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Assessment of genetic diversity on populations of three *satureja* species in Iran using ISSR markers

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ABSTRACT

As we know, Savory herb is one of the most important medicinal plants of Iran that belongs to Lamiaceae family which about 284 species have been identified in the world. Iran, as one of the most important repositories of Savory germplasm in the world, has about 16 species of Satureja genus. Because of so many useful medicinal properties of Savory, this plant has been considered in many recent studies. In this study, 10 different populations of three species (Satureja rechingeri, S. khuzistanica and S. spicigera) related to the different area of Ilam, Lorestan and Gilan provinces have been presented. In order to evaluate the genetic diversity in Satureja genus, the Inter Simple Sequence Repeats method (ISSR) was exploited for 10 populations. After screening of 10 ISSR primers three primers that produced clear and reproducible fragments were selected for further analysis. The three primers generated 19 bands ranging in size from 450 to 1700 bp, corresponding to an average of 6.3 bands per primer. A set of three anchored primers amplified a total of 19 bands out of which 100 % were polymorphic among 49 individuals. Polymorphic information content (PIC value) ranged from 0.22 to 0.36 and marker index (MI) ranged from 1.1 to 2.88 per primer. The primer UBC820 had the highest PIC (0.36) and MI (2.88) values. A maximum genetic similarity value of 0.99 was observed between populations 5 (S. rechingeri) and 8 (S. khuzistanica) and a minimum similarity value of 0.57 was observed between populations 6 (S. rechingeri) and 10 (S. spicigera). The mean coefficient of gene differentiation (Gst) was 0.425, indicating that 72% of the genetic diversity resided within the population. Clustering analysis using WARD algorithm based on Nei's Unbiased Measures of Genetic distance, classified the Satureja populations into four major groups. The PCOA data confirmed the results of clustering. The results of this study revealed that ISSR markers could be efficiently used for genetic differentiation of the Satureja accessions. The primer UBC820 is useful to detection of a high level of polymorphism and it can be used to guide future breeding studies and management of Satureja genus.

Keywords: Genetic diversity, ISSR Markers, *Satureja*.

INTRODUCTION

Satureja is a genus of aromatic plants of the family Lamiaceae (the mint family). The genus has been a subject of much discussion among taxonomists and is variously treated. In Flora Europaea, Heywood & Richardson [20] recognized 5 genera in the region including *Calamintha, Acinos, Clinopodium, Micromeria* and *Satureja* . In the Flora of USSR [39],China [24], Turkey [8] and Iranica [36] a similar classification was adopted considering to some specific genera which were included on the basis of geographical distribution. *Satureja* in its narrow concept is a

genus comprising 30 species, mainly distributed in Mediterranean Region but also extended to Irano-Turanian phytogeographical region. *Satureja* belongs to the tribe Mentheae within the subfamily Nepetoideae and includes about 284 species in the world[30].This genus is represented in Iranian flora by 16 species, nine of which are endemic for the country (*S. macrosiphonia ,S. bachtiarica ,S. rechingeri ,S. isophylla S. atropatana ,S. sahendica ,S. khuzistanica ,S. intermedia and S. edmondi*) [21].There are about 30 species called savories, of which summer savory and winter savory are the most important in cultivation. *Satureja* species are native to warm temperate regions and may be annual or perennial. They are low-growing herbs and subshrubs, reaching heights of 15–50 cm.The leaves are 1 to 3 cm long, with flowers forming in whorls on the stem, white to pale pink-violet. Both summer savory and winter savory are used to flavor food. The former is preferred by cooks but as an annual is only available in summer, winter savory is an evergreen perennial. Certain organic chemicals are derived from these species, which are useful to humans. They have usually well known, and will be used by native inhabitants as spice, medicinal plant or source of essential oils. Medicinal properties and a large variety of specimens in the species increase the importance of diversity studies in this genus. Within the *Satureja* genus the genetic diversity has been dealt with using morphological characters[17],[23],[10] Enzyme electrophoresis [1],[2],[19] chloroplast DNA restriction site analysis [7] and molecular markers (RAPD, SAMPL and AFLP) [15],[16],[18],[4],[5],[6] ,[40].Molecular markers offer a powerful means of analyzing genetic diversity and relationships. Among molecular markers, the inter simple sequence repeats (ISSRs) have been successfully applied in many crop species [45], [29], [34], [44], [13]. ISSRs have proved to be useful in population genetic studies [3], [9], [42], [45]. In fact, PCR was used to amplify the sequence of DNA between two microsatellites using two types of primers: the unanchored one consists of a simple microsatellite sequence whereas the anchored primers are added with extra nucleotides in the 3'- or the 5'-end of a microsatellite sequence. Our aims were to specify genetic diversity for investigation of differences or resemblances within and among different populations from different geographical regions and clarify their phylogenic relationship in order to establish breeding programs. In this paper, we have applied the ISSR technique in 10 populations of three different species using unanchored and anchored primers.

MATERIALS AND METHODS

Plant materials and DNA extraction

10 populations (49 individuals) of three species of *Satureja* genus were collected from Research Institute of Forest and Rangelands (Table 1). Total DNA was extracted from young leaves following the modified sodium dodecyl sulfate (SDS) method [14]. Approximately 0.5 gr. leaves was ground to a fine powder in a mortar with liquid nitrogen and then transferred to a 2 ml Eppendorf tube filled with 900 µl of extraction buffer(100 mM Tris -HCl, 50 mM EDTA , 1M NaCl, 0.2% mercaptoethanol and 1.5% PVP) after being incubated at 65◦C, the homogenate was mixed with 130 µl SDS 10%(w/v) and incubate the samples at 65◦C for 15-20 minutes and mix every 5 min and then add 300 µl Sodium acetate and centrifuge for 10 min at 11000 rpm and transfer supernatant into a labeled new tube and add 0.25 volume LiCl , mixed thoroughly by inversion, incubate sample on ice for 10 min centrifuge for 10 min, at 12000rpm and transfer the supernatant in to new tube and add 0.7 volume cold isopropiolic alcohol 70% and mixed few minutes, centrifuge for 12 min, at 12000rpm, dissolved pellets in 250 µl TE (10:1)buffer and then 2 µl RNase A solution (100 mg/ml) were added, and mixed well and incubate at 37◦C for 1h. The homogenate was then extracted with 300 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and was centrifuged at 13,000 rpm for 12 min, The supernatant was transferred into a new 2 ml clear-colored tube and were added equal volume of isoamylalcohol /chloroform and mixed well, incubate sample on ice for 5 min, centrifuge for 10 min, at 13000 rpm, transfer the supernatant in to new tube and added 0.1 volume Sodium acetate and 2 volume chilled absolute ethanol and incubate sample on ice for 1h,and then centrifuge for 10 min at 14000 rpm. Discard the supernatant and were added 300 µl of 70 % ethanol into each tube and washed the pellet. Then centrifuge for 3 min. at 14000 rpm and discard the supernatant and The pellet was dried in an Eppendorf Vacfuge™ (Eppendorf North America, Hauppauge, NY, USA) at 37°C for 15 min. Re-suspend the pellet in 250-300 µl of TE (10:0.1)buffer and was stored at -80◦C.

DNA quantity and quality

Purified DNA was quantified using a NanoDrop® ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) to measure the DNA concentration and the absorbance ratio (A260/A280). A pure sample of DNA has the ratio at 1.8 and is relatively free from protein contamination. The purified total DNA was qualified by 0.8 % agarose gel electrophoresis and the DNA samples were diluted to 5ng/µl and stored at -20 ºC.

Primers

Primers were purchased in lyophilized form from TIB MOLBIOL-Germany 10 ISSR primers were tested in this research (Table 2).

ISSR assay

A total of 10 primers were tested to amplify DNA and among them, three primers with considerable polymorphism and reproducibility were selected for further analysis (Table 2). PCR were performed in 50 µl volume consisted of 1X PCR buffer, 1mM MgCl2, 0.2 mM each of dNTPs, 0.5 µM primer, 2.5U of Taq DNA polymerase (Qiagen-Germany), 5 % formamide and 15 ng of template DNA. Amplification was performed in a Eppendorf Mastercycler gradient Thermal Cycler under the following conditions: 5 min at 95ºC for 1 cycle, followed by 60 s at 95ºC, 60 s at 35◦C, 38◦C or 43◦C (Depending on the primer), and 2 min at 72ºC for 40 cycles, and 10 min at 72ºC for a final extension. Amplification products were separated on 1% agarose gels run at 50 V in 1 × TAE, visualized by staining with ethidium bromide, and photographed ultraviolet light. Molecular weights were estimated using a 100 bp DNA ladder.

Table 1- Geographical Characteristics of Sampled Populations of three species of *Satureja* genus used in the study

population Code	Species	Main Place of collection	Code	Number of Samples
Pop1	*S. rechingeri*(1)	Ilam - Howeian to Nasrian	15	5
Pop2	*S. rechingeri*(2)	Ilam - Zarrin Abad	16	5
Pop3	*S. rechingeri*(3)	Ilam, Mehran(1)	30	5
Pop4	*S. rechingeri* (4)	Ilam, Mehran(2)	22	5
Pop5	*S. rechingeri* (5)	Ilam, Mehran(3)	24	5
Pop6	*S. rechingeri* (6)	unknown	25	5
Pop7	*S. khuzistanica* (1)	Lorestan Poldokhtar - Andimeshk	13	5
Pop8	*S. khuzistanica* (2)	Lorestan Poldokhtar - Paalm	14	4
Pop9	*S. spicigera* (1)	Gillan -Rudbar	32	5
Pop10	*S. spicigera* (2)	Gillan -Rudbar-Ganjeh	35	5

Table 2- ISSR primer with their sequences and annealing temperature (TM) (R=A/G, Y=C/T)

No.	Primer Code	Sequence(5'-3')	Annealing Temp.(◦C)
1	UBC808	(AG)8 C	48
2	UBC 820	(GT)8 C	43
3	UBC 844	(CT)8 RC	38
4	UBC 801	(AT)8 T	30
5	UBC 802	(AT)8 G	32
6	UBC 803	(AT)8 C	32
7	UBC 804	(TA)8 A	30
8	UBC 805	(TA)8 C	32
9	UBC 806	(TA)8 G	32
10	UBC 807	(AG)8 YT	35

Data analysis

Marker indices were calculated for the ISSR markers to characterize the capacity of each primer to detect polymorphic loci among the populations and individuals. As such, the marker index was the sum of the polymorphism information content (PIC) values or Average heterozygosity for all the selected markers produced by a particular primer. The PIC value was calculated using the formula PICi=2Pi (1-Pi), as proposed by Roldan-Ruiz [37], where PICi is the PIC of the marker i, Pi is the frequency of the amplified allele (band present) and (1-Pi) is the frequency of the null allele. And the other was MI. MI was calculated as the product of two functions: PIC and EMR (Effective Multiplex Ratio). EMR of a primer is defined as "the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay" [28]. Only reproducible and clear bands in the replications were considered as potential polymorphic markers. The data obtained by scoring the presence (1) or absence (0) of amplified fragments from the ISSR and assembled in a data matrix. POPGENE software 1.31 [43] and GenAlex [35] were utilized to generate the single population gene frequencies and the grouped population gene frequencies as well as Nei's (1972) [31] genetic distances matrix between the populations from the 0, 1, data matrix. The results of distance matrix used to construct a (Ward) phenogram for the 10 populations using JMP software [38]. Also

observed number of alleles (na); Number of effective alleles (ne); Nei's (1973) [32] genetic diversity index; Shannon's Information index (I); the total Heterozygosity (Ht); the Expected Heterozygosity within subpopulations (Hs); the coefficient of genetic differentiation (Gst) and estimate of gene flow from Gst (Nm) were analyzed with POPGENE and GenAlex software's. The ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA), as described by Excoffier [11]. The analysis of AMOVA was performed using GenALEx software. GenAlex was also used to calculate a Principal Coordinates Analysis (PCOA) that plots the relationship between distance matrix elements based on their first two principal coordinates.

RESULTS

ISSR is an efficient, cost effective method for genotype identification and assessment of genetic relationships among the populations. For present investigation 10 populations obtained from three different species were analyzed using ISSR primers. Three selected ISSR primers amplified a total of 19 scorable bands ranging in size from 450 to 1700 bp, corresponding to an average of 6.3 bands per primer (Table 3 and Fig 1). The highest number of bands was scored for *S. spicigera* (2) of which 73.68 % were polymorphic, followed by *S. rechingeri* (5) with 52.63 % polymorphic bands. The least polymorphism (31.57%) was observed in *S. rechingeri* (1 and 6).

Table 3- ISSR primers successfully used in this study and the number of total and polymorphic bands amplified in 10 populations with Polymorphism information content (PIC) and marker index (MI)values. Y= C /T, R = A /G

MI	PIC	Percentage of polymorphism	Number of polymorphic bands	Band amplitude produced	The total number of bands	Sequence (5'-3')	primer
1.1	0.22	100	5	450-1300	5	$(AG)_8YT$	UBC807
2.88	0.36	100	8	600-1700	8	$(GT)_8C$	UBC820
1.86	0.31	100	6	500-1500	6	$(CT)_8RC$	UBC844
1.95	0.296	Mean	19		19	Total	

Table 4- Overall genetic variability across all the populations. Nm = estimate of gene flow from Gst. E.g., Nm = 0.5 (1 - Gst)/Gst.

Locus	Observed No. of alleles	Effective No. of alleles	Nei's gene Diversity (h)	Shannon's information index (I)	Ht	Hs	Gst	Nm
Loc807-1	2	1.0471	0.0449	0.1095	0.0441	0.0349	0.2075	1.9092
Loc807-2	2	1.044	0.0422	0.1040	0.0413	0.0378	0.0863	5.2951
Loc807-3	2	1.8523	0.4601	0.6527	0.4617	0.2332	0.4948	0.5105
Loc807-4	2	1.695	0.4100	0.6003	0.4136	0.2943	0.2885	1.2331
Loc807-5	2	1.1373	0.1207	0.2393	0.1185	0.0465	0.6076	0.3229
Loc844-1	2	1.599	0.3746	0.5619	0.3755	0.3191	0.1502	2.8282
Loc844-2	2	1.6096	0.3787	0.5664	0.3737	0.1773	0.5254	0.4517
Loc844-3	2	1.9597	0.4897	0.6828	0.4927	0.2806	0.4305	0.6613
Loc844-4	2	1.4743	0.3217	0.5024	0.3169	0.1148	0.6377	0.2841
Loc844-5	2	1.0902	0.0828	0.1782	0.0861	0.0799	0.0722	6.4252
Loc844-6	2	1.2029	0.1687	0.3094	0.17	0.1424	0.1625	2.5769
Loc820-1	2	1.1012	0.0919	0.1934	0.0901	0.0654	0.2748	1.3197
Loc820-2	2	1.6864	0.4070	0.5971	0.4072	0.0909	0.7768	0.1436
Loc820-3	2	1.9307	0.4821	0.6751	0.4863	0.2457	0.4948	0.5105
Loc820-4	2	1.8787	0.4677	0.6605	0.469	0.239	0.4904	0.5197
Loc820-5	2	1.7067	0.4141	0.6046	0.4091	0.2937	0.2821	1.2722
Loc820-6	2	1.6295	0.3863	0.5747	0.3813	0.2327	0.3897	0.7831
Loc820-7	2	1.9631	0.4906	0.6837	0.491	0.2957	0.3978	0.7569
Loc820-8	2	1.1929	0.1617	0.2996	0.1588	0.1061	0.3318	1.0070
Mean	2	1.5158	0.305	0.4629	0.3046	0.1753	0.4246	0.6777
Std	0	0.3402	0.1676	0.213	0.0283	0.0102		

Polymorphic information content (PIC value) ranged from 0.22 to 0.36 and marker index (MI) ranged from 1.1 to 2.88 per primer. The primer UBC820 had the highest PIC (0.36) and MI (2.88) values (Table 3). The total mean values of gene diversity (h) and Shannon's Information index (I) of the 49 individuals of different populations for three ISSR markers determined were 0.305± 0.167 and 0.463± 0.213 respectively (Table 4). The highest mean value of h and I of the 49 individuals was found for UBC820 ISSR marker (0.38±0.15 and 0.57±0.19). Among 10 populations, just P10 (*S. spicigera*) had the highest values of h and I (0.27±0.19 and 0.40±0.27) for three ISSR markers. The mean value of effective alleles, Shannon's index , genetic diversity, total Heterozygosity (Ht), Expected Heterozygosity within subpopulations (Hs), coefficient of genetic differentiation (Gst) and estimate of gene flow from Gst (Nm) based on ISSR data were 1.516, 0.463 , 0.305, 0.305, 0.175, 0.425 and 0.678 respectively,

while UBC820-7 locus showed the highest number of effective alleles (1.963), Shannon's index (0.684), genetic diversity value (0.491), Total Heterozygosity (0.491) and Expected Heterozygosity (0.296) (Table 4). The amount of gene flow among 19 scorable bands, estimated as Nm = 0.5(1 -Gst)/Gst was found to be ranges from 0.1436 to 6.4252 (Table 4).

Table 5- Nei's Original Measures of Genetic Identity and Genetic distance. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Population	1	2	3	4	5	6	7	8	9	10
1	0	0.9091	0.8544	0.8703	0.8497	0.8474	0.8561	0.8188	0.7309	0.6909
2	0.0953	0	0.9735	0.9346	0.8881	0.9098	0.923	0.8142	0.7183	0.7301
3	0.1573	0.0269	0	0.9706	0.9474	0.9522	0.9234	0.8826	0.7865	0.7002
4	0.139	0.0676	0.0298	0	0.9689	0.9234	0.8839	0.8991	0.7344	0.7108
5	0.1629	0.1186	0.054	0.0316	0	0.924	0.8998	**0.9923**	0.9038	0.7632
6	0.1656	0.0945	0.049	0.0797	0.079	0	0.9206	0.873	0.8062	**0.5736**
7	0.1554	0.0801	0.0797	0.1234	0.1056	0.0828	0	0.8578	0.8161	0.7745
8	0.1999	0.2055	0.1249	0.1063	**0.0078**	0.1359	0.1533	0	0.9321	0.7091
9	0.3135	0.3309	0.2402	0.3087	0.1012	0.2155	0.2033	0.0703	0	0.7088
10	0.3698	0.3145	0.3565	0.3413	0.2702	**0.5558**	0.2555	0.3438	0.3442	0

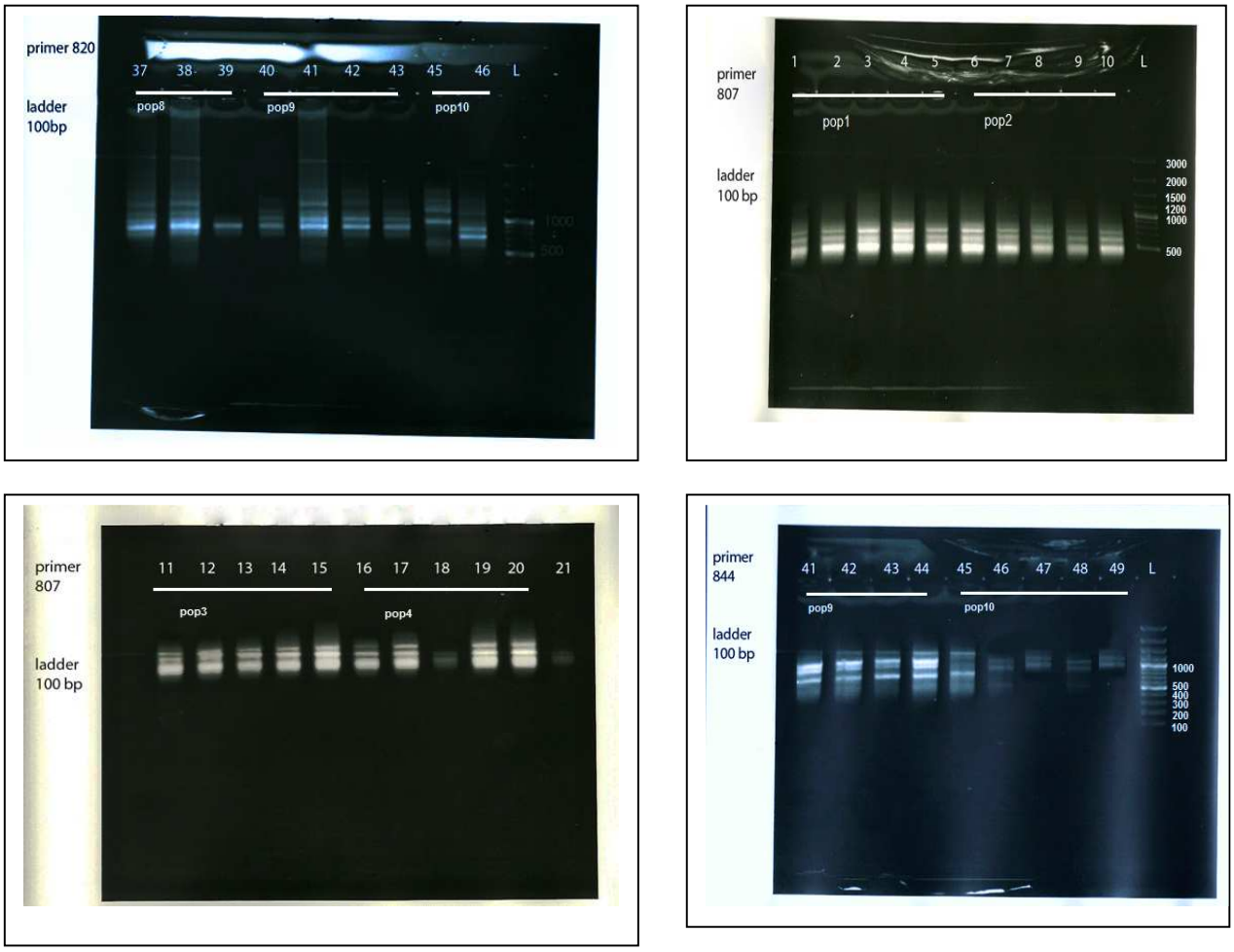

Figure 1- ISSR marker profiles of some of the 10 populations (49 individuals) of *Satureja* species generated by primers UBC820, UBC807 and UBC844 in 1% agarose gel. Lane (L): 100bp DNA ladder.

Nei's [33] unbiased genetic identity and genetic distance for ISSR data determined among the *Satureja* populations are given in Table 5. The value of genetic identity varied from 0.574 between *S. spicigera*(2) and *S. rechingeri*(6) to 0.992 between *S. rechingeri*(5) and *S. khuzistanica* (2).

Table 6- Analysis of molecular variance (AMOVA) for 10 populations of different *Satureja* species based on ISSR markers.

Sources	Degrees of freedom (df)	Sum of squared (SSD) deviations	Mean square MSD	Variance components	Percentage of variance
Among populations	9	53.712	5.698**	0.795	28%
Within populations	39	80.9	2.074**	2.074	72%
Total	48	134.612		2.869	100%

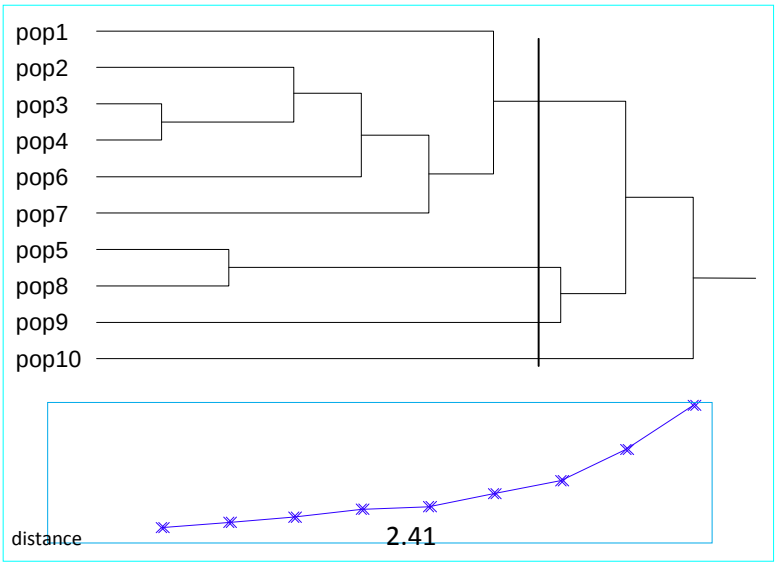

Figure 2- Dendrogram constructed from Nei's unbiased genetic distance matrix among 10 populations, clustered with the Ward method (r=0.96) based on ISSR data.

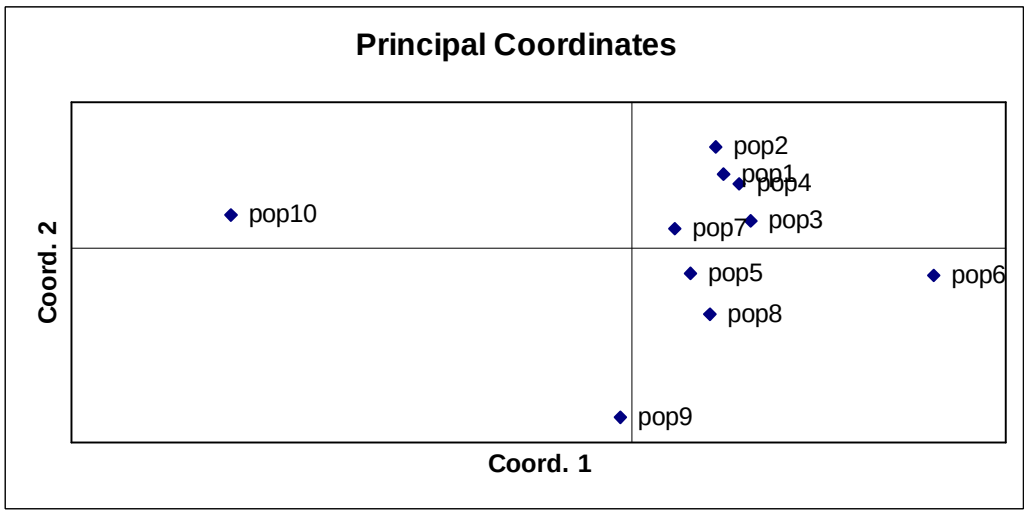

Figure 3- Scatter plot of 10 populations for the first two PCO analyses

The mean coefficient of gene differentiation (Gst) was 0.425, indicating that 72% of the genetic diversity resided within the populations. Clustering analysis using WARD algorithm based on Nei's Unbiased Measures of Genetic distance, classified the *Satureja* populations into four major groups. The cophenetic correlation of Ward tree was about (r = 0.96). The rate of genetic affinities and relatedness of the three taxa under consideration can also be observed between the 10 populations (Fig. 2). Two clusters, one consisting of *S. spicigera* (Gillan –Rudbar) and the other consisting of the same species *S. spicigera* (Gillan -Rudbar-Ganjeh) from nearby areas can be observed separately.
The ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA), as described by Excoffier [11].The results of AMOVA analysis showed that 28% and 72 % of genetic diversity resided between and within the populations respectively and genetic variation among and within populations were significant at 1% level (Table 6 and Fig 2).

PCOA analysis of ISSR data showed that the first three factors comprised about 95.40% of total variance when the first, second and third axis comprised about 62, 27 and 6% of total variance respectively. Ward dendrogram clustering of ISSR data produced similar results supported by PCOA ordination plot (Figures 2 and 3).

The results of this study revealed that ISSR markers could be efficiently used for genetic differentiation of the *Satureja* accessions. The primer UBC820 is useful to detection of a high level of polymorphism and it can be used to guide future breeding studies and management of *Satureja* germplasm.

DISCUSSION

Genetic variation is a basic requirement for plant breeding, whereas a high genetic variation is needed for genetic improvement of plants. Exploration and evaluation of diversity among and within populations would be of great significance for in situ conservation and *Satureja* species breeding programs. In recent years, genetic markers are increasingly used for the study of genetic diversity. Moreover, the polymorphism determined by these markers is one of the valuable parameters for study of populations and understanding of their genetic differences. The high reproducibility of ISSR markers may be because of using longer primers and higher annealing temperature than those used for RAPD. Based on its unique characters, ISSR technique can detect more genetic loci than isozyme and has higher stability than RAPD [45]. Our work is the first application of this method to characterize and to evaluate the genetic diversity within and among some *Satureja* species.

The Savory landraces constitutes a rich source of biodiversity and their conservation and utilization requires that their genetic structure is well characterized and understood. DNA fingerprinting is a routine method employed to study the extent of genetic diversity across a set of germplasm or cultivars and group them into specific categories. Comparative studies in *Satureja* species involving RAPD, AFLP, and SAMPL marker systems were used by very limited researchers [16], [18]. The discriminative power of DNA markers used as tool to characterize the *Satureja* genus is very important because they can be used to assess the genetic diversity among the *Satureja* species and populations.

The analyses were conducted using four individuals and one bulk of each population. Bulk analyses are useful to obtain information on genetic variability between different populations [26]. ISSR fingerprints were previously amplified, with more or less success, in animal and higher plants using different approaches.

Among 10 ISSR primers surveyed across the *Satureja* species only three primers with considerable polymorphism and reproducibility were selected for further analysis (Table 1 and Figure 1). Hadian, *et al.* detected 83 % of polymorphism in 28 accessions of *Satureja* hortensis L., collected from different parts of Iran with RAPDs [15]. Hadian, *et al.* also reported phylogenetic relationships and genetic diversity of different Iranian *Satureja* species using AFLP and SAMPL markers [16], [18]. In both marker systems, all accessions were grouped according to their species with high bootstrap values.

In this study the mean level of polymorphism revealed by ISSR (100 %) is higher than RAPD (83%) and AFLP (48.05 %) or SAMPL (42.57 %) methods. ISSR primers generated 6 to 14 bands with average of 8.5 bands per populations. The distribution of different microsatellite sequences in different populations determines the possibility of using this method for DNA fingerprinting. Comparison of PIC values for three primers (a parameter associated with the discriminating power of markers) indicated that the range of PIC values was from 0.22 (UBC807) to 0.36

(UBC820). Primer $(GT)_8$ C proved to be the best one due to the sharpness of its patterns and the high number of polymorphic markers provided.

The experimental results of this study will provide evidence for the reliability and usefulness of ISSR markers, to estimate genetic diversity within and between native *Satureja* populations. The average amount of total heterozygosity that was calculated in this study was almost high (0.3046±0.028) (Table 4) and the mean value of Hs (Nei's genetic diversity within subpopulations) and the mean coefficient of gene differentiation (Gst) in this study were (0.1753±0.01) and 0.4246 respectively. Our results showed that based on the Shannon's information index, genetic diversity in *S. spicigera* (2) is higher than the others (0.27±0.19) and that the *S. rechingeri* (1) had the lower one (0.12±0.19).

Cluster analysis was carried out on marker profiling data based on ISSR. The results based on all the DNA marker profiles broadly grouped the 10 populations into four clusters. The high similarity among S. *rechingeri* populations was noticed in ISSR analysis indicating that these populations are closely related except Pop5. The pattern of sub clustering of the cluster, which included 10 populations correctly, grouped the species except one mismatching with initial identification of populations. There was close relationship between some of the populations used in this study; presumably they might have been collected from similar locations. Probably, one of the reasons of mismatching in cluster grouping is hybridization between two species. Inclusion of genotypes bred for specific objectives like yield and quality parameters over different geographical location resulting in narrowing of genetic base and the marker system used could be the reason for clustering most of the cultivars or populations in one cluster.

In ISSR analysis, the *Satureja* genotypes were grouped into four sub clusters revealing sufficient amount of diversity within the cluster.

When compared to RAPD, another multi-loci and PCR-based method (AFLP and SAMPL) [15], ISSR amplifications gave more markers and showed a higher level of polymorphism between *Satureja* species. So the results indicated that ISSR could be a better tool than RAPD, AFLP and SAMPL markers for diversity studies in *Satureja* species.

These observations are in agreement with many studies showing the higher reproducibility and efficiency of ISSR markers [12], [22], [25], [27], [40]. This study confirms the superiority and usefulness of ISSR over AFLP, SAMPL and RAPD to survey of *Satureja* species. ISSR technique is also more economical than other molecular marker fingerprinting methods (RAPD, RFLP, AFLP, SSCP or SSR). Also ISSR fingerprints appeared to be a useful and quick molecular tool to solve the problems of morphological identification and individual characterization of *Satureja* species. ISSR fingerprints clearly distinguished all the tested species. They allowed identification from genus level to geographical species level and allowed to separate populations and individuals of the same species with different chemical compounds (data not shown).

ISSR-PCR gave complete, very reliable, reproducible and highly polymorphic fingerprints within and among populations of *Satureja* species. ISSR amplifications also open new and interesting possibilities in the *Satureja* genus characterization field. In the future, the use of ISSR should be enlarged, for example, 1) to study genetic relationships among more species and genera to compare results with all the previous data, 2) to the management of species collections especially for identification, 3) to screen quickly the most abundant SSR motifs in order to develop microsatellite markers.

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