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Original Article

Genetic Diversity of *Salvia* Species Assessed by ISSR and RAPD Markers

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Abstract

The genus *Salvia* includes an enormous assemblage of nearly 1,000 species dispersed around the world. Due to possible threats to this genus, there is an immediate requirement to evaluate the diversity of its wild populations. ISSR and RAPD molecular techniques were used to evaluate the genetic relationships among twenty-one ecotypes of eight *Salvia* species. Amplification of genomic DNA using 23 primers (15 RAPD and eight ISSR) produced 280 bands, of which 91% were polymorphic. The results of marker parameters showed no clear difference between two marker systems. It was generally observed that both ISSR and RAPD markers had similar efficiency in detecting genetic polymorphisms with remarkable ability to differentiate the closely related ecotypes of *Salvia*. Nei's similarity coefficients for these techniques ranged from 0.48 to 0.98. Based on the results of clustering, PCoA and AMOVA, the genetic diversity between and within species was confirmed. So, conservation and domestication of the genus *Salvia* must be due to levels of genetic variations.

Keywords: AMOVA, Clustering, Marker parameters, PCoA, Polymorphism

Introduction

The genus *Salvia* (tribe Mentheae, Lamiaceae) includes an enormous assemblage of nearly 1,000 species displaying a notable range of variation around the world: Central and South America (500 spp.), central Asia/Mediterranean (250 spp.), and eastern Asia (90 spp.) (Erbano *et al*., 2015). Exploitation of the genus *Salvia* has been widely increased in the industries such as pharmaceutical, food and spices production (Wang *et al*., 2011). Additionally, the abundance of metabolites as principal chemical components in *Salvia* species, gives them distinctive pharmacological specifications including, anti-inflammatory, antiplatelet and antithrombotic effects (Erbano *et al*., 2015). *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 (Sepehry Javan *et al*., 2012).

Sepehry Javan *et al*. (2012) mentioned that three major factors influencing genetic variations in *Salvia* are: species, geographical distribution and selection. These factors along with cross-pollination make the taxonomy and genetic relationships of *Salvia* species unclear (Wang *et al*., 2011). Morphological characteristics are easily affected by environment that makes identification of species more complex (Chen *et al*., 2013). The conservation and suitable use of plant genetic resources require accurate monitoring of their accessions. So, genetic characterization is essential to manifest the extent of plant genetic diversity, and also to discover better genotypes; especially in the geographically differentiated genus such as *Salvia* (Song *et al*., 2010; Peng *et al*., 2014). In the era of modern biotechnology, several DNA-based molecular markers have been developed either for cultivar identification or assessment of genetic relationships among plant species. These techniques include properties like cost effectiveness, abundance, stability, high polymorphism and independency from environmental influences (Kharazian *et al*., 2015).

RAPD markers provide a convenient tool for studying genetic differences even at lower intraspecific levels.. Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) technique is an efficient system for identification of plant genetic diversity. It is also suitable to address evolutionary processes at inter- and intra-specific level (Peng *et al*., 2014; Kharazian *et al*., 2015). This technique involves primers composed of a microsatellite sequence anchored at 3′ or 5′ ends by 2-4 arbitrary nucleotides and higher annealing temperatures. Also, ISSRs are easier to use due to their reproducibility; but they differentiate mostly as dominant following simple Mendelian inheritance like RAPDs (Song *et al*., 2010).

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Table 1. Identity of *Salvia* species used for genetic diversity analysis

Scientific name	Abbreviation	Collection site	State	Latitude	Longitude	Altitude (m)
Salvia aethiopis	AJ	Jadeh Chalous	Mazandaran	36°22′12″N	51°07′12″W	1200
	AR	Roudbar	Gilan	36°47′06″N	49°49′52″W	1880
	AK	Khalkhal	Ardabil	37°38′53″N	48°36′11″W	1958
Salvia macrosiphon	MK	Khatam	Yazd	30°07′24″N	53°59′06″W	2178
	MD	Dashtestan	Boushehr	28°57′22″N	51°28′31″W	430
	MJ	Jahrom	Fars	29°20′07″N	51°52′08″W	1610
Salvia nemorosa	NI	Khansar	Isfahan	33°10′00″N	50°23′00″W	2400
	NK	Khalkhal	Ardabil	37°39′51″N	48°18′55″W	1782
	NQ	Qazvin	Qazvin	36°33′36″N	50°26′20″W	2250
Salvia officinalis	OH	Hamedan	Hamedan	34°46′10″N	48°30′00″W	1870
	ON	Najafabad	Isfahan	32°36′93″N	51°27′90″W	1642
Salvia reuterana	RK	Khoramabad	Lorestan	33°29′07″N	48°22′12″W	2040
	RT	Tehran	Tehran	35°43′41″N	51°35′32″W	1618
Salvia sclarea	SR	Rostamabad	Gilan	36°55′10″N	49°24′30″W	1290
	SM	Meshkinshahr	Ardabil	36°21′12″N	48°14′28″W	1260
	SQ	Qazvin	Qazvin	36°33′89″N	50°18′70″W	2100
Salvia verticillata	VQ	Qazvin	Qazvin	36°22′34″N	50°32′19″W	1836
	VK	Karaj	Tehran	35°58′11″N	51°21′15″W	2170
Salvia virgata	ViJ	Jadeh Chalous	Mazandaran	36°22′12″N	51°07′12″W	1200
	ViN	Najafabad	Isfahan	32°36′93″N	51°27′90″W	1642
	ViQ	Qazvin	Qazvin	36°33′36″N	51°26′20″W	2250

Plant genetic diversity is an important factor for their domestication and breeding. Accordingly, some researchers have tried to assess this variability by ISSR and RAPD techniques in different *Salvia* species (Song *et al*., 2010; Wang *et al*., 2011; Sepehry Javan *et al*., 2012; Zhang *et al*., 2013; Peng *et al*., 2014; Erbano *et al*., 2015). Genetic variability was also investigated in a genus of Lamiacea family as *Ocimum*, with the application of these markers (Chen *et al*., 2013; Patel *et al*., 2014). Germplasm variation detected by ISSRs has been reported in the genus *Stachys*, due to the rich genetic resources of the collection regions; which are important in conservation purposes (Kharazian *et al*., 2015).

Given the possible threats to Iran's natural landscapes; where *Salvia* is one of the threatened component genera, there is an immediate requirement to evaluate the diversity of wild populations, especially those with economic values. The present research was undertaken with the aims of evaluating the genetic diversity of the eight species of *Salvia*.

Materials and Methods

Plant materials

Twenty-one ecotypes of eight *Salvia* species were selected from different geographical regions of Iran (Table 1). The seeds of these ecotypes were provided by the Gene Bank of Iran, Research Institute of Forest and Rangelands; and sowed in a greenhouse for sampling.

Isolation and evaluation of the quantity/quality of genomic DNA

Young leaves of each ecotype were ground as bulk into a fine powder in liquid nitrogen. Powders (250 mg) were immediately transferred to a tube adding 1 ml of preheated extraction buffer [2% CTAB, 100 mM Tris-HCl (pH = 8), 25 mM EDTA (pH = 8), 2.5M NaCl, 2% polyvinylpyrrolidone (PVP), and 1% β-mercaptoethanol]. The mixture was shaken and incubated at 65 °C for 1 h in a water bath. Then the tubes were centrifuged at 9,720 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 9,720 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 19,000 rcf for 10 min at 4 °C and supernatant was removed. DNA pellets were washed with 500 µl of 70% ethanol. The DNA pellets were dried and dissolved in 200 µl TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. The quality and quantity of the DNAs were evaluated by 0.8% agarose gel and NanoDrop Spectrophotometer.

PCR amplification

A set of 13 ISSR primers from UBC Company (the University of British Columbia), and 30 decamer RAPD primers from Operon Technologies Inc. (USA) were screened for their repeatable amplification (Table 1). For each primer, Polymerase Chain Reaction (PCR) in volume 25 µ was composed of *Taq* DNA polymerase enzyme (1 u/µl), PCR Buffer (1X), $Mgcl_2$ (2 mM), dNTP (200 µM), primer (0.5 µM), DNA (50 ngr) and ddH_2O. Amplifications were carried out using a DNA thermal cycler (Tech Model TC-5000) with the following program: initial denaturation for 3 min at 95 °C followed by 35 cycles of denaturation for 30s at 95 °C, annealing of 30 s at 34 °C for RAPD and at 30-43°C for ISSR, extension at 72 °C for 2 min, and final elongation at 72 °C for 10 min. Each amplified PCR products (in three replications), were run on 1.5% agarose gel visualized by ethidium bromide staining, and the images were documented using Gel Documentation System (USA) under U.V. lights.

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 loci (TB), number of polymorphic loci (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 these parameters: Polymorphism index (PI) and Polymorphism Information Content (PIC) were calculated as explained by Rizza *et al*. (2007). Effective Multiplex Ratio or $EMR = n \times PPB\%$; where n is the average number of fragments amplified by

accessions, and Marker Index ($MI = PIC \times \text{EMR}$) were estimated to characterize the capacity of each primer to detect polymorphic loci among the genotypes (Powell *et al*., 1996). Also, the Resolving Power (RP) of each primer was scored by $RP = \sum Ib$; where Ib represents the informative fragments as $Ib = 1 - (2 \times |0.5 - p_i|)$ (Chen *et al*., 2013). Simpson's Index was evaluated based on Manica-Cattani *et al*. (2009) as $SI = \sum(1 - p_i^2) / N$. In latter equations, p_i is the frequency of the i^{th} allele, and N is the number of polymorphic loci detected by each primer. The data matrix was submitted to determine observed number of alleles (N_o), expected number of alleles (N_e), Nei's gene diversity (h), Shannon's information index of genetic diversity (I), total heterozygosity (H_t), average heterozygosity within populations (H_s), degree of genetic differentiation for polymorphism between populations (G_{st}) and indirect estimation of gene flow between populations (N_m) using the software POPGENE ver. 1.32. The data matrix of both markers was converted into genetic similarity matrix using pairwise Nei's unbiased genetic distance (Nei, 1978). The genetic relatedness among the ecotypes and species was analyzed by an unweighted pair group method with arithmetic average (UPGMA) using TFPGA software, and related dendrograms were constructed by resampling with 1,000 replacements over loci. Bootstrap values were considered as a frequency to form a particular group. Analysis of Molecular Variance (AMOVA) was used to calculate variation among and within population using GenAlEx 6.501 ver. 6.41. As well, Principal Coordinate Analysis (PCoA) was performed using GenAlEx to highlight the resolving power of the ordination based on similarity coefficients. Mantel test was performed using 1,000 permutations in XLSTAT, 2015 software (α = 0.05), to compute the correlation (r) between the similarity matrices to test the congruence of fit between RAPD and ISSR markers.

Results

ISSR analysis

The total numbers of amplified fragments generated by eight ISSRs were 98, with sizes ranging from 300 to 3,000 bp. All 85 (86.7%) loci were polymorphic (Fig. 3-a, Table 2). The minimum number of amplified bands (TB) as well the smallest number of polymorph loci (PB) were both obtained with UBC-825 (10 and 8, respectively). As well, the maximum number of TB and PB were recorded for UBC-823 (14 and 13, respectively). The averages of 12 and 10 loci per primer were obtained for TB and PB, respectively. The percentage of polymorphism (PPB) varied from 77% for UBC-841-Y to 93% for UBC-823 (Table 2). High PIC value of 0.46 (UBC-855) and low PIC value of 0.38 (UBC-846), with an average value of PIC per primer 0.43 were obtained. In this experiment, the highest effective multiplex ratio (EMR, 11.14) was observed with the primer UBC-823 and the lowest amount of this ratio (7.16) was detected by the primer UBC-873 with an average EMR of 8.49 per primer. The highest value of marker index (MI) was recorded with the primer UBC-823 as 5.1 and the lowest MI was observed in the primer UBC-846 as 2.8, totally with an average of MI per primer that was calculated as 3.7. The highest resolving power (RP) value was for the primer UBC-823 (10.0), and the lowest RP observed by UBC-825 as 5.3 with an average RP of 7.4 per primer. The most values of Simpson's index were for UBC-873 (SI=0.80), while the lowest value of this index was 0.66 for UBC-823 (Table 2). The Nei's gene diversity (h) and Shannon index (I) among all studied ecotypes of *Salvia* about ISSR marker data were calculated as 0.37 ± 0.16 and 0.53 ± 0.22, respectively. Mean coefficient of gene differentiation (G_{st}) was found as 0.80, while the estimation of gene flow (N_m) in the population was 0.12 (Table 3). The

Table 2. Marker parameters calculated for each tested primer among *Salvia* ecotypes

Locus name	Sequence 5'–3'	TB	PB	PPB (%)	PI	PIC	EMR	MI	RP	SI
UBC-823	(TC)8C	14	13	93	5.91	0.455	11.14	5.07	10.00	0.66
UBC-825	(AC)8T	10	8	80	3.40	0.425	7.50	3.19	5.33	0.77
UBC-841-Y	(GA)8YC	13	10	77	4.21	0.421	7.46	3.14	6.76	0.75
UBC-846	(CA)8RT	11	10	91	3.76	0.376	7.45	2.81	5.52	0.79
UBC-855	(AC)8YT	12	10	83	4.58	0.458	9.58	4.39	7.62	0.68
UBC-856	(GGAGA)3	13	12	92	5.42	0.452	8.77	3.96	8.86	0.77
UBC-873	(GACA)4	13	11	85	4.55	0.414	7.16	2.96	7.05	0.80
UBC-887	DVD(TC)7	12	11	92	4.97	0.452	8.84	3.99	8.10	0.77
ISSR	Sub-Total	98	85	-	-	-	-	-	-	-
	Avg./primer	12.25	10.63	86.73	4.60	0.432	8.49	3.69	7.41	0.75
P13	CCT GGG TGG A	15	14	93	6.14	0.439	11.00	4.83	9.52	0.66
P21	ACC GGG TTT C	14	13	93	5.59	0.430	7.22	3.10	8.95	0.84
P31	CCG GCC TTC C	16	14	88	5.65	0.404	8.06	3.25	9.05	0.76
P40	TTA CCT GGG C	12	12	100	5.34	0.445	10.33	4.60	8.67	0.73
P42	TTA ACC CGG C	11	11	100	4.18	0.380	8.45	3.21	6.19	0.79
P46	TTA AGG GGG C	10	10	100	3.76	0.376	9.70	3.65	5.33	0.73
P47	TTC CCC AAG C	9	9	100	3.55	0.394	9.33	3.68	5.43	0.75
P54	GTC CCA GAG C	16	16	100	6.71	0.420	7.81	3.28	10.67	0.83
P62	TTC CCC GTC G	9	9	100	3.76	0.417	10.00	4.17	6.00	0.73
P77	GAG CAC CAG G	14	11	79	4.30	0.391	5.78	2.26	6.86	0.84
P78	GAG CAC TAG C	15	14	93	5.79	0.413	7.56	3.12	9.14	0.82
P81	GAG CAC GGG G	11	9	82	3.95	0.438	7.00	3.07	6.38	0.81
P84	GGG CGC GAG T	10	9	90	3.80	0.422	4.69	1.98	6.19	0.68
P86	GGG GGG AAG G	12	12	100	4.58	0.382	6.58	2.51	6.86	0.88
P95	GGG GGG TTG G	8	8	100	3.79	0.474	11.50	5.45	6.38	0.69
RAPD	Sub-Total	182	171	-	-	-	-	-	-	-
	Avg./primer	12.13	11.4	93.96	4.73	0.415	8.33	3.48	7.44	0.77
ISSR + RAPD		280	256	91.43	4.67	0.423	8.41	3.59	7.43	0.76

Note: TB - the number of total bands, PB: the number of polymorphic bands, PB (%): the percentage of polymorphic bands, EMR: effective multiplex ratio, PI: polymorphism index, PIC: polymorphic information content, MI: marker index, RP: resolving power of primer, SI: Simpson's index

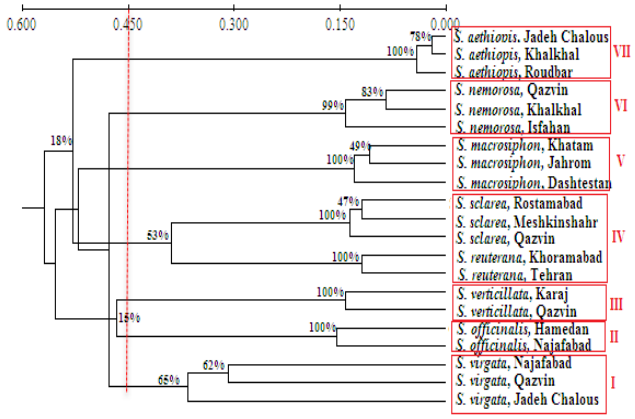

Fig. 1. Dendrogram of *Salvia* ecotypes based on Nei's genetic distance obtained from ISSR markers using the UPGMA algorithm. Numbers on branches refer to bootstrap values (1,000 replications)

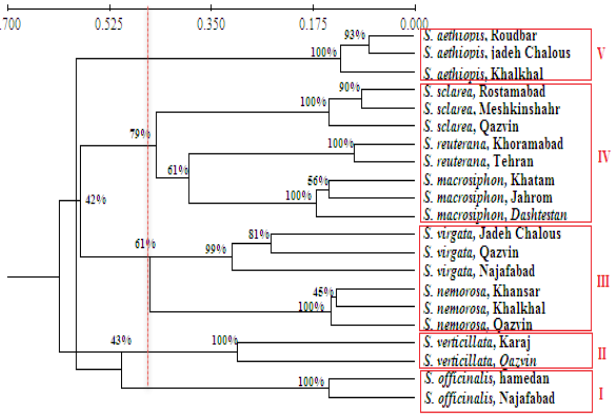

Fig. 2. Dendrogram of *Salvia* ecotypes based on Nei's genetic distance obtained from RAPD markers using the UPGMA algorithm. Numbers on branches refer to bootstrap values (1,000 replications)

results of AMOVA analysis showed that 68% of variance occurred among populations, and 32% variance occurred within populations. The similarity matrix revealed that Nei's similarity index ranged from 0.48 (among *S. virgata*, Najafabad and *S. reuterana*, Tehran) to 0.98 (between Chalous and Khalkhal ecotypes of *S. aethiopis*) with mean value of 0.61 (Data not shown). The UPGMA clustering algorithm for ISSR data based on Nei's genetic distances grouped 21 *Salvia* ecotypes, into seven groups at an average cutoff value of 0.45 (Fig. 1). Bootstrapping values demonstrated the confidence of tree topology and cutoff point was considered based on the confidence values more than 0.50. It was concluded that the ecotypes from each *Salvia* species were grouped together into subclusters. Cluster I has three ecotypes, all from the *S. virgata*. Cluster II and III have four ecotypes belonged to *S. officinalis* and *S. verticillata*., respectively. Cluster IV includes *S. reuterana* and *S. sclarea* ecotypes. Cluster V, VI and VII grouped separately the ecotypes of *S. macrosiphon*, *S. nemorosa* and *S. aethiopis*, respectively (Fig. 1).

RAPD analysis

For RAPD analysis, 182 loci were produced by 15 RAPDs ranging from 300 to 3000 bp. Of which, 171 loci (93.9%) were polymorphic (Fig. 3-b, Table 2). The number of TB detected with RAPDs ranged from eight (P95) to 16 (P31 and P54) with an average of 12.1 loci per primer. The number of PB was eight (P95) to 16 (P54) with an average of 11.40 polymorphic loci per primer. The percentage of polymorphism (PB%) varied from 79 % for P77 to 100 % for primers P40, P42, P46, P47, P54, P62, P86 and P95 (Table 2). P95 showed the highest PIC value (0.47) and P46 gave the lowest (0.38) with an average PIC of 0.415. Also, the highest EMR (11.5) was observed by primer P95, and the lowest was detected by the primer P84 (4.7) with an average of 8.3 per primer. The highest value of MI was devoted to primer P95 as 5.45 and the lowest MI observed in the primer P84 as 1.98 with an average of 3.48. The maximum RP belonged to primer P54 (10.7) and the lowest RP observed by P46 as 5.33 with an average of 7.4 per primer. Also, the highest value of Simpson's index (SI = 0.88) was observed in P86 and the lowest value, in P13 as 0.66 (Table 2). The Nei's gene diversity (h) and Shannon index (I) among all studied ecotypes of *Salvia* for RAPD data were calculated as 0.38 ± 0.14 and 0.55 ± 0.18, respectively. Mean coefficient of gene differentiation (G_{st}) was found as 0.78; while the estimation of gene flow (N_m) in the population was 0.14 (Table 3). The results of AMOVA analysis showed that 64% and 36% variance occurred among and within species, respectively. The similarity matrix indicated that Nei's similarity index ranged from 0.48 (between *S. aethiopis*, Khalkhal and *S. sclarea*, Meshkinshahr) to 0.92 (between ecotypes Roudbar and Chalous of *S. aethiopis*) with mean value of 0.59 (Data not shown). The UPGMA clustering algorithm for RAPD data classified 21 *Salvia* ecotypes into five groups at cutoff value of 0.45 (Fig. 2). As ISSR dendrogram, it was concluded that the ecotypes from each *Salvia* species were placed together into subclusters. Cluster I and II each has two ecotypes from *S. officinalis* and *S. verticillata*, respectively. Cluster III includes *S. nemorosa* and *S. virgata* each with their evaluated ecotypes. Cluster IV has *S. macrosiphon*, *S. reuterana* and *S. sclarea* ecotypes. Cluster V shows the ecotypes of *S. aethiopis* (Fig. 2).

RAPD and ISSR-based combined analysis

A total of 23 primers showed 91.4% polymorphism across all the ecotypes of *Salvia*. The mean values of PIC, EMR, MI, RP and SI observed for all the studied primers were 0.42, 8.41, 3.59, 7.43, 0.76, respectively (Table 2). Based on combined data from ISSR and RAPD, the minimum genetic similarity (0.50) was observed between *S. sclarea*, Qazvin and *S. officinalis*, Najafabad; while the maximum of this coefficient (0.93) was recorded between ecotypes Roudbar and Chalous of *S. aethiopis* (data not shown). The Mantel correlation coefficient (r) between the Nei's genetic similarity matrices of ISSRs and RAPDs was high (r = 0.82). The results of species clustering based on UPGMA algorithm and Nei's similarity coefficients of combined data (ISSR and RAPD) at an average cutoff value of 0.45 determined three major groups, including *S. aethiopis* as the first group, *S. officinalis* and *S. verticillata* as the second group and five other species as the third group that has two sub-clusters *S. macrosiphon*, *S. sclarea* and *S. reuterana* in the first sub-cluster as well as *S. nemorosa* and *S. virgata* in the second sub-cluster (Fig. 5). Principal Coordinate Analysis (PCoA) was performed to provide spatial diagram of the relative genetic distances among ecotypes. PCoA revealed that the first three principal coordinate components accounted for 42.7% variation of the genetic similarity. In accordance to cluster

Table 3. Summary of various genetic diversity indices analyzed in *Salvia* ecotypes

Diversity indices		N_o	N_e	h	I	H_t	H_s	G_{st}	N_m
Mean value ±std.	ISSR	1.87±0.34	1.67±0.33	0.37±0.16	0.53±0.22	0.37±0.03	0.072±0.004	0.804	0.122
deviation	RAPD	1.94±0.24	1.67±0.30	0.38±0.14	0.55±0.18	0.38±0.02	0.082±0.005	0.785	0.137

Note: N_o: observed number of alleles, N_e: expected number of alleles, h: Nei's gene diversity, I: Shannon's information index, H_t: heterozygosity, H_s: average heterozygosity, G_{st}: degree of genetic differentiation, N_m: estimate of gene flow

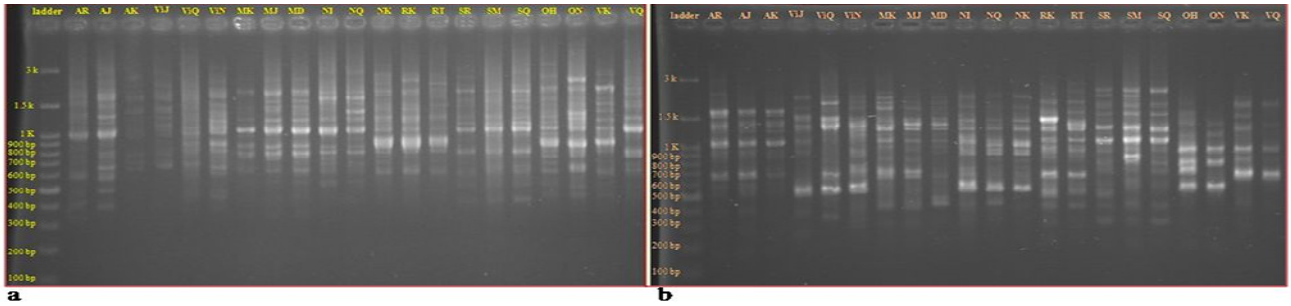

Fig. 3. ISSR (a, Primer UCB-873) and RAPD (b, Primer P13) markers pattern for *Salvia* species, respectively. DNA Ladder 100-3000 bp (DM2300-SMOBiO). Abbreviations of each ecotype are as listed in Table 1

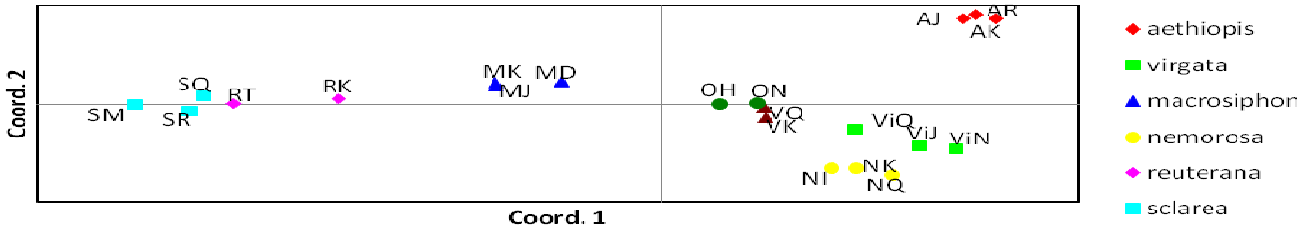

Fig. 4. A two-dimensional plot of the Principal Coordinate Analysis (PCoA) for ISSR and RAPD combined data showing the assortment of *Salvia* ecotypes. The first and second principal coordinates account for 15.6% and 15.5% of total variation, respectively. Abbreviations of each ecotype are as listed in Table 1

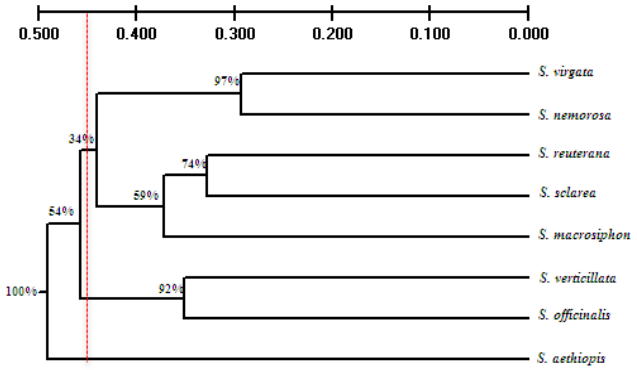

Fig. 5. Nei's similarity dendrogram of eight *Salvia* species generated by UPGMA algorithm based on combined data (ISSR and RAPD). Numbers on branches refer to bootstrap values (1,000 replications)

analysis, the ecotypes of the species were somewhat close to each other with the exception of two species, *S. nemorosa* and *S. virgata*; that are far from species *S. macrosiphon*, *S. reuterana* and *S. sclarea*. The three latter species were in the sub-clusters of third class in cluster analysis (Figs. 4 and 5).

Discussion

Most medicinal plants like *Salvia* have originated from wild herbs, so information on their genetic characters is essential for their domestication and conservation. Factors like reproductive systems, seed dispersal mechanism, and transferring to other regions are of the most important agents for genetic diversity of plant. The limitations of morphological studies can be overcome through the utilization of molecular markers (Zhang *et al*., 2013). DNA profiling methods used in this research contain regions of genome with substantially different evolutionary background. In the present study, of the eight ISSR and 15 RAPD tested primers, about 12 bands were averagely generated; RAPD markers had more polymorphic bands as 11.4 (93.9%). Since the RAPD markers usually represent widely distributed portions of the genome and the ISSR profiles are produced from its microsatellite-rich regions, this high polymorphism is expected. The similar results reported in *Salvia miltiorrhiza* based on ISSRs (Zhang *et al*., 2013) and other *Salvia* species using AFLP markers (Sajadi *et al*., 2010) as 95% and 99% polymorphism, respectively. Also, polymorphism index (PI) in RAPD primers was higher; whereas, other indices like PIC, EMR and MI were somewhat high in ISSRs. On the other hand, RP index was approximately equal in both techniques. In general, small differences in terms of calculated indices showed that both techniques had similar efficiency to differentiate the closely related ecotypes of *Salvia*. Chen *et al*. (2013) reported PIC values about 0.20 in *ocimum* species by ISSR and RAPD markers and also showed the RP values as 1.39 and 5.13, respectively. PIC analysis can be used to select the most appropriate markers for genetic mapping. Also, the high MI reflects the marker efficiency to simultaneously analyze a large number of bands (Powell *et al*., 1996; Patel *et al*., 2014). The high average Simpson's coefficients (about 0.80) indicate high genetic variability among studied accessions of *Salvia*, too. This finding was similar to the study by Manica-Cattani *et al*. (2009) on

accessions of *Lippia alba* by ISSR and RAPD. In their study on *Salvia lachnostachys* ecotypes by ISSR primers, Erbano *et al*. (2015) showed a range of 0.66-0.86 for Simpson's index.

Comparison of Nei's similarity coefficients between ISSRs and RAPDs showed that both markers had high diagnostic capability. This is consistent with the results of ISSR markers in Mint accessions by Kang *et al*. (2013) and *Salvia miltiorrhiza* germplasms studied by Zhang *et al*. (2013); while the genetic similarity derived from SRAPs and ISSRs represented high proximity among *Salvia miltiorrhiza* populations (Song *et al*., 2010). Cluster analysis could group all 21 ecotypes and the results showed reasonable congruency in RAPD and ISSR in terms of species topology. Zhang *et al*. (2013) showed five major clusters for *S. miltiorrhiza* germplasms based on Nei's similarity coefficient for ISSRs; which did not indicate any clear pattern according to their locations. Patel *et al*. (2014) reported that in dendrograms of ISSR and RAPD, the genotypes of each *Ocimum* species were grouped, separately.

Based on species phylogenetic clustering, it can be concluded that the species located in the common group may have closer genetic relationships. Nearby, populations of genus *Salvia* pollinated by bees may share many alleles and have gene flow; leading to genetic similarity of such species. Sepehri Javan *et al*. (2012) illustrated *S. hydrangea* and *S. nemorosa* as the most divergent species and good candidates for breeding programs. In the present study, *S. aethiopis* can be considered a divergent species.

The high Mantel correlation between two studied techniques was confirmed by Ma *et al*. (2012); Patel *et al*. (2014). So, there could be positive coincidence for ISSR- and RAPD-based similarities in *Salvia*. This high correlation has been reported between ISSRs and SRAPs in *Salvia miltiorrhiza*, too (Song *et al*., 2010; Peng *et al*., 2014).

Conclusions

The present experiment would provide useful genetic information for developing conservation strategies and domestication of *Salvia* species and ecotypes. The results showed that both studied techniques could separate species well via their ecotypes.

References

Chen SY, Dai TX, Chang YT, Wang SS, Ou SL, Chuang WL, Cheng CY, Lin YH, Lin LY, Ku HM (2013). Genetic diversity among *Ocimum* species based on ISSR, RAPD and SRAP markers. Australian Journal of Crop Science 7(10):1463-1471.

Erbano M, Schühli GS, Santos ÉP (2015). Genetic Variability and Population Structure of *Salvia lachnostachys*: Implications for Breeding and Conservation Programs. International Journal of Molecular Sciences 16(4):7839-7850.

Kang MJ, Sundan S, Lee GA, Ko HC, Chung JW, Huh YC, Gwag JG, Oh SJ, Kim YG, Cho GT (2013). Genetic Diversity and Population Structure of Korean Mint *Agastache rugosa* (Fisch & Meyer) Kuntze (Lamiaceae) Using ISSR Markers. Korean Journal of Plant Resources 26(3):362-369.

Kharazian N, Rahimi S, Shiran B (2015). Genetic diversity and morphological variability of fifteen *Stachys* (Lamiaceae) species from Iran using morphological and ISSR molecular markers. Biologia 70(4):438-452.

Liu J, Shi S, Chang E, Yang W, Jiang Z (2013). Genetic diversity of the critically endangered *Thuja sutchuenensis* revealed by ISSR markers and the implications for conservation. International Journal of Molecular Sciences 14:14860-14871.

Ma X, Chen SY, Bai SQ, Zhang XQ, Li DX, Zhang CB, Yan JJ (2012). RAPD analysis of genetic diversity and population structure of *Elymus sibiricus* (Poaceae) native to the southeastern Qinghai-Tibet Plateau, China. Genetics and Molecular Research 11(3):2708-2718.

Manica-Cattani MF, Zacaria J., Pauletti G, Atti-Serafii L, Echeverrigaray S (2009). Genetic variation among South Brazilian accessions of *Lippia alba* Mill. (Verbenaceae) detected by ISSR and RAPD markers. Brazilian Journal of Biology 69(2):375-380.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583-590.

Patel HK, Fougat RS, Kumar S, Mistry JG, Kumar M (2014). Detection of genetic variation in *Ocimum* species using RAPD and ISSR markers. 3 Biotech 5(5):697-707.

Peng L, Ru M, Wang B, Wang Y, Li B, Yu J, Liang Z (2014). Genetic diversity assessment of a germplasm collection of *Salvia miltiorrhiza* Bunge. based on morphology, ISSR and SRAP markers. Biochemical Systematics and Ecology 55:84-92.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding 2(3):225-238.

Rizza MD, Real D, Reyno R, Porro V, Burgueno J, Errico E, Quesenberry KH (2007). Genetic diversity and DNA content of three South American and three Eurasiatic *Trifolium* species. Genetics and Molecular Biology 30(4): 1118-1124.

Sajadi SS, Shiran B, Kharazian N, Houshmand S, Sorkheh K (2010). Genetic diversity of *Salvia* species from Chaharmahal va Bakhtiari and Isfahan province using AFLP molecular markers. Iranian Journal of Horticultural Sciences 40:79-88.

Sepehry Javan Z, Rahmani F, Heidari R (2012). Assessment of genetic variation of genus *Salvia* by RAPD and ISSR markers. Australian Journal of Crop Science 6(6):1068-1073.

Song Z, Li X, Wang H, Wang J (2010). Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Genetica 138(2):241-249.

Wang M, Li J, Zhang L, Yang RW, Ding CB, Zhou YH, Yin ZQ (2011). Genetic diversity among *Salvia miltiorrhiza* Bunge and related species using morphological traits and RAPD markers. Journal of Medicinal Plants Research 5(13):2687-2694.

Zhang Y, Li X, Wang Z (2013). Diversity evaluation of *Salvia miltiorrhiza* using ISSR markers. Biochemical Genetics 51(9):707-721.