Research Article

Determination of genetic relationships among *Salvia* species by RAPD and ISSR analyses

Salvia türleri arasındaki genetik ilişkilerin RAPD ve ISSR analizleriyle belirlenmesi

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ABSTRACT

INTRODUCTION: *Salvia* L. represents the biggest genus of Lamiaceae family, which includes approximately 1000 species. According to recent studies, 100 *Salvia* species in total grow in Turkey. At the same time, 53% of them are endemic. For the purpose of investigating genetic relationships among fifteen *Salvia* species, which were grown in wild conditions in Turkey's Eastern Anatolia region

METHODS: Genetic relationships among fifteen *Salvia* species, Inter-Simple Sequence Repeat and Random Amplified Polymorphic-DNA profiles were utilized in the present study. Thirteen ISSR primers and eleven RAPD primers were utilized. The combination of the ISSR and RAPD data was performed to construct the UPGMA cluster.

RESULTS: According to the results of the RAPD and ISSR data, *Salvia* species were classified into six groups. As a result of the combined analysis, it was shown that similarities of the species varied between 0.54 (*S. rosifolia*- *S. sclarea*, *S. rosifolia*- *S. limbata* and *S. staminea*- *S. verticillata*) and 0.93 (*S. sclera*- *S. divaricata*).

DISCUSSION AND CONCLUSION: The findings show that two markers represent powerful instruments used to assess the genetic diversity and relations among *Salvia* species.

Keywords: *Salvia* species, Genetic diversity, ISSR, RAPD

ÖZ

GİRİŞ ve AMAÇ: *Salvia* L., yaklaşık 1000 tür içeren Lamiaceae familyasının en büyük cinsini temsil etmektedir. Son çalışmalara göre, Türkiye'de toplam 100 *Salvia* türü yetişmektedir. Aynı zamanda, türlerin % 53'ü endemiktir. Bu çalışmada, Türkiye'nin Doğu Anadolu Bölgesi'nde doğal olarak yetişen on beş *Salvia* türü arasındaki genetik çeşitliliğin değerlendirilmesi amaçlanmıştır.

YÖNTEM ve GEREÇLER: On beş *Salvia* türü arasındaki genetik çeşitlilik, RAPD ve ISSR DNA profilleri kullanılarak araştırıldı. On üç ISSR primer ve on bir RAPD primeri kullanıldı. UPGMA kümelmesini oluşturmak için ISSR ve RAPD verilerinin kombinasyonu gerçekleştirildi.

BULGULAR: RAPD ve ISSR verilerinin sonuçlarına göre, *Salvia* türleri altı gruba ayrılmıştır. Kombine analiz sonucunda türlerin benzerliklerinin 0.54 (*S. rosifolia*- *S. sclarea*, *S. rosifolia*- *S. limbata* and *S. staminea*- *S. verticillata*) ve 0.93 (*S. sclera*- *S. divaricata*) arasında değiştiği görülmüştür.

TARTIŞMA ve SONUÇ: Bu çalışmada, hem RAPD hem de ISSR belirteçlerinin *Salvia* türleri arasındaki genetik bağıntıları incelemek için kullanılabileceği gösterilmiştir.

Anahtar Kelimeler: *Salvia* türleri, Genetik çeşitlilik, ISSR, RAPD

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Introduction

Salvia L. represents the biggest genus of Lamiaceae family. Central and South America, Western Asia (Iran, Afghanistan, Turkey and Russia), Eastern Asia, Africa, and Europe are the main distribution regions of *Salvia* species. Western Asia is the second richest region with ca. 200 species of the genus after America with ca. 500 species [1, 2]. Turkey takes place among the important diversity centers of *Salvia* [1]. According to the Flora of Turkey there are 86 species [3]. According to recent studies, 100 *Salvia* species in total grow in Turkey. At the same time, 53% of them are endemic [2].

Species have been utilized as traditional medication for the mild sickness of the stomach and the common cold since ancient ages [4]. Moreover, their volatile oils are utilized as an anti-inflammatory agent and antiseptic, and at the same time, a pleasurable sensory feeling is produced by them in the mouth and throat [5, 6]. *Salvia* species have antibacterial, antifungal, antimycobacterial, cytotoxic, antitumor, cardiovascular, antifeedant and insecticidal effects. [7]. *Salvia* species have the following essential oil composition: α -pinene, β -pinene, 1,8-cineole, camphor, borneol, and β -thujone, thymol, caryophyllene, caryophyllene oxide and 1-octadecanol [5, 8]. Several *Salvia* species were also studied to investigate antioxidant and antibacterial properties [5, 7, 9, 10]. Different properties of *Salvia* species growing around Erzincan province were investigated with some studies. Polat et al. (2015) [11] used three endemic *Salvia* species in their study to investigate their micromorphological and anatomical characteristics. Ethnobotanical uses of some *Salvia* species were investigated in and around Erzincan by Korkmaz and Karakuş (2015) [12]. It has been confirmed that molecular markers are highly estimable for judicial, biodiversity and mapping practices. Sufficiently high polymorphism enables the component bands of the fingerprints to function as genetic markers, with the distinction and recombination of the markers, which are utilized for the building of genetic maps [13].

Various kinds of molecular markers, for example, random amplified polymorphic DNA (RAPD [14]), inter-simple sequence repeats (ISSR [15]) and amplified fragment length polymorphism (AFLP [16]), have been improved and utilized widely in the investigation of genetic relationship, germplasm management and genetic diversity, together with the developments experienced in plant molecular biology. Scientists extensively utilize ISSR, which takes place among the above-mentioned molecular markers, in different spheres of plant improvement due to its being simple and cost-effective [17]. Variation in species is also studied by RAPD [14].

In this study, it was also aimed to evaluate the genetic diversity present in 15 *Salvia* species, employing two marker systems.

Material and Methods

The material used

In the present study, the leaf specimens of 15 *Salvia* taxa from Turkey were examined. The following species were analyzed: *Salvia cryptantha* Montbret & Aucher *Salvia caespitosa* *Salvia candidissima* *Salvia nemorosa* L., *Salvia sclarea* L., *Salvia verticillata* L., *Salvia verticillata* L. subsp. *amasiaca* (Freyn & Bornm.) Bornm., *Salvia staminea* Montbret & Aucher ex Benth., *Salvia multicaulis* Vahl, *Salvia limbata* C.A. Mey., *Salvia aethiopis* L., *Salvia rosifolia* Sm., *Salvia virgata* Jacq., *Salvia pachystachya* Trauv., and *Salvia divaricata* Montbret & Aucher ex Benth. 11 examples of the Irano-Turanian elements, 1 of the Euro-Siberian, and 3 of the unknown region take place among the samples examined. Table 1 includes information on the phytogeographic regions, endemism, and record numbers of *Salvia* taxa.

In field studies, plant specimens gathered from various regions of Turkey represent the species. Scientific names of the plant specimens were determined with the help of Davis (1982) [18], Güner (2012)[19] after herbarium studies had been carried out. All of the taxa names were checked from the literature [20-21]. Furthermore, the identification of the phytogeographical regions and endemic taxa was performed. Irano-Turanian elements constitute the majority of the taxa (11 taxa). There were 4 endemic taxa (27%). Herbarium samples of all taxa were stored in the Herbarium of Erzincan University.

Chemicals

The DNA isolation of *Salvia* species was performed by the combination with CTAB isolation methods with minor changes [22]. Nanodrop (QIAGEN) was used for the determination of the quantity and purity of genomic DNA and 0.8% agarose gel electrophoresis was utilized against the known concentrations to prove this.

RAPD

Ten primers out of thirty-two were amplified polymorphic amplicons and used for the analyses of genetic diversity in RAPD-PCR reactions (Table.2). PCR amplifications were performed in SENSEQUEST Thermal Cycle in a total volume of 20 µl, 1× PCR buffer (without MgCl₂) 0.25-µM dNTP, 0.5 mM primer, 2.5 mM MgCl₂ and 1.5 U Taq DNA polymerase (BioLab M0267S). An initial denaturation at the temperature of 95°C for the period of 5 min, with the following 46 cycles at 94°C for the period of 1 min, at various annealing temperatures for all primers for the period of 1 min, 72°C for the period of 2 min, penultimate step of 15 min at the temperature of 72°C and a final extension of 10 min at the temperature of 4°C constituted the amplification profile. The PCR products (20ml) were blended with 6 gel loading buffer (3ml) and exposed to agarose. Then, electrophoresis was applied to separate them by means of 1% agarose gel in 0.5 × TBE buffer with 80 V constant voltages for the period of 150 min. The gels were dyed with Etd-Br visualized under the UV light and the gel visualization system was used to take the photographs of them.

ISSR

Fifteen primers were used for ISSR amplifications. Two primers of these (ISSR UBC844B and ISSR UBC822) did not give amplification in PCR reactions. Other thirteen primers were amplified polymorphic amplicons (Table 3). The PCR mixture (20µl) was prepared as follows: 2.0 µl of 10× PCR buffer, 0.5µl of dNTPs (10mM), 2µl of magnesium chloride (25 mM), 1.0 µl of primer (5 mM), 0.5 µl of polymerase enzyme (Taq) (250 unit), 13µl of distilled water, 1.0 µl of genomic DNA sample (100 ng/µl). An initial denaturation at the temperature of 94°C for the period of 4 min, with the following 35 cycles at 94°C for the period of 0.30 min, at various annealing temperatures for all primers for the period of 0:35 min, 72°C for the period of 1 min, penultimate step of 5 min at the temperature of 72°C and a final extension of 10 min at

the temperature of 4°C constituted the amplification profile. The PCR products (20ml) were blended with 6X gel loading buffer (3ml) and exposed to agarose. Then, electrophoresis was applied to separate them by means of 2% agarose gel in 0.5 × TBE buffer with 80 V constant voltage for the period of 150 min. The gels were dyed with Etd-Br visualized under the UV light and the gel visualization system was used to take the photographs of them.

Data analysis

The Total Lab TL120 program was utilized for the assessment of the ISSR and RAPD patterns. The scoring of PCR products was performed as presence (1) and absence (0) of bands. The Jaccard (1908) similarity index was calculated by using the data, and a dendrogram was performed based on the UPGMA (unweighted pair group method using arithmetic average) technique.

Results and discussion

First, screening of twenty-three RAPD primers was performed against *Salvia* species. 143 distinct reproducible bands in total with 10.2 bands on average per primer could be produced by ten primers. The products amplified varied between 183 and 2941 bp in size. All (100%) of the 143 bands acquired were polymorphic. Their division into four clusters was enabled by the construction of a dendrogram in accordance with the RAPD data of *Salvia* species (Fig.1).

The first cluster included *salvia cryptantha*, *S. candidissima*, *S. nemorosa*, *S. sclarea*, *S. verticillata*, *S. multicaulis*, *S. limbata*, *S. aethiopis*, *S. virgata*, *S. pachhystachya* and *S. divaricata*.

The second one included *S. caespitosa*.

The third one included *S. verticillata* subsp. *amasiaca* and *S. staminea*.

The fourth cluster included *S. rosifolia*.

The most significant likeness was identified between *S. rosifolia* and *S. caespitosa* species (0.50), while the most significant difference was determined between *S. divaricata* and *S. sclarea* (0.90).

According to the results of the RAPD data, it was observed that most of the species (seven from 11 species) in the first cluster were Irano-Turanian phytogeographic region elements of Turkey (Table 1; Figure 1). Both of the two most similar species (*S. rosifolia* and *S. caespitosa*) are Irano-Turanian phytogeographic region elements and endemic species.

To perform cultivar identification, thirteen ISSR primers, which demonstrated reproducible and polymorphic patterns, were selected and produced 160 bands (polymorphic) in total, with 12.3 bands on average per primer. There was a variation from 241 to 3483 base pair in size. The ISSR data of *Salvia* species were used to construct a dendrogram, which enabled their division into six clusters (Fig.2).

The first cluster included *Salvia cryptantha*, *S. candidissima*, *S. sclarea*, *S. multicaulis*, *S. limbata*, *S. aethiopis*, *S. virgata*, *S. pachhystachya*, *S. divaricata*.

The second one included *S. verticillata*.

The third cluster included *S. nemorosa*.

The fourth cluster included *S. verticillata* subsp. *amasiaca* and *S. staminea*.

The fifth cluster included *S. rosifolia*.

The sixth cluster included *S. caespitosa*.

The most significant likeness was identified between *S. caespitosa* and *S. divaricata* (0.47) species, while the most significant difference was determined between *S. divaricata* and *S. sclera* (0.95) species.

According to the results of the ISSR data, it was observed that most of the species (seven from nine species) in the first cluster were Irano-Turanian phytogeographic region elements of Turkey (Table 1; Figure 2). Both of the most similar two species (*S. divaricata* and *S. caespitosa*) are Irano-Turanian phytogeographic region elements and endemic species.

The construction of a dendrogram was performed according to the combined data acquired from the RAPD and ISSR marker analyses (Fig.3).

The first cluster included *Salvia cryptantha*, *S. candidissima*, *S. sclarea*, *S. multicaulis*, *S. limbata*, *S. aethiopis*, *S. virgate*, *S. pachystachya*, *S. divaricata*.

The second cluster included *S. verticillata*.

The third cluster included *S. nemorosa*.

The fourth cluster included *S. staminea* and *S. verticillata* subsp. *amasiaca*.

The fifth cluster included *S. caespitosa*.

The sixth cluster included *S. rosifolia*.

As a result of the combined analysis, it was determined that similarities of the species varied between 0.54 (*S. rosifolia*- *S. sclarea*, *S. rosifolia*- *S. limbata* and *S. staminea*- *S. verticillata*) and 0.93 (*S. sclera*- *S. divaricata*).

The most significant difference was determined between *S. sclera* and *S. divaricata* species in RAPD, ISSR and the combined data produced from RAPD and ISSR.

Differently from *S. sclera*, *S. divaricata* is an Irano-Turanian phytogeographic region element and endemic species of Turkey.

The descriptions of morphological, agronomic properties and isozyme examination have constituted for evaluating the relationship among *Salvia* species. Morphological examinations have a number of limitations, which can be eliminated by the usage of molecular markers, with good reproducibility and high sensitivity [23]. Molecular markers showing polymorphism at the level of DNA have been regarded to be an important instrument used to assess plant genetic diversity characterization [24, 25].

To characterize *Salvia* species, different types of molecular markers, for example, random amplified polymorphic-DNA (RAPD) inter-simple sequence repeat (ISSR) [26, 27], amplified fragment length polymorphisms (AFLP) [28, 29], simple sequence repeat (SSR) [28] and sequence-related amplified polymorphism (SRAP) [27], have been employed with a great success. Agar et al. [26] used randomly amplified polymorphic DNA (RAPD) profiles to study genetic relationships in eight *Salvia* taxa. In this study, they shown that RAPD profiles were useful method for determination of genetic profiles that can be used to identify *Salvia* species. In another study, Songet et al. [27] used simple sequence repeats (ISSR) and sequence-associated amplified polymorphism (SRAP) markers to assess the level of genetic diversity in *S. miltiorrhiza*. The results showed that these markers were effective and reliable in evaluating the degree of genetic variation of *S. miltiorrhiza*.

RAPD and ISSR represent an easy and effective marker system used to assess and determine genetic diversity among plant species. The ISSR technique has a number of benefits, such as combining the majority of the advantages of AFLP and SSR markers, providing higher reproducibility when compared to RAPD, identifying a higher rate of genomic polymorphisms when compared to RFLP, and being more cost-efficient when compared to AFLP [29,30, 31].

RAPD and ISSR markers were used to measure the genetic diversity among eight species of *Salvia* collected from different locations in Iran (Javan et al) [32]. In our study, RAPD and ISSR combined data obtained from marker assay showed that *S. limbata* and *S. aethiopis* species were in the same group just like in this study. *S. verticillata* and *S. nemorosa* species were also observed in separate groups. ISSR and RAPD markers revealed findings, which were almost independent of each other, among *Salvia* species. Therefore, an especially positive correlation was determined for the ISSR and RAPD analysis of genetic relations among *Salvia* species.

Conclusions

The findings show that two markers represent powerful instruments used to assess the genetic diversity and relations among *Salvia* species.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: Taxonomic information about studied *Salvia* species

N o	Taxon name	Record no.	Locality	Phyto-geographic region	Endemic
1	<i>S. cryptantha</i> Montbret & Aucher ex Benth.	Korkmaz 3649	Erzincan, Çayırılı	Ir.-Tur.	+
2	<i>S. caespitosa</i> Montbret & Aucher ex Benth.	Korkmaz 3891	Erzincan, Çayırılı	Ir.-Tur.	+
3	<i>S. candidissima</i> Vahl	Korkmaz 2887; 3896	Erzincan, Çayırılı	Ir.-Tur.	-
4	<i>S. nemorosa</i> L.	Korkmaz 2805	Erzincan, Çayırılı	-	-
5	<i>S. sclarea</i> L.	Korkmaz 2795	Erzincan, Çayırılı	-	-
6	<i>S. verticillata</i> L.	Korkmaz 2994; 2792	Erzincan, Çayırılı	Eur.-Sib.	-
7	<i>S. verticillata</i> L. subsp. <i>amasiaca</i> (Freyn & Bornm.) Bornm.	Korkmaz 107	Erzincan, Çayırılı	Ir.-Tur.	-
8	<i>S. staminea</i> Montbret & Aucher ex Benth.	Korkmaz 3858	Erzincan, Çayırılı	Ir.-Tur.	-
9	<i>S. multicaulis</i> Vahl	Korkmaz 3516	Erzincan, Çayırılı	Ir.-Tur.	-
10	<i>S. limbata</i> C.A. Mey.	Korkmaz 2992	Erzincan, Çayırılı	Ir.-Tur.	-
11	<i>S. aethiopsis</i> L.	Korkmaz 3221	Erzincan, Çayırılı	-	-
12	<i>S. rosifolia</i> Sm.	Korkmaz 3268	Erzincan, Çayırılı	Ir.-Tur.	+
13	<i>S. virgata</i> Jacq.	Korkmaz 3217	Erzincan, Çayırılı	Ir.-Tur.	-
14	<i>S. pachystachya</i> Trautv.	Korkmaz 2228	Erzincan, Ergen Mountain	Ir.-Tur.	-
15	<i>S. divaricata</i> Montbret & Aucher ex Benth.	Korkmaz 2641	Erzincan, Ergen Mountain	Ir.-Tur.	+

Table 2: Used primers and sequences in RAPD amplification

RAPD Primers	Sequence (5'-3')	Length of amplified bands	No. of bands	No. of monomorphic bands	No. of polymorphic bands	Polymorphism ratio (%)
OPA-1	CAGGCCCTTC	2238-444	15	0	15	100
OPA-2	TGCCGAGCTG	2941-433	16	0	16	100
OPA-4	TGCCGAGCTG	1649-400	12	0	12	100
OPA-6	AATCGGGCTG	2088-600	11	0	11	100
OPB-8	CTGCTGGGAC	2439-419	18	0	18	100
OPB-10	GGTCCCTGAC	1500-232	11	0	11	100
OPH-18	GTCCACACGG	958-183	8	0	8	100
OPW-8	GAATCGGCCA	1945-566	13	0	13	100
OPY-6	GACTGCCTCT	1873-353	15	0	15	100
OPY-16	AAGGCTCACC	1174-200	10	0	10	100
OPH-17	CACTCTCTCTC	1790-351	14	0	14	100

Table 3: Used primers and sequences in ISSR amplification

ISSR Primers	Sequence (5'-3')	Length of amplified bands	No. of bands	No. of monomorphic bands	No. of polymorphic bands	Polymorphism ratio (%)
ISSR UBC811	GAGAGAGAGAGAGAGAC	1935-276	15	0	15	100
ISSR UBC815	CTCTCTCTCTCTCTG	1246-408	11	0	11	100
ISSR UBC826	ACACACACACACACC	936-362	7	0	7	100
ISSR UBC840	GAGAGAGAGAGAGAGATT	1303-241	12	0	12	100
ISSR UBC844A	CTCTCTCTCTCTCTG	1592-378	13	0	13	100
ISSR UBC845	CTCTCTCTCTCTCTTT	2878-391	18	0	18	100
ISSR UBC852	TCTTCTCTCTCTCTCAA	1432-592	7	0	7	100
ISSR 8081	GAGAGAGAGAGAGAGAC	2040-303	14	0	14	100
ISSR 8082	CTCTCTCTCTCTCTCTG	3483-441	14	0	14	100
ISSR 17889A	GTGTGTGTGTGTCC	1268-337	17	0	17	100
ISSR HB12	CAGCAGCAGGC	1392-408	10	0	10	100
ISSR HBS10	GAGAGAGAGAGACC	1362-344	11	0	11	100
ISSR UBC834	AGAGAGAGAGAGAGATT	1442-458	11	0	11	100

Figure 1: RAPD marker-based UPGMA clustering for *Salvia* species

1. *Salvia cryptantha*, 2. *S. caespitosa*, 3. *S. candidissima*, 4. *S. nemorosa*, 5. *S. sclarea*, 6. *S. verticillata*, 7. *S. verticillata* subsp. *amasiaca*, 8. *S. staminea*, 9. *S. multicaulis*, 10. *S. limbata*, 11. *S. aethiopis*, 12. *S. rosifolia*, 13. *S. virgata*, 14. *S. pachystachya*, 15. *S. divaricata*.

Figure 2: ISSR marker-based UPGMA clustering for *Salvia* species

1. *Salvia cryptantha*, 2. *S. caespitosa*, 3. *S. candidissima*, 4. *S. nemorosa*, 5. *S. sclarea*, 6. *S. verticillata*, 7. *S. verticillata* subsp. *amasiaca*, 8. *S. staminea*, 9. *S. multicaulis*, 10. *S. limbata*, 11. *S. aethiopis*, 12. *S. rosifolia*, 13. *S. virgata*, 14. *S. pachystachya*, 15. *S. divaricata*.

Figure 3: RAPD-ISSR marker-based UPGMA clustering for *Salvia* species

1. *Salvia cryptantha*, 2. *S. caespitosa*, 3. *S. candidissima*, 4. *S. nemorosa*, 5. *S. sclarea*, 6. *S. verticillata*, 7. *S. verticillata* subsp. *amasiaca*, 8. *S. staminea*, 9. *S. multicaulis*, 10. *S. limbata*, 11. *S. aethiopsis*, 12. *S. rosifolia*, 13. *S. virgata*, 14. *S. pachystachya*, 15. *S. divaricata*.