Short Communication

Investigation of Genetic Diversity in Different Species of *Salvia* Medicinal Plant by ISSR Markers

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Abstract

Salvia is the largest genus in the *Lamiaceae* family in the world and Iran contains 58 species. This genus contains over 900 species of annual and perennial herbaceous plants and differs from other lamias in the unusual structure of their plumage. Information on important medicinal plants' genetic diversity and population structure is well documented in the literature. Increased genetic diversity can reduce the negative effects of inbreeding on populations. We investigated genetic variation among eight *Salvia* species using ISSR molecular markers. ISSR is a molecular marker amplified by PCR using microsatellite primers. Samples were gathered from various locations in Iran. Most samples were assigned to Ardabil city. Twenty randomized ISSR primers were used, generating different polymorphic bands. The 20 ISSR primers generated 225 valuable bands and 221 bands were polymorphic (98/2%). Pairwise genetic distances ranged from 0.083 to 0.577. Dendrograms were generated using the UPGMA method using NTSYSpc 2.02i software to identify seven major groups from eight *Salvia* species. The polymorphism levels observed in the present study represent a high degree of genetic diversity among *Salvia* species. Following the first study on the genetic relationships of eight species of *Salvia* in Iran using RAPD and ISSR molecular markers, this study was performed using the ISSR molecular marker to investigate the genetic relationships of eight other species of *Salvia*. The results of this research represented that the molecular markers of the ISSR are suitable for assessing genetic variation and evolutionary relationships among *Salvia* species, showing a wide range of dispersal.

***Keywords*:** Primer, ISSR, *Salvia*, UPGMA, dendrogram

Introduction

Many plant species that serve as important food and medicinal resources are threatened by large-scale anthropogenic change and destruction of natural habitats (that is, more than 30% of the Earth's land surface) (Bakhshipour et al., 2019; Lienert, 2004). Habitat disturbance poses a serious threat to plant species as it reduces populations and causes geographical isolation. This may allow a deeper understanding of genetic variation given its association with extinction risk for plant species (Lee et al., 2018; Mafakheri et al., 2020; Méndez et al., 2014). Information on important medicinal plants' genetic diversity and population structure is well documented in the literature (Mafakheri et al., 2020). Increasing genetic diversity can reduce the negative effects of inbreeding on populations. Higher levels of genetic diversity in plant genetics promote resilience to adverse environmental changes, community organization, and ecosystem function (Hoban et al., 2021; Hughes et al., 2008). The mint family is an important medicinal plant. *Salvia* is one of the most widespread plants in the world, with 58 species in Iran. This genus contains over 900 species of annual and perennial herbaceous plants, the largest genus in the Lamiaceae family. *Salvia* means medicine in Latin, and this genus has always been valued for its healing properties for both health and disease (Kilic et al., 2005). *Salvia* extract has, antioxidant, antibacterial, antitumor, and antidiabetic properties to treat various ailments (Ebrahimzadegan and Maroufi, 2022). This genus has a high environmental tolerance (Song et al., 2010), and is distributed worldwide, especially in moderate and tropical regions (Delamare et al., 2007). Iran has seventeen endemic species of *Salvia* (Mozaffarian, 1996). This genus differs from other lamia in an unusual wing structure (Walker et al., 2004). Seeds of *Salvia* have medicinal, nutritional, and oily properties. The influence of environmental and genetic factors on essential fats in *Salvia* has

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been demonstrated. Species, geographical area, and human selection are the three main factors affecting genetic diversity in *Salvia* (Joseph, 2004). In addition, plant breeders can use genetically distinct parents in breeding programs to improve the productivity of agricultural and horticultural crop varieties. (Raffard et al., 2019). Among many interesting techniques, DNA-based techniques are more reliable than other current approaches and are widely used in plant development projects. Unlike other markers, molecular markers are not affected by environmental changes or plant developmental stages (Cao et al., 2022). Molecular markers provide practical tools for describing and managing germplasm. ISSR is another molecular marker amplified by PCR using microsatellite primers. Using these markers has many advantages as no prior genomic information about the wild genome sequence is required. The results of this method are highly reproducible and produce a high degree of polymorphism in most systems. After conducting the first study to investigate the genetic relationship between eight *Salvia* species using RAPD and ISSR molecular markers (Javan and Heidari, 2012), another study was followed in Iran using ISSR molecular markers. This is his second study done with eight species of *Salvia*.

Materials and Methods

Plant materials

Seeds of eight species of *Salvia* were collected from different regions of Iran. These seeds were planted in pots and the pots were placed in the Phytotron system. About a month after planting, the leaves were ready to harvest. Fresh and young leaves were selected and after covering them with aluminum foil, they were placed in liquid nitrogen, then transferred to a freezer at -80^{oc} and kept there until the experiments were performed.

DNA extraction

The leaves were ground to a fine powder using a mortar and pestle chilled in liquid nitrogen. Genomic DNA was extracted using the CTAB method of Doyle and Doyle (Doyle and Doyle, 1987).

ISSR amplification

25 ISSR primers were purchased from a commercial company (Cinnagen, Tehran, Iran), and 20 of these primers were selected after testing (Table 1). Polymerase chain reaction (PCR) was performed using 1 µl DNA (30 ng), 0.25 µl Taq polymerase enzyme (5 U/µl), 0.5 µl dNTPs (10 mM), 2.5 µl 10x PCR buffer, 0.75 µl Mgcl2 (50 mM), 19.7 µl ddH2O and 0.3 µl primer in Veriti 96-well thermal cycler (Applied Biosystem). The PCR program started with an initial stage of 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes, and a final elongation of 72°C for 10 minutes.

Bands profile

PCR results were run at 70V on a 2% agarose gel using a 0.5x TBE buffer system and stained with ethidium bromide. Each gel was photographically documented under UV light using a Gel Logic 212 Pro Imaging System "Carestream, USA". The molecular size of the amplified products was estimated using 100-3000 bp DNA ladder markers (Fermentas).

Data analysis

A clear and reproducible highlighted band was selected for analysis. The presence of a band is indicated by (1) and the absence by (0). Data obtained through individual and collective evaluation of ISSR markers were subjected to similarity matrix computation using Jaccard coefficients. Similarity scores were used for cluster analysis. Sequential agglomerative hierarchical non-overlapping (SAHN) clustering was applied using the unweighted pair-groups method with arithmetic mean (UPGMA). Dendrograms were generated using the NTSYSpc 2.02 software (Rohlf, 1998).

Results and Discussion

This study follows the first study that used the molecular markers RAPD and ISSR to analyze the genetic connectivity among eight Iranian *Salvia* species (Javan and Heidari, 2012). As a result, molecular ISSR markers were used to define the genetic relationships of eight different *salvia* species. Twenty randomized ISSR primers were used to generate all the different polymorphic bands (Table 1). Results obtained using 848 primers are shown in Figure 1.

221 out of 225 response bands generated by ISSR primers were polymorphic. As a result, it was found that *Salvia* species show great genetic diversity. The genetic similarity between *Salvia* species is 0.423 (*S. Macrosiphon* and *S. urmiensis*) to 0.917% (*S. Verbenaka* and *S. oligophylla*), (Table 2).

Table 1. Titles and sequences of primers used in PCR.

Sequence (5'→3′)	Primer	Number of polymorphic bands	Number of generated bands	Polymorphism percent
CACACACACACACACAAGC	848	6	6	100
CACACACACACACACAGT	846	10	10	100
GAGAGAGAGAGAGAGACC	841	9	9	100
AGAGAGAGAGAGAGAGCTT	834	10	10	100
TGTGTGTGTGTGTGTGC	829	11	11	100
ACACACACACACACACG	827	10	10	100
ACACACACACACACACC	826	11	11	100
ACACACACACACACACT	825	10	10	100
TCTCTCTCTCTCTCTCG	824	12	12	100
GTGTGTGTGTGTGTGTC	821	10	10	100
GTGTGTGTGTGTGTGTC	820	12	12	100
CACACACACACACACAT	816	14	14	100
CTCTCTCTCTCTCTCTA	814	14	14	100
GAGAGAGAGAGAGAGAC	811	12	12	100
GAGAGAGAGAGAGAGAT	810	9	9	100
AGAGAGAGAGAGAGAGC	808	14	14	100
AGAGAGAGAGAGAGAGT	807	12	12	100
AGAGAGAGAGAGAGAGCA	836	20	21	95.24
CTCTCTCTCTCTCTCTAGG	845	7	8	87.5
ACACACACACACACACCTA	856	8	9	89
Total		221	253	98.2

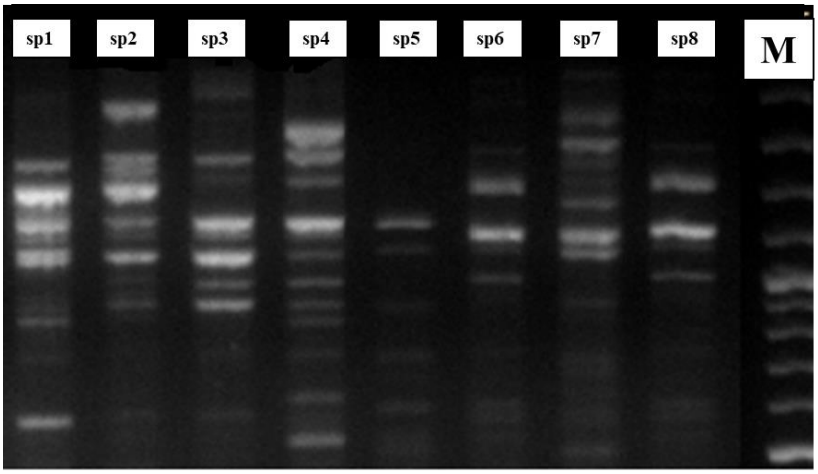

Figure 1. Agarose gel electrophoresis of ISSR marker with 848 primers. sp1 = *S. verbenaca*, sp2 = *S. macrosiphon*, sp3 = *S. brachyantha*, sp4 = *S. aristata*, sp5 = *S. urmiensis*, sp6 = *S. sahendica*, sp7 = *S. bazmanica*, sp8 = *S. oligophylla*.

The genetic distance between species ranges from 0.083 to 0.577. Dendrograms were created using the UPGMA method (Figure 2).

Table 2. Species similarity matrix based on Jaccard's similarity coefficients.

	sp1	**sp2**	**sp3**	**sp4**	**sp5**	**sp6**	**sp7**	**sp8**
S.verbenaca	1.0000							
S.macrosiphon	0.5455	1.0000						
S.brachyantha	0.5731	0.4743	1.0000					
S.aristata	0.5810	0.4822	0.5573	1.0000				
S.urmiensis	0.5455	0.4229	0.6087	0.5217	1.0000			
S.sahendica	0.4466	0.5296	0.4387	0.4545	0.4427	1.0000		
S.bazmanica	0.6008	0.5731	0.4822	0.5534	0.4625	0.4664	1.0000	
S.oligophylla	0.9170	0.5731	0.5692	0.5929	0.5494	0.4664	0.5968	1.0000

sp1 = *S. verbenaca*, sp2 = *S. macrosiphon*, sp3 = *S. brachyantha*, sp4 = *S. aristata*, sp5 = *S. urmiensis*, sp6 = *S. sahendica*, sp7 = *S. bazmanica*, sp8 = *S. oligophylla*.

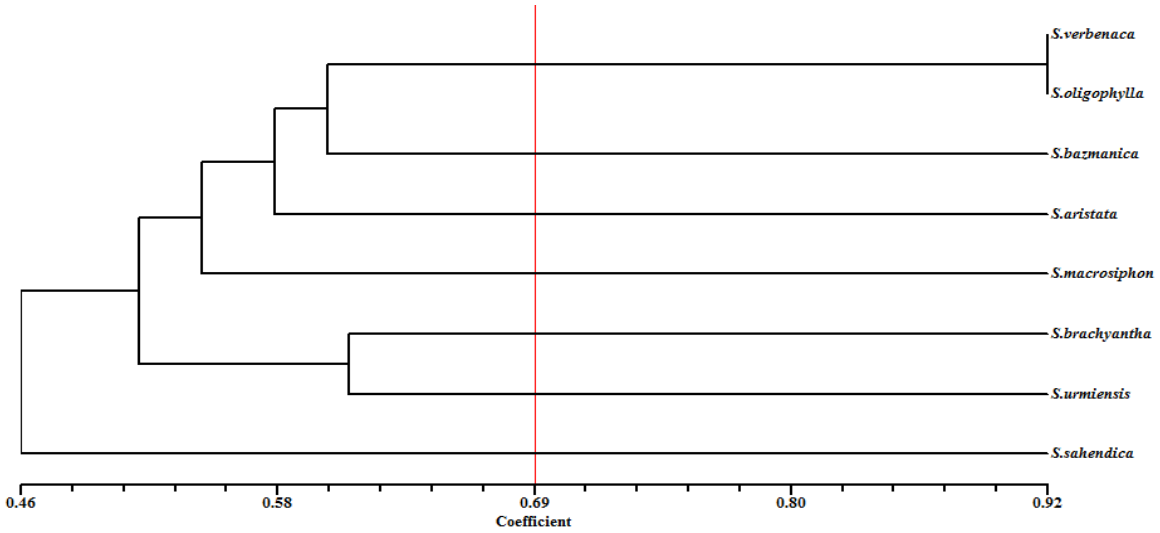

Figure 2. Dendrogram of the genetic distance of 8 species of *Salvia*. The dendrogram describes the seven main groups of the eight species of *Salvia* as follows: (1) *S. verbenaca* and *S. oligophylla*, (2) *S. bazmanica*, (3) *S. aristata*, (4) *S. macrosiphon*, (5) *S. brachyantha*, (6) *S. urmiensi*, and (7) *S. sahendica* (Fig. 2).

The results of this study demonstrated that the ISSR molecular marker is useful for assessing genetic variation and evolutionary relationships among *Salvia* species and exhibits a wide range of distribution. A high value of the cophenetic correlation coefficient indicates a strong relationship between the similarity matrix and the generated dendrogram. The genetic diversity of *S. miltiorrhiza* has been assessed using various molecular markers such as AFLP, CoRAP, and RAPD (Guo et al., 1994; Wang et al., 2007), SRAP and ISSR (Song et al., 2010). AFLP is commonly used for genomic fingerprinting because of its speed, reliability, and high number of polymorphisms. However, the two main drawbacks of this technique are its prohibitive cost and time-consuming procedure (Wang et al., 2007). CoRAP produced lesser polymorphic bands in *Salvia*, leading to the depreciation of

polymorphism and genetic diversity (Wang et al., 2007). SRAP was another molecular marker that was first proposed (Li and Quiros, 2001) and has been widely used in genetic linkage mapping and genetic diversity studies (Ding et al., 2008; Ferriol et al., 2003; Guo and Luo, 2006; Li and Quiros, 2001). SRAP and ISSR were developed as beneficial and dependable genetic markers to accurately measure the level of genetic diversity in *S. miltiorrhoza* (Song et al., 2010). Average genetic distances generated from SRAP markers revealed more genetic differences than average genetic distances obtained from ISSR markers, although both showed that ISSR was associated with diversity in other molecular markers and genes We showed that introgression analysis, compared with germplasm identification, may be useful in genetics research. RAPD markers showed high genetic similarity in cultured populations of *Salvia miltiorrhoza* (Guo et al., 1994). Our data from a single marker system indicated that the species studied had limited genetic similarity at the interspecies level. Finally, the 30 selected markers provided a good overview of the relations among the eight *Salvia* species, allowing PCR-based fingerprinting techniques to estimate the degree of genetic diversity and determine patterns of genetic relatedness. I have shown that it helps RAPD and ISSR analyses were first used in Iran to obtain basic information about the genetic profile of this plant (Javan and Heidari, 2012). Our results demonstrate that DNA markers are effective and reliable molecular markers for assessing genetic diversity. The current findings may pave the way for future research on more potent markers such as Sequence Related Amplified Polymorphism (SRAP) and Simple Sequence Repeat (SSR). We hope that our research may be valuable for germplasm management activities to extend the hereditary differences of *Salvia* in Iran.

Conclusion

According to the results of this research, the level of polymorphism that was detected indicates a high genetic distance at the interspecies level and introduces the ISSR marker as an efficient marker to evaluate the genetic relationship in *Salvia*. In addition, the analysis we performed to assess genetic diversity can provide useful information for the use of these materials, especially for genetic improvement.

Conflict of interest

The author declares that she has no conflict of interest to disclose.

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References

Bakhshipour M., Mafakheri M., Kordrostami M., Zakir A., Rahimi N., Feizi F. and Mohseni M. (2019) In vitro multiplication, genetic fidelity and phytochemical potentials of Vaccinium arctostaphylos L.: An endangered medicinal plant. Industrial Crops and Products 141:111812.

Cao J., Leng G., Yang P., Zhou Q. and Wu W. (2022) Variability in Crop Response to Spatiotemporal Variation in Climate in China, 1980–2014. Land 11:1152.

Delamare A. P. L., Moschen-Pistorello I. T., Artico L., Atti-Serafini L. and Echeverrigaray S. (2007) Antibacterial activity of the essential oils of Salvia officinalis L. and Salvia triloba L. cultivated in South Brazil. Food chemistry 100:603-608.

Ding G., Zhang D., Ding X., Zhou Q., Zhang W. and Li X. (2008) Genetic variation and conservation of the endangered Chinese endemic herb Dendrobium officinale based on SRAP analysis. Plant Systematics and Evolution 276:149-156.

Doyle J. J. and Doyle J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin.

Ebrahimzadegan R. and Maroufi A. (2022) In vitro regeneration and Agrobacterium-mediated genetic transformation of Dragon's Head plant (Lallemantia iberica). Scientific Reports 12:1784.

Ferriol M., Picó B. and Nuez F. (2003) Genetic diversity of a germplasm collection of Cucurbita pepo using SRAP and AFLP markers. Theoretical and Applied Genetics 107:271-282.

Guo B., Lin S., Feng Y. and Zhao Y. (1994) Primary research on genetic relationship among main populations of Salvia miltiorrhiza and genuineness of herb. Chinese Traditional and Herbal Drugs.

Guo D.-L. and Luo Z.-R. (2006) Genetic relationships of some PCNA persimmons (Diospyros kaki Thunb.) from China and Japan revealed by SRAP analysis. Genetic Resources and Crop Evolution 53:1597-1603.

Hoban S., Campbell C. D., da Silva J. M., Ekblom R., Funk W. C., Garner B. A., Godoy J. A., Kershaw F., MacDonald A. J. and Mergeay J. (2021) Genetic diversity is considered important but interpreted narrowly in country reports to the Convention on Biological Diversity: Current actions and indicators are insufficient. Biological Conservation 261:109233.

Hughes A. R., Inouye B. D., Johnson M. T., Underwood N. and Vellend M. (2008) Ecological consequences of genetic diversity. Ecology letters 11:609-623.

Javan Z. S. and Heidari R. (2012) Assessment of genetic variation of genus' Salvia'by RAPD and ISSR markers. Australian Journal of Crop Science 6:1068-1073.

Joseph C. P. (2004) Genetic diversity among varieties of chia (Salvia hispanica L.). Genetic Resources and Crop Evolution 51:773-781.

Kilic T., Dirmenci T., Satil F., Bilsel G., Kocagoz T., Altun M. and Goren A. (2005) Fatty acid compositions of seed oils of three Turkish Salvia species and biological activities. Chemistry of Natural Compounds 41:276-279.

Lee S.-R., Choi J.-E., Lee B.-Y., Yu J.-N. and Lim C. E. (2018) Genetic diversity and structure of an endangered medicinal herb: implications for conservation. AoB Plants 10:ply021.

Li G. and Quiros C. F. (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theoretical and Applied Genetics 103:455-461.

Lienert J. (2004) Habitat fragmentation effects on fitness of plant populations–a review. Journal for nature conservation 12:53-72.

Mafakheri M., Kordrostami M., Rahimi M. and Matthews P. D. (2020) Evaluating genetic diversity and structure of a wild hop (Humulus lupulus L.) germplasm using morphological and molecular characteristics. Euphytica 216:1-19.

Méndez M., Vögeli M., Tella J. L. and Godoy J. A. (2014) Joint effects of population size and isolation on genetic erosion in fragmented populations: finding fragmentation thresholds for management. Evolutionary Applications 7:506-518.

Mozaffarian V. (1996) A dictionary of Iranian plant names. Tehran: Farhang Moaser 396:396-398.

Rohlf F. J. (1998) On applications of geometric morphometrics to studies of ontogeny and phylogeny. Systematic biology 47:147-158.

Song Z., Li X., Wang H. and Wang J. (2010) Genetic diversity and population structure of Salvia miltiorrhiza Bge in China revealed by ISSR and SRAP. Genetica 138:241-249.

Walker J. B., Sytsma K. J., Treutlein J. and 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 B., Zhang Y., Chen C.-B., Li X.-L., Chen R.-Y. and Chen L. (2007) Analysis on genetic diversity of different Salvia miltiorrhiza geographical populations in China. Zhongguo Zhong yao za zhi= Zhongguo Zhongyao Zazhi= China Journal of Chinese Materia Medica 32:1988-1991.