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Assessment of genetic variation of genus *Salvia* by RAPD and ISSR markers

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Abstract

RAPD and ISSR markers were used to evaluate the genetic variation among eight species of *Salvia* collected from different locations of Ardebil in Iran. Fifteen RAPD primers produced 220 scorable electrophoretic bands which all were polymorphic. Fifteen ISSR primers also produced 245 amplified bands of which 241 were polymorphic. The pair-wise Jaccard genetic similarity varied from 0.0725 to 0.3529 for RAPD and from 0.1692 to 0.3478 for ISSR data. The dendrogram was constructed using UPGMA method with the help of NTSYSpc 2.02 software, which distinguished five and four main groups among eight species of *Salvia* based on RAPD and ISSR analyses, respectively. In the present study, detected polymorphism level represents high genetic distance at inter-species level and introduces the RAPD and ISSR as efficient markers for genetic relatedness assessment in *Salvia*. Furthermore, our genetic diversity analysis could provide useful information for utilization of these materials, especially for genetic improvement.

Keywords: Dendrogram, geneticvariation, ISSR, RAPD, *Salvia.*
Abbreviations: CTAB, Cetyl-Trimethyl Ammonium Bromide; ISSR, Inter Simple Sequence Repeats; PCR, Polymerase Chain Reaction; RAPD, Random Amplified Polymorphic DNA; UPGMA, Unweighted Pair-Group Method using Arithmetic Average.

Introduction

Salvia is an important and the largest genus of the lamiaceae family which includes 900 species spread throughout the world. Fifty eight annual or perennial species of the genus are found in Iran of which 17 are endemic (Mozaffarian, 1996). The genus is named "*Salvia*", derived from Latin "Salveo", which means to save, or to recover (Aktas et al., 2009). *Salvia* is one of the most appreciated herbs for its essential oil richness and biologically active compounds (Penso, 1983). The essential oil composition of *Salvia* is highly influenced by genetic and environmental factors (Dean and Ritchine, 1987; Piccaglia and Marottu, 1993). *Salvia* has been used to treat variety of diseases (Vukovic´-Gačic´ et al., 2006) such as acquired immunodeficiency syndrome (AIDS) (Abd- Elazem et al., 2002), diabetic nephropathy (Jung et al., 2002), liver (Ping et al., 2002) and Alzheimer (Akhondzadeh et al., 2003) diseases. They also have economical value and are used as spices and flavoring agents in perfumery and cosmetics (Delamare et al., 2007).Three predominant factors influencing genetic diversity in *Salvia* are: species, geographical range and human selection (Cahill, 2004). Molecular markers provide a powerful tool for proper characterization of germplasm and their management. Among developed genetic markers, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers have been widely used for diversity analyses (Thimmappaiah et al., 2009). RAPD technique is quick, easy and requires no prior sequence information, which detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence (Williams et al., 1990). ISSR marker is involving 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 (Zietkiewicz et al., 1994). The technique is rapid, simple, inexpensive and more reproducible than RAPD (Pharmawati et al., 2004). RAPD and ISSR markers have been extensively used for DNA finger-printing (Moreno et al., 1998), genetic diversity (S'anchez de la Hoz et al., 1996; Esselman et al., 1999), population genetic (Wolfe et al., 1998; Nebauer et al., 1999) and phylogenetic studies (Hess et al., 2000). According to literature information, no report has been recorded on diversity of *Salvia* based on these two molecular marker systems. The objective of present study was to estimate the genetic relationship among eight species of *Salvia* including *S. hydrangea*, *S. xanthocheila*, *S. limbata*, *S. aethiopis*, *S. macrochlamys*, *S. nemorosa*, *S. sclarea* and *S. verticillata* using RAPD and ISSR genetic markers.

Results

RAPD analysis

Fifteen RAPD primers amplified distinct polymorphic products. Fig 1a is representative of RAPD amplification of 8 *Salvia* species by OPD-11 primer. The size of the amplified products ranged from 200 to 3000 bp. Fifteen random primers generated 220 scorable loci, which all were polymorphic (100%). The number of bands varied from 10 (primer OPA-07) to 21 (primer OPB-01) with an average of 14.66 markers per primer (Table 3). The polymorphism level, calculated as the number of polymorphic bands per primer was 100% among all 15 primers. Jaccard's similarity coefficient among different *Salvia* species ranged from 0.0725 (between *S. verticillata* and *S. nemorosa*) to 0.3529 (between *S. limbata* and *S. xanthocheila*) for RAPD marker (Table 1). UPGMA

Table 1. Similarity matrix obtained for: (A) RAPD, (B) ISSR, (C) RAPD and ISSR.

A

	S. hydrangea	*S. xanthocheila*	*S. limbata*	*S. aethiopis*	*S. macrochlamys*	*S. nemorosa*	*S. sclarea*	*S. verticillata*
S. hydrangea	1.000							
S.xanthocheila	0.2047	1.000						
S. limbata	0.1557	0.3529	1.000					
S. aethiopis	0.1908	0.2857	0.3178	1.000				
S.macrochlamys	0.2131	0.2288	0.2667	0.2231	1.000			
S. nemorosa	0.0753	0.0899	0.1039	0.1111	0.1084	1.000		
S. sclarea	0.1509	0.2020	0.2299	0.1509	0.2128	0.1579	1.000	
S. verticillata	0.1504	0.1140	0.1386	0.0833	0.1619	0.0725	0.1163	1.000

B

	S. hydrangea	*S. xanthocheila*	*S. limbata*	*S. aethiopis*	*S. macrochlamys*	*S. nemorosa*	*S. sclarea*	*S. verticillata*
S. hydrangea	1.000							
S.xanthocheila	0.2303	1.000						
S. limbata	0.2378	0.3380	1.000					
S. aethiopis	0.2195	0.3453	0.2808	1.000				
S.macrochlamys	0.2547	0.2770	0.2685	0.2400	1.000			
S. nemorosa	0.1702	0.1692	0.2358	0.1732	0.3017	1.000		
S. sclarea	0.2765	0.2740	0.3478	0.2979	0.3309	0.2333	1.000	
S. verticillata	0.2342	0.3188	0.3285	0.2260	0.2746	0.2101	0.2899	1.000

C

	S. hydrangea	*S. xanthocheila*	*S. limbata*	*S. aethiopis*	*S. macrochlamys*	*S. nemorosa*	*S. sclarea*	*S. verticillata*
S. hydrangea	1.000							
S. xanthocheila	0.2277	1.000						
S. limbata	0.2049	0.3457	1.000					
S. aethiopis	0.2062	0.3216	0.2960	1.000				
S. macrochlamys	0.2384	0.2556	0.2688	0.2351	1.000			
S. nemorosa	0.1342	0.1376	0.1869	0.1502	0.2222	1.000		
S. sclarea	0.2278	0.2459	0.3049	0.2387	0.2845	0.2045	1.000	
S. verticillata	0.2007	0.2262	0.2489	0.1635	0.2267	0.1604	0.2242	1.000

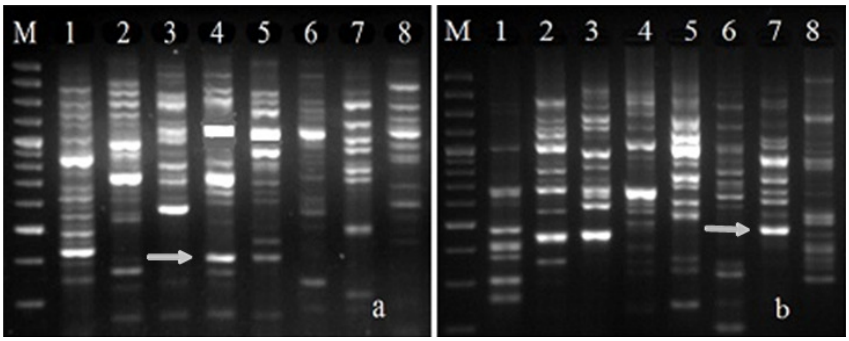


Fig 1. (a) RAPD marker profiles of primer OPD-11, (b) ISSR marker profiles of primer 825. M= Ladder 100 bp, 1= *S. hydrangea*, 2= *S. xanthocheila*, 3= *S. limbata*, 4= *S. aethiopis*, 5= *S. macrochlamys*, 6= *S. nemorosa*, 7= *S. sclarea*, 8= *S. verticillata*
Arrows are representative of polymorphic bands

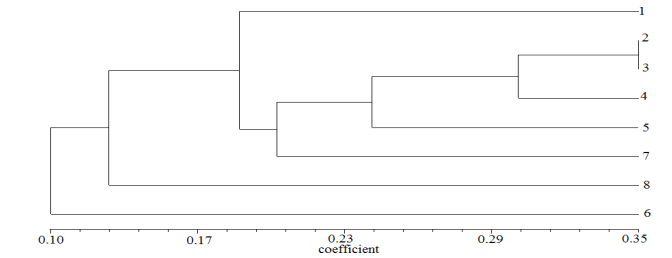
cluster analysis revealed five main clusters (Fig 2a). Cluster I consisted of only *S. hydrangea*. Cluster II was divided into 2 sub clusters. The first sub cluster comprised of *S. xanthocheila, S. limbata and S. aethiopis*. The second sub cluster contained only *S. macrochlamys*. Cluster III included *S. sclarea*. The *S. verticillata* and *S. nemorosa* were grouped in clusters IV and V, respectively. A high cophenetic correlation coefficient (r=0.94) was obtained between original similarity matrix and dendrogram.

ISSR analysis

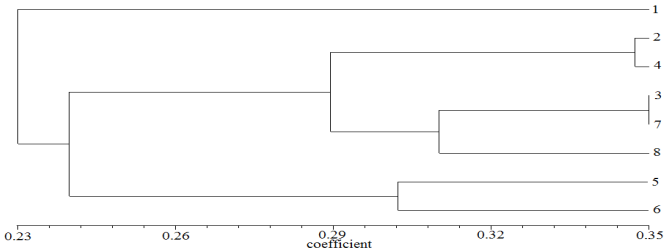
Fifteen out of 18 ISSR primers amplified distinct polymorphic products. Fig 1b is representative of ISSR amplification of eight *Salvia* species by primer 825. The size of the amplified products ranged from 200- 3000 bp. Fifteen ISSR primers generated 245 scorable loci, which 241 loci were polymorphic (98.4%). The number of bands varied from 7 (primers 834 and 888) to 28 (primer 825) with an average of 16.3 markers per

Table 2. *Salvia* species with their collection sites.

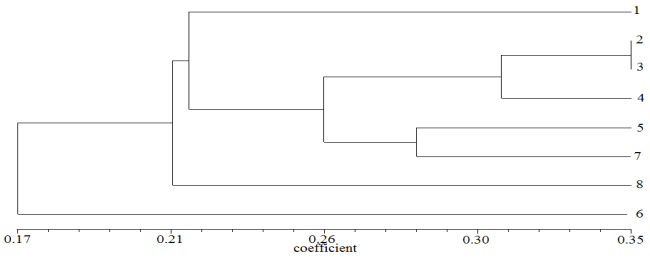
Plant samples	Origin
S. hydrangea	Khalkhal, Ardebil, Iran
S. xanthocheila	Sabalan mountain, Shabil, Ardebil, Iran
S. limbata	Ahar-Meshginshahr road, Ardebil, Iran
S. aethiopis	Sardabeh, Aradebil, Iran
S. macrochlamys	Germi, Ardebil, Iran
S. nemorosa	Ahar-Meshginshahr road, Ardebil, Iran
S. sclarea	Khalkhal, Ardebil, Iran
S. verticillata	Sardabeh, Ardebil, Iran



(A)



(B)



(C)

Fig 2. Dendrogram generated using UPGMA analysis, showing relationships among *Salvia* species, using RAPD, ISSR and combining RAPD and ISSR data. (A) RAPD. (B) ISSR. (C) RAPD and ISSR.1= S. *hydrangea*, 2= *S. xanthocheila*, 3= *S. limbata*, 4= *S. aethiopis*, 5= *S. macrochlamys*, 6= *S. nemorosa*, 7= *S. sclarea*, 8= *S. verticillata.*

primer. The polymorphism level, calculated as the number of polymorphic bands per primer ranged from 71.43% to 100%. The average number of polymorphic bands per primer was 16.06. Minimum and maximum number of polymorphic bands were obtained with the primer 888 (5) and 825 (28), respectively (Table 3). Jaccard's similarity coefficient among different *Salvia* species ranged from 0.1692 (between *S. nemorosa* and *S. xanthocheila*) to 0.3478 (between *S. sclarea* and *S. limbata*) for ISSR marker (Table 1). Plotted dendrogram revealed four main clusters. Cluster I included only *S. hydrangea*. Cluster II comprised of *S. xanthocheila* and *S. aethiopis*. The *S. limbata*, *S. sclarea* and *S. verticillata* were grouped in cluster III and cluster IV contained *S. macrochlamys* and *S. nemorosa*. The cophenetic correlation coefficient of 0.7 was computed.

Combined RAPD and ISSR analysis

The RAPD and ISSR data were combined for similarity and cluster analyses. Jaccard's similarity coefficient ranged from 0.1342 (between *S. nemorosa* and *S. hydrangea*) to 0.3457 (between *S. limbata* and *S. xanthocheila*) (Table 3). Cluster analysis performed on combining data of both markers which generated a dendrogram that separated the species into five distinct clusters (Fig 2c). Cluster I included only *S. hydrangea*. Cluster II comprised of *S. xanthocheila*, *S. limbata and S. aethiopis*. Cluster III consisted of *S. macrochlamys* and *S. sclarea*. *The S. verticillata* and *S. nemorosa* were grouped in clusters IV and V, respectively. The cophenetic correlation coefficient between Jaccard's similarity matrix and the plotted dendrogram was 0.9.

Discussion

In the present study, RAPD and ISSR markers were applied to assess the genetic diversity at inter-specific level among *Salvia* species. The ISSR primers generated more polymorphic bands (241) than RAPD primers (220). Dendrograms based on UPGMA analyses revealed five and four main clusters for RAPD and ISSR, respectively. Cluster analysis was also carried out on two sets of marker profiling data based on RAPD and ISSR combination which grouped the eight *Salvia* species into five clusters. First cluster included *S. hydrangea* in all of three studied marker systems. Based on morphological traits, we expected high genetic distance between S. *hydrangea* and rest of studied species. Variations in DNA sequences lead to polymorphism and greater polymorphism are indicative of greater genetic diversity. The high similarity between *S.xanthocheila* and *S.limbata* was obtained in RAPD (0.3529) and RAPD+ISSR combined (0.3457) analyses. The ISSR analysis also revealed high genetic similarity between *S. limbata* and *S. xanthocheila* (0.3380), too. In fact, after *S. limbata* & *S. sclarea* with similarity of 0.3478 and *S. xanthocheila* & *S. aethiopis* with similarity of o.3453, *S. limbata* and *S. xanthocheila* are the closet species according to ISSR analysis. These results indicate that these two species are closely related. Variation between RAPD and ISSR may be due to the fact that PCR profiles are amplified from different non-repetitive and repetitive regions of the genome in the two marker systems (Thormann et al., 1994). The RAPD, ISSR and combined RAPD+ISSR analyses showed *S.hydrangea* and *S.nemorosa* as the most divergent ones, which reveal the existence of consistency between these two marker systems for estimation of genetic variation in *Salvia*. Our analyses revealed similarity of 0.0753, 0.1702 and 0.1342 between *S.hydrangea* and *S.nemorosa* based on RAPD, ISSR and combined analysis, respectively. This result also provides the presence of sufficient amount of genetic variability among *Salvia* species in Iran. According to our results, the pair-wise genetic distance varied from 0.6471 to 0.9275 for RAPD, from 0.6522 to 0.8308 for ISSR and from 0.6543 to 0.8658 for RAPD+ISSR. The high values of cophenetic correlation coefficient also represent a good correlation between similarity matrices and constructed dendrograms. Studies about the genetic diversity of *S.miltiorrhiza* have been undertaken using different molecular markers such as RAPD,

Table 3. List of RAPD and ISSR primers used to detect polymorphism.

Primer	Sequence (5'→ 3')	Number of bands	Number of polymorphic bands	Polymorphism %
OPA-03	AGTCAGCCAC	14	14	100
OPA-04	AATCGGGCTG	16	16	100
OPA-05	AGGGGTCTTG	12	12	100
OPA-06	GGTCCCTGAC	11	11	100
OPA-07	GAAACGGGTG	10	10	100
OPB-01	GTTTCGCTCC	21	21	100
OPB-02	TGATCCCTGG	11	11	100
OPB-03	CATCCCCCAG	17	17	100
OPB-04	GGACTGGAGT	15	15	100
OPB-05	TGCGCCCTTC	14	14	100
OPD-02	GGACCCAACC	17	17	100
OPD-03	GTCGCCGTCA	12	12	100
OPD-05	TGAGCGGACA	14	14	100
OPD-08	GTGTGCCCCA	16	16	100
OPD-11	AGCGCCATTG	20	20	100
RAPD	Sub-Total	220	220	100
811	(GA)8C	8	8	100
814	(CT)8A	26	26	100
818	(CA)8G	14	14	100
825	(AC)8T	28	28	100
826	(AC)8C	8	6	75
827	(AC)8G	20	20	100
834	(AG)8CTT	7	7	100
836	(AG)8CA	13	13	100
841	(GA)8CC	27	27	100
845	(CT)8AGG	14	14	100
846	(CA)8AGT	17	17	100
848	(CA)8AGC	23	23	100
856	(AC)8CTA	23	23	100
868	CGTAGTCGT(CA)7	10	10	100
888	GAG(CA)7	7	5	71.43
ISSR	Sub-Total	245	241	98.4
RAPD+ISSR	Total	465	461	99.14

AFLP, CoRAP (Bao-Lin et al., 2002; Wang et al, 2007, 2009) SRAP and ISSR (Song et al., 2010). AFLP is 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 underestimation of polymorphism and genetic diversity level (Wang et al., 2009). SRAP is another molecular marker, first introduced by Li and Quiros (2001) and had been extensively applied in genetic linkage map construction and genetic diversity analyses (Li and Quiros, 2001; Ferriol et al., 2003; Guo and Luo, 2006; Ding et al., 2008). SRAP and ISSR were introduced as effective and reliable genetic markers for accurate assessing the degree of genetic variation of *S. miltiorrhoza* (Song et al., 2010). The average genetic distance derived from SRAP markers showed higher genetic differences compared to the average genetic distance of ISSR markers, but both represented high similarity and minor genetic differences among 5 populations of *S.miltiorrhiza* (Song et al., 2010). Compared to other molecular markers, ISSR could reveal high polymorphism and found to be potentially useful for studying genetic diversity, introgression analysis and identification of germplasm. High genetic similarity was also reported by RAPD markers in cultivated populations of *Salvia miltiorrhoza* (Bao-Lin et al., 2002). Our data based on two marker systems reveals the existence of low genetic similarity at inter-specific level among studied species. In conclusion, thirty selected markers introduced a sufficient overview of the relationships among the eight *Salvia* species and revealed that PCR based fingerprinting techniques were informative for estimating the extent of genetic diversity as well as determining the pattern of genetic relationships. RAPD and ISSR analyses were applied for the first time to obtain preliminary information on genetic profile of this plant in Iran. Our data suggests both DNA markers are effective and reliable molecular markers for accurate assessment of genetic variation. Our analyses revealed *S.hydrangea* and *S.nemorosa* as the most divergent ones. Therefore, they could be considered as good candidates for breeding programs. The present study could be the start of further investigations using more powerful markers such as Simple Sequence Repeat (SSR) and Sequence Related Amplified Polymorphism (SRAP). We hope our findings could be beneficial in germplasm management activities to maximize genetic diversity of *Salvia* in Iran.

Materials and methods

Plant materials

Seeds of eight species of *Salvia* from Gene bank collection of Ardebil (Iran) were cultivated in Phytotron system (Table 2). After twenty days of growth under controlled conditions,

leaves were cut and frozen in liquid nitrogen for DNA extraction.

DNA Extraction

Leaves were ground in liquid nitrogen to a fine powder with a chilled mortar and pestle. Genomic DNA was extracted using Doyle and Doyle (1987) CTAB method. The quantity and quality of DNA were determined by spectrophotometer and electrophoresis on 1% agarose gel, respectively.

RAPD amplification

Twenty arbitrary RAPD primers were purchased from commercial source (Cinnagen, Tehran, Iran). Fifteen primers with reproducible and scorable amplifications were chosen for further studies (Table 3). The PCR amplifications were carried out in Veriti 96-Well Thermal Cycler (Applied Biosystem) in final volume of 25 µl containing 12.5 µl of Master mix (Cinnagen, Tehran, Iran), 1 µl of genomic DNA (30 ng), 11.2 µl of ddH2O and 0.3 µl of primer. The PCR program started with an initial phase of 3 min at 95°c, followed by 35 cycles of 30 s at 95°c, 30 s at 39°c, 2 min at 72°c and 10 min final elongation at 72°c.

ISSR amplification

Eighteen ISSR primers were purchased from commercial source (Cinnagen, Tehran, Iran). After initial tests, fifteen primers were chosen for further examinations (Table 3). The PCR amplifications were performed in a final volume of 25 µl containing 0.25 µl Taq polymerase enzyme (5u/µl), 2.5 µl 10x PCR Buffer , 0.75 µl Mgcl2 (50 mM), 0.5 µl dNTP (10 mM), 1 µl DNA (30 ng), 0.3 µl primer and 19.7 µl ddH2O in Veriti 96-Well Thermal cycler (Applied Biosystem). The PCR program started with an initial phase of 3 min at 95°c, followed by 35 cycles of 30 s at 95°c, 30 s at 50°c, 2 min at 72°c and 10 min final elongation at 72°c.

Bands profile

PCR products were run on 2% agarose gel with a 0.5x TBE Buffer system at 70 V and stained with ethidium bromide. Each gel was photo documented under UV using Gel Logic 212 Pro Imaging System (Carestream, USA). Molecular sizes of amplified products were estimated using a 100-3000 bp DNA ladder marker (Fermentas).

Data analysis

The DNA profiles were scored visually from gel photographs. Clear and reproducible amplified bands were chosen in the analyses. The presence of a band was designated as (1) and absence as (0). The data obtained by scoring the RAPD and ISSR profiles individually as well as collectively were subjected to the calculation of similarity matrix using Jaccard's coefficients. The similarity values were used for cluster analyses. Sequential agglomerative hierarchical non-overlapping (SAHN) clustering was applied using unweighted pair group method with arithmetic averages (UPGMA) method. Dendrograms were plotted using NTSYSpc 2.02 software (Rohlf, 1998).

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References

Abd-Elazem I, Chen H, Bates R, Huang R (2002) Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from *Salvia miltiorrhiza*. Antiviral Res 55:91–106.

Akhondzadeh S, Noroozian M, Mohammadi M, Ohadinia S, Jamshidi AH, Khani M (2003) *Salvia officinalis* extract in the treatment of patients with mild to moderate Alzeimer's disease: a double blind, randomized and placebo-controlled trial. Clin Pharmacol Ther 28:53–59.

Aktas k. Ozdemir C, Ozcan M, Yurdanur A, Pelin B (2009) Morphological and anatomical characteristics of *Salvia tchihatcheffii* endemic to Turkey. Afr J Biotechnol 8 (18):4519–4528.

Bao-Lin G, Sheng L, Yu-Xiu F, Yang-Jing Z (2002) Primary research on genetic relationship among main populations of *Salvia miltiorrhiza* and genuineness of herb. Chin Tradit Herb Drugs 33:1113–1116.

Cahill JP (2004) Genetic diversity among varieties of Chia (*Salvia hispanica L.*). Genet Resour Crop Evol 51(7):773–781.

Dean SG, Ritchie G (1987) Antibacterial properties of plant essential oils. Inter J Food Microbiol 5:165–180.

Delamare APL, Moschen-Pistorello IT, Artico L, Atti-Serafini L, Echeverrigaray S (2007) Antibacterial activity of the essential oils of *Salvia officinalis* L. and *Salvia triloba* L. cultivated in South Brazil. Food Chem 100(2):603–608.

Ding G, Zhang DZ, Ding XY, Zhou Q, Zhang WC, Li XX (2008) Genetic variation and conservation of the endangered Chinese endemic herb *Dendrobium officinale* based on SRAP analysis. Plant Syst Evol 276:149–156.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull Bot Soc Amer 19:11–15.

Esselman EJ, Jianqiang L, Crawford DL, Windus JL, Wolfe AD (1999) Clonal diversity in the rare *Calamagrostis porteri* spp. Insperata (Poaceae): Comparative result for allozymes and random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. Mol Ecol 8:443–451.

Ferriol M, Pico B, Nuez F (2003) Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers. Theor Appl Genet 107:271–282.

Guo DL, Luo ZR (2006) Genetic relationships of some PCNA persimmons (*Diospyros kaki Thunb*.) from China and Japan revealed by SRAP analysis. Genet Resour Crop Evol 53:1597–1603.

Hess J, Kadereit JW, Vargas P (2000) The colonization history of *Olea europea* L. in Macaronesia based on internal transcribed apacer 1 (ITS-1) sequence, randomly amplified polymorphic DNAs (RAPD) and inter-simple sequence repeats (ISSR). Mol Ecol 9:857–868.

Jung M, Lee HC, Ahn CW, Park W, Choi S, Kim H (2002) Effective isolation of magnesium lithospermate B and its inhibition of aldose reductase and fibronectin on mesangial cell line. Chem Pharm Bull 50:1135–1136.

Li G, Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor Appl Genet 103:455–461.

Moreno S, Martin JP, Ortiz JM (1998) Inter-simple sequence repeats PCR for characterization of closely related grapevine germplasm. Euphytica 101:117–125.

Mozaffarian VA (1996) Dictionary of Iranian plant names. Farhang moaser, Tehran p.477.

Nebauer SG, Agudo LDC, Segura J (1999) RAPD variation within and among natural populations of outcrossing willow-leaved foxglove (*Digitalis obscura* L.). Theor Appl Genet 98:985–994.

Penso G (1983) Index Plantarum Medicinalium Totius Mundi Eorumgue Synonymorum. Milano: OEMF.

Pharmawati M, Yan G, McFarlane IJ (2004). Application of RAPD and ISSR markers to analyse molecular relationships in *Grevillea* (Proteaceae). Aust Syst Bot 17:49–61.

Piccaglia R, Marottu M (1993) Characterization of several aromatic plants grows in northern Italy. Flavour Frag J 8:115–122.

Ping L, Cheng-Hai L, Hai-Nan W, Yi-Yang H, Cheng L (2002) Effect of salvianolic acid B on collagen production and mitogen-activated protein kinase activity in rat hepatic stellate cells. Acta Pharmacol Sin 23(8):733–738.

Rohlf FJ (1998) On applications of geometric morphometrics to studies of ontogeny and phylogeny. Syst Biol 47:147–158.

S'anchez de la Hoz MP, Davilla JA, Loarce Y, Ferrer E (1996) Simple sequence repeat primers used in polymerase chain reaction amplification to study genetic diversity in barley. Genome 39:112–117.

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 138:241–249.

Thimmappaiah, Santhosh WG, Shobha D, Melwyn GS (2009) Assessment of genetic diversity in cashew germplasm using RAPD and ISSR markers. Scientia Hortic 120:411–417.

Thormann CE, Ferreira ME, Camargo LEA, Tivang JG, Osborn TC (1994) Comparison of RFLP and RAPD markers to estimating genetic relationships within and among Cruciferous species. Theor Appl Genet, 88: 973-980.

Vukovic´-Gacˇic´ B, Nikcˇevic´ S, Beric´-Bjedov T, Knezˇevic´-Vukcˇevic´J, Simic´D (2006) Antimutagenic effect of essential oil of sage (*Salvia officinalis* L.) and its monoterpenes against UV-induced mutations in *Escherichia coli* and *Saccharomyces cerevisiae*. Food Chem Toxicol 44:1730–1738.

Wang B, Zhang Y, Chen CB, Li XL, Chen RY, Chen L (2007) Analysis on genetic diversity of different *Salvia miltiorrhiza* geographical populations in China. Chin J Chin Mater Medica 32:1988–1991.

Wang QH, Zhang BL, Lu QS (2009) Conserved region amplification polymorphism (CoRAP), a novel marker technique for plant genotyping in *Salvia miltiorrhiza*. Plant Mol Biol Rep 27:139–143.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res 18: 6531–6535.

Wolfe AD, Xiang QY, Kephart SR (1998) Assessing hybridization in natural populations of *Penstemon* (Svrophulariaceae) using hypervariable inter-simple sequence repeat (ISSR) bands. Mol Ecol 7:1107–1125.

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183.