World Applied Sciences Journal 6 (2): 238-241, 2009
ISSN 1818-4952

Determination of Genetic Relations among Four *Salvia* L. Species Using RAPD Analysis

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Abstract: The phylogenic relationship among four species of *Salvia* (*S. macrosiphon* Boiss., *S. Aethiopis* L., *S. brachyantha* (Bordz.) Pobed. and *S. sclarea* L.) and one species of *Nepeta* (*N. saccharata* Bunge) was investigated by Randomly Amplyfied Polymorphic DNA analysis (RAPD). All the species were clearly identified using 20 primers tested (Z1- Z20). The total number of amplification products produced with these primers was (548). Genetic distance was calculated in order to construct phylogenic dendrogram of closely related samples. Results indicated that *N. saccharata* Bunge had the closest relationship with two samples of *S. macrosiphon* and *S. Aethiopis*. The species *S. brachyantha* have not only showed farther genetic distance from *S. macrosiphon* and *S. Aethiopis* but also different patterns of RAPD-PCR compare to others. Dendrogram showed one main cluster included five tested species of *Salvia* and *Nepeta* .

Key words: *Salvia macrosiphon* · *Salvia aethiopis* · *Salvia sclarea* · *Salvia brachyantha* · *Nepeta saccharata* · RAPD analysis

INTRODUCTION

Salvia, with over 900 species from both the Old and New World, is the largest genus in the Lamiaceae family and comprises 58 species in Iran. Some of them like *S.bazmanica* Rech.f.& Esfand. and *S. eremophila* excursively grow in Iran but others also grow in Iraq, Armenia, Turkey, Saudi Arabia and south-west of Africa [1, 2]. *Salvia* is not monophyletic, *Rosmarinus* and *Perovskia* together are sister to an Old World clade of *Salvia* [1]. *Salvia*, like *Nepeta,* has an important center of diversity in our area. It exhibits a particularly interesting range of morphological variation which is as great as, if not more than, anywhere in the Old World with regard to habit, calyx structure, corolla form, staminal structure and nutlet characters [3].

In this study, we focused on four species of *salvia* (*Salvia macrosiphon, Salvia Aethiopis, Salvia sclarea* and *Salvia brachyantha*) belonging to third group which, are often suffruticose with large corollas and staminal connectives clearly longer than filaments [3]. Also, we tried to determine the relationship among those mentioned *Salvia* species and *Nepeta saccharata* Bunge. Because of the closely morphological characters of these plants, they have been used alternatively as the medicinal plants and remedies in folk and traditional medicine in Iran.

Many species of *Salvia* have been used as medicinal plants and show several activities. Some of the important activities are bactericidal, virucidal, fungistatic, spasmolytic and anti-hypertention effects. Recently, some phenolic acids (caffeic acid and chlorogenic acid), flavonoids (genkwanin methyl ether, genkgwanin, apigenin and luteolin), diterpenoids (carnosolic acid, rosmanol and safficinolide) and triterpenoid compounds (ursolic acid) have been separated and identified from *Salvia* genus [4, 5, 6]. Therefore, determination of genetic relationship among *Salvia* species, together with the chemotaxonomical analyses, will help to further understand the biological and medicinal functions of those mentioned plants.

In addition, literature reviews show that there is no report to study genetic relationship among Iranian

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Salvia species. Here, the phylogenic relationship among *Salvia* (four species) and *Nepeta* (one species), grows all around Iran, has been investigated by RAPD-PCR for the first time. RAPD technique is ideally suited for fingerprinting applications because it is fast, requires little materials and technically easy. The wide availability of commercial primers makes this technique widespread, inexpensive and yields large numbers of markers [7].

MATERIALS AND METHODS

Plant Material: All the plant materials (leaves), used in this study, gathered around Damavand on the way of Tehran- Firuzkouh road during full flowering stage (June, 2005). Voucher specimens of these plants are all deposited at the Herbarium of the Faculty of Pharmacy and Medicinal Plant Research Center, Tehran University of Medical Sciences. Plant specimens were identified by Dr. Kholam Reza Amin from the same institute (Table 1).

Total DNA Extraction: Dried leaves of the species were used for total DNA extraction. Plant samples grind to fine powder and then the DNA extraction was carried out by GMO DNA extraction Kit (Bioneer, Cat No.K3031) according to the manufacturer's instruction. The extracted DNAs were precipitated in ethanol (96- 100%) and the precipitates were rinsed with 70% ethanol (EtOH). The nucleic acids were purified by the phenol- $CHCl_3$ extraction method and precipitated again in EtOH. The remained pellets were dissolved in double distilled water (ddH_2O) and kept at 4°C [8].

RAPD Analysis: In order to use single primers for DNA amplification, twenty 10-mer oligonucleatides (Alpha DNA, Canada) were applied for PCR (Table 2). Amplification mixtures contained 2.5il buffer, 0.5 il dNTP mixture (10mM of each dATP, dTTP, dCTP and dGTP), 0.25 il Supertaq DNA polymerase (AB gene), 40ng template DNA and ddH_2O up to 25 il.

The PCR program was: 94°C (20") 36°C + 0.1°C /sec (4'), 72°C (3') for 30 cycles in a Primus thermal cycler. PCR products were analyzed on 1% agarose gel which, visualized under ultraviolet light at 254 nm. Molecular sizes of amplification products were estimated by using a 100 bp DNA ladder marker (Sigma Chemical Company, St. Louis).

Statistical Analysis and Data Scoring: The DNA profiles were scored visually from photographs of the gels. Reproducible bands (at least two times) were used in the analysis. To trace the matrix and calculate the genetic distance among *Salvia* species, single matching coefficient (Ssm) was calculated for each pair of samples, based on the presence or absence of the unique and shared fragments, regardless of their intensity [8, 9, 10].

Dendrogram was constructed based on the similarity matrix data (Fig. 1), by applying unweighted pair- group method with arithmetical average (UPGMA) using cluster

Table 1: *Salvia* and *Nepeta* samples with their origin, voucher specimens and date of harvesting

Plant samples	Origin	Date	Voucher No.
S. macrosiphon Boiss.	Damavand, Tehran	June, 2007	6674- THE
S. Aethiopis L.	Kiasar, Mazandaran	July, 2007	1142- MPRC
S. brachyantha (Bordz.) Pobed.	Savadkuh, Mazandaran	July, 2007	1145- MPRC
S. sclarea L.	Firuzkuh, Mazandaran	June, 2007	1146- MPRC
N. saccharata Bunge	Savadkuh, Mazandaran	June, 2007	1208- MPRC

Table 2: Oligonucleotids used as 10-mer primers in PCR

Code	5' to 3'	Code	5' to 3'
Z1	GGTCGGAGAA	Z2	TCGGACGTGA
Z3	AGACGTCCAC	Z4	GGAAGTCGCC
Z5	AGTCGTCCCC	Z6	CTGCATCGTG
Z7	GAAACACCCC	Z8	TGTAGCTGGG
Z9	ACGCGCATGT	Z10	GACGCCACAC
Z11	ACCAGGTTGG	Z12	AATGGCGCAG
Z13	CACTCTCCTC	Z14	GAATCGGCCA
Z15	CTGACCAGCC	Z16	GGGAGACATC
Z17	ACAACGCGAG	Z18	CCGCCTAGTC
Z19	GGAGGAGAGG	Z20	TCATCCGAGG

analysis in the NTSYS pc program version 2.1 by Rohlf (Exeter, software, New York).

RESULTS AND DISCUSSION

Pair-wise comparison of all RAPD profiles revealed a similarity matrix. Simple matching coefficient (Ssm) and genetic distance (d), derived from RAPD banding patterns, are shown in Table 3. The genetic distance between the two samples of *S. macrosiphon* and *S. Aethiopis* was considered to be short (0.839) and their RAPD banding patterns were quite similar to each other also there is a close relationship between these two samples of *Salvia* with *N. saccharata* (0.854). The dendrogram constructed base on genetic distances, derived from RAPD analysis, shown in Fig. 1. Clustering analysis was based on UPGMA.

In this dendrogram, *N. saccharata* represents the closest relationship with two samples of *S. macrosiphon* and *S. Aethiopis*. Phylogenetic distances show that *S. brachyantha* is not only far from *S. macrosiphon* and *S. Aethiopis* but also, has completely different patterns of RAPD profiles compare to other samples.

Comparison of the main components in the volatile oils and the morphological characters of the *Salvia* species (Table 4) show that *S. Aethiopis* and *S. sclarea* are similar to each other, especially because of the presence of caryophylene derivatives and sesquiterpene compounds in their oils [11, 12]. Although, *S. macrosiphon* is the closest species to *S. Aethiopis* phylogenetically, its volatile oil composition (rich of fatty acid ester) shows no similarity to *S. Aethiopis* [13].

N. saccharata has a different volatile compounds pattern compare to *Salvia* species, for example, decanal and dodecanal are the main components of *N. saccharata* [14]. Although, most species of *Nepeta* have remarkably similar apigenin type flavonoid profiles with cirsimaritin (the major surface flavone and its 4'-methyl ether, salvigenin), *N. saccharata* have not contained flavonol 3-methyl ethers [15]. This is in agreement with *Salvia* species in which, flavonols are rarely accumulated [16]. But luteolin derivatives are higher in number than those of the apigenin series (contain 6-methoxy derivatives), exhibiting a considerable degree of methylation [17].

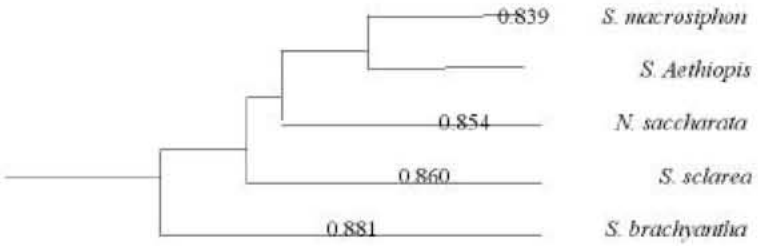

Fig. 1: Dendrogram of genetic relationships between four samples of *Salvia* and one *Nepeta*, generated by UPGMA cluster analysis of the genetic distances

Table 3: Simple matching coefficient (Ssm, above the diagonal) and genetic distances (d, below the diagonal) between pairs of *Salvia* and *Nepeta* plants based on RAPD-PCR

	N. accharata	*S. clarea*	*S. acrosiphon*	*S. rachyantha*	*S. ethiopis*
N. saccharata	------	0.282	0.286	0.178	0.255
S. sclarea 0.847	------	0.236	0.214	0.284	
S. macrosiphon	0.845	0.874	------	0.202	0.296
S. brachyantha	0.907	0.886	0.893	------	0.244
S. Aethiopis	0.863	0.846	0.839	0.869	------

Table 4: Comparative main components in the essential oils of *Salvia* species and their morphologicals characters

Species	Main volatile components	Out standing morphological characters [3]
S. Aethiopis	β-caryophyllene, α-copaene and germacrene D [5, 11]	The very sturdy stems, lanate indumentums and stiffly candelabriform inflorescence with white flowers.
S. sclarea	linalool, linalyl acetate, β-caryophyllene, a-terpineol, geraniol, neryl acetate, sclareol and germacrene D [12]	Large and colorful bracts.
S. macrosiphon	Linalool, hexyl hexanoate, hexyl isovalerate, hexyl-2-methyl-butanoate, sclareol and hexyl octanoate [13]	Narrow tubular calyces, long corolla tubes*.
S. brachyantha	There is no report about the volatile components of this species.	Eglandular arachnoids indumentums throughout, violet-tinged inflorescences.

* It is a polymorphic species with a wide variation in indumentums type and density, bract size and leaf shapes

Anyhow, *N. saccharata* show similarity with *Salvia* species especially in morphological characteristic and flavonoid contents. But the main constituents of its volatile oil have differences with *Salvia*. Hence, the identification of plant material is so important for preparation of medicinal plant remedies (contain *Salvia* plants) to reach the appropriate pharmacological functions.

In conclusion, molecular biological assays show that *S. macrosiphon* and *S. Aethiopis* are very similar to each other and *S. brachyantha* has a far genetic distance from other species of *Salvia*. Although, there is a close relationship (genetically and morphologically) between some species of *Salvia* and *Nepeta* species, chemotaxonomic characters are different in their volatile oils.

ACKNOWLEDGMENT

This research has been supported by Tehran University of Medical Sciences and Health Services grant (No. 4624). The authors wish to thank Research Council and Vice chancellor of TUMS.

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