Hedge 1982, Sheidai et al. 2010). Some of these species are very distinct while others show close affinity to each other, and some of the species are in a state of evolutionary flux (Hedge 1982). Interspecific hybridization is suspected to be operative in this genus leading to a great morphological diversity (Hedge 1982). The occurrences of both naturally formed interspecific hybrids as well as artificial crosses have been reported in *Salvia*. Successful crosses usually occur between closely related species. These hybrids usually have intermediate morphological features (Tychonievich and Warner 2011, Radosavljević et al. 2012).

Different molecular markers have been used in different investigations related to *Salvia* taxa: DNA barcoding meth-

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od (rpoB, rbcL, matK and trnH-psbA) (De Mattia et al. 2011), combination of chloroplast and nuclear ribosomal DNA sequences and allozyme (Sudarmono and Okada 2008), random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers (Wang et al. 2011, Sepehry-Javan et al. 2012), chloroplast DNA regions of *rbcL* and *trnL-F* (Walker et al. 2004), nrDNA ITS sequences (Zhan et al. 2012), PCR-RFLP (Karaca et al. 2008) and simple sequence repeats (SSR) markers (Radosavljević et al. 2011, 2012).

The present study was performed to investigate genetic diversity and morphological variation in six *Salvia* species with the aim of producing data regarding their inter- and intra-population genetic structure and possible interspecies gene flow. Such information will be important in the conservation of these medicinal plants and also in the provision of information about the evolution of the genus.

We used ISSR molecular markers for genetic diversity analysis and for studying the species relationship, as these molecular markers were reported to be suitable for such investigations (Wang et al. 2011, Sepehry-Javan et al. 2012).

Material and methods

Plant material

Thirty-nine accessions of six *Salvia* species were collected from natural habitats in Iran (Tab. 1). Sampling was done in the Central Alborz region during 2013. *Salvia* species studied are: *S. hypoleuca* Benth., *S. limbata* C. A. Mey., *S. nemorosa* L., *S. xanthocheiala* Boiss. ex. Benth. (from the species group 1), and *S. spinosa* L., *S. reuterana* Boiss. (from the species group 3). The voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU) (Tab. 1).

Morphological studies

Morphological characters studied are: pedicel length, calyx length, stem leaf length, stem leaf width, bract length, filament length, anther length, corolla length, nut length, nut width, basal leaf length, basal leaf width, corolla color, corolla shape, bract shape, seed color, seed shape, bract color, corolla latex, leaf surface, calyx shape, and basal leaf shape.

DNA extraction and ISSR assay

Fresh leaves were collected randomly from plant specimens and dried in silica gel powder. Genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) activated charcoal protocol (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 $(GT)_7CA$ custom synthesized 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 $MgCl_2$, 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 amplification reactions were performed in a Techne thermocycler (Germany) with the following program: a 5 min initial denaturation step at 94 °C, 30 s at 94 °C; 1 min at 50 °C and 1 min at 72 °C. The reaction was completed with a 7 min extension step 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 analyses

Principal coordinate analysis (PCoA) was performed to group the plant specimens according to morphological characters and a principal components analysis (PCA biplot) was used to identify the most variable morphological characters among the studied species (Podani 2000). Morphological data were standardized (mean = 0, variance = 1) for these analyses (Podani 2000).

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). Genetic diversity parameters were determined in each population. These parameters were Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Weising 2005, Freeland et al. 2011). Nei's genetic distance was determined among the studied populations and used for clustering (Freeland et al. 2011, Weising 2005). For grouping of the plant specimens, the neighbor joining (NJ) clustering method and the neighbor-net method of networking were performed after bootstrapping 100 times (Huson and Bryant 2006, Freeland et al. 2011).

The Mantel test was performed to check correlation between the geographical distance and the genetic distance of the studied species (Podani 2000). PAST ver. 2.17 (Hamer et al. 2001), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) programs were used for these analyses. Significant genetic differences among the studied populations and provinces were determined by: 1 – Analysis of molecular variance (AMOVA) test (with 1000 permutations) with the use of GenAlex 6.4 (Peakall and Smouse 2006), and 2 – Nei's Gst analysis of GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004). Furthermore, populations' 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 et al. 2003), was used to estimate parameters related to genetic structure (theta B value). Three runs were conducted with default sampling parameters (burn-in = 50,000, sample= 250,000, thin = 50) to ensure consistency of results (Tero et al. 2003).

The genetic structure of geographical populations and provinces was studied with two methods. First we carried out a Bayesian based model STRUCTURE analysis (Pritchard et al. 2000). For this analysis, data were scored as dominant markers (Falush et al. 2007). An Evanno test

Tab 1. *Salvia* species studied, their locality and voucher number.

Species	Locality	Latitude	Longitude	Height	Voucher Number
S. hypoleuca Benth.	Porkan, Chalous	51.04.03	36.56.05	1613	HSBU 2012131
S. hypoleuca	Polur1, Mazandaran	52.25.12	35.50.43	2273	HSBU 2012132
S. hypoleuca	Polur2, Mazandaran	52.25.12	35.50.43	2273	HSBU 2012133
S. hypoleuca	Chanar gharb, Firoozkooh	52.85.12	35.39.35	1572	HSBU 2012134
S. hypoleuca	Dizin1, Tehran	51.25.19	36.00.58	3476	HSBU 2012135
S. hypoleuca	Dizin2, Tehran	51.25.19	36.00.58	3476	HSBU 2012136
S. hypoleuca	Jagrod1, Alborz	51.47.09	35.35.32	1235	HSBU 2012137
S. hypoleuca	Jagrod2, Alborz	51.47.09	35.35.32	1235	HSBU 2012138
S. nemorosa L.	Polur, Mazandaran	52.25.12	35.50.43	2273	HSBU 2012139
S. nemorosa	Damavand1, Alborz	52.32.50	35.50.46	2221	HSBU 2012140
S. nemorosa	Damavand2, Alborz	52.32.50	35.50.46	2221	HSBU 2012141
S. nemorosa	Khojir1, Firoozkooh	51.43.19	35.39.71	1300	HSBU 2012142
S. nemorosa	Khojir2, Firoozkooh	51.43.19	35.39.71	1300	HSBU 2012143
S. nemorosa	Dizin1,Tehran	51.25.19	36.00.58	3476	HSBU 2012144
S. nemorosa	Dizin2,Tehran	51.25.19	36.00.58	3476	HSBU 2012145
S. nemorosa	Chanargharb1, Firoozkooh	52.85.12	35.39.35	1572	HSBU 2012146
S. limbata C.A.Mey	Chanargharb2, Firoozkooh	52.85.12	35.39.35	1572	HSBU 2012147
S. limbata	Khojir1, Firoozkooh	51.43.19	35.39.71	1300	HSBU 2012148
S. limbata	Khojir2, Firoozkooh	51.43.19.	35.39.71	1300	HSBU 2012149
S. limbata	Tochal1,Tehran	51.24.02	35.49.14	1857	HSBU 2012150
S. limbata	Tochal2,Tehran	51.24.02	35.49.14	1857	HSBU 2012151
S. limbata	Gulahak, Firoozkooh	52.07.35	35.39.31	2022	HSBU 2012152
S. xanthocheila Boiss.	Laar1, Alborz	53.03.22	35.51.19	2163	HSBU 2012153
S. xanthocheila	Laar2, Alborz	53.03.22	35.51.19	2163	HSBU 2012154
S. xanthocheila	Dizin1,Tehran	51.25.19	36.00.58	3476	HSBU 2012155
S. xanthocheila	Dizin2,Tehran	51.25.19	36.00.58	3476	HSBU 2012156
S. xanthocheila	Emamzadeh Hashem, Mazandaran	52.20.21	35.46.48	2699	HSBU 2012157
S. spinosa L.	Khojir1, Firoozkooh	51.43.19	35.39.71	1300	HSBU 2012158
S. spinosa	Khojir2, Firoozkooh	51.43.19	35.39.71	1300	HSBU 2012159
S. spinosa	Chitgar1, Tehran	51.12.29	35.46.47	1285	HSBU 2012160
S. spinosa	Chitgar2, Tehran	51.12.29	35.46.47	1285	HSBU 2012161
S. spinosa	Hesar1, Chalous	51.01.53	35.49.24	1394	HSBU 2012162
S. spinosa	Hesar2, Chalous	51.01.53	35.49.24	1394	HSBU 2012163
S. reuteriana Boiss.	Tochal1,Tehran	51.24.02	35.49.14	1857	HSBU 2012164
S. reuteriana	Tochal2,Tehran	51.24.02	35.49.14	1857	HSBU 2012165
S. reuteriana	Darakah, Tehran	51.23.48	48.25.45	1697	HSBU 2012166
S. reuteriana	Porkan1, Chalous	51.04.03	36.56.05	1613	HSBU 2012167
S. reuteriana	Porkan2, Chalous	51.04.03	36.56.05	1613	HSBU 2012168
S. reuteriana	Chanar gharb, Firoozkooh	52.85.12	35.39.35	1572	HSBU 2012169

was performed on the STRUCTURE result to find the proper number of K by using delta K value (Evanno et al. 2005).

Secondly, we performed K-Means clustering as performed in GenoDive ver. 2. (2013). In this analysis, the optimal clustering is the one with the smallest amount of variation within clusters. This is calculated by 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 (Meirmans 2012). We used two summary statistics to present K-Means clustering, 1- pseudo-F (Caliński and Harabasz 1974), and 2- Bayesian information criterion (BIC) (Schwarz 1978). The clustering with the highest value for pseudo-F is regarded as providing the best fit, while clustering with the lowest value for BIC is considered to provide the best fit (Meirmans 2012). Similarly, non-metric multidimensional scaling (MDS)

(Podani 2000) was performed to study the genetic distinctness of the provinces.

Recently Frichot et al. (2013) introduced the statistical model called "latent factor mixed models (LFMM)", which 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 the ISSR markers used here show any correlation with the environmental features of the species studied. The analysis was done with the LFMM program Version: 1.2 (2013).

Results

Morphometry

The analysis of variance (ANOVA) test showed a significant difference ($p < 0.05$) for quantitative morphological characters among *Salvia* species. Moreover, PCoA plot of both quantitative and qualitative morphological characters separated the studied species into two distinct groups (Fig. 1). *S. reuterana* and *S. spinosa* of the species group 3, were placed close to each other and much separated from the other studied species of the species group 1 according to Flora Iranica (Hedge 1982).

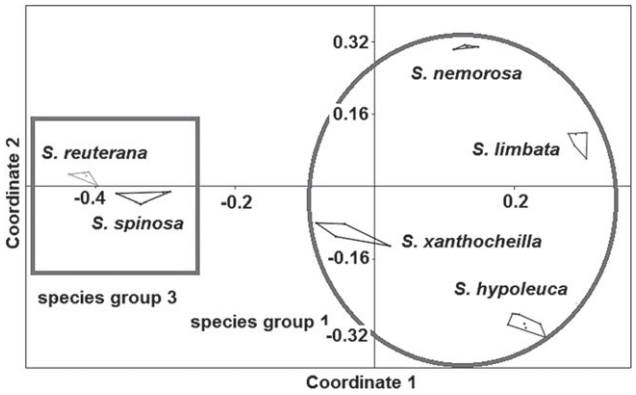


Fig. 1. Principal coordinate analysis plot of morphological characters, separating two *Salvia* species groups from each other.

Within the species group 3, *S. hypoleuca* showed morphological similarity with *S. nemorosa*, while, *S. limbata* and *S. xanthocheila*, were placed closer to each other.

PCA analysis revealed that the first 3 components comprised about 86% of total morphological variability. In the first PCA components with about 45% of total variation, characters like calyx length, bract length, seed shape, calyx shape, and basal leaf shape showed the highest positive correlation (> 0.80). These morphological characters mainly separated *S. spinosa* and *S. reuterana* (species group 3) from the other species (Fig. 2).

The nut width, basal leaf length, seed color, and leaf surface showed the highest positive correlation (> 0.70) with the second PCA component. These characters separated *S. hypoleuca* and *S. limbata* from *S. nemorosa* and *S. xanthocheila* of the species group 1 (Fig. 2).

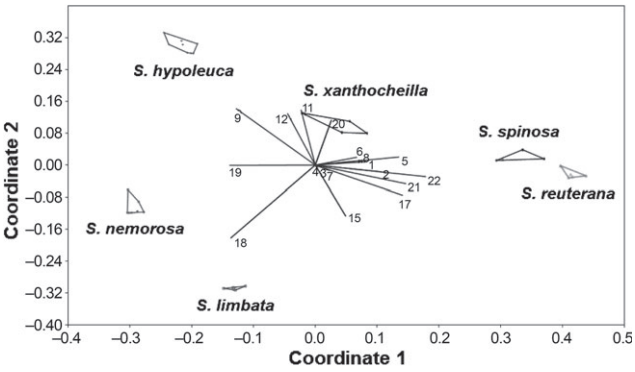


Fig. 2. Principal components analysis biplot of morphological characters in *Salvia* species. Character numbers: 1) pedicel length, 2) calyx length, 3) stem leaf length, 4) stem leaf width, 5) bract length, 6) filament length, 7) anther length, 8) corolla length, 9) nut length, nut width, 10) basal leaf length, 11) basal leaf width, 12) corolla color, 13) corolla shape, 14) bract shape, 15) seed color, 16) seed shape, 17) bract color, 18) corolla latex, 19) leaf surface, 20) calyx shape, and 21) basal leaf shape, respectively.

ISSR analysis

We obtained reproducible bands from almost all ISSR primers used and finally, a data matrix was formed. The detrended correspondence analysis (DCA) plot revealed (not shown) scattered distribution of the ISSR loci studied, which indicated these loci are not linked and are suitable for population genetic structure analysis.

Genetic diversity parameters determined in 6 studied species (Tab. 2) revealed that *S. limbata* had the highest level of genetic polymorphism (57.14%), while the lowest level of genetic polymorphism (28.57%) occurred in *S. reuterana*. *S. limbata* also had the highest values for effective number of alleles (Ne = 1.256) and Shannon information index (I = 0.25).

The AMOVA test revealed significant molecular differences (P = 0.01) among the studied species. It also revealed that 21% of total genetic variability occurred among the studied populations while 79% occurred within these species. Furthermore, pair-wise AMOVA test as well as non-metric MDS analysis revealed that most of the paired samples comparisons differed significantly from each other (P = 0.01) (Fig. 3).

Tab. 2. Genetic diversity parameters determined in *Salvia* species. Na – no. of different alleles, Ne – no. of effective alleles, I – Shannon's information index, He – expected heterozygosity, UHe – unbiased expected heterozygosity, %P – percentage of polymorphic loci.

Species	N	Na	Ne	I	He	UHe	%P
S. hypoleuca	8	0.78	1.119	0.14	0.083	0.088	38.46%
S. nemorosa	7	0.96	1.183	0.20	0.125	0.134	47.25%
S. limbata	7	1.15	1.256	0.26	0.163	0.175	57.14%
S. xanthocheila	5	1.00	1.192	0.21	0.132	0.146	49.45%
S. spinosa	6	0.87	1.237	0.22	0.145	0.158	42.86%
S. reuterana	6	0.67	1.169	0.15	0.098	0.107	28.57%

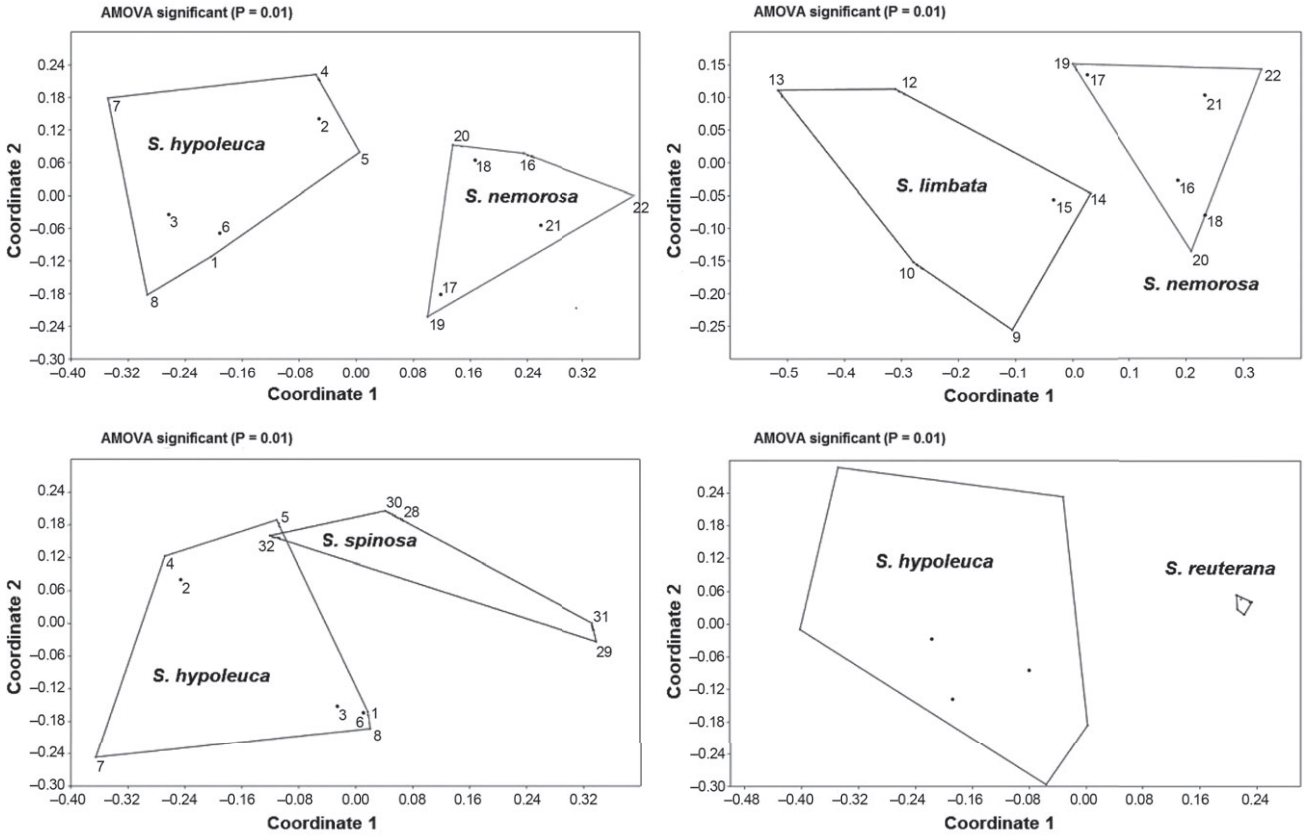


Fig. 3. Multidimentional scaling plots showing genetic distinctness of *Salvia* species.

The Hickory test we used also produced a Theta B value of 0.25, which is a significant value. Gst value (0.18, P = 0.001), and Hedrick, standardised fixation index (G'st = 0.23, P = 0.001) as well as the Jost, differentiation index (D-est = 0.06, P = 0.001) showed that *Salvia* species are genetically differentiated.

Nei's genetic identity and the genetic distance determined among the studied species are presented in On-line Suppl. Tab. 1. The results showed that the highest degree of genetic similarity (0.989) occurred between *S. hypoleuca* and *S. limbata* and then between *S. limbata* and *S. spinosa* (0.981). The lowest degree of genetic similarity occurred between *S. nemorosa* and *S. reuterana* (0.877).

NJ tree based on Nei's genetic distance (Fig. 4), showed that *S. reuterana* differed genetically from the other studied species, as it stands far from them. This dendrogram showed close genetic affinity between *S. hypoleuca* and *S. nemorosa*, while *S. xanthocheila* joined them with some distance.

The neighbor-net network obtained for all plant specimens revealed more detailed information about intraspecies genetic variability as well as the genetic affinity between the studied species (Fig. 5). It showed that all plant specimens of *S. reuterana* were placed close to each other. This holds true for plant specimens of *S. limbata*. However, specimens studied from the other species showed genetic variability and were placed in different clusters.

The network also showed genetic affinity between specimens of *S. hypoleuca* and *S. nemorosa* (coded 1 and 2 respectively), and between *S. xanthocheila* and *S. spinosa* (coded 4 and 5 respectively). These results are in agreement with the NJ tree result.

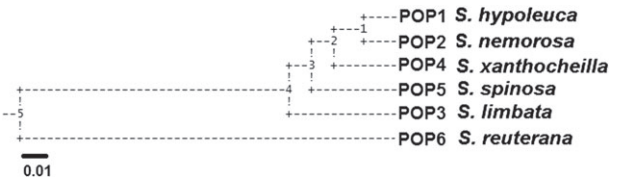


Fig. 4. Neighbor joining tree of inter simple sequence repeats data in the studied *Salvia* species.

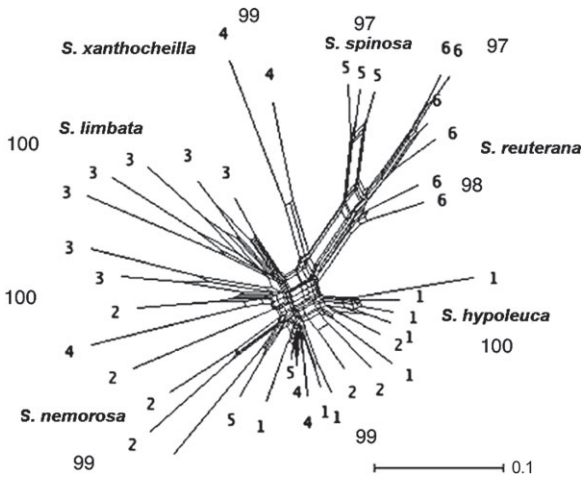


Fig. 5. NeighborNet tree of inter simple sequence repeats data in the studied *Salvia* species. Numbers at the tip of splits are bootstrap values.

A Mantel test did not produce significant correlation (r = 0.01, p = 0.63) between geographical distance and genetic distance of these species and therefore, no isolation by distance (IBD) exists between them.

Salvia species studied are placed in two different species groups (namely species group 1 and 3 in Flora Iranica). In order to check if we have also two distinct genetic structures, we performed K-Means clustering and Bayesian based method of STRUCTURE analysis.

K-Means clustering result (On-line Suppl. Tab. 2), showed that the optimum number of genetic groups (number of K) present in our data according to Calinski & Harabasz' pseudo-F is K = 2 (the highest value of pseudo-F = 7.218). On the other hand, according to Bayesian information criterion, K = 3 (the lowest value of BIC = 235.0).

STRUCTURE analysis followed by Evanno test also produced delta K = 3 (On-line Suppl. Fig. 1). Therefore, we do have at least 2–3 genetic groups in the studied species.

STRUCTURE plot (On-line Suppl. Fig. 2), which is based on Bayesian analysis, recognized 3 distinct genetic: 1- specimens having mostly blue colored segments (allelic combination) including *S. hypoleuca* and *S. limbata*; 2- specimens with blue and red segments, including *S. nemorosa* and *S. xanthocheila*; and 3- specimens having mostly green colored segments, including *S. spinosa* and *S. reuterana*. This genetic grouping is in agreement with the delta k of the Evanno test and K-Means clustering.

Reticulation analysis (On-line Suppl. Fig. 3) showed that some degree of inter-specific gene flow occurs among members of the studied species and not a single species is entirely isolated from all other studied species.

The analysis done by LFMM program produced a very low value of -log10 (p-value) not higher than 0.20 which were all non-significant values (p >0.05). These results are summarized in a Manhattan plot, which is presented in Online Suppl. Fig. 4.

Discussion

Morphological analyses of the studied *Salvia* species showed that they are well differentiated from each other both in quantitative measures (the ANOVA test result) and qualitative characters (The PCoA plot result). Moreover, the present study supports the taxonomic treatment of these taxa and the placing of them in two different species groups in Flora Iranica (Hedge 1982).

In addition, PCA analysis suggests that characters like calyx length, bract length, seed shape, calyx shape, and basal leaf shape, could be used in species groups delimitation.

AMOVA test revealed significant molecular difference (P = 0.01) among the studied species. It also revealed that 21 % of total genetic variability occurred among the studied populations while, 79% occurred within these species. The possible reason for high within-species genetic variability is due to new mutations that occur and gradually accumulate within populations of the certain species. Similar results were reported in *Salvia miltiorrhiza* Bunge (Song et al. 2009). Both ISSR and SRAP molecular markers revealed the presence of a greater proportion of total genetic variation within *S. miltiorrhiza* populations, rather than among populations.

The significant pair-wise AMOVA test, as well as the non-metric MDS result, revealed the genetic distinctness of *Salvia* species studied. However, neighbor-net network and NJ tree results indicated a higher degree of genetic uniformity within *S. reuterana* and *S. limbata* than in the other studied species.

An interesting result was obtained from K-Means clustering and STRUCTURE analysis, which showed that 2–3 genetic groups are present among the species studied. This is in agreement with species groups identified in *Salvia* and also supports the AMOVA result showing genetic distinctness of the studied taxa.

As we presented before, morphological and ISSR groupings separated *S. spinosa*, and *S. reuterana* of the species group 3 from the other studied species of the species group 1. However, the ISSR based grouping showed closer affinity between *S. hypoleuca* and *S. limbata*, while a morphological grouping showed closer similarity between *S. hypoleuca* and *S. nemorosa*. Differences between the two groupings might be due to various reasons, such as the gene expression, environment, and gene introgressions as also reported in *S. miltiorrhiza* accessions and its varieties (Wang et al. 2011). Similar studies in populations of *S. japonica* and some other *Salvia* species (Sudarmono and Okada 2008) did not show correlation between morphological variations and allozyme and DNA sequences. It was concluded that *S. japonica* is still at the early stage of speciation process.

Sympatry or co-occurrence of closely related species can either result from a sympatric speciation process or from secondary contact due to range expansion after speciation. Under the allopatric scenario, genetic variation tends to be uniform across the genome due to a large proportion of the genome changing through a combination of divergent selection, differential response to similar selective pressures and genetic drift (see for example Strasburg et al. 2012). In contrast, in the extreme case of sympatric speciation, gene flow between the incipient species can homogenize most of the genome, except for loci that experience strong divergent selection pressures or regions that are tightly linked with these loci (see for example, Strasburg et al. 2012, Via 2012).

Different mechanisms, including isolation by distance, lack of gene flow, local adaptation, and genetic drift followed by strong selection pressure, are responsible for species/population differentiation and genetic divergence (Tero et al. 2003, Freeland et al. 2011, Frichot et al. 2013). In the present study, a Mantel test did not produce a significant correlation between the geographical and the genetic distance of these species and therefore, no isolation by distance (IBD) exists between them. Moreover, the STRUCTURE result indicated that a high degree of infra-specific genetic variability is present in *Salvia* species due to change in the allelic frequency and gene flow/admixture. However, a Manhattan plot did not show correlation between ISSR loci and environmental features of *Salvia* taxa. These re-

sults indicate that *Salvia* species differentiation is not solely due to genetic isolation and lack of gene flow. However, we are not sure about the main evolutionary mechanism for *Salvia* species diversification at present and further investigation is needed and possibly in many cases speciation is underway.

References

Calinski, R. B., Harabasz, J., 1974: A dendrite method for cluster analysis. Communications in Statistics 3, 1–27.

De Mattia, F., Bruni,I., Galimberti, A., Cattaneo, F., Casiraghi, M., Labra, M., 2011: A comparative study of different DNA barcoding markers for the identification of some members of Lamiacaea. Food Research International 44, 693–702.

Evanno, G., Regnaut, S., Goudet, J., 2005: Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14, 2611–2620.

Falush, D., Stephens, M., Pritchard, J. K., 2007: Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7, 574–578.

Freeland, J. R., Kirk, H., Peterson, S. D., 2011: Molecular ecology (2nd ed). Wiley-Blackwell, UK.

Frichot, E., Schoville, S. D., Bouchard, G., Francois, O., 2013: Testing for associations between loci and environmental gradients using latent factor mixed models. Molecular Biology and Evolution 30, 1687–1699.

Hamer, Ø., Harper, D. A. T., Ryan, P. D., 2001: PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4, 9.

Hedge, I. C., 1982: Labiatae. In: Rechinger, C. H. (ed.), Flora Iranica Vol. 150, 403–476. Akademische Druk-U. Verlagsanstalt, Graz, Austria.

Hedrick, P. W., 2005: A standardized genetic differentiation measure. Evolution 59, 1633–1638.

Holsinger, K. E., Lewis, P. O., 2003: Hickory: a package for analysis of population genetic data V1.0. Retrieved 2003 from http://www.eeb.uconn.edu.

Huson, D. H., Bryant, D., 2006: Application of phylogenetic networks in evolutionary studies, Molecular Biology and Evolution 23, 254–267.

Jost, L., 2008: GST and its relatives do not measure differentiation. Molecular Ecology 17, 4015–4026.

Karaca, M., Ince, A. G., Ay, S. T., Turgut, K., Onus, A. N., 2008: PCT-RFLP and DAMD-PCR genotyping for *Salvia* species. Journal of Science and Food Agriculture 88, 2508–2516.

Kharazian, N., 2011: Karyotypic study of some *Salvia* (Lamiaceae) species from Iran. Journal of Applied Biological Science 5, 21–25.

Meirmans, P. G., 2012: AMOVA-based clustering of population genetic data. Journal of Heredity 103, 744–750.

Meirmans, P. G., Van Tienderen, P. H., 2004: GENOTYPE AND GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes 4, 792–794.

Özdemir, C., Senel, G., 1999: The morphological, anatomical and karyological properties of *Salvia sclarea* L. Turkish Journal of Botany 23, 7–18.

Peakall, R., Smouse, P. E., 2006: GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6, 288–295.

Podani, J., 2000: Introduction to the exploration of multivariate data [English translation], Leide, Netherlands: Backhuyes,

Pritchard, J. K., Stephens, M., Donnelly, P., 2000: Inference of population structure using multilocus genotype Data. Genetics 155, 945–959.

Radosavlijević, I., Jakse, J., Javornik, B., Šatović, Z., Liber, Z., 2011: New microsatellite markers for *Salvia officinalis* (Lamiaceae) and cross-amplification in closely related species. American Journal of Botany, e316–e318.

Radosavlijević, I., Bogdanović, S., Šatović, Z., Liber, Z., 2012: Natural hybridization between *Salvia officinalis* L. and *Salvia fruiticosa* Mill. (Lamiaceae) as revealed by microsattelite markers. In: Rešetnik I., Bogdanović S., Alegro A. (eds.), International symposium on "Evolution of Balkan biodiversity". Zagreb, BalkBioDiv Consortium and Croatian Botanic Society, 65–65.

Salehi-Sormaghi, M. H., Amin, G. H., Samadai, N., Hemmati, F., Sarkhail, P., 2006: Chemical composition and antimicrobial activity of essential oils of *Salvia spinosa* L. Asian Journal of Plant Sciences 5, 654–656.

Schwarz, G. 1978. Estimating the dimension of a model. Annals of Statistics 6, 461–464.

Sepehry-Javan, Z., Rahmani, F., Heidari, R., 2012: Assessment of genetic variation of genus *Salvia* by RAPD and ISSR markers. Australian Journal of Crop Science 6, 1068–1073.

Sheidai, M., Alijanpoor, B., Khayyami, M., 2010: Contribution to cytology of genus *Salvia* L. (Lamiaceae) in Iran. Caryologia 63, 405–410.

Sheidai, M., Zanganeh, S. Haji-Ramezanali, R., Nouroozi, M., Noormohammadi, Z., Ghsemzadeh-Baraki, S., 2013: Genetic diversity and population structure in four *Cirsium* (Asteraceae) species. Biologia 68, 384–397.

Song, Z., Li, X., Wang, H., Wang, J., 2009: Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Genetica 138, 241–249.

Standley, P., Williams, L., 1973: Labiateae. Fieldiana, Botany 24, 237–317.

Strasburg, J. L., Sherman, N. A., Wright, K., MMoyle, L. C., Willis, J.H., Rieseberg, L. H., 2012: What can patterns of differentiation across plant genomes tell us about adaptation and speciation? Philosophical Transaction of Royal Society B 367,364–373.

Sudarmono, Okada, H., 2008: Genetic differentiations among the populations of *Salvia japonica* (Lamiaceae) and its related species. Hayati Journal of Bioscience 15, 18–26.

Tero, N., Aspi J., Siikamaki, P., Jakalaniemi, A., Tuomi, J., 2003: Genetic structure and gene flow in a metapopulation of an endangered plant species, *Silene tatarica*. Molecular Ecology 12, 2073–2085.

Tychonievich, J., Warner, R. M., 2011: Interspecific crossability of selected *Salvia* species and potential use for crop improvement. Journal of American Society of Horticultural Science 136, 41–47.

Via, S., 2012. Divergence hitchhiking and the spread of genomic isolation during ecological speciation-with-gene-flow. Philosophical Transactions of Royal Society, B 367, 451–460.

Walker, J. B., Sytsma K. J., Treutlein, J., Wink, M., 2004: *Salvia* (Lamiaceae) is not monophyletic: Implications for the systematics, radiation, and ecological specializations of *Salvia* and tribe Mentheae. American Journal of Botany 91, 1115–1125.

Wang M., Li J., Zhang, L., Yang. R. W., Ding, C. B., Zhou, Y. H., Yin, Z. Q., 2011: Genetic diversity among *Salvia miltiorrhiza* Bunge and related species using morphological traits and RAPD markers. Journal of Medicinal Plants Research 5, 2687–2694.

Weising, K., Nybom, H., Wolff, K., Kahl, G., 2005: DNA Fingerprinting in plants. Principles, Methods, and Applications. (2nd ed.), CRC Press, Boca Rayton, Fl., USA.

Wu, C. Y., Li, H. W., 1982: On the evolution and distribution in Labiateae. Acta Botanica Yunnanica 4, 97–118.

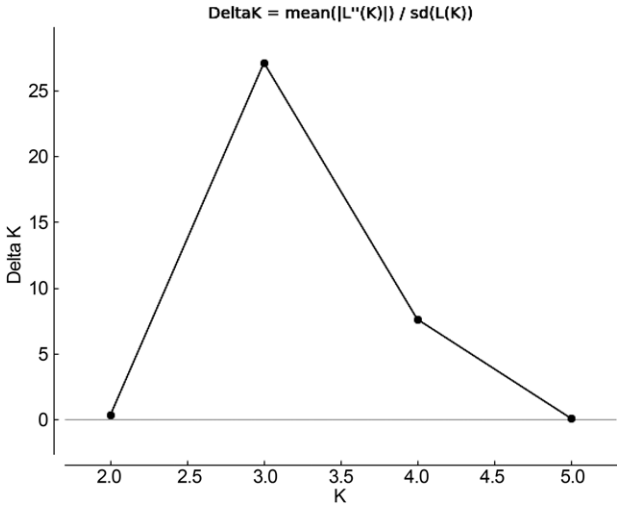
Zhan, L., Zhao, H. X, Fan, X., Wang, M., Ding, C. B., Yang, R. W., Yin, Z. Q., Xie, X. L., Zhou, Y. H., Wan, D. G., 2012: Genetic diversity among *Salvia miltiorrhiza* Bunge and related species inferred from nrDNA ITS sequences. Turkish Journal of Biology 36, 319–326.

On-line Suppl. Tab. 1. Nei's genetic identity (above diagonal) and genetic distance (below diagonal). Population ID: 1 – S*alvia. hypoleuca*, 2 – *S. limbata*, 3 – *S.nemorosa,* 4 – *S. xanthocheila*, 5 – *S. spinosa*, 6 – *S. reuterana*.

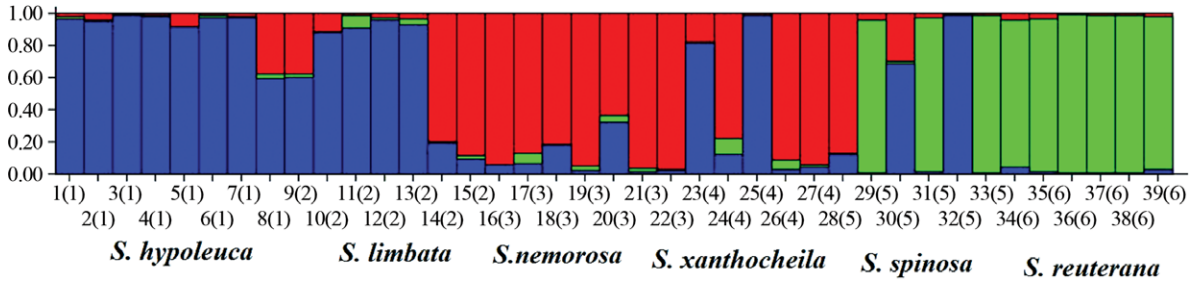
Population	1	2	3	4	5	6
1	0.000	0.989	0.965	0.978	0.972	0.889
2	0.010	0.000	0.969	0.981	0.977	0.892
3	0.035	0.031	0.000	0.967	0.966	0.877
4	0.021	0.018	0.033	0.000	0.973	0.888
5	0.027	0.022	0.033	0.027	0.000	0.921
6	0.117	0.113	0.130	0.117	0.081	0.000

On-line Suppl. Tab. 2. K-Means clustering of inter simple sequence repeats (ISSR) data. Clustering statistics from k = 2 to k = 6. Asterisk (*) denotes best clustering according to Calinski & Harabasz' pseudo-F: k = 2. Symbol "&" denotes best clustering according to Bayesian information criterion: k = 3.

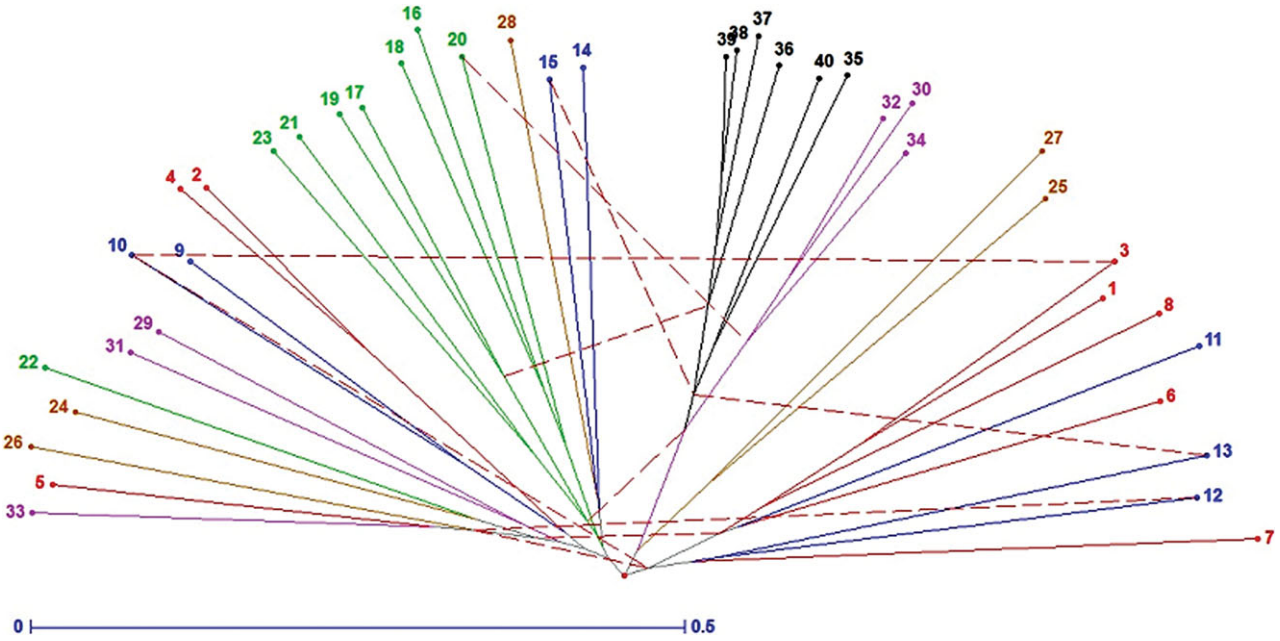
k	SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	BIC
2*	410.974	67.085	343.889	0.163	7.218	235.100
3&	410.974	98.270	312.704	0.239	5.657	235.056
4	410.974	121.766	289.208	0.296	4.912	235.673
5	410.974	142.352	268.623	0.346	4.504	236.457
6	410.974	162.407	248.567	0.395	4.312	237.094



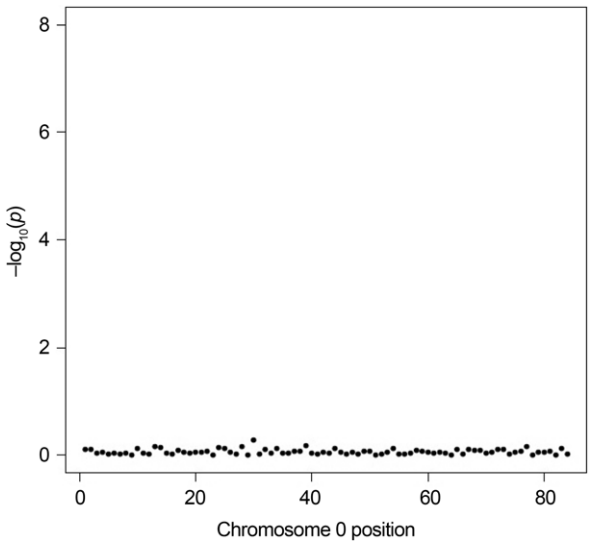
On-line Suppl. Fig. 1. Delta K value of Evanno test.



On-line Suppl. Fig. 2. STRUCTURE plot of *Salvia* species showing interspecific genetic variability and admixture.



On-line Supplement Fig. 3. Reticulogram of *Salvia* species.
Species numbers are: 1-8 = *S. hypoleuca*, 9-15 = *S. limbata*, 16-22 = *S. nemorosa,* 23-27 = *S. spinosa,* 28-33 = *S. xanthocheila*, 34-39 = *S. reuterana*, respectively.



On-line Supplement Fig. 4. Manhattan plot of inter simple sequence repeats (ISSR) data.