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Research article/Araştırma makalesi

Determination of genetic relationships between some endemic *Salvia* species using RAPD markers

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Abstract

In this study, randomly amplified polymorphic DNA (RAPD) analysis was used to determine genetic relationship among four endemic *Salvia* species. A total of 15 primers were screened, among them 7 primers produced polymorphic and reproducible bands. Eighty one DNA bands were amplified, among which 80 bands (98.7%) were polymorphic. The number of bands amplified per primer varied from 7 to 14. Similarity coefficients were calculated and a dendrogram was constructed by using UPGMA algorithm. In dendrogram, the four *Salvia* species were divided into two major clusters. Cluster I included two subclusters, one containing all accessions of *S. wiedemannii* and the other one containing accessions of *S. tchihatcheffii*. Cluster II was also separated into two subclusters. The first subcluster contained all accessions belonging to *S. crypthanta* and the second subcluster contained *S. cyanescens*. The genetic relationships estimated by the RAPD analysis are basically in agreement with morphological data. Thus, RAPD technique is a reliable marker system that can be used to study genetic relationship in the genus *Salvia*.

Key words: *Salvia*, Genetic relationship, RAPD, PCR, Endemic plant

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Bazı endemik *Salvia* türleri arasındaki genetik akrabalığın RAPD belirteçleri ile saptanması

Özet

Bu çalışmada, rastgele çoğaltılmış polimorfik DNA (RAPD) analizi 4 endemik *Salvia* türü arasındaki genetik akrabalığı belirlemek amacıyla kullanılmıştır. Toplamda 15 primer denenmiş bunlardan 7'si polimorfik ve tekrar çoğaltılabilen bantlar oluşturmuştur. Çoğaltılan 81 banttan 80'i (%98.7) polimorfiktir. Primer başına çoğaltılan bant sayısı 7 ila 14 arasında değişmiştir. Benzerlik katsayısı hesaplanarak UPGMA işlemi ile dendrogram oluşturulmuştur. Bu dendrogramda 4 *Salvia* türü iki ana kümeye ayrılmıştır. Birinci küme iki alt kümeye ayrılarak ilki *S. wiedemannii'*ye ait bireyleri, diğer alt küme ise *S. tchihatcheffii*'ye ait bireyleri içermiştir. İkinci küme de iki alt kümeye ayrılmıştır. İlk alt küme *S. crypthanta*'ya ait bireyleri içerirken ikinci alt küme *S. cyanescens*'i içermiştir. RAPD analizi ile belirlenen bu genetik ilişki temelde morfolojik verilerle uyum göstermektedir. Dolayısıyla, RAPD tekniği *Salvia* cinsi içerisinde genetik akrabalık çalışmalarında kullanılabilir, güvenilir bir belirteç sistemidir.

Anahtar kelimeler: *Salvia*, genetik ilişki, RAPD, PCR, endemik bitki

1. Introduction

Salvia, with over 900 species from both the Old and New World, is the largest genus in the Lamiaceae (Walker et al., 2004). In Turkey, *Salvia* genus is represented by 89 species and 94 taxa and about 50% of which are endemic (Hedge, 1982). Many species of the Lamiaceae are aromatic and are often used as herbs, herbal tea, spices, folk medicines, antioxidant, and fragrances. All of these properties make the *Salvia* very important in the food and drug industry. In addition, *Salvia* species are grown in parks and gardens as ornamental plants (Nakipoglu, 1993).

Information about genetic diversity is important not only for the study of the flora, but also to elaborate strategies of conservation and rational use of genetic resources. Despite its economical importance, *Salvia* has been poorly studied genetically. DNA markers are considered the best tools for determining genetic relationships, as they are unlimited in number, show high polymorphism and are independent of environmental interaction. Several marker

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systems have been developed for DNA fingerprinting such as restriction fragment length polymorphism (RFLP) (Botstein et al. 1980), simple sequence repeats (SSRs) (Tautz 1989), randomly amplified polymorphic DNAs (RAPD) (Williams et al. 1990), inter simple sequence repeats (ISSR; Zietkiewicz *et al*., 1994), and amplified fragment length polymorphism (AFLP; Vos *et al*., 1995). Among these, RAPD is an inexpensive and rapid method not requiring any information regarding the genome, and has been widely used to study the genetic diversity in several plants (Belaj et al. 2001; Deshwall et al. 2005). Within the Lamiaceae family, RAPD markers were used to study intraspecific variations in several aromatic and medicinal species such as *Salvia fruticosa* Mill. (Skoula et al. 1999), *Thymus vulgaris* L. (Echeverrigaray et al. 2001), and *Cunila galioides* (Fracaro et al. 2005).

In this study, we have focused on four *Salvia* species, *Salvia cryptantha*, *S. cyanescens*, *S. tchihatcheffii* and *S. wiedemannii*, which are all endemic, and they were recorded as either near threatened (*S. tchihatcheffii*) or least concern in Turkish Red Data Book (Ekim et al. 2000). Until today, no molecular study was performed on these four *Salvia* species, there are only few reports about their chemical composition (Başer, 1995; 2000; Topçu ve ark. 1997) and seed germination characteristics (Yücel and Yılmaz, 2009; Yücel, 2000). Based on their potential as aromatic and medicinal plants, the objective of the present study was to examine genetic relationships among these endemic *Salvia* species growing in Turkey by using RAPD markers.

2. Materials and methods

2.1. Plant material

Four endemic *Salvia* species (*Salvia crypthanta*, *S. cyanescens*, *S. tchihatcheffii* and *S. wiedemannii*) were used in this study. Fresh leaves and whole plant samples were collected at different locations in Eskişehir (Table 1) between June 2002 and July 2003. The sampled leaves were kept at 0 °C in plastic bags during field work and then stored at -20 °C until DNA extraction. Herbarium specimens were taxonomically characterized and deposited at the Herbarium of Anadolu University (ANES).

Table 1. List of *Salvia* genotypes and details of collection sites

Species	**Population No.**	**Sample size**	**Locality**	**Latitude, longitude, altitude**
Salvia crypthanta	1	10	**402** Eskişehir, Alpu, Doğanoğlu village, Karasakal cemetery	South, N 39° 49’ 110”, E 31° 09’ 575”, 850 m.
	2	1	**406** Eskişehir, Mihalıççık, Ahırköy	West, N 39° 47’ 121”, E 31° 31’ 933”, 848 m.
	3	9	**408** Eskişehir, Mihalıççık, Üçbaşlı village	East, N 39° 47’ 668”, E 31° 39’ 629”, 942 m.
	4	1	**413** Eskişehir, Akkaya village	East, N 39° 37’ 592”, E 30° 23’ 352, 857 m.
	5	5	**414** Eskişehir, Gökçekısık village	East, N 39° 40’ 358”, E 30° 24’ 006”, 851 m.
Salvia tchihatcheffii	1	1	**402** Eskişehir, Alpu, Doğanoğlu village, Karasakal cemetery	South, N 39° 49’ 110”, E 31° 09’ 575”, 850 m.
	2	10	**406** Eskişehir, Mihalıççık, Ahırköy	West, N 39° 47’ 121”, E 31° 31’ 933”, 848 m.
	3	10	**407** Eskişehir, Mihalıççık, Üçbaşlı village	East, N 39° 46’ 424”, E 31° 39’ 392”, 848 m.
	4	5	**414** Eskişehir, Gökçekısık village	East, N 39° 40’ 358”, E 30° 24’ 006”, 851 m.
	5	2	**415** Eskişehir, Musaözü	North, N 39° 41’ 316”, E 30° 18’ 446”, 938 m.
Salvia wiedemannii	1	10	**402** Eskişehir, Alpu, Doğanoğlu village, Karasakal cemetery	South, N 39° 49’ 110”, E 31° 09’ 575”, 850 m.
	2	10	**409** Eskişehir, Mihalıççık, Üçbaşlı village, Kızılbel	North, N 39° 45’ 550”, E 31° 37’ 525”, 762 m.
	3	5	**413** Eskişehir, Akkaya village	East, N 39° 37’ 592”, E 30° 23’ 352”, 857 m.
	4	5	**416** Eskişehir, Akçayır village	North, N 39° 44’ 159”, E 30° 23’ 225”, 918 m.
S. cyanescens	1	1	Eskişehir-Ankara highway 40.km.	

2.2. DNA isolation and RAPD-PCR

Genomic DNA was extracted from 0.5 g powdered leaf tissue by using Genomic DNA Purification Kit (Fermentas, Maryland, USA). The quantity and quality of DNA were determined with a Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA). DNA samples were diluted to 5µl with DNase-RNase free water (AppliChem, Darmstadt, Germany) and stored at -20°C.

The decamer oligonucleotide primers (Kit AB) used for PCR amplification were purchased from Operon Technologies (Alamada, USA) (Table 2). PCR amplifications were performed in a 25 µl reaction mixture containing 15 ng of template DNA, 1X Taq polymerase buffer and 1 U of Taq polymerase (Fermentas, Maryland, USA), and 2.5 mM $MgCl_2$, 1 mM dNTP, 1 µM primer. Amplifications were carried out in a Progene Thermal Cycler (Techne Inc., Burlington, USA). The PCR amplification cycle was programmed for an initial denaturation step of 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 36 °C for 30 s, 72 °C for 2 min, and a final elongation at 72 °C for 7 min. The PCR products were separated on a 1.5 % agarose gel containing ethidium bromide (0.5 µg/ml) and digitally photographed with the UVIpro gel documentation system (UVItec, Cambridge, UK). A negative control with no DNA template was also included in each PCR amplification in order to verify the absence of contamination. Duplicate amplifications were conducted for each RAPD primer in order to ensure reproducible results.

2.3. Data Analysis

The presence (1) or absence (0) of the amplified bands were scored for each primer. Faintly stained bands were not considered. The genetic similarity between *Salvia* species was calculated according to the Jaccard's coefficient (Jaccard, 1908) and a dendrogram was generated by Unweighted Pair Group Method with Arithmetic Mean (UPGMA; Sneath & Sokal, 1973) and the SAHN clustering analysis. All analyses were done using the NTSYS-pc 2.01 software package (Rohlf, 1998).

Table 2. List of RAPD primers used in this study

Code	**5'-3' Nucleotide sequence**	**Annealing temperature (°C)**
AB-01	CCGTCGGTAG	34
AB-02	GGAAACCCCT	32
AB-03	TGGCGCACAC	34
AB-04	GGCACGCGTT	34
AB-05	CCCGAAGCGA	34
AB-06	GTGGCTTGGA	32
AB-07	GTAAACCGCC	32
AB-08	GTTACGGACC	32
AB-09	GGGCGACTAC	34
AB-10	TTCCCTCCCA	32
AB-11	GTGCGCAATG	32
AB-12	CCTGTACCGA	32
AB-14	AAGTGCGACC	32
AB-15	CCTCCTTCTC	32
AB-16	CCCGGATGGT	34
AB-17	TCGCATCCAG	32
AB-18	CTGGCGTGTC	34
AB-20	CTTCTCGGAC	32

3. Results

As an initial step, a total of 15 arbitrary 10-mer primers was screened. All primers amplified *Salvia* DNA, but only 7 of them gave consistently reproducible banding patterns among samples (Table 2). A total of 81 bands were scored of which 80 (98.7%) were polymorphic. Six primers (OPAB 2, 3, 5, 7, 9, 11 and 14) amplified 100% polymorphic bands. Of the total bands generated, only one was found to be monomorphic across all genotypes (OPAB-9, 450 bp). The number of bands amplified per primer varied from 7 to 14, a minimum of 7 bands were generated by the primer OPAB 2 while the maximum of 14 bands were amplified with primer OPAB 9. The size of the amplified products varied from 200 to 2400 bp. RAPD profiles of a representative primer OPAB 9 are shown in Fig 1.

Pairwise genetic similarities (Table 3) generated using Jaccard's similarity coefficient ranged from as low as 0.130 between populations of *S. tchihatcheffii* and *S. crypthanta*, to as high as 0.667 between two populations of *S. wiedemannii*. At the interspecific level, the highest similarity value was 0.400 between *S. wiedemanii* and *S. crypthanta*, while the lowest value was 0.130 between *S. tchihatcheffii* and *S. crypthanta*.

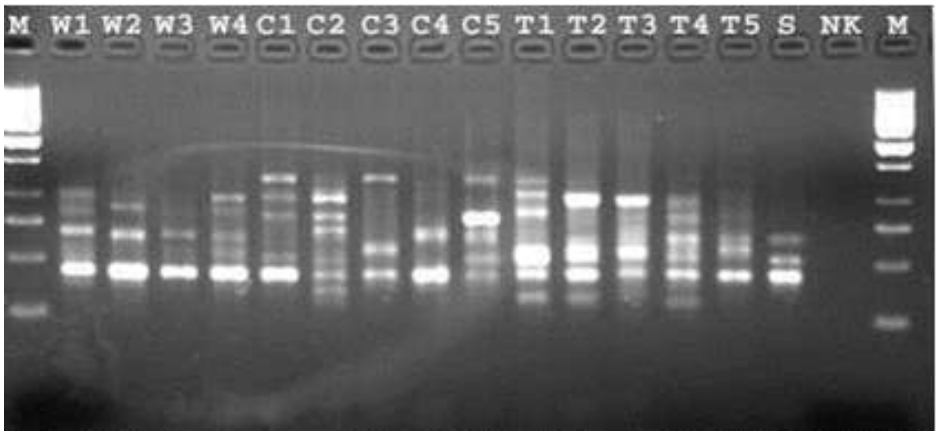

Figure 1. RAPD band profile produced with OPAB-9 primer
M, 1kb DNA ladder (Fermentas), W1-4, *S. wiedemannii*; C1-5, *S. cryptantha*; T1-5, *S. tchihatcheffii*; S, *S. cyanescens*, NK, negative control.

The mean genetic similarity was highest among *S. wiedemannii* at 0.527±0.1, followed by *S. tchihatcheffii* at 0.447±0.09, and *S. crypthanta* at 0.395±0.08. Since only a single accession was used, the mean genetic similarity value for *S. cyanescence* could not be obtained.

Table 3. Jaccard similarity estimates among *Salvia* species computed from RAPD band profiles

	SW1	SW2	SW3	SW4	SC1	SC2	SC3	SC4	SC5	ST1	ST2	ST3	ST4	ST5	SC
SW1	1.000														
SW2	0.667	1.000													
SW3	0.476	0.600	1.000												
SW4	0.461	0.560	0.400	1.000											
SC1	0.400	0.303	0.345	0.278	1.000										
SC2	0.242	0.200	0.226	0.184	0.324	1.000									
SC3	0.267	0.258	0.250	0.235	0.437	0.323	1.000								
SC4	0.226	0.258	0.296	0.167	0.394	0.323	0.400	1.000							
SC5	0.258	0.212	0.286	0.162	0.516	0.353	0.344	0.536	1.000						
ST1	0.216	0.243	0.235	0.256	0.233	0.182	0.139	0.167	0.250	1.000					
ST2	0.244	0.268	0.231	0.279	0.255	0.289	0.196	0.196	0.273	0.512	1.000				
ST3	0.343	0.371	0.257	0.308	0.250	0.286	0.159	0.186	0.182	0.381	0.600	1.000			
ST4	0.297	0.290	0.216	0.238	0.273	0.250	0.130	0.209	0.233	0.372	0.354	0.356	1.000		
ST5	0.237	0.333	0.294	0.308	0.250	0.174	0.186	0.244	0.182	0.381	0.454	0.538	0.525	1.000	
SC	0.243	0.237	0.265	0.250	0.227	0.204	0.282	0.219	0.244	0.212	0.286	0.229	0.277	0.255	1.000

UPGMA cluster analysis of the genetic similarity values generated a dendrogram illustrating the overall genetic relationships between the species studied and the accessions within those species (Fig. 2). UPGMA dendrogram revealed two distinct clusters, the first cluster was further divided into two subclusters, one comprising all accessions of *S. wiedemannii* and the other one contained all accessions belonging to *S. tchihatcheffii*. The second cluster was also separated into two subclusters. The first subcluster included all accessions belonging to *S. crypthanta* and the second subcluster contained *S. cyanescens*.

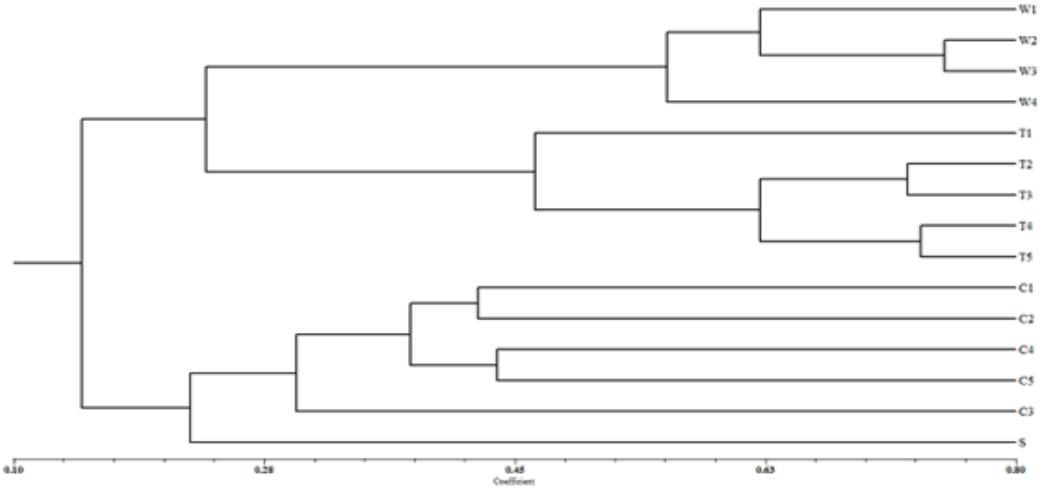

Figure 2. UPGMA dendrogram of 15 *Salvia* accessions generated by using Jaccard similarity coefficient based on RAPD analysis. W: *S. wiedemanniana*, T: *S. tchihatcheffii*, C: *S. crypthanta*, S: *S. cyanescens*

4. Conclusions

RAPD polymorphism detected in four endemic *Salvia* species revealed a high level of variability suggesting that RAPD technique is an efficient approach for genetic diversity studies at inter-specific level. This technique can quickly and cost-effectively generate markers for species without genomic sequence information. Despite of some limitations such as low reproducibility and dominance, the technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationship in several plant genera (Li et al., 1999; Kapteyn et al., 2002; Agostini et al., 2008; Ogunkanmi et al., 2010).

In this study, the polymorphism level detected between four *Salvia* species was 98.7%. This result emphasizes the significant genotypic divergences among the species studied. However, when a single species was considered, the percentage of polymorphic bands was 83.9% for *S. tchihatcheffii*, 87.6 % for *S. wiedemanii*, 93.8% for *S. crypthanta*. This percentage of polymorphism is higher than those previously reported at the intraspecific level for other medicinal and aromatic species of the Lamiaceae family: 63.8 and 59.8% for thyme (Echeverrigaray et al., 2001) and dittany (Fracaro et al., 2005), respectively. Genetic relationships between commercial cultivars and Brazilian accessions of *Salvia officinalis* L. were also estimated by using RAPD markers (Echeverrigaray and Agostini, 2006). It was found that in *S. sclarea* 73% of the bands were polymorphic. However, in *S. officinalis* the percentage of polymorphic bands was 59.5 %. A wide genetic variation was also obtained among some other *Salvia* species: balsamic sage (*Salvia tomentosa* Mill.), European sage (*Salvia sclarea* L.), meadow sage (*Salvia virgata* Jacq.), Anatolian sage (*Salvia fruticosa* Mill.) and Turkish sage (*Salvia dichroantha* Stapf.) using combined PCR-RFLP and DAMD-PCR techniques (Karaca et al., 2008).

Cluster analysis indicated that all 15 samples could be distinguished by RAPD markers. In UPGMA dendrogram the first cluster included accessions of *S. wiedemannii* and *S. tchihatcheffii* in different subclusters. On the other hand, the first subcluster of second cluster contained all accessions of *S. crypthanta*, while the second subcluster contained *S. cyanescence*. These results were concordant with morphological data. For example, in flora of Turkey *S. tchihatcheffii* and *S. wiedemannii* were grouped together (Hedge, 1982). They both show suffruticose stems and linear-oblong or shortly obovate-cuneate terminal leaflet characteristics. Moreover, these two species have another common morphological characters; their calyces are expanded in fruit and not membranous-reticulate, and the upper lip is clearly 3-lobed and toothed. *S. crypthanta* which formed the second cluster, has calyces that are expanded in fruit, membranous-reticulate, the upper lip subentire or indistincly toothed. On the other hand, molecular data did not support morphological data for *S. cyanescence* which constituted other subcluster of the second cluster. This species is characterised by several cauline leaves and many-flowered inflorescence expected to form another distinct cluster in the dendrogram. Using only a single accession, could be one of the reason for discrepancy between molecular and morphological data for. *S. cyanescence*.

In conclusion, RAPD markers are an efficient marker system that can be used to determine genetic relationships among closely related *Salvia* species as well as identification of *Salvia* genotypes..

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