Genetic Relationship among *Salvia* Species in the Syrian Coast Assessed by ISSR and RAPD Markers

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Abstract

The genus Salvia includes an enormous assemblage of nearly 1000 species dispersed around the World. Most medicinal plants like Salvia have originated from wild herbs, so information on their genetic characters is essential for their conservation and domestication. Hence, it is important to evaluate the genetic diversity of Salvia in Syria. ISSR and RAPD markers were used to evaluate the genetic relationship among fifteen ecotypes of seven Salvia species collected from different locations of the Syrian Coast (Lattakia and Tartous). Amplification of genomic DNA using 28 primers (14 RAPD and 14 ISSR) produced 133 bands, of which 114 were polymorphic (85.7%).

The Nei's dissimilarity index varied from 0.07 to 0.40 for RAPD and from 0.12 to 0.52 for ISSR data. The dendrogram was constructed using UPGMA method with bootstrapping 100 times using the iTOL software V.2.3, which distinguished four main clusters among seven species of Salvia based on RAPD and ISSR analyses, respectively. It was generally observed that both ISSR and RAPD markers had similar efficiency in detecting genetic polymorphisms but ISSR wasmore reproducible than RAPD with remarkable ability to differentiate the closely related ecotypes of Salvia.

Based on the results of clustering and AMOVA, the genetic diversity among the species was confirmed. Furthermore, our genetic diversity analysis could provide useful information for utilization of these species, especially for genetic improvement.

Keywords - *Salvia, genetic relationship, RAPD, ISSR, dendrogram, polymorphism, clustering, AMOVA*

I. INTRODUCTION

Salvia is the largest genus of the Lamiaceae family which includes 1000 species spread throughout the World: central and south America (500 spp.), central Asia- Mediterranean (250 spp.), and eastern Asia (90 spp.) [1].

Thirty two annual or perennial species of the genus are found in Syria and Lebanon [2]. The genus name 'Salvia' is derived from Latin 'Salvio' which means to save, or to recover [3]. *Salvia* is one of the most appreciated herbs for its essential oil richness and biologically active compounds [4]. Using of the genus *Salvia* has been widely increased in the industries such as pharmaceutical because it has pharmacological specifications including, anti-inflammatory, antiplatelet effects [1]. *Salvia* has also been used to treat a number of different diseases such as acquired immunodeficiency syndrome (AIDS), diabetes, liver malfunction and Alzheimer's disease [5]. They also have economical value in the industry of perfumery and cosmetics, and are used as spices and flavoring agents [6]. Molecular markers provide a powerful tool for studying the genetic diversity. Among advanced genetic markers, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers have been widely used for diversity analyses [7]. RAPD technique is quick, easy and requires no prior sequence information. The technique detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence [8]. ISSR marker involves PCR amplification of DNA by a single 16-18 bp. long primer composed of a repeated sequence anchored at the 3' or 5' end of 2-4 arbitrary nucleotides [9].The technique is rapid, simple, inexpensive and more reproducible than RAPD [10]. RAPD and ISSR markers have been extensively used for finger-printing [11], genetic diversity [12], and phylogenetic studies [13]. Accordingly, some researchers have tried to assess this variability by ISSR and RAPD techniques in different *Salvia* species [14, 15, 5, 16, 17, 1], genetic diversity was also investigated among eight species of *Salvia* in Iran by using RAPD and ISSR markers and produced 280 bands, of which 91% were polymorphic [18].

No study was found investigating the genetic relationship among *Salvia* species in Syria based on these two molecular marker systems. The objective of present study was to estimate the genetic relationship among seven species of *Salvia*including*S. viridis, S. fruticosa, S.verbenaca, S. viscosa, S. sclarea, S. tomentosa, S. judaica* using RAPD and ISSR genetic markers.

II. MATERIALS AND METHODS

A. Plant materials

Fifteen ecotypes of seven *Salvia* species were selected from different geographical regions of the Syrian Coast which is composed of two provinces: Lattakia and Tartous (Table 1).

B. DNA extraction

Fresh and young leaves of each ecotype were ground in liquid nitrogen to a fine powder with a chilled mortar and pestle. Genomic DNA was extracted using Doyle and Doyle [19] CTAB method.Powders (250 mg) were immediately transferred to a tube and 1µL of preheated extraction buffer [2% CTAB, 100 mMTris-HCl (pH = 8), 25mM EDTA (pH =8), 2.5M NaCl, 2% polyvinylpyrrolidone (PVP), and 1% R-mercaptoethanol] was added. The mixture was shaken and incubated at 65°C for 1h in a water bath. Then the tubes were centrifuged at 10000 rcf for 10 min at 4°C and the transparent upper phase was transferred into a fresh tube. An equal volume of chlorophorm: isoamyl alcohol (24:1) was added and mixed slowly. Samples were centrifuged at 10000 rcf for 10 min at 4°C and the aqueous phase was transferred into a new tube. Isopropanol was added to precipitate nucleic acids, tubes were shaken and incubated at -20°C for 30 min. Precipitated DNA was centrifuged at 12,000 rcf for 10 min at 4°C and supernatant was removed. DNA pellets were washed with 500µL of 70% ethanol. The DNA pellets were dried and dissolved in 200µL TE buffer [10 mMTris-HCl (pH 8.0), 1mM EDTA (pH 8.0)]. The quantity and quality of DNA were determined by spectrophotometer and electrophoresis on 1% agarose gel, respectively.

Table 1. Fifteen ecotypes of seven *Salvia* species from different geographical regions of the Syrian Coast and various attributes of the collection sites

Scientific name	Ecotype abbreviation	Collection site	Province	Latitude	Longitude	Altitude
Salvia viridis	S1	Alkhrnoba	Lattakia	35°31_47″N	036°01_43″E	80m
	S8	Fdyo	Lattakia	35°30_11″N	035°52_02 E	82.25m
Salvia fruticosa	S2	Almzeraa	Lattakia	35°31_59″N	036°04_07″E	645m
	S3	Almzeraa	Lattakia	35°31_59″N	036°04_07″E	645m
	S9	Slonfeh	Lattakia	35°35_73″N	036°06_12 E	650 m
Salvia verbenaca	S4	Drekesh	Tartous	34°55_90″N	036°09_64″E	493 m
	S13	Jablah	Lattakia	35°13_88″N	036°22_12″E	530 m
Salvia viscosa	S5	Drekesh	Tartous	34°56_47″N	036°12_50″E	545 m
	S6	Banyas	Tartous	35°06_34″N	036°03_28″E	427 m
Salvia sclarea	S7	Drekesh	Tartous	34°55_90″N	036°09_64″E	493 m
	S10	Slonfeh	Lattakia	35°37_82″N	036°10_13 E	1020 m
	S15	Fjlet	Tartous	34°50_90″N	036°09_60″E	540 m
Salvia judaica	S11	Slonfeh	Lattakia	35°35_73″N	036°06_12″E	650 m
	S14	Jablah	Lattakia	35°15_02″N	036°01_66″E	385 m
Salvia tomentosa	S12	Jablah	Lattakia	35°14_02″N	036°06_99″E	744 m

C. RAPD amplification

Fifteen arbitrary RAPD primers were purchased from commercial source. Fourteen primers with reproducible and scorable amplifications were chosen for further studies (Table 2). The PCR amplifications were carried out in Veriti 96-Well Thermal Cycler (TECNE-Flexigene) in final volume of 25µLcontaining 2.5µL of PCR buffer 10x, 1µL of $MgCl_2$(50mM), 0.5µL of dNTP (10 mM), 3µL of primer (10pmol/µL) (Primer formulas are listed in Table 2), and 0.25µL taq polymerase enzyme (5 units/µL), 5µL DNA (10 ng/µL) and 12.75µL ddH_2O.The PCR program started with an initial phase of 10 min at 95°C, followed by 40 cycles of 45s at 95°C, 1min at 36°C, 1.5min at 72°C and 10 min final elongation at 72°C.The PCR products were run on 1.5% agarose gel with a 1x TBE buffer at 85V for 75 min and stained with ethidium bromide. Each gel was photo documented under UV using gel documentation system (UVITEC-Cambrige).Molecular sizes of amplified products were estimated using a 100-3000 bp DNA ladder.

D. ISSR amplification

Ninteen ISSR primers were purchased from commercial source. After initial tests, fourteen primers were chosen for further examinations (Table 2). The PCR amplifications were performed in a final volume of 25µL containing 0.3µL taq polymerase enzyme (5 unites/µL), 2.5µL of PCR buffer10x, 1µL $Mgcl_2$ (50 mM), 0.5µL dNTPS (10 mM), 8µL DNA (10 ng/µL), 3µL primer (10pmol/µL) and 9.7µL ddH_2O in 96-Well thermal cycler (TECNE-Flexigene). The PCR program started with an initial phase of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1min at (40-56 °C), 2 min at 72°C and 10 min final elongation at 72°C. The PCR products were run on 1.8% agarose gel with a 1x TBE buffer at 85V for 90 min and stained with ethidium bromide. Each gel was photo documented under UV using gel documentation system (UVITEC-Cambrige). Molecular sizes of amplified products were estimated using a 100-3000 bp DNA ladder.

E. Statistical analysis

Reproducible patterns of each ISSR and RAPD primers were selected for manual band scoring

as '0' (no band) and '1'(presence of band) binary data matrix. Number of total bands (TB), number of polymorphic bands (PB) and polymorphism percentage (PPB%) were calculated for each primer. The suitability of both the ISSR and RAPD markers to evaluate genetic profiles of *Salvia* ecotypes was measured using the aforementioned parameters. The software Power Marker [20]was used to calculate genetic diversity (GD), polymorphism information content (PIC), genetic distance coefficients according to [21].The distance matrix was used to construct a typical cluster scheme revealing associations among species based on the unweighted pair group method with arithmetic averages (UPGMA) with bootstrapping 100 times using the iTOL software V.2.3 [22] .Analysis of Molecular Variance (AMOVA) was used to calculate variation among and within species.

Table 2. Marker parameters calculated for each tested primer (ISSR+RAPD) among *Salvia* ecotypes

Locus name	Sequence 5'-3'	TB	PB	PPB (%)	PIG	GD
811	(GA)8C	2	2	100%	0.2157	0.2578
814	(CT)8A	4	4	100%	0.3168	0.4000
818	(CA)8G	2	1	50%	0.1869	0.2489
826	(AC)8C	5	4	80%	0.2688	0.3200
827	(AC)8G	14	14	100%	0.3598	0.4711
834	(AG)8CTT	6	5	83.3%	0.2366	0.2756
836	(AG)8CA	7	7	100%	0.3168	0.4000
841	(AG)8CC	7	7	100%	0.3598	0.4711
845	(CT)8AGG	3	3	100%	0.2366	0.2756
848	(CA)8AGC	4	4	100%	0.3302	0.4178
856	(AC)8CTA	6	6	100%	0.2595	0.3111
868	(CA)7CGTAGTCGT	4	4	100%	0.2688	0.3200
UBC-823	(TC)8C	5	5	100%	0.3598	0.4711
UBC-855	(AC)8YT	4	4	100%	0.3302	0.4178
	Sub Total ISSR	**73**	**70**	**95.89 %**		
OPD-03	GTCGCCGTCA	4	3	75%	0.2508	0.3200
OPB-03	CATCCCCCAG	6	5	83%	0.2323	0.2889
OPD-02	GGACCCAACC	3	2	67%	0.1659	0.2011
OPB-02	TGATCCCTGG	1	1	100%	0.1167	0.1244
OPB-01	GTTTCGCTCC	5	3	60%	0.1459	0.1778
OPA-07	GAAACGGGTG	7	7	100%	0.3073	0.4000
OPA-06	GGTCCCTGAC	7	7	100%	0.3050	0.3956
OPD-08	GTGTGCCCCA	3	2	67%	0.2138	0.2678
OPA-03	AGTCAGCCAC	2	0	0%	0.0534	0.0669
OPA-04	AATCGGGCTG	4	3	75%	0.1370	0.1600
OPA-05	AGGGGTCTTG	4	2	50%	0.1156	0.1422
OPB-04	GGACTGGAGT	4	4	100%	0.3073	0.4000
OPB-05	TGCGCCCTTC	5	2	40%	0.0803	0.0889
OPD-05	TGAGCGGACA	5	3	60%	0.1078	0.1289
	Sub Total RAPD	**60**	**44**	**73.3 %**		

Note: TB - the number of total bands, PB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PIC: polymorphic information content, GD: the genetic diversity.

III.RESULTS AND DISCUSSION

A. RAPD analysis

The total number of amplified fragments generated by fourteen RAPDs was 60, with sizes ranging from 300 to 2500 bp. of which 44 bands (73.3%) were polymorphic (Fig. 1, Table 2). The number of TB detected with RAPDs ranged from 1 (OPB-02) to 7 (OPA-07 and OPA-06) with an average of 4.28 bands per primer. The number of PB was one (OPB-02) to 7 (OPA-07 and OPA-06) with an average of 3.14 polymorphic bands per primer. The percentage of polymorphism (PB%) varied from 40 % for OPB-05 to 100 % for primers OPB-02, OPA-07, OPA-06 and OPB-04 (Table 2). OPA-07 and OPB-04 showed the highest PIC value (0.307) and OPA-03 gave the lowest (0.05) with an average PIC of 0.180. The genetic diversity (GD) ranged from 0.06 to 0.40. The dissimilarity matrix indicated that Nei's dissimilarity index ranged from 0.07(between *S. fruticosa* and *S. viridis*) to 0.40 (between *S. tomentosa* and *S. viridis*) with mean value of 0.13(Data not shown). The UPGMA clustering algorithm for RAPD data classified 7 *Salvia* species into four clusters. Cluster I has *S. verbenaca*, cluster II has *S. judaica* and *S. tomentosa*, cluster III includes *S.sclarea* and *S. viscosa*, cluster IV includes *S. fruticosa* and *S. viridis*(Fig. 2).

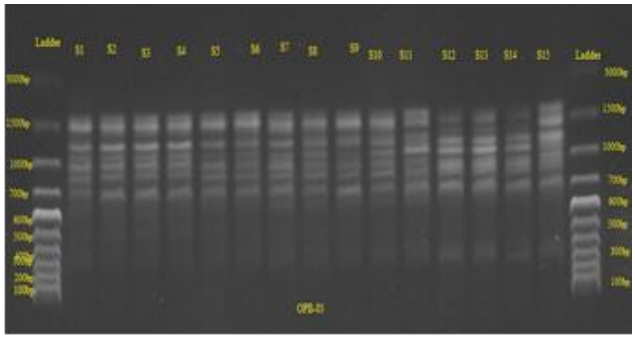

Fig. 1. RAPD (Primer OPB-03). DNALadder 100-3000 bp. Abbreviations of each ecotype are as listed in Table1

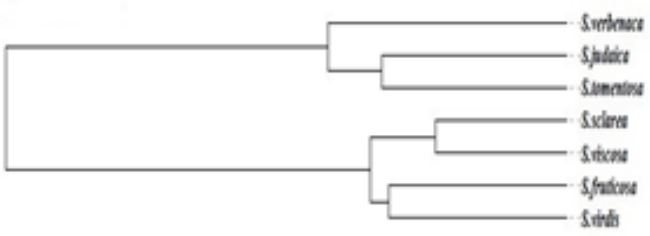

Fig. 2.Dendrogram of seven *Salvia* species based on Nei's genetic distance obtained from RAPD markers using the UPGMA algorithm. Numbers on branches refer to bootstrap values (1,000 replications)

B. ISSR analysis

The total number of amplified fragments generated by fourteen ISSRs was 73, with sizes ranging from 300 to 2500 bp. All 70 (95.89%) bands were polymorphic (Fig. 3, Table 2). The minimum number of amplified bands (TB) as well the smallest number of polymorph bands (PB) were both obtained with 818(2 and 1, respectively). As well, the maximum number of TB and PB were recorded for 827 (14 and 14, respectively). The averages of 5.21 and 5 bands per primer were obtained for TB and PB, respectively. The percentage of polymorphism (PPB) varied from 50% for 818 to 100% for 811, 814, 827, 836, 841, 845, 848, 856, 868, UBC- 823 and UBC-855 (Table 2). High PIC value of 0.359 (827) and low PIC value of 0.186 (818), with an average value of PIC per primer 0.35 were obtained. The genetic diversity (GD) ranged from 0.24 to 0.47. The dissimilarity matrix revealed that Nei's dissimilarity index ranged from 0.12 (between *S. judaica* and *S.verbenaca*) to 0.52 (between *S. tomentosa*and*S.viridis*) with mean value of 0.19 (Data not shown). The UPGMA clustering algorithm for ISSR data based on Nei's genetic distances grouped 7 *Salvia* species into four clusters.

Cluster has *S. tomentosa*, cluster IIincludes *S. judaica* and *S. verbenaca*, cluster III includes*S.sclarea*and *S. viridis*, cluster IV has *S. fruticosa* and *S. viscosa*, respectively. (Fig. 4).

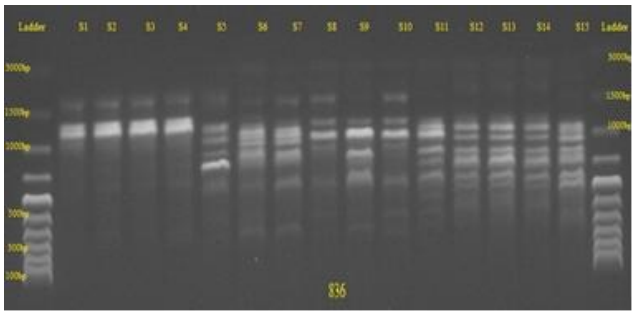

Fig. 3. ISSR (Primer 836). DNA Ladder 100-3000 bp. Abbreviations of each ecotype are as listed in Table 1

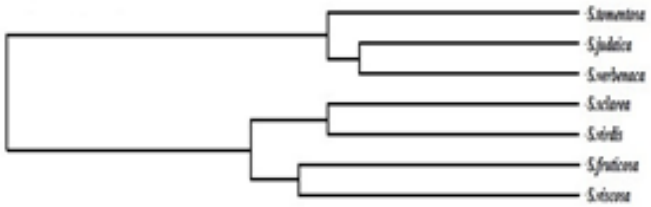

Fig. 4. Dendrogram of seven *Salvia* species based on Nei's genetic distance obtained from ISSR markers using the UPGMA algorithm. Numbers on branches refer to bootstrap values (1,000 replications)

C. RAPD and ISSR-based combined analysis

A total of 28 primers showed 85.7% polymorphism across all the species of *Salvia*. The mean values of PIC observed for all the studied primers were 0.23, respectively. Based on combined data from ISSR and RAPD, the minimum genetic dissimilarity (0.25) was observed between *S. judaica* and *S.tomentosa* while the maximum of this coefficient (0.79) was recorded between *S. tomentosa* and *S. viridis*. The results of AMOVA analysis showed that 23 % of variance occurred among species, and 77 % ofvariance occurred within species.

The UPGMA clustering algorithm based on Nei's genetic distance of combined data (ISSR and RAPD) at an average value of 0.34 determined fivemajor clusters, including *S. verbenaca* as the first cluster, *S. judaica* and *S. tomentosa* as the second cluster, *S. sclarea* as the third cluster, *S. viridis* as the fourth cluster, and*S. fruticosa* and *S.viscosa* as the fifth cluster (Fig.5). Based on species phylogenetic clustering it can be concluded that the species located in the common group may have closer genetic relationships, which showed that both studied techniques could separate species via their ecotypes.

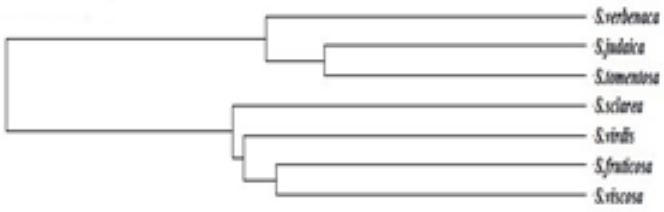

Fig. 5.Dendrogram of seven *Salvia* species based on Nei's genetic distance generated by UPGMA algorithm based on combined data (ISSR and RAPD). Numbers on branches refer to bootstrap values (1,000 replications)

IV.CONCLUSIONS

This is the first report on the assessment of genetic relationship among *Salvia* species in the Syrian Coast (Lattakia and Tartous) using molecular markers. In this study, RAPD and ISSR markers were used to assess the genetic diversity among *Salvia* species. Cluster analysis based on both ISSR and RAPD markers divided the seven species into five distinct clusters with a high level of polymorphism.

The present study could be the start of further investigation using more powerful markers such as Simple Sequence Repeat (SSR) and Sequence Related Amplification Polymorphism (SRAP) and we hope our findings could be beneficial in the determination ofthe genetic diversity of *Salvia* in Syria.

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