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Phenotypic and Genetic Variation of Some *Salvia* Species Grown in Eastern Anatolia Region of Turkey

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Fatty acid and random amplified polymorphic-DNA (RAPD) profiles were used to examine phenotypic and genetic relationships among 8 *Salvia* taxa. All the *Salvia* taxa were used separated based on the presence and composition of 85 different fatty acids. Ten decamer primers were used to find out polymorphism. A total of 33 amplicons in the size range of 360 bp to 2500 bp were produced by ten different primers from the eight *Salvia* taxa. The results suggested that *S. argentea* is completely different that other species. Three genetically distinct groups were among the species of *Salvia* species with high genetic variation. All of *Salvia* species tested in this study was separated with unique FAME profiles including 86 different fatty acids. This is the first study showing that RAPD and FAME profiles are useful methods for determination of genetic and phenotypic profiles which may be used for identification and characterization of *Salvia* species.

Key Words: *Salvia* spp., Genetic variation, RAPD, FAMEs.

INTRODUCTION

Salvia is an important genus consisting of 900 species in the family Lamiaceae (formally Labiatae) and some species of *Salvia* have been cultivated worldwide for use in folk medicines and for culinary purposes[1]. A total of 88 species in the genus *Salvia* is present in Turkey[2,3]. Eight of the *Salvia* species including *S. argentea* L., *S. candidissima* Vahl., *S. ceratophyla* L., *S. microstegia* Boiss. Et Bal., *S. multicauhes* Vahl., *S. nemerosa* L., *S. staminea* Montbret & Aucher ex Bentham and *S. verticillata* L. are commonly grown and used in Eastern Anatolia region of Turkey. They are considered quite diverse in terms of morphological characteristics. The phenotypic parameters

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used in classification can sometimes be influenced by environmental conditions[4]. Therefore, biochemical and isoezyme studies are providing alternative approaches for evaluating diversity in plants[5-7]. However, biochemical and limited number of isoenzyme markers available are known to be affected by different stages of plant development. Recent advances in the field of molecular biology and gene technology are creating exciting possibilities for the rapid and accurate determination of genetic variation within and between plant species. The developments are based on the molecular structure (fatty acids) and specific characteristics of nucleic acids (DNA and RNA). Within last two decades, nucleic acid based techniques, particularly Random Amplified Polymorphic DNA (RAPD) technique, have been successfully used for evaluation of genetic relationship between plants species since 1990s[8-14]. Genetic diversity of some *Salvia* species including *S. fruticosa* Miller and *S. hispanica* L. have been assessed in the previous studies[15,16]. Fatty acids of *Salvia* species haven't been studied until now. There has been no attempt to study the genetic variation between *Salvia* species grown and used in the Eastern Anatolia region of Turkey up to now. The main goal of the present work was to study the relationships between eight *Salvia* species, *S. argentea, S. candidissima, S. ceratophyla, S. microstegia, S. multicauhes, S. nemerosa, S. staminea* and *S. verticillata* wildly grown in Eastern Anatolia region of Turkey using FAME and RAPD analysis.

EXPERIMENTAL

Sample collection: Plant samples of 6 *Salvia* taxa were collected at flowering stage from different locations in the vicinity of Erzurum, located in the eastern Anatolia, Turkey (Table-1). The taxonomic identifications were confirmed by Dr. Meryem Sengul, in Department of Biology, Atatürk University, Erzurum. Collected plant materials were dried in shade. The leaves were detached from the stems and ground in a grinder with a 2 mm diameter mesh. The ground material was used for DNA extraction and FAMEs. Voucher specimens were deposited at the Herbarium of the Department of Biology, Atatürk University; Erzurum (Table-1).

Extraction and analysis of FAMEs: Preparation and analysis of FAMEs from whole cell fatty acids were performed according to the method described by the manufacturer (Sherlock Microbial Identification System version 4.5, MIDI, Inc., Newark, DE). Plant leaves were powdered after lyophilization in liquid nitrogen. Approximately 40 mg of powdered cells from each sample was added to 1 mL 1.2 M NaOH in 50 % aqueous methanol with 5 glass beads (3 mm in diam) in a screw cap tube, then incubated at 100 ºC for 0.5 h in a water bath. After the saponified samples were cooled at room temperature for 25 min, they were acidified and methylated by adding 2 mL 54 % 6 N HCl in 46 % aqueous methanol and incubated at 80 ºC

for 10 min in a water bath. After rapid cooling, methylated fatty acids were extracted with 1.25 mL 50 % methyl-*tert* butyl ether (MTBE) in hexane. Each sample was mixed for 10 min and the bottom phase was removed with a Pasteur pipette. The top phase was washed with 3 mL of 0.3 M NaOH. After mixing for 5 min, the top phase was removed for analysis. Following the base wash step, the extract (FAMEs) was cleaned in anhydrous sodium sulfate and then transferred into a GC sample vial for analysis.

FAMEs were separated using gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) in a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5 % phenyl methyl silicone. The operating parameters for the study were set and controlled automatically by computer program. The chromatograms with peak retention times and areas were produced on the recording integrator and were electronically transferred to the computer for analysis, storage and report generation. Peak naming and column performance was achieved by using Eukary calibration standard mix (Microbial ID 1201-A) containing nC9-nC30 saturated and 2 and 3 hydroxy fatty acids. Cellular fatty acids were identified on the basis of equivalent chain length data. FAME profiles of each plant species tested were identified by comparing the commercial databases (Eukary) with the MIS software package.

DNA Extraction: Genomic DNA was extracted from powdered plant materials using a modified method described by Lin *et al.*[17].

Random Amplified Polymorphic-DNA (RAPDs): Samples were screened for RAPD variation using standard 10-base primers supplied by Operon. 30 µL of reaction cocktail was prepared as follows: 10 × Buffer 3.0 µL, dNTPs (10 mM) 1.2 µL, magnesium chloride (25 mM) 1.2 µL, primer (5 µM) 2.0 µL, taq polymerase (5 unit) 0.4 µL, water 19.2 µL sample DNA 3.0 µL (100 ng/µL). Total 10 RAPD primers were tested in this study.

Three of these primers produced amplicons for all of the species of *Salvia* tested were selected and used further studies based on the preliminary test results (Table-2).

The thermal cycle was: 2 min at 95 °C; 2 cycles of 30 s at 95 °C, 1 min at 37 °C, 2 min at 72 °C; 2 cycles of 30 s at 95 °C, 1 min at 35 °C, 2 min at 72 °C; 41 cycles of 30 s at 94 °C, 1 min at 35 °C, 2 min at 72 °C; followed by a final 5 min extension at 72 °C then brought down to 4 ºC.

Electrophoresis: The PCR products (27 µL) were mixed with 6 × gel loading buffer (3 µL) and loaded onto an agarose (1.5 % w/v) gel electrophoresis in 0.5 XTBE (*Tris*-Borate-EDTA) buffer at 70 V for 2.5 h. Amplification products separated by gel was stained in ethidium bromide solution (2 µL Etbr/100 mL 1xTBE buffer) for 40 min. The amplified DNA products were detected by using the Bio Doc Image Analysis System with Uvisoft analysis package (Cambrige, UK).

TABLE-1
NAME OF PLANT USED

OTUs	Abbreviation	Herbarium number of the vouchers	Locality	Status	Altitude (m)
Saliva argentea	AR	9623	Askale, Erzurum	Wild	2150
Saliva candidissima	CA	9653	Askale, Erzurum	Wild	2350
Saliva ceratophyla	CE	9778	Narman, Erzurum	Wild	2200
Saliva microstegia	MI	9643	Askale, Erzurum	Wild	2150
Saliva multicauhes	MU	9629	Askale, Erzurum	Wild	2200
Saliva nemerosa	NE	9775	Tortum, Erzurum	Wild	1550
Saliva staminea	ST	9779	Bayburt, Erzurum	Wild	1500
Saliva verticillata	VE	9777	Tortum, Erzurum	Wild	1550

TABLE-2
NUMBER OF AMPLIFICATION PRODUCTS GENERATED WITH 10 ARBITRARY OLIGONUCLEOTIDE PRIMERS IN *Salvia*

Primer	Total number of RAPD products per primer	Total number of polymorphic RAPD products	Number of polymorphic amplification products								Sequence of primer 5'→3'
			AA	BB	CC	DD	EE	FF	GG	HH	
OP A01	3	3	NA	NA	NA	NA	NA	1	2	NA	CAGGCCCTTC
OP B01	2	2	NA	NA	NA	NA	NA	1	1	1	GTTTCGCTCC
OP B02	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	TGATCCCTGG
OP B03	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	CATCCCCCTG
OP B04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	GGACTGGAGT
OP B05	3	3	NA	NA	NA	NA	NA	3	2	2	TGCGCCCTTC
OP B07	13	13	2	1	2	4	NA	4	4	4	GGTGACGCAG
OP B08	6	6	NA	NA	NA	NA	NA	3	N.A	3	GTCCACACGG
OP B12	4	4	NA	NA	NA	NA	NA	2	3	NA	CCTTGACGCA
OP B14	2	2	NA	NA	NA	NA	NA	NA	NA	2	TCCGCTCTGG
Total	33	33	2	1	2	4	0	14	12	12	
Total % polymorphism			6.06	3.03	6.06	12.12	0	42.42	36.36	36.36	

AA = *S. ceratophylla;* BB = *S. nemerosa;* CC = *S. staminea;* DD = *S. verticillata;* EE = *S. candidissima;* FF = *S. argentea;* GG = *S. microstegia;* HH = *S. Multicauhes;* NA = No Amplification.

Data analysis: PCR products were scored as presence (1) and absence (0) of band for each of the 8 accessions analyzed. Only reproducible bands were scored. For FAME analysis, fatty acids of each plant species were scored as presence (0.1-100 %) and absence (0 %). Data were used to calculate a Jaccard (1908) similarity index from which a UPGMA dendrogram was constructed. All experiments were repeated at least two times.

RESULTS AND DISCUSSION

The selected ten primers didn't amplify with *S. candidissima*. DNA bands among the eight *Salvia* species tested (Figs. 1-5, Table-2). The size of the amplicons produced in the present study varied from 360 bp to 2500 bp. Primer OPBO7 produced more RAPD products than those of others. *S. argentea* compared with the other species gave the highest DNA bands with all ten primers tested (Figs. 1-5, Table-2). *S. ceratophylla, S. nemerosa, S. staminea* and *S. verticillata* species gave amplification with only primer OPBO7. All eight *Salvia* species showed completely different RAPD patterns using the same primers (Figs. 1-5). *S. argentea* was completely different than the other species tested. *S. microstegia, S. cearatophyla* and *S. candidissima* were clustered together and separated from other cluster including the remaining four *Salvia* species tested (Fig. 6). The least variation was observed between *S. multicauhes* and *S. nemerosa* (Figs. 1-5, Table-2). RAPD markers have been used to determine genetic relationship at the species and subspecies level of plants. It is particularly useful for resolving relationship between closely related species and populations of genetically variable species. Therefore, RAPD markers have been used to find out genetic diversity in many plant species such as *Lolium*[18], *Hordeum*[19], *Ixora*[20], *Tripsacum*[21], *Clivia*[11], *Astragalus*[22] and *Vicia*[23] *etc*. RAPD is also used to assess genetic variation in the species of two *Salvia* species including *S. fruticosa* and *S. hispanica*[15,16].

All of eight *Salvia* species tested in this study were also separated based on the presence and composition of 86 different fatty acids (Table-3). Three of *Salvia* species including *S. nemerosa, S. multicauhes* and *S. staminea* were found to have more fatty acids (up to 45 fatty acids) than the others. The relative proportions of two fatty acids (16:0 and 18:1ω 8c) were higher (19-59 %) in these four *Salvia* species (Table-3). The remaining species have limited number of fatty acids with unique FAMEs profiles. FAME profiles have been used to find out phenotypic in many plant species *Astragalus*[22], *Cicer*[24], *Hypericum*[14], *Vicia*[23], *Lathyrus*[25,26] species in the previous studies. However, this is the first study to determine FAME profiles of *Salvia* species, demonstrating that FAME profile can be used to determine phenotypic differences between *Salvia* species closely related.

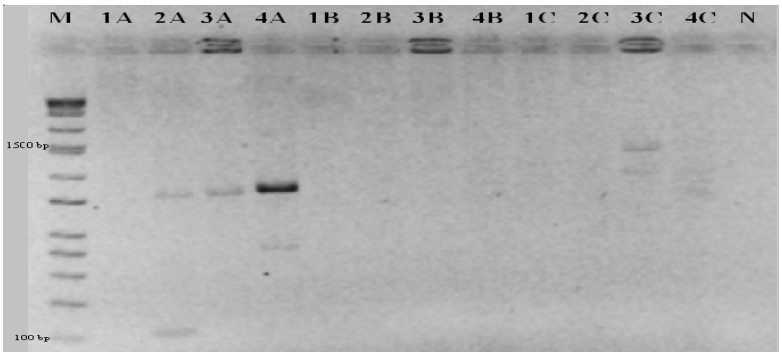

Fig. 1. RAPD profiles generated with the primers OPB01 (A), OPB02 (B) and OPB03 (C) respectively. Lanes: 1) *S. candidissima*; 2) *S. argantea*; 3) *S. microstegia*; 4) *S. multicauhes*; N; Negativ control M) Molecular Marker (10 kb)

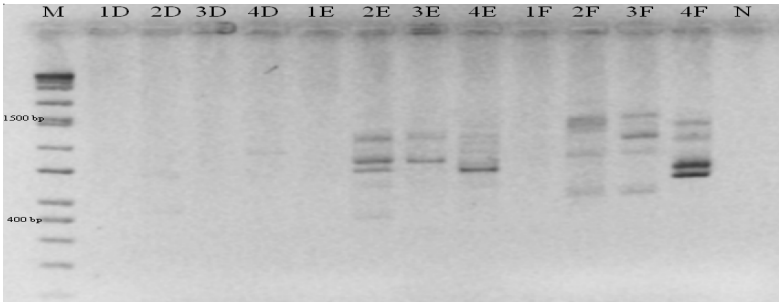

Fig. 2. RAPD profiles generated with the primers OPB04 (D), OPB05 (E) and OPB07 (F) respectively. Lanes: 1) *S. candidissima*; 2) *S. argantea*; 3) *S. microstegia*; 4) *S. multicauhes*; N; Negativ control M) Molecular Marker (10 kb)

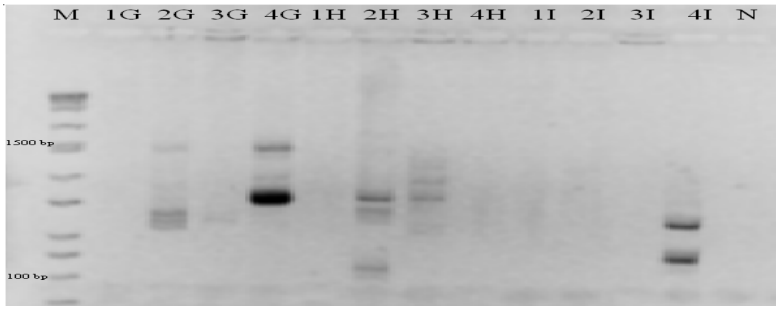

Fig. 3. RAPD profiles generated with the primers OPB08 (G), OPB12 (H) and OPB14 (I) respectively. Lanes: 1) *S. candidissima*; 2) *S. argantea*; 3) *S. microstegia*; 4) *S. multicauhes*; N; Negativ control M) Molecular Marker (10 kb)

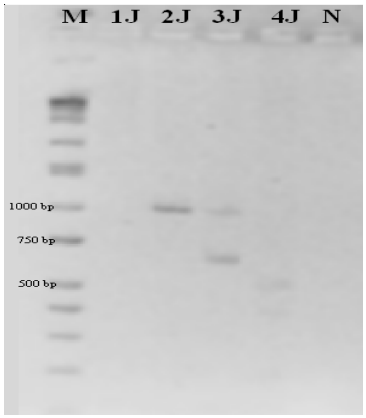

Fig. 4. RAPD profiles generated with the primer OPA01 (J) respectively. Lanes: 1) *S. candidissima*; 2) *S. argantea*; 3) *S. microstegia*; 4) *S. multicauhes*; N; Negativ control M) Molecular Marker (10 kb)

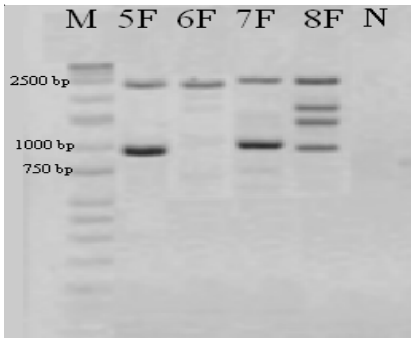

Fig. 5. RAPD profiles generated with the primer OPB07 (F) respectively. Lanes : 1) *S. ceratophylla*; 2) *S. nemerosa*; 3) *S. staminea*; 4) *S. verticillata*; N; Negativ control M) Molecular Marker (10 kb)

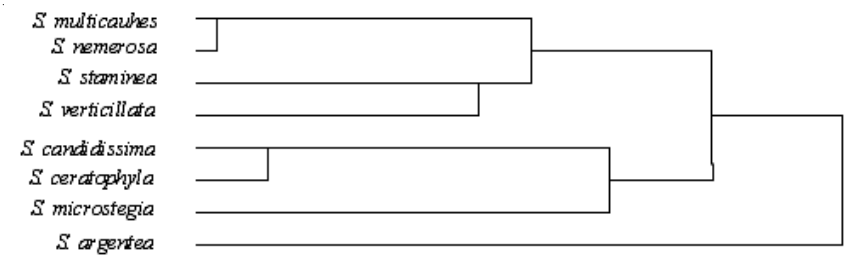

Fig. 6. UPGMA dendrogram showing the relationship of *Salvia*

TABLE–3
COMPOSITION OF FATTY ACIDS IN *Salvia* SPECIES

	Fatty acid concentration (%)							
Fatty acids	AA	BB	CC	DD	EE	FF	GG	HH
9 Dicarboxylic acid	–	–	–	–	–	3.83	4.41	–
10 Dicarbox (Sebacic)	–	1.44	–	–	4.43	1.32	–	–
10:0 3OH	–	–	–	–	3.33	–	–	10.80
11:0 iso	10.24	–	1.27	–	–	0.49	1.29	–
11:0 iso 3OH	–	–	–	–	–	–	1.68	–
12:0	–	–	–	–	–	1.66	3.04	–
12:0 2OH	–	–	–	–	–	0.42	–	–
12:0 3OH	–	–	–	–	–	0.12	–	–
12:0 2–Me	–	–	–	–	1.99	–	–	–
12:0 iso 3OH	–	–	–	–	3.69	–	–	–
12:0 iso	–	–	–	–	5.73	–	–	8.38
12:0 Dimethyl acetal	–	–	–	–	–	–	–	17.71
12 Primary alcohol	–	–	–	–	–	–	–	7.75
12:0 ALDE	–	–	–	–	–	0.63	–	–
13:0	28.81	–	–	–	–	–	–	–
13:0 iso	–	1.18	–	–	–	–	–	–
13:0 iso 3OH	–	–	–	–	1.80	–	–	–
14:0	–	2.88	2.34	1.90	–	1.64	4.25	–
14 N Alcohol	–	–	–	–	–	0.17	–	–
15:0	–	–	–	–	–	–	1.57	–
15:0 iso	–	–	2.59	–	–	–	–	–
15:0 anteiso	–	–	1.53	–	–	–	–	–
16:0	–	18.74	24.10	17.96	–	23.70	31.50	–
16:0 3OH	–	–	–	–	–	1.22	–	–
17:0 iso	–	–	2.08	–	–	–	–	–
17:0 anteiso	–	–	1.31	–	–	–	–	–
18:0	–	–	2.72	1.47	–	1.65	1.69	–
18 N Alcohol	–	3.00	3.24	2.38	–	2.82	–	–
18:0 *cis* 9,10 epoxy	–	–	–	–	–	0.60	1.45	–
19:0	–	–	–	–	–	–	1.58	–
19:0 3OH	–	–	–	1.96	–	–	–	–
20:0	–	–	–	–	–	0.53	1.38	–
20:0 3OH	–	–	–	1.91	–	0.63	–	–
20 N Alcohol	–	–	–	–	–	0.87	2.57	–
21:0 iso	–	–	–	–	–	2.30	–	–
22:0	–	–	–	–	3.57	0.24	–	–
23:0 2OH	–	–	–	–	–	0.18	–	–
24:0	–	–	–	–	–	0.39	–	–
24:0 2OH	–	–	–	–	–	0.88	–	–
24:0 3OH	–	1.63	–	–	–	–	–	–
25:0	–	–	–	–	–	0.43	–	–
25:0 3OH	–	–	–	–	–	0.66	–	–
25 N Alcohol	18.67	–	–	–	–	0.20	–	–
30:0	–	–	–	–	–	0.67	–	–

Fatty acid concentration (%)								
Fatty acids	AA	BB	CC	DD	EE	FF	GG	HH
12:1 3OH	–	2.20	–	–	–	–	–	–
12:1:ω 9c	–	–	–	–	–	0.17	–	–
12:2:ω 6c	–	–	–	–	–	0.78	–	–
13:1:ω 5c	–	2.22	–	–	–	–	–	–
13:1:ω 9c	–	–	–	–	–	0.16	–	–
14:1 iso E	–	–	–	–	–	0.63	–	–
14:1 *cis* 7 DMA	–	2.08	–	–	–	–	–	–
14:1:ω 6c	–	–	–	–	4.71	–	–	–
14:1:ω 8c	–	–	–	–	43.53	–	–	14.31
14:2:ω 6c	–	–	–	–	2.73	–	–	–
15:1 iso AT 5	–	–	–	–	0.30			
15:1 iso F	–	–	–	–	0.08	–	–	–
15:1:ω 6c	–	–	–	–	3.70	–	–	–
16:1:ω 6c	–	–	–	1.86	–	0.94	–	–
16:1:ω 7c	–	–	–	–	–	1.03	1.04	–
16:1:ω 7c Alcohol	–	–	–	–	–	1.04	0.86	–
16:1 *cis* 7DMA (ω9)	–	2.43	–	1.29	–	2.54	2.83	–
17:1 Alcohol (ω8?)	–	–	–	–	1.12	–	–	–
17:1:ω 3c	–	–	–	–	–	0.33	–	–
18:1:ω 9t Alcohol	–	–	3.07	1.96	–	5.00	4.44	–
18:1:ω 8c	–	59.08	38.02	55.95	–	18.88	–	–
18:1:ω 9c	–	–	–	–	–	–	24.08	–
18:1:ω 9c 12OH	–	–	–	–	3.17	–	–	–
18:1:ω 9c DMA	–	–	–	1.05	–	0.30	–	–
18:2:ω 6c	–	–	10.01	5.34	–	4.91	–	–
18:3:ω 6c	–	–	–	–	–	–	–	17.94
19:1:ω 6c	–	–	2.55	3.55	–	5.91	–	–
20:1:ω 11c	–	–	–	–	2.97	–	–	–
20:1:ω 9t	–	–	–	–	3.42	–	–	–
20:3:ω 6c	–	–	–	–	–	1.56	–	–
24:1:ω 9c Nervonic	8.86	–	–	–	–	–	–	–
24:5:ω 3	2.98	–	–	–	–	–	–	–
11.097	–	–	–	–	–	–	0.97	–
12.112	–	–	–	–	–	0.40	–	–
12.486	–	–	–	–	1.87	–	–	–
12.553 “B”	–	–	–	–	3.41	0.24	1.24	15.03
18.197	–	–	–	–	1.94	–	–	–
20.343 “D”	–	–	1.42	–	–	1.43	3.02	–
23.283 “C”	–	–	–	–	2.49	–	–	–
24.407 “D”	–	–	–	–	–	–	–	8.08
25.052	30.45	–	–	–	–	0.87	–	–

AA = *S. argentea*; BB = *S. candidissima*; CC = *S. ceratophyla*;
DD = *S. microstegia*; EE = *S. multicauhes*; FF = *S. nemerosa*;
GG = *S. stamine*; HH = *S. verticillata*

In conclusion, the results demonstrated that RAPD and FAME analysis are found to be useful for differentiation of *Salvia* species tested in the present study. A further study is necessary to determine the sequences of the polymorphic RAPD bands for each *Salvia* species tested and used for their identification and characterization studies in the future.

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