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Genetic Variation Among *Salvia* Species Based on Sequence-Related Amplified Polymorphism (SRAP) Marker

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Abstract
In this study, SRAP molecular maker approach was performed to investigate genetic diversity in the *Salvia* genus. A total of 205 DNA bands were produced from PCR amplification of 11 *Salvia* species and populations using 25 selective primer combinations, of which 204 polymorphic genetic loci accounted. The total number of amplified fragments ranged from 3 to 15. The genetic similarities of 11 collections were estimated from 0.0968 to 0.6949. According to the SRAP marker analysis, *S.verticillata2* and *S.nemorosa2* had the lowest similarity and the species of *S.verticillata2* and *S.verticillata3* had the highest similarity. Unweighted pair-group method with arithmetic averages clustering algorithm categorized *Salvia* species into six major groups. In general, SRAP markers were able to detect high polymorphism among *Salvia* genotypes. Hence, the technique is regarded as a reliable tool for differentiation of *Salvia* at inter and intra-species level.

Keywords: Cluster analysis; Genetic diversity; *Salvia*; SRAP molecular marker

Introduction

The genus *Salvia* represents an enormous and cosmopolitan assemblage of nearly one thousand species in the world, which is by far the largest genus in the Lamiaceae family. *Salvia* as the biggest genus is now widely cultivated in various parts of the world particularly in tropical areas such as China (84 species), Iran (58 species), Mediterranean area, southeast of Africa, central and south America (Baricevic *et al*. 2001; Barnes *et al*. 2002). In the past decade, this popular herb has been intensively studied for its anti-oxidative components as well as for the treatment of many kinds of ailments (Botstein *et al*. 1980; Litt and Luty 1989; Hohmann *et al*. 1999). This plant has a wide range of biological activities including antibacterial and antifungal effects as well as estrogenic and tannin-based astringent activities (Farag *et al*. 1986; Li and Quiros 2001). More important, sage essential oils have also been identified to be hypotensive and have antispasmodic activities as well as nervous system-depressant actions (Lu and Foo 2000).

Various DNA-based molecular markers and DNA fingerprinting techniques have been applied in wide range of plants species for identifying genetic relationships amongn species and polymorphism analysis. Some of these developed techniques include restriction fragment length polymorphism (RFLP) (Moore *et al*. 1991), random amplified polymorphic DNA (RAPD) (Sang *et al*. 2010; Tautz 1989), simple sequence repeats (SSR) (Tautz 1989; Vos *et al*. 1995) and amplified fragment length polymorphisms (AFLP) (Wang *et al*. 2009; Wang *et al*. 2011). However, these techniques have an important defect in high

false positive genotyping due to relatively low initiating annealing temperature (Welsh and Mcclelland 1990). In addition, some systems have poor consistency and low multiplexing output (RAPD), while some others are considerably expensive and time-consuming (RFLP). In this context, a novel marker system dubbed as sequence related amplified polymorphism (SRAP) that has provided a simple and efficient marker system applicable to a variety of fields including map construction, genomic and cDNA fingerprinting (Willershausen *et al*. 1991).

In previous studies using RAPD and ISSR techniques, genetic diversity of eight species of *Salvia* has been studied (Sepehry *et al*. 2012). In this report, SRAP marker was applied to assess the level and pattern of genetic diversity in different important cultivated species of *Salvia*.

Materials and Methods

In this study, seeds of 11 species and populations of *Salvia* were prepared from gene bank of Ardabil and cultured in phytotron under growth conditions (Table1). The DNA was extracted by CTAB method (Saghai-Maroof *et al*. 1984). The quantity and quality of the extracted DNA were evaluated using spectrophotometer and agarose gel electrophoresis, respectively. Each 25 µl PCR reaction mixture consisted of 3 µl genomic DNA (10 ng/ µl), 0.5 µl primer (100µM), 0.75 µl dNTP (10mM), 1 µl MgCl2 (50 mM), 0.5 µl of Taq DNA polymerase (5U/ µl), 2.5 µl of 10X PCR Buffer and 16.25 µl deionized sterile water. DNA amplification was carried out under the following conditions: initial denaturation at 94°C for 3 min, followed by five cycles comprising 1 min denaturation at 94 °C, 1 min annealing at 32°C, and 1.5 min of elongation at 72°C. In the following 35 cycles, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1.5 min were carried out, ending with an elongation step for 10 min at 72°C. The amplified products were stored at 4°C until they were loaded on to the gel for analysis. Twenty five selective primer combinations (Table 2) were used in these amplification experiments. Electrophoresis of PCR products was performed using 3% agarose gel running at 50 V for 5 hours. After staining with ethidium bromide, DNA was visualized and photographed under ultraviolet light using Gel Logic 212 Pro Imaging System (Carestream, USA). The presence or absence of reproducible amplified DNA bands was determined for each isolate and scored as 1 and 0, respectively. To evaluate the relationship between accessions, unweighted pair-group method with arithmetic average (UPGMA) clustering analysis was performed by NTSYS-pc package, version 2.02, using Jaccard coefficient of similarity, and the dendrogram was constructed.

Results

Twenty five selective SRAP primer combinations generated 205 PCR amplified bands of which 204 were polymorphic and the polymorphism rate was 99.51 %. The size of the amplified products ranged from 100 to 3000 bp. The total number of amplified fragments was in the range of 3 to 15. The lowest number of bands was produced by ME4-EM6 primer combinations, while the highest number was produced by ME3-EM3 (Table 2). Figure 1 illustrates banding pattern of

Table 1. Salvia species and populations with their sampling origin

Species and populations	Origin
S. hydrangea	Khalkhal, Ardebil
S. xanthocheila	Sabalan mountain, Shabil, Ardebil
S. limbata	Ahar-Meshginshahr road
S. aethiopis	Sardabeh, Aradebil
S. macrochlamys	Germi, Ardebil
S. nemorosa2	Ahar-Meshginshahr road
S. sclarea	Khalkhal, Ardebil
S. verticillata1	Sardabeh, Ardebil
S.verticillata2	Ahar-Meshginshahr road
S.verticillata3	Khalkhal, Ardebil
S.nemorosa1	Urmia, East Azarbaijan

ME3-EM4 primer by SRAP marker profile. Jaccard's similarity coefficient was estimated from 0.0968 to 0.6949. According to the SRAP marker analysis, the species of *S.verticillata2* and *S.nemorosa2* had the lowest similarity and the *S.verticillata2* and *S.verticillata3* populations showed the highest similarity. Constructed dendrogram using the UPGMA method clustered 11 Salvia species and populations into six major groups (Figure 2). Cluster 1 included *S. sclarea*; cluster 2 consisted of two species of *S. limbata, S. Xanthocheila;* Cluster 3 was divided into 2 subclusters. The first subcluster comprised of *S. nemorosa1* and *S. nemorosa2* and the second subcluster comprised of *S. athiopis.* Within first subcluster, *S. nemorosa1,* and *S. nemorosa2* appeared to be closer to each other, with a 0.584 similarity coefficient (Table 3). Cluster 4 and 5 only included *S. hydrangea* and *S. macrochlamys,* respectively; cluster 6 composed of three populations of *S.verticillata1*, *S.verticillata2*, and *S.verticillata3*. The polymorphism level, calculated as the number of polymorphic bands per primer, ranged from 90% to 100%.

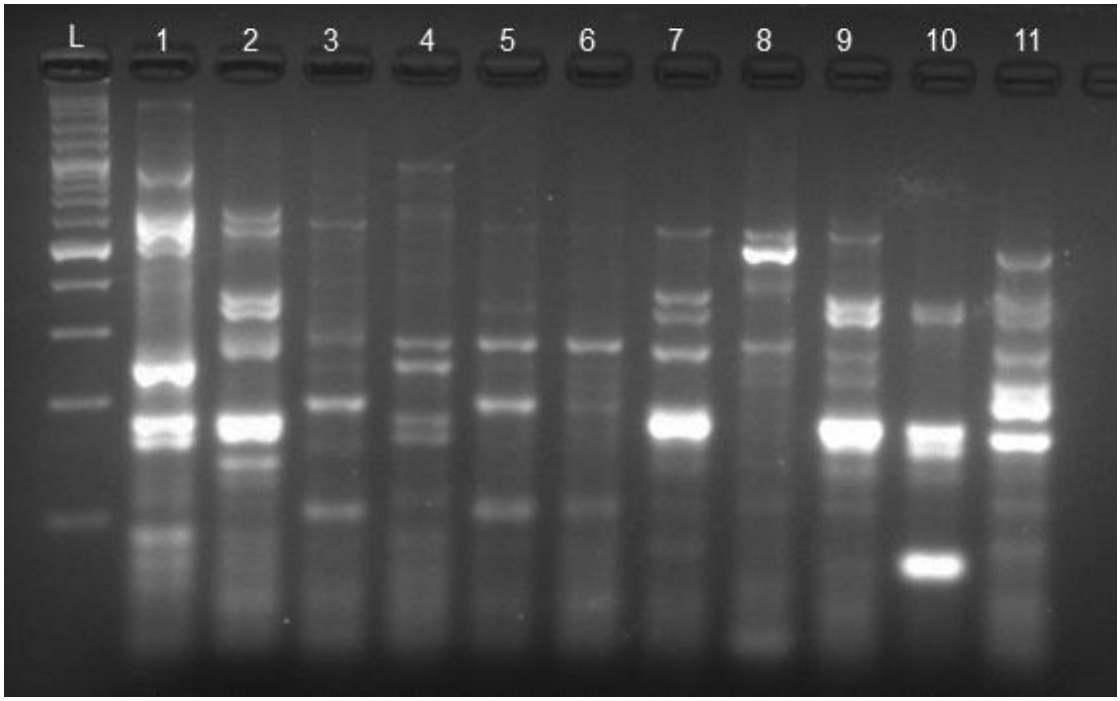

Figure 1. SRAP profile based on Me3-Em4 primer
L: DNA ladder, 1=*S. nemorosa2*, 2=*S. athiopis*, 3=*S.verticillata1*, 4=*S.nemorosa1*, 5=*S.verticillata2*, 6=*S.verticillata3*, 7=*S.xanthocheila*, 8=*S.macrochlamys*, 9=*S.limbata*, 10=*S.sclarea*, 11=*S.hydrangea*

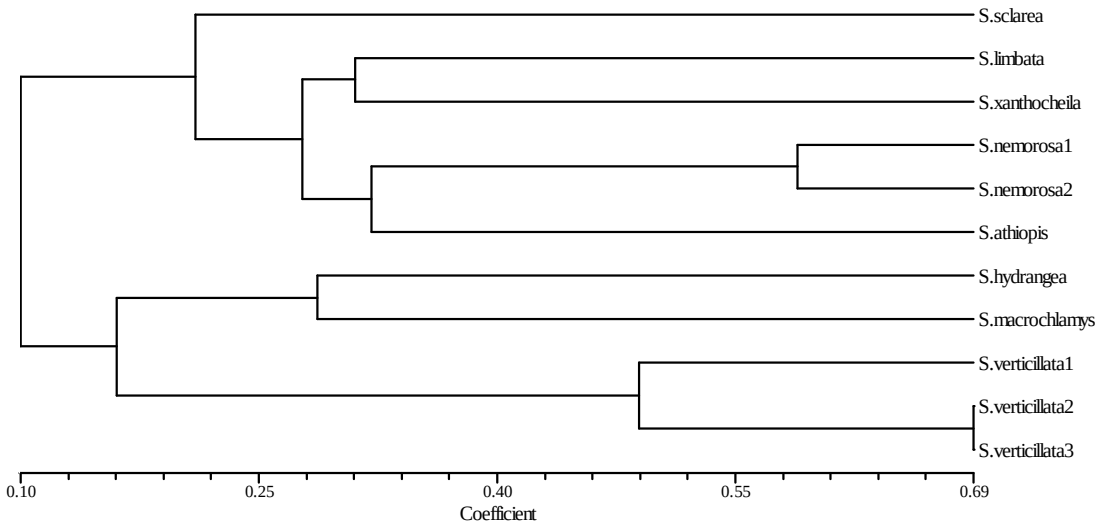

Figure 2. Dendrogram of 11 *Salvia* species and populations constructed by the SRAP technique. The dendrogram was constructed using the Unweighted Pair-Group Method with Arithmetic Averages

Table 2. The number of amplified fragments for each primer pair

Primer-pair	Total amplified fragments	Number of polymorph fragments	Polymorphism
Me3-Em1	10	10	100%
Me3-Em3	15	15	100%
Me3-Em4	12	12	100%
Me3-Em6	10	10	100%
Me1-Em3	8	8	100%
Me1-Em4	11	11	100%
Me1-Em6	10	10	100%
Me2-Em3	10	10	100%
Me2-Em4	11	11	100%
Me2-Em6	10	9	90%
Me4-Em2	11	11	100%
Me4-Em3	7	7	100%
Me4-Em6	3	3	100%
Me4-Em1	10	10	100%
Me4-Em4	4	4	100%
Me3-Em20	7	7	100%
Me2-Em19	4	4	100%
Me3-Em17	8	8	100%
Me3-Em18	4	4	100%
Me3-Em19	5	5	100%
Me2-Em17	12	12	100%
Me2-Em18	6	6	100%
Me4-Em17	7	7	100%
Me4-Em18	4	4	100%
Me1-Em17	6	6	100%
total	205	204	99.51%

Discussion

So far, various DNA-based molecular markers and DNA fingerprinting techniques have been applied in the wide range of plants species for identifying genetic relationships among species and, also for the polymorphism analysis of these plants. Several PCR biomarker systems are available varying in reliability, complexity and information generating capacity. In the current study, a recently described molecular approach, namely sequence-related amplified polymorphism (SRAP), which preferentially amplifies open reading frames (ORFs), was applied to assess the level and pattern of genetic diversities in different important cultivated species of genus *Salvia.* SRAP system combines simplicity, reliability, moderate throughput ratio and easier sequencing of selected bands. Furthermore, this system targets coding sequences in the genome. With the ability to sequence from complex profiles, a specific marker tagging a given trait is important for marker-assisted selection. In this regard and because most SRAP markers can produce clear high-intensity bands rarely overlapping, it is easier than other markers to sequence polymorphic loci by cutting directly from the gels (Williams *et al*. 1990).

Table 3. Similarity matrix obtained for SRAP marker

	S.sclarea	*S.hydrangea*	*S.limbata*	*S.macrochlamys*	*S.xanthocheila*	*S.verticillata1*	*S.verticillata2*	*S.nemorosa1*	*S.verticillata3*	*S.nemorosa2*	*S.athiopis*
S.sclarea	1.000										
S.hydrangea	0.185	1.000									
S.limbata	0.243	0.170	1.000								
S.macrochlamys	0.211	0.283	0.229	1.000							
S.xanthocheila	0.239	0.186	0.306	0.156	1.000						
S.verticillata1	0.139	0.166	0.169	0.191	0.219	1.000					
S.verticillata2	0.141	0.156	0.108	0.193	0.222	0.491	1.000				
S.nemorosa1	0.218	0.214	0.291	0.168	0.298	0.122	0.123	1.000			
S.verticillata3	0.138	0.163	0.166	0.245	0.211	0.485	0.694	0.138	1.000		
S.nemorosa2	0.206	0.250	0.273	0.172	0.3059	0.107	0.096	0.584	0.120	1.000	
S.athiopis	0.261	0.215	0.290	0.182	0.312	0.212	0.156	0.347	0.191	0.311	1.000

In this study, we isolated 204 polymorphic bands resulting from the amplification of the *Salvia*, using 25 different primer combinations. The high similarity between *S.verticillata2* and *S.verticillata3* was noticed in SRAP analysis indicating that these populations are closely related, Furthermore, the SRAP marker showed the *S.verticillata3* and *S.nemorosa2* as most divergent populations, which reveals the existence of sufficient amount of genetic variability among the *Salvia* species. Studies about the genetic diversity of *S.miltiorrhiza* have been undertaken using different molecular markers such as RAPD, AFLP, CoRAP (Bao-lin *et al.* 2002; Wang *et al*. 2009). AFLP is now widely used for genomic fingerprinting due to its rapidity, reproducibility and numerous polymorphisms. But, two major disadvantages associated with this technique are prohibitive expenses and tedious procedures (Wang *et al*. 2007). The CoRAP produces fewer polymorphism bands in *Salvia*, which would lead to underestimate polymorphism analysis and genetic diversity level (Wang *et al*. 2007). In the study by Song *et al*. (2010) the level and pattern of genetic diversity in five important cultivated populations of *S.miltiorrhiza* was assessed using SRAP markers. Among these populations, 110 polymorphic bands (90.16%) were observed in 122 bands amplified by six SRAP primers. High genetic similarity was also reported by RAPD markers in cultivated populations of *Salvia miltiorrhiza* (Bao-lin *et al.* 2002). In a recent

study by Sepehry *et al.* (2012) genetic variation of *Salvia* based on RAPD and ISSR markers was assessed and 30 selected markers introduced a sufficient overview of the relationships among the eight *Salvia* species. They suggested that both RAPD and ISSR markers are effective and reliable for accurate assessment of genetic variation (Tautz 1989). Our study indicated that SRAP markers could detect high polymorphism among *Salvia* genotypes (99.5%). The significant cophenetic correlation coefficient (r= 0.96) indicated high correspondence of the similarity matrix with the constructed dendrogram. The average genetic distance derived from SRAP markers showed higher genetic differences compared with the average genetic distance of other markers. On the other hand, this marker was effective and reliable for assessing the degree of genetic variation as well as differentiation of *Salvia* at inter and intra-species level. We hope our findings assist the breeders to maximize genetic diversity and manage the germplasm of this medicinal plant.

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