Determination of the Genetic Relationships Among Salvia Species by RAPD and ISSR Analyses

Salvia Türleri Arasındaki Genetik İlişkilerin RAPD ve ISSR Analizleriyle Belirlenmesi

OBJECTIVES: Salvia L. is the largest genus of the family Lamiaceae, 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. The purpose of this study was to investigate the genetic relationships among 15 Salvia species that grow in wild conditions in Turkey's Eastern Anatolia region.

MATERIALS AND METHODS: The genetic relationships among 15 Salvia species were investigated using inter-simple sequence repeat (ISSR) and random amplified polymorphic-DNA (RAPD) profiles in the present study. Thirteen ISSR primers and 11 RAPD primers were utilized. The ISSR and RAPD data were combined to construct the unweighted pair group method using arithmetic average cluster.

RESULTS: Based on the RAPD and ISSR data, the Salvia species were classified into six groups. As a result of the combined analysis, it was shown that similarities between 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).

CONCLUSION: The findings show that the two markers represent powerful instruments for assessing the genetic diversity and relationships among Salvia species.

KEY WORDS: Salvia species, genetic diversity, ISSR, RAPD

ÖZ

Amaç: Salvia L., yaklaşık 1000 tür içeren Lamiaceae familyasının en büyük cinsidir. Son çalışmalarla göre, Türkiye’de toplam 100 Salvia türü yetişmektedir. Ayrıca zamanda, türlerin %53’ü endemiktir. Bu çalışmada, Türkiye’den Doğu Anadolu Bölgesi’nde doğal olarak yetişen 15 Salvia türü arasındaki genetik çeşitliliği araştırılmıştır.

Gereç ve Yöntemler: Çalışmada, Salvia türlerindeki genetik çeşitlilik, İnter-Simple Sequence Repeat (ISSR) ve Random Amplified Polymorphic-DNA (RAPD) analizleri kullanılarak incelenmiştir. Aritmetik ortalamayı kullanarak, RAPD ve ISSR sonuçlarının birleştirilmesi için birbirlerine ait tüm verilerin analizi yapılmıştır.

Bulgular: ISSR ve RAPD analizleri, Salvia türlerin genetik çeşitliliğini, doyurucu bir şekilde ortaya koydu. Çalışmada, RAPD ve ISSR sonuçlarının birleştirilmesiyle, Salvia türlerinin genetik çeşitliliği araştırılırken kullanılabileceğini gösterilmiştir.

Anahtar kelimeler: Salvia türleri, genetik çeşitlilik, ISSR, RAPD

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INTRODUCTION

Salvia L. is the largest genus of the family Lamiaceae. 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 is 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 times.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, α-thujone, thymol, caryophyllene, caryophyllene oxide, and 1-octadecanol.5,8 Several Salvia species were also studied to investigate their antioxidant and antibacterial properties.5,7,9,10 Different properties of Salvia species growing around Erzincan Province were investigated in previous studies. Polat et al.11 investigated the micromorphological and anatomical characteristics of three endemic Salvia species. The ethnobotanical uses of some Salvia species were investigated in and around Erzincan by Korkmaz and Karakuş.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 (ISSRs),15 and amplified fragment length polymorphism (AFLP),16 have been improved and utilized widely in the investigation of genetic relationships, germplasm management, and genetic diversity, together with the developments achieved in plant molecular biology. Scientists extensively utilize ISSRs, which take place among the above-mentioned molecular markers, in different spheres of plant improvement due to their being simple and cost effective.17 Variation in species is also studied by RAPD.14 The aim of the present study was to evaluate the genetic diversity present in 15 Salvia species, employing two marker systems.

MATERIALS AND METHODS

The material used

Leaf specimens of 15 Salvia taxa from Turkey were examined. The following species were analyzed: S. cryptantha Montbret and Aucher, S. caespitosa, S. candidissima, S. nemorosa L., S. sclarea L., S. verticillata L., S. verticillata L. subsp. amasiaca (Freyn & Bornm.) Bornm., S. staminea Montbret and Aucher ex Benth., S. multicaulis Vahl., S. limbata C.A.Mey., S. æthiopis L., S. rosifolia Sm., S. virgata Jacq., S. pachystachya Trauv., and S. divaricata Montbret and Aucher ex Benth. Eleven examples of Irano-Turanian elements, 1 Euro-Siberian, and 3 of unknown region were among the samples examined. Table 1 includes information on the phytogeographical 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 Davis18 and Güner19 after herbarium studies had been carried out. All of the taxon names were checked in the literature.20,21 Furthermore, 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 deposited at the Herbarium of Erzincan University.

Chemicals

The DNA isolation of Salvia species was performed by the combination of cetyltrimethyl ammonium bromide 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.

Random amplified polymorphic DNA

Eleven primers out of 23 were amplified polymorphic amplicons and used for the analyses of genetic diversity in RAPD-polymerase chain reactions (PCR) (Table 2). PCR amplifications were performed in SENSEQUEST Thermal Cycle in a total volume of 20 µL, 1X PCR buffer (without MgCl2) 0.25-µM deoxyribonucleoside triphosphate (dNTP), 0.5 mM primmer, 2.5 mM MgCl2, and 1.5 U Tag DNA polymerase (BioLab M0267S). Initial denaturation at 95 °C for 5 min with the following 46 cycles at 94 °C for 1 min at various annealing temperatures for all primers for 1 min, 72 °C for 2 min, a penultimate step of 15 min at 72 °C, and a final extension of 10 min at 4 °C constituted the amplification profile. The PCR products (20 µL) were blended with 6X gel loading buffer (3 mL) and exposed to agarose. Then electrophoresis was applied to separate them by means of 1% agarose gel in 0.5X tris, borate, and EDTA (TBE) buffer with 80 V constant voltages for 150 min. The gels were dyed with Etde-Br visualized under ultraviolet light and the gel visualization system was used to take photographs of them.

Inter-simple sequence repeats

Fifteen primers were used for ISSR amplifications. Two primers of these (ISSR UBC844B and ISSR UBC822) did not give amplification in PCR reactions. The other 13 primers were amplified polymorphic amplicons (Table 3). The PCR mixture (20 µL) was prepared as follows: 2.0 µL of 10X PCR buffer, 0.5 µL of dNTPs (10 mM), 2 µL of magnesium chloride (25 mM), 1.0 µL of primer (5 mM), 0.5 µL of polymerase enzyme (Taq)
(250 units), 13 µL of distilled water, and 1.0 µL of genomic DNA sample (100 ng/µL). Initial denaturation at 94 °C for 4 min with the following 35 cycles at 94 °C for 0.30 min, at various annealing temperatures for all primers for 0.35 min, 72 °C for 1 min, a penultimate step of 5 min at 72 °C, and a final extension of 10 min at 4 °C constituted the amplification profile. The PCR products (20 mL) were blended with 6X gel loading buffer (3 mL) and exposed to agarose. Then electrophoresis was applied to separate them by means of 2% agarose gel in 0.5X TBE buffer with 80 V constant voltage for 150 min. The gels were dyed with Etd-Br visualized under UV light and the gel visualization system was used to take photographs of them.
Statistical 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 created based on the unweighted pair group method using arithmetic average.

RESULTS AND DISCUSSION

First, screening of 23 RAPD primers was performed against Salvia species, and 143 distinct reproducible bands in total with 10.2 bands on average per primer were produced by 11 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 (Figure 1).

The first cluster included S. cryptantha, S. candidissima, S. nemorosa, S. sclarea, S. verticillata, S. multicaulis, S. limbata, S. aethiopis, S. virgata, S. pachystachya, 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 (0.50), while the most significant difference was determined between S. divaricata and S. sclarea (0.90).

According to the RAPD data, most of the species (seven from 11 species) in the first cluster were Irano-Turanian phytogeographical region elements of Turkey (Table 1; Figure 1).

To perform cultivar identification, 13 ISSR primers that 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 pairs in size. The ISSR data of Salvia species were used to construct a dendrogram, which enabled their division into six clusters (Figure 2).

The first cluster included S. cryptha, S. candidissima, S. nemorosa, S. sclarea, S. verticillata, S. multicaulis, S. limbata, S. aethiopis, S. virgata, S. pachystachya, and S. divaricata.

The second one included S. caespitosa.

The third cluster included S. verticillata subsp. amasiaca and S. staminea.

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

The fifth cluster included S. rosifolia.

Table 3. Primers and sequences used 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  | CTCTCTCTCTCTCTCTG | 1246-408                  | 11           | 0                        | 11                       | 100                    |
| ISSR UBC826  | ACACACACACACACACC| 936-362                   | 7            | 0                        | 7                        | 100                    |
| ISSR UBC840  | GAGAGAGAGAGAGAGATT| 1303-241                  | 12           | 0                        | 12                       | 100                    |
| ISSR UBC844A | CTCTCTCTCTCTCTCTG | 1592-378                  | 13           | 0                        | 13                       | 100                    |
| ISSR UBC845  | CTCTCTCTCTCTCTT   | 2878-391                  | 18           | 0                        | 18                       | 100                    |
| ISSR UBC852  | TCTCTCTCTCTCTCTCA | 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    | CACGACGACGCC     | 1392-408                  | 10           | 0                        | 10                       | 100                    |
| ISSR HBS10   | GAGAGAGAGAGACC   | 1362-344                  | 11           | 0                        | 11                       | 100                    |
| ISSR UBC834  | AGAGAGAGAGAGAGATT| 1442-458                  | 11           | 0                        | 11                       | 100                    |

ISSR: Inter-simple sequence repeat

The first cluster included S. cryptantha, S. candidissima, S. nemorosa, S. sclarea, S. verticillata, S. multicaulis, S. limbata, S. aethiopis, S. virgata, S. pachystachya, 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 (0.50), while the most significant difference was determined between S. divaricata and S. sclarea (0.90).

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

To perform cultivar identification, 13 ISSR primers that 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 pairs in size. The ISSR data of Salvia species were used to construct a dendrogram, which enabled their division into six clusters (Figure 2).

The first cluster included S. cryptantha, S. candidissima, S. nemorosa, S. sclarea, S. verticillata, S. multicaulis, S. limbata, S. aethiopis, S. virgata, S. pachystachya, and 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), while the most significant difference was determined between *S. divaricata* and *S. sclera* (0.95).

According to the ISSR data, most of the species (seven from nine species) in the first cluster were Irano-Turanian phytogeographical region elements of Turkey (Table 1; Figure 2). Both of the most similar species (*S. divaricata* and *S. caespitosa*) are Irano-Turanian phytogeographical region elements and endemic species. The first cluster included *S. cryptantha*, *S. candidissima*, *S. sclera*, *S. multicaulis*, *S. limbata*, *S. aethiops*, *S. virgate*, *S. pachyystachya*, and *S. divaricata*.
The second cluster included *S. verticillata*.
The third cluster included *S. nemorosa*.
The fourth cluster included *S. staminea* and *S. verticillata* subsp. *amosica*.

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. sclera*, *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* in RAPD, ISSR, and the combined data produced from RAPD and ISSR. Differently from *S. sclera*, *S. divaricata* is an Irano-Turanian phytogeographical region element and endemic species of Turkey.
The descriptions of morphological and agronomic properties and isozyme examination were constituted for evaluating the relationship among *Salvia* species. Morphological examinations have a number of limitations, which can be eliminated by the use of molecular markers with good reproducibility and high sensitivity. Molecular markers showing polymorphism at the level of DNA have been regarded as an important instrument used to assess plant genetic diversity characterization.

To characterize *Salvia* species, different types of molecular markers, for example, RAPD, ISSR, AFLP, SSR, and sequence-related amplified polymorphism (SRAP), have been employed with great success. Agar et al. used RAPD profiles to study genetic relationships in eight *Salvia* taxa. They showed that RAPD profiles were useful for the determination of genetic profiles that can be used to identify *Salvia* species. In another study, Song et al. used ISSR and 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 in *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.

RAPD and ISSR markers were used to measure the genetic diversity among eight species of *Salvia* collected from different locations in Iran. In our study, RAPD and ISSR combined data obtained from marker assays showed that *S. limbata* and *S. aethiops* were in the same group just like in that study. *S. verticillata* and *S. nemorosa* species were also observed in separate groups.

ISSR and RAPD markers revealed findings that 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.

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