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Chemical and Genetic Relationships among Sage (*Salvia officinalis* L.) Cultivars and Judean Sage (*Salvia judaica* Boiss.)

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The essential oil composition and genetic variability of common sage (*Salvia officinalis* L.) and its three ornamental cultivars ('Purpurascens', 'Tricolor', and 'Kew Gold') as well as Judean sage (*Salvia judaica* Boiss.) were analyzed by GC-FID, GC-MS, and random amplified polymorphic DNA (RAPD). Common sage and its cultivars contained the same volatile compounds; only the ratio of compounds differed. The main compounds were the sesquiterpene α-humulene and the monoterpenes β-pinene, eucalyptol, and camphor. Judean sage contained mainly the sesquiterpenes β-cubebene and ledol. All of the samples exhibited characteristic RAPD patterns that allowed their identification. Cluster analyses based on oil composition and RAPD markers corresponded very well to each other, suggesting that there is a strong relationship between the chemical profile and the genetic variability.

KEYWORDS: Essential oil; gas chromatography; mass spectroscopy; RAPD; genetic distances; cluster analysis

INTRODUCTION

Common sage (*Salvia officinalis* L., Lamiaceae) is one of the most important medicinal plants in the mint family. Sages are frequent components in several multiherb products and food supplements such as teas, tinctures, liquid extracts, rheuma patches, and cosmetics (*1*, *2*). Sage is commonly used also as a culinary herb, listed by the Council of Europe (*3*) as a natural source of food flavoring. It can be added to foodstuffs providing that the concentration of thujones present in the final product does not exceed 0.5 mg/kg. In the United States sage is listed as "generally recognized as safe" (GRAS) (*4*). Its drug and tincture are official in several Pharmacopoeias (e.g., Pharmacopoeia Hungarica, 8th ed.; European Pharmacopoeia, 6th ed.) (*5*, *6*).

The plant is reported to have a wide range of biological activities, such as antibacterial, fungistatic, virustatic, estrogenic, anticholinesterase, adstringent, eupeptic, and antihydrotic effects (*7*, *8*). The antimicrobial properties as well as the tannin-based astringent activities of sage (active ingredient of dental-care herbal medicinal preparations) reduce plaque growth, inhibit gingival inflammation, and have positive effects on caries prophylaxis (*9*). The leaves of sage are well-known for their antioxidative properties; other experimental studies on sage extracts or sage essential oil showed hypotensive, central nervous system-depressant actions, and antispasmodic activities. Furthermore, sage is externally used for the treatment of insect bites (*10*, *11*).

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The strongest active constituents of sage are within its essential oil (1−2.8%), comprising the monoterpenes α- and β-thujone, camphor, cineole, and borneol as well as the sesquiterpenes α-humulene and β-caryophyllene in larger amounts, whereas the leaf contains di- and triterpenes, as well. Due to the presence of toxic thujone components, sage oil should be used with caution. Other constituents of the herb that contribute to its antioxidant effect are phenolic acids (caffeic, chlorogenic, rosmarinic, ferulic), flavonoids, and tannins (*12*−*14*).

The chemical composition of sage essential oil has been investigated in various countries (*15*, *16*), and the essential oils were divided into five groups according to the amount of the major constituents:

1. camphor > α-thujone > 1,8-cineole > β-thujone
2. camphor > α-thujone > β-thujone > 1,8-cineole
3. β-thujone > camphor > 1,8-cineole > α-thujone
4. 1,8-cineole > camphor > α-thujone > β-thujone
5. α-thujone > camphor > β-thujone > 1,8-cineole

Further authors mentioned viridiflorol (sesquiterpene-alcohol) as one of the five major components (*17*). ISO standard 9909:1997 for the essential oil composition of common sage prescribes the following: α-thujone, 18.0−43.0%; β-thujone, 3.0−8.5%; camphor, 4.5−24.5%; 1,8-cineole, 5.5−13.0%; α-humulene, 0−12%; α-pinene, 1.0−6.5%; camphene, 1.5−7.0%; limonene, 0.5−3.0%; linalool and its esters, < 1%; and bornyl acetate, < 2.5% (*18*).

Variation in essential oil yield and constitution can be due to both environmental and genetic factors. Echeverrigaray et al. (*19*) found, for example, that the population level diversity in essential oil composition of dittany (*Cunila galioides* Benth.) is

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affected by the genetic background rather than environmental circumstances.

DNA-based molecular markers have been used in a wide range of plant species either for cultivar identification or assessment of genetic relationships between individuals and species. Random amplified polymorphic DNA (RAPD) markers provide a convenient and rapid tool in assessing genetic differences between genotypes, even at lower intraspecific taxonomic levels. RAPD markers proved to be of high value in the genetic analysis of various taxa belonging to the Lamiaceae family, such as thyme (*Thymus vulgaris* L.) cultivars (*20*), wild basil (*Ocimum gratissimum* L.) accessions (*21*), and dittany populations (*22*).

Skoula et al. (*23*) analyzed 48 clones of Greek sage (*Salvia fruticosa* Mill.) for their essential oil and genetic profiles using RAPD markers. The patterns of relatedness observed in chemical profiles corresponded with the genetic profiles generated by RAPDs, suggesting that there may be a genetic basis for the chemical features.

Cahill (*24*) assessed the genetic diversity among 38 wild and domesticated accessions of chia (*Salvia hispanica* L.) collected throughout Mesoamerica by using RAPD markers. He found that genetic diversity was higher among wild varieties than all domesticated varieties and modern commercial domesticated varieties, suggesting loss of diversity accompanying domestication.

The objective of the present work was to evaluate the genetic and chemical variability among ornamental sage cultivars and to establish if diversity in essential oil composition can be related to genetic variability. The study may also provide a basis for including the studied cultivars in breeding programs directed at the improvement of essential oil composition.

MATERIALS AND METHODS

Plant Material. Leaf samples of the five studied sage taxa belonging to family Lamiaceae (*Salvia officinalis* L., *S. officinalis* cvs. 'Purpurascens', 'Tricolor', and 'Kew Gold', and *Salvia judaica* Boiss.) were collected in the botanic garden of the University of Pécs, Hungary, in 2007 and 2008, before, during, and after the flowering period. Samples and herbarium specimens have been deposited at the University of Pécs, Department of Pharmacognosy (herbarium numbers *Salvia officinalis*, 200; *S. officinalis* cv. 'Purpurascens', 200A; *S. officinalis* cv. 'Tricolor', 200B; *S. officinalis* cv. 'Kew Gold', 200C; *Salvia judaica*, 201). Fresh leaf samples were used immediately for DNA extraction, whereas the rest of the leaves was dried at room temperature for essential oil extraction.

Essential Oil Extraction. Essential oils were obtained by water steam distillation for 3 h, using 30 g of dried, powdered plant material using the apparatus prescribed in the seventh Hungarian Pharmacopoeia (*25*). Oil content was measured directly by volumetric method.

Chemical Composition of the Essential Oil. Analysis of composition of essential oils was performed by gas chromatography accomplished with a Fisons 8000 gas chromatograph, equipped with a flame ionization detector. An Rt-β-DEXm capillary column, 30 m long, 0.25 mm id., 0.25 μm film thickness was used. Carrier gas was nitrogen at 6.86 mL/min flow rate; 0.2 μL was injected (5 μL of essential oil in 2 mL of chloroform). Splitless injection was made at 10 s. The temperatures of the injector and detector were 210 and 240 °C, respectively. Oven temperature increased at a rate of 8 °C/min from 60 to 230 °C, with a final isotherm at 230 °C for 5 min. Percentage evaluation of compounds was carried out by area normalization; identification of peaks was made by comparison of retention times of standards and co-addition of standards. All measurement were made in duplicate.

GC-MS was performed with a coupled system Agilent 6890N GC, 5973N mass selective detector, the Chrom Card Server ver. 1.2. equipped with A HP-5MS capillary column, 30 m long, 0.25 mm id., 0.25 μm film thickness was used. Carrier gas was helium (p_{He} was 0.20 MPa), at 1 mL/min flow rate; 1 μL (10 μL/mL essential oil in ethanol) was injected at 0.7 mg/mL velocity, splitless type with an Agilent 7683 autosampler. The temperature of the injector was 280 °C, and the temperature of the transfer line was 275 °C. Oven temperature was programmed initially at 60 °C for 3 min, then increased with a rate of 8 °C/min to 200 °C, then kept at 200 °C for 2 min, and also increased at a rate of 10 °C/min to 250 °C with a final isotherm at 250 °C for 15 min. MS conditions: ionization energy, 70 eV; mass range, m/z 40−500; 1 analysis/min. Identification of peaks was carried out by comparison with MS and retention data of standards and spectra from the NIST library (*26*).

Statistical Analysis of Oil Composition. To evaluate the relationship between cultivars based on essential oil composition, the unweighted pair-group method with arithmetic average (UPGMA) was used, with the Bray−Curtis coefficient and the Euclidian distance. Applying either coefficient similar dendrograms were created, but the tree based on the Bray−Curtis coefficient was used, because it is more suitable for representing slight quantitative differences between taxa regarding essential oil components. Analyses were made using the SYNTAX 2000 program (*27*, *28*).

Isolation of Genomic DNA. One hundred milligrams of fresh harvested leaf samples was ground into a fine powder with liquid nitrogen using a sterile mortar and pestle. Extraction proceeded by using a DNeasy Plant Mini Kit (Qiagen). DNA quality and concentration were checked with lambda DNA standards on agarose gels. A dilution test was carried out to determine the optimal amount of DNA for amplification.

PCR Amplification. PCR reactions were performed in a total reaction volume of 12 μL. The PCR mix contained 25 mM $MgCl_2$, 10× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 100 μM each dATP, dCTP, dGTP, and dTTP, 0.5 μM primer, 1 unit of Taq DNA polymerase, and distilled water. Fifty-nine random decamer oligonucleotide primers (OP-A, 1−20; OP-B, 2−20; OP-N, 4; and OP-O, 1−19; Operon Technologies Inc.) were used for amplifications. Reaction mixtures were amplified in a PTC-200 thermal cycler (Perkin-Elmer). The thermal cycle used was 2 min at 94 °C, followed by 36 cycles of 10 s at 94 °C, 30 s at 36 °C, and 1 min at 72 °C min. A final cycle of 2 min at 72 °C completed extension of remaining products prior to holding the samples at 4 °C for analysis. Amplified fragments, along with a 100 bp DNA Ladder Plus (Fermentas), were separated by electrophoresis on horizontal 1.5% agarose gels in 0.5× TBE buffer (0.45 M Tris−borate, 0.01 M EDTA, pH 8.3). Gels were stained with ethidium bromide, visualized under UV light, and photographed using a BioDoc-It System UV Transilluminator (UVP Inc.).

Data Analysis. Because RAPDs have often been criticized for low reproducibility, highly constant conditions were maintained throughout the experiments and all reactions were repeated at least twice. Bands were scored as a binary variable: '1' for the presence and '0' for the absence of a band at a particular position. Only distinct well-resolved stable bands were included in the analysis. The above scores were used for calculation of pairwise genetic distances using Jaccard's coefficient. A dendrogram was constructed using UPGMA to estimate relationships among sage taxa. The software SYNTAX 2000 was used to perform the cluster analysis.

RESULTS AND DISCUSSION

Essential oil yields in the different common sage cultivars changed between 0.2 and 1.3 mL/100 g of dried material. Higher yields were obtained from *S. officinalis* itself and cv. 'Kew Gold' (0.9 ± 0.2 and 0.95 ± 0.55 mL/100 g, respectively), whereas lower yields were characteristic for cvs. 'Purpurascens' and 'Tricolor' (0.55 ± 0.15 and 0.45 ± 0.5 mL/100 g, respectively). Judean sage contained essential oil only in traces.

Analysis of the essential oil composition (**Table 1**) showed that all *S. officinalis* cultivars contained basically the same compounds, and differences could be observed only in their ratios. The ratio of the essential oil components in common sage corresponded well to ISO standards (*18*), the main component being α-thujone (34.80%), whereas in sage cultivars it was α-humulene (14.55−33.24%). Further major compounds included β-pinene, 1,8-cineole, and camphor. As expected, the essential oil composition of Judean sage differed significantly from that of common sage, containing mainly β-cubebene (9.45%) and ledol (12.00%).

Table 1. Percentage Distribution of Volatile Compounds in Essential Oil of *Salvia* Taxa[a]

		percentage of compounds				
compound	retention time (min)	*S. officinalis*	*S. officinalis* cv. 'Purpurascens'	*S. officinalis* cv. 'Tricolor'	*S. officinalis* cv. 'Kew Gold'	*S. judaica*
α-thujene	5.38	0.07	0.12	0.36	2.82	
α-pinene	5.74	0.24	1.31	3.47	2.57	
camphene	6.18	0.68	1.66	4.62	2.58	
sabinene	6.46	0.50	0.49	0.72	0.63	
β-pinene	6.63	1.00	4.57	11.33	8.55	
β-myrcene	7.06	0.12	0.16	0.09	0.14	
β-cymene	7.29	0.83	1.17	1.10	1.06	
limonene	7.58	0.41	0.29	0.39	0.25	
1,8-cineole	7.88	4.45	1.30	0.66	1.40	2.88
trans-β-ocimene	8.18		0.67	0.13	0.16	
γ-terpinene	8.29	0.20	0.27	0.17	0.20	
terpinolene	9.26		0.07	0.07	0.07	4.60
β-terpinene	9.75	0.21	0.13	0.13		3.14
α-thujone	10.30	34.80	8.63	12.35	10.32	0.29
β-thujone	10.56	2.97	1.66	1.78	1.38	0.19
cis-sabinol	10.84	0.14	2.92	0.13	0.11	0.14
camphore	11.41	11.06	10.99	7.32	9.17	0.16
pinocamphone	11.60	0.80	1.40	1.96	1.12	0.04
isopinocamphone	11.48	0.17	0.20	0.23	0.27	0.04
borneol	12.32	1.65	2.75	2.67	7.69	
bornyl acetate	12.77	0.21	0.65	0.49	0.45	
myrtenyl acetate	12.84	1.60	2.82	3.00	3.71	
α-cubebene	13.04	0.07	0.65	0.43	0.66	
α-copaene	14.13	0.05	0.50	0.37	0.88	
β-caryophyllene	14.69	8.38	2.64	1.97	3.51	6.56
(+)-aromadendrene	14.81	0.20	1.55	0.35	1.79	1.22
α-humulene	15.34	15.10	33.24	23.38	14.55	8.52
γ-muurolene	15.43	1.88	1.54	1.23	2.03	
(+)-ledene	15.66		2.04	0.53	2.53	
β-cubebene	15.71					9.45
δ-cadinene	16.19	0.17	3.33	2.35	3.16	2.31
palustrol	18.00	-	0.65	0.42	0.92	
ledol	18.75	2.77	4.11	4.20	1.19	12.00
caryophyllene oxide	18.97	0.76	0.60	1.24	1.06	1.17
palmitic acid	25.38					9.44
sclareol	26.19	5.18	2.86	3.08	2.16	7.01
epimanool	26.55					6.21
monoterpenes		62.14	44.23	53.17	54.08	11.48
sesquiterpenes		29.38	50.84	33.35	32.28	41.23
diterpenes		5.18	2.86	3.08	2.16	13.22
nonoxygenated terpenes		32.91	60.30	54.27	48.63	57.24
oxygenated terpenes		63.79	37.63	35.33	39.89	18.13
alcohols		6.97	9.18	6.30	10.88	13.36
ethers		5.21	1.90	1.90	2.59	4.05
ketones		49.80	23.08	23.64	22.26	0.72
esters		1.81	3.47	3.49	4.16	
total		96.7	97.93	89.60	88.52	75.35

[a] Average content of volatile compounds by three collection periods. Standard deviations (SD) were between 0.01 and 4.44.

Common sage cultivars and Judean sage contained higher ratios of nonoxygenated terpenes (48.63−60.30%) than common sage itself, the essential oil of which was dominated by oxygenated terpenes (63.79%), alcohols, ethers, esters, and ketones. A higher ratio of sesquiterpenes (50.84%) was observed in cv. 'Purpurascens', whereas the rest of the cultivars were characterized mainly by monoterpenes (53.17−62.14%), but each contained the diterpene sclareol. Sesquiterpene compounds (41.23%) dominated in the essential oil of Judean sage, but mono- and diterpenes (sclareol and epimanool) could also be detected.

As expected, the cluster analysis separated *S. officinalis* and its cultivars from Judean sage, due to greater similarity in the essential oil composition of the *S. officinalis* group, compared to *S. judaica* (**Figure 1**). The common sage cluster can be divided into two further groups, the first including *S. officinalis* itself and the other composed by its cultivars. The major components in the background of this distinction were *trans*-β-ocimene, terpinolene, and (+)-ledene, which were absent from the essential oil of common sage, but could be detected in all three cultivars.

Parallel to the essential oil studies, the five sage taxa were examined for the extent of genetic variability by extracting genomic DNA and generating a RAPD marker profile. The investigated taxa included common sage and its three ornamental cultivars, as well as Judean sage as an outgroup species.

From 59 primers of kits A, B, N, and O of Operon Technologies, 39 oligonucleotide primers were selected for the evaluation of relationships. Using the 39 selected primers, 311 RAPD bands were scored, ranging in size from 150 to 1500 bp. The number of amplification products per primer varied from 2 (OPB-16) to 15 (OPB-12). An example of the banding patterns is shown in

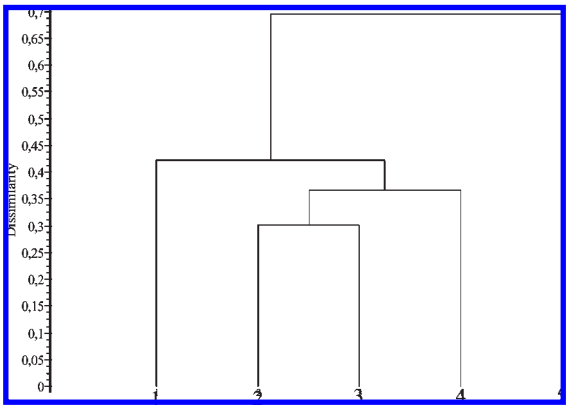

Figure 1. Dendrogram obtained by unweighted pair-group method with arithmetic average (UPGMA), using the Bray−Curtis coefficient, based on the chemical composition of essential oil of common sage cultivars and Judean sage. 1, *Salvia officinalis*; 2, *S. officinalis* cv. 'Purpurascens'; 3, *S. officinalis* cv. 'Tricolor'; 4, *S. officinalis* cv. 'Kew Gold'; 5, *Salvia judaica*.

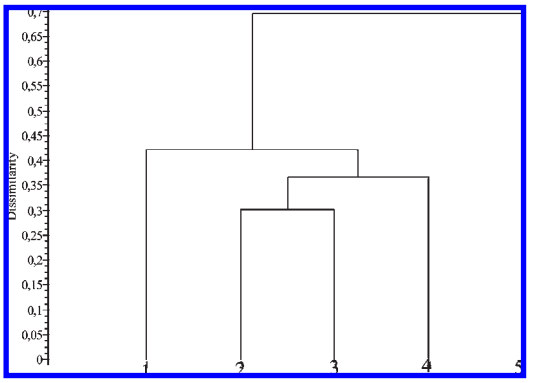

Figure 3. Dendrogram showing genetic relationships among common sage cultivars and Judean sage, determined by the analysis of 311 RAPD markers, generated by unweighted pair-group method with arithmetic average (UPGMA). 1, *Salvia officinalis*; 2, *S. officinalis* cv. 'Purpurascens'; 3, *S. officinalis* cv. 'Tricolor'; 4, *S. officinalis* cv. 'Kew Gold'; 5, *Salvia judaica*.

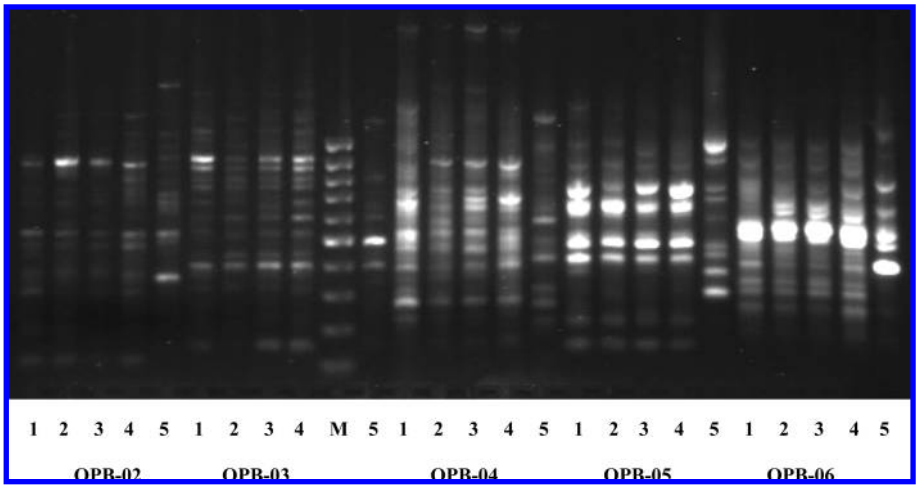

Figure 2. RAPD patterns of common sage cultivars and Judean sage obtained with primers OPB-02, OPB-03, OPB-04, OPB-05, and OPB-06. 1, *Salvia officinalis*; 2, *S. officinalis* cv. 'Purpurascens'; 3, *S. officinalis* cv. 'Tricolor'; 4. *S. officinalis* cv. 'Kew Gold'; 5. *Salvia judaica*; M, marker (100 bp DNA Ladder Plus).

Figure 2. The number of polymorphic bands, regardless of band intensity, varied among primers from 0 to 12 when excluding and including *S. judaica* in the analysis, respectively. When Judean sage was included in the analysis, 83.6% of the bands were polymorphic. However, when only common sage and its cultivars were considered, the percentage of polymorphic bands was 57.2%. This percentage of polymorphism is similar to those previously reported at the intraspecific level for other medicinal and aromatic species of the Lamiaceae family: 63.8 and 59.8% for thyme (*20*) and dittany (*22*), respectively.

Hierarchical cluster analysis of the taxa, using RAPD markers, produced a dendrogram of genetic distances as shown in **Figure 3**. As expected, Judean sage, which was considered to be an outgroup, segregated from common sage and its cultivars at a high level of dissimilarity (69.54%). The various *S. officinalis* taxa fell into two clusters: one of them was formed by common sage itself and the other by its ornamental cultivars. The cvs. 'Purpurascens' and 'Tricolor' exhibited the closest relationship (30.14% dissimilarity) and were tightly clustered. Cv. 'Kew Gold' was also closely related to the other two ornamental cultivars, with a level of 36.75% dissimilarity.

The cluster analyses based on oil composition and on RAPD markers corresponded very well to each other (**Figures 1** and **3**), suggesting that there is a strong relationship between the chemical profile and the genetic pools. Further studies should confirm that there is an actual linkage between oil components and specific genes. Similar correlation between volatile oil components and genetic background was reported for Greek sage (*23*) and other Lamiaceae species, for example, thyme (*19*, *29*).

Complex morphological, phytochemical, and genetic studies carried out simultaneously can provide sufficient information for the chemotaxonomic identification of medicinal and aromatic plants at the inter- and, especially, the intraspecific levels. In accordance with studies conducted on other sage species (*23*, *24*), our results also confirm that the chemical profile, as well as the RAPD markers, can be used efficiently to evaluate relationships of sage cultivars. The data presented in this study can also provide future breeding programs with valuable data.

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