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Genetic Diversity Analysis in Four Species of *Satureja Spp* and *Gontcharovia Popovii* using Morphological Traits and Molecular RAPD Markers

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ABSTRACT

In order to evaluate the genetic diversity in 4 species of *Satureja* Spp and one species of *Gontcharovia Popovii*, 90 genotypes of 5 species were exploited using morphological traits and molecular RAPD markers. These species included *Satureja bachtiarica* Bunge (15 genotypes), *Satureja mutica* (15 genotypes), *Satureja sahendica* (12 genotypes), *Satureja hortensis* (24 genotypes) and *Gontcharovia Popovii* (24 genotypes). DNA was isolated from young leaflet tissue of the plant. Next, DNA was quantified using spectrophotometer (Pharmacia) and its quality was determined by electrophoresis on 0.7 % agarose gel. Finally, qualified DNAs were diluted for 25 µL reaction. After completion of DNA amplification in the polymerase chain reaction, DNA loaded by electrophoresis on 1.2 % agarose gel in 95 V TAE 1X buffer for 70 minutes. Genotypes were grouped into two and three clusters based on morphological traits and polymorphic obtained by RAPD markers, respectively. Out of 40 RAPD primers, 11 appropriate polymorphic primers were observed. A total of 49 polymorphic DNA bands generated with a mean of 4.45 bands per primer was obtained. The most polymorphic and the least polymorphic bands were seen in P_7 and P_1 primers, respectively. In this study, the average number of polymorphic bands per primer was 0.156. The highest (0.240) and lowest(0.060) number of polymorphic bands was achieved with P_2 and P_9 primers, respectively. Average marker index any primer in this test 1.449 was calculated so that the primer P_7 (2.589) the maximum amount of primer P_9 (0.781) were allocated to the lowest. According to the obtained data, RAPD markers could be useful tools for investigation of genetic diversity among species of *Satureja* Spp.

KEYWORDS: genetic diversity, morphological traits, grouping, *Satureja* Spp, RAPD molecular marker.

INTRODUCTION

Different types of markers can be used to study the genetic diversity. Available morphological and protein markers, compared to DNA markers, are less used in the grouping due to the level of polymorphism. DNA markers have more genetic distinctiveness than the morphological and protein markers (Smith and Smith, 1992). This is because DNA markers can detect differences between non-coding rows and adjacent sequences in the genome in addition to showing existing differences in the coding rows. In the past decade, the introduction of polymerase chain reaction is used as another powerful tool for genomic studies, especially for showing DNA polymorphism. Moreover, this method is used for fingerprinting of cultivars, philogenetic relationships, determination of similarities between inbred lines and genome mapping and classification of organisms cultivars (Saiki et al., 1985; Ghareyazie et al., 1985). It has been always usual to evaluate the genetic diversity in plants using morphological or biochemical traits (Chtourou-Ghorbel et al., 2002). Conscious utilization of genetic resources for use in various studies including genetic analysis requires a detailed knowledge of the genetic relationships of herbal materials and determining the level of genetic diversity (Liu et al., 2003).

Knowledge of the genetic diversity of germplasm and genetic relationship among genotypes is too important to protect and use the germplasm resources (Matus and Hayes, 2002). This information can help researchers to select suitable parent compounds for maximum utilization of available genetic materials and production of high-yielding hybrids and populations that are being separated (Reif et al., 2004).

Genetic Variation Analysis is a process that represents differences or similarities between species, populations or individuals using specific statistical methods and models based on morphological traits, genealogical information or molecular characterization of individuals (Mohammadi and Prasanna, 2003). Molecular markers are useful tools for reformers to study the plant genes (Francis, 1999).

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The genetic variety of 28 populations of *Satureja hortensis* from different parts of Iran was investigated using horticultural traits and polymorphic components of molecular RAPD markers. Statistical analysis showed that there are significant differences among species in terms of horticultural traits. It also became clear that the diversity of molecular RAPD markers along with evaluation of horticultural traits in the long term seems appropriate with high accuracy in fingerprinting and assessment of genetic relationships in the *Satureja* Spp varieties (Hadian et al., 2008).

A study was conducted using SAMPLE molecular marker and related species were studied in three main groups. The study of relationship between *Satureja* Spp species showed that cluster analysis is somewhat proportional to the geographical distribution (Hadian et al., 2010).

The purpose of this study was to investigate the genetic diversity in 90 genotypes of five species of *Satureja hortensis, Satureja mutica, Satureja sahendica, Satureja bachtiarica and Gontcharovia Popovii* using RAPD markers and morphological traits (plant height, leaf width and leaf length).

MATERIALS AND METHODS

In this study, 90 genotypes collected from five species of Labiatae in the different provinces of Fars (Estahban), Zanjan, Kurdistan and Khorasan were studied (table 1). This study was conducted at Estahban Islamic Azad University in 2011, lat 29.05 'N, long 54.02 'E, altitude 1760 m, max and min temperature of 40.2 and -5.6 ° C During the growth season.

Table1. Names of the studied cultivars

	Persian Name	Scientific name	Location of data collection
1	Marzeh Bakhtiari	*Satureja bachtiarica*	Kurdistan province
2	Marzeh Motika	*Satureja mutica*	Khorasan Province
3	Marzeh Sahandi	*Satureja Sahendica*	Zanjan province
4	Marzeh Baghi	*Satureja hortensis*	Herbs website of Estahban Islamic Azad University
5	Gontsharovia Popovi	*Gontcharovia Popovii*	Mountains of Estahban

DNA was extracted from young leaves of the plant using Krizman et al (2006) method. The quality and quantity of DNA using a spectrophotometer with a wavelength of 260 and 280 nm was set, it was tested by 0.7% agarose gel and electrophoresis (Figure 1). Finally, qualified DNAs were diluted for polymerase chain reaction of 25 µL.

Polymerase chain reaction (PCR) was conducted in the final volume of 25 µL including a 2.5 µL reaction of PCR, 2.5 µL Mgcl2 (magnesium chloride), 0.5 µL dNTP Mix (mixed nucleotides), 1 µL primer, 1 unit of DNA single polymerase enzyme , 2 µL DNA and 15.5 µL double distilled water.

PCR thermal conditions, thermal cycles consisted of an initial denaturing cycle of in the temperature of 94°C for 2 minutes, 42 cycles each consisting of 30 seconds denaturing at a temperature of 94 °C, 30 seconds annealing at a temperature of 30 °C and 1minutes and 30 seconds extension at 72°C, and finally, one final extension cycle in 7 min at 72 °C were applied on samples.

After completion of DNA amplification in the polymerase chain reaction, DNA loaded by electrophoresis on 1.2 % agarose gel in 95 V TAE 1X buffer for 70 minutes. Then gel was stained in 0.05 percent ethidium bromide solution for 20 min. After staining, the gel put at distilled water for 3 minutes. Next, it was placed in the Gel Document device and was visible under UV light (figure 2). The obtained bands were scored for presence (1) or absence (0) and entered into Excel software. Molecular and morphological data analysis was performed using NTSYSpc, SAS and Mega 4 software.

Calculation of polymorphism information (PIC) and marker index (MI), as criterion to separate the primers, was conducted using the following equations.

$PIC= \Sigma[2p_i (1-p_i)]$

$MI= PIC \times B$

In this study, pi and B are frequency of ith band and number of polymorphic bands, respectively.

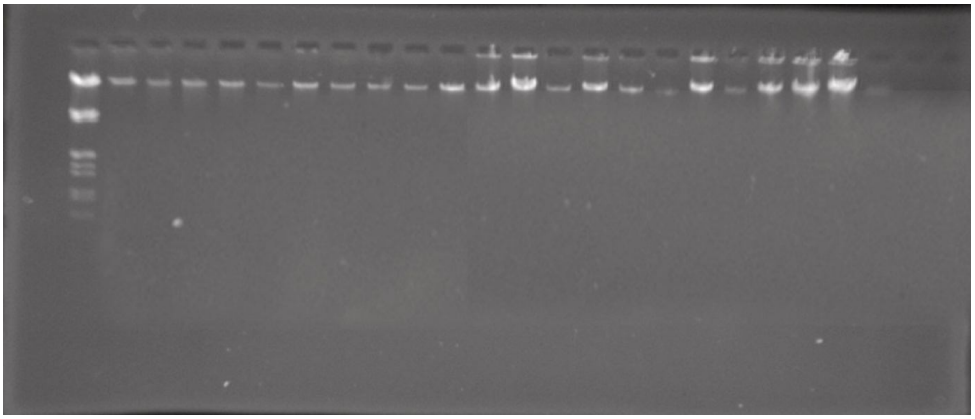

Figure1. Quality of some studied DNA genotypes

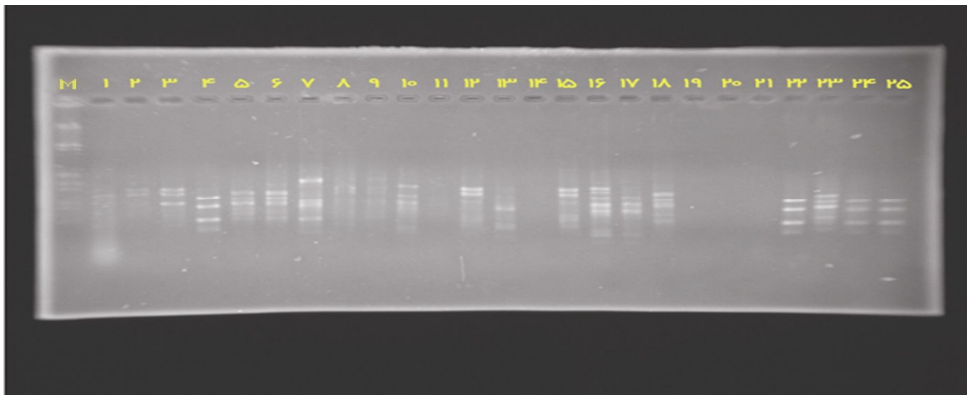

Figure 2. An example of obtained bands related to O_6 primer for 25 genotypes

Results of variance analysis

Morphological analysis of variance showed a significant difference between genotypes at the 5% level (Table 2).

In the plant height trait, 5 species were divided into 3 groups in which there was no difference between *Satureja bachtiarica*, *Satureja hortensis* and *Gontcharovia Popovii* species in terms of plant height while *Satureja mutica* and *Satureja Sahendica* species were in different groups. The highest and lowest height was observed in the *Satureja mutica* and *Satureja Sahendica* species, respectively. The trait of leaf width divided into 4 groups. *Satureja bachtiarica* and *Satureja mutica* were not different in terms of leaf width but other species showed differences because they were in the different groups. The highest and lowest leaf width was observed in the *Satureja bachtiarica* and *Satureja Sahendica* species, respectively. The leaf length trait divided into five different groups that were different in terms of leaf length so that the highest and lowest leaf length was observed in the *Satureja mutica* and *Satureja bachtiarica* species, respectively (Table 3).

Table 2 - Variance analysis of the studied traits

Plant height	Leaf width	Leaf length	Degrees of freedom	Sources of change
0. 49*	0.04*	0.37*	2	Block
84.95*	2.46*	284.64 *	4	Treatment
3.32	0.01	0.26	8	Error
367.41	10.08	1141.48	14	Total
6.42	2.74	2.43		Coefficient of Variation

** And * are significant at 1% and 5% levels, respectively.

Table 3 - Comparison of traits average based on Duncan test

Leaf length (Mm)	Leaf width (Mm)	Plant height (Cm)	Species
9.74 e	5.03 a	27.78b	*Satureja bachtiarica*
29.66 a	4.84 a	36.23a	*Satureja mutica*
11.50 d	2.77 d	21.28c	*Satureja Sahendica*
27.09 c	4.61 b	28.90 b	*Satureja hortensis*
28.12 b	4.18 c	27.59 b	*Gontcharovia Popovii*

Similar letters in each column are not significantly different.

Genetic diversity

Obtained results based on morphological traits

90 genotypes of 5 species were exploited. These species included *Satureja bachtiarica* Bunge (15 genotypes), *Satureja mutica* (15 genotypes), *Satureja sahendica* (12 genotypes), *Satureja hortensis* (24 genotypes) and *Gontcharovia Popovii* (24 genotypes). They were divided into two groups based on morphological traits (Figure 3). The first group included *Satureja bachtiarica and Satureja Sahendica* while the second group included *Satureja hortensis, Satureja mutica and Gontcharovia Popovii* species. Results indicate that species clustered in the same group of are closer together in terms of morphological traits.

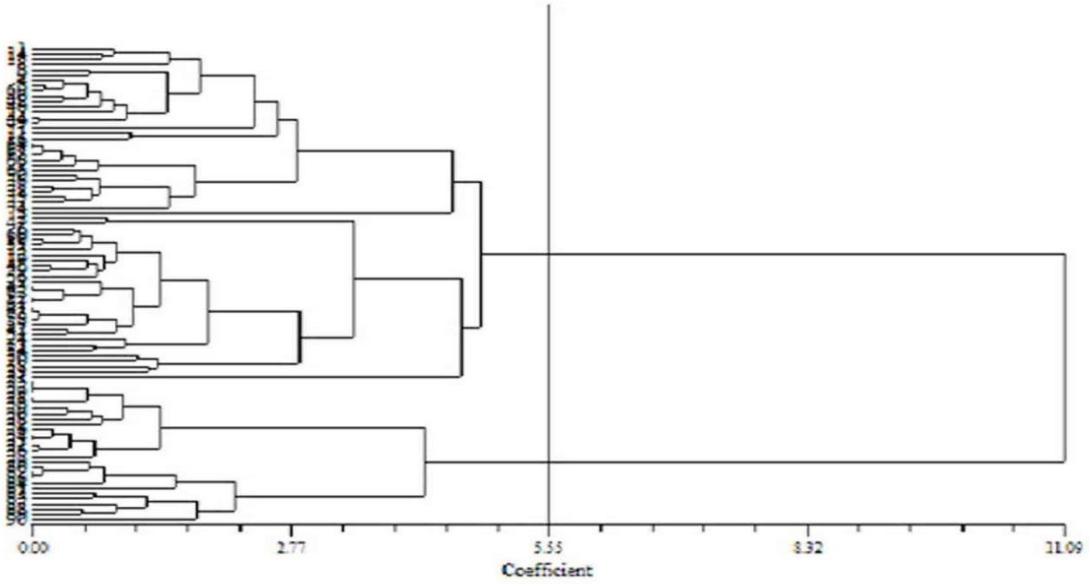

Figure 3 - Grouping of 5 studied species based on morphological traits

Obtained results based on the polymorphism of molecular RAPD marker

Out of 40 randomly selected primers used in this study, 11 primers generated appropriate polymorphic bands. Generated markers in this experiment were 49 random amplified bands so that the average number of polymorphic bands for each primer was 4.45. The highest and lowest polymorphic bands observed in the P_7 and P_1 primers, respectively There was (Table 4). In this study, the average polymorphic information for each primer was 0.156. The highest and lowest polymorphic information was related to P_2 (0.240) and P_9 (0.060) primers, respectively. In this experiment, the average index marker for each primer was calculated (1.449) so that the highest and lowest index marker observed in the P_7 (2.589) and P_9 (0.781) primers, respectively (Table 5).

Table 4 - Name, sequence and characteristics of primers

Number	Primers	Name of primer	Sequence (5'→ 3 ')	Percent GC	Total number of bands	Number of polymorphic bands	The percentage of polymorphic bands	PIC	MI
1	P_1	S_1	CTA CTG CGC T	60%	5	2	40%	0.157	0.788
2	P_2	O_6	CCA CGG GAA G	70%	10	3	30%	0.240	2.401
3	P_3	AD_3	TCT CGC CTA C	60%	7	3	42.85%	0.129	0.909
4	P_4	M_6	CTG GGC AAC T	60%	9	4	44.44%	0.134	1.213
5	P_5	S_{11}	AGT CGG GTG G	70%	9	3	33.33%	0.170	1.537
6	P_6	**OPB-10**	CTG CTG GGA C	70%	9	6	66.66%	0.154	1.388
7	P_7	OPY_{-7}	AGA GCC GTC A	60%	19	10	52.63%	0.136	2.589
8	P_8	OPA_{-13}	CAG CAC CCA C	70%	9	3	33.33%	0.196	1.772
9	P_9	OPA_{-19}	CAA ACG TCG G	60%	13	9	69.23%	0.060	0.781
10	P_{10}	OPA_{-08}	GTG ACG TAG G	60%	8	3	37.5%	0.165	1.326
11	P_{11}	OPH_{-20}	GGG AGA CAT C	60%	7	3	42.85%	0.176	1.234

Table 5 – Statistical information of primers

Number of polymorphic bands	MI	PIC	
4.45	1.449	0.156	Average
10 - P_7 Primer	2.589 (P_7 Primer)	0.240(P_2 Primer)	Maximum
2 - P_1 Primer	0.781(P_9 Primer)	0.060(P_9 Primer)	Minimum

The obtained dendrogram was at 78% level of similarity so that investigated genotypes were divided into 3 groups based on RAPD marker (Figure 4). First group included genotypes 28 and 25, both of which related to *Satureja bachtiarica*. Second group included six genotypes of *Satureja bachtiarica* (26-27-29-32-34-35). Third group included 82 genotypes of *Satureja mutica, Satureja Sahendica, Satureja hortensis and Gontcharovia Popovii* species while seven other genotypes included *Satureja bachtiarica*.

Given that some of the genotypes with high similarity were clustered in the same group (69 and 70 genotype of *Satureja mutica* species), possible transfer of some species from one region to another and their renaming would be occurred; though having a common parent for them is not unexpected. Results indicate that *Satureja bachtiarica* has more genetic variation than other species because some genotypes are placed in separate groups. *Gontcharovia Popovii* was genetically similar to *Satureja mutica, Satureja Sahendica and Satureja hortensis* species and morphologicaly similar to *Satureja mutica* and Satureja *hortensis* species. Therefore, *Satureja mutica* and *Satureja hortensis* species were more morphologically and moleculary similar to *Gontcharovia Popovii* species.

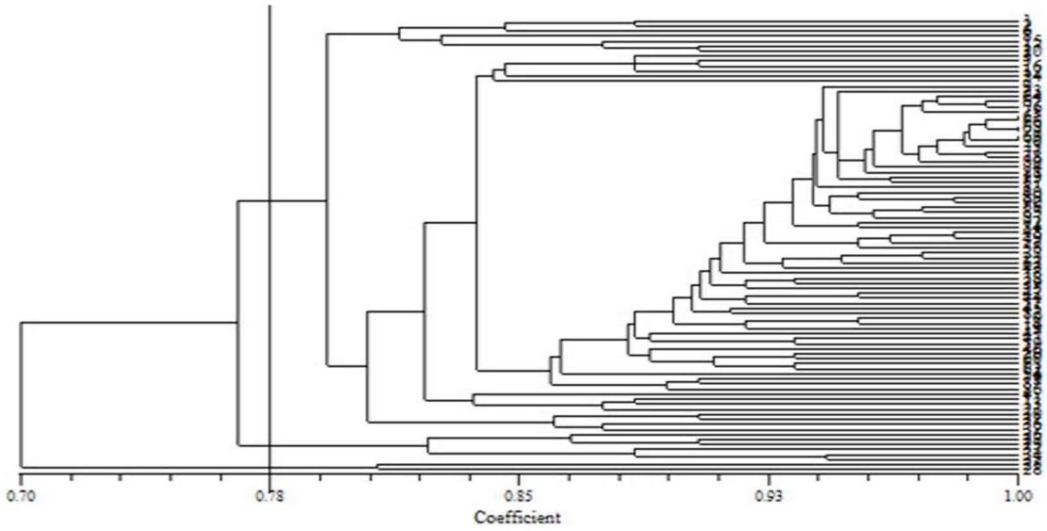

Figure 4 - Dendrogram of 90 investigated genotypes related to obtained data of RAPD markers.

Moreover, molecular information was used to form the investigated genotypes based on Mega 4 Software analysis in two main groups. The group 1 with 13 genotypes was related to *Satureja bachtiarica* while other genotypes placed in the group 2 (Figure 5). This represents that *Satureja bachtiarica* is genetically less similar to other species but it has more analogy to other species in terms of genetic diversity. Obtained results of Mega4 are consistent with the results of dendrogram obtained from RAPD markers. This confirms the results of this study.

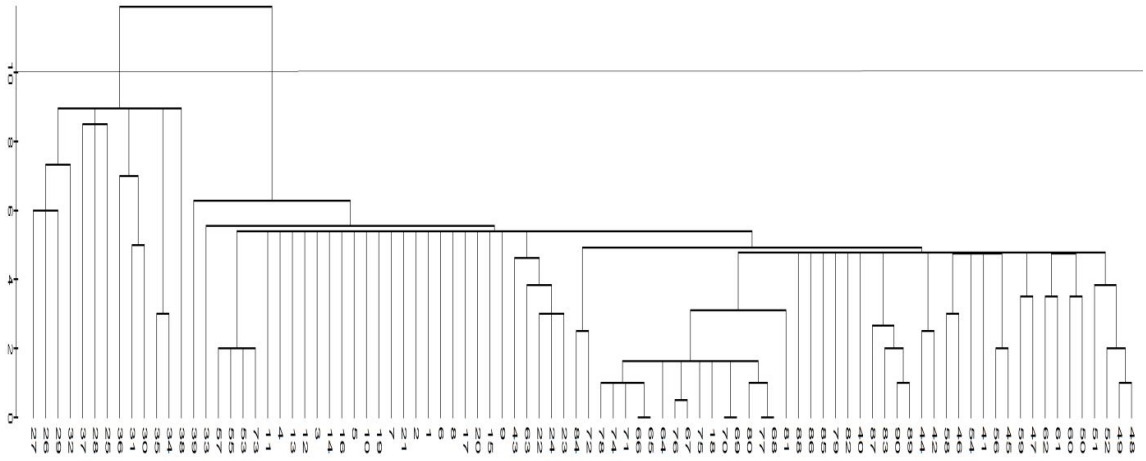

Figure 5 - Dendrogram of 90 investigated genotypes related to obtained data of Mega4

Some differences and similarities were observed by examining the dendrogram obtained from morphological charactersand RAPD markers. For example, genotypes (types 69 and 70) of *Satureja mutica* were most similar to the grouping of molecular data while the two genotypes in the dendrogram obtained from morphological characters were far from each other. Given dendrogram correlation Coefficient (r=0.1) and its insignificancy, the obtained groupings showed no statistically significant correlation.

In addition, since most of the morphological traits are controlled by many genes , they are strongly influenced by environmental conditions and sequences of RAPD molecular markers are randomly distributed throughout the genome (even off points of the genome). They mainly have no phenotypic occurrence that seems to be reasonable (Dey et al., 2006).

Moreover, although the RAPD marker is used widely in the studies of samples classification and detection of cultivars and genetic diversity (Konstantinos et al., 2008), its repeatable feature has always been questioned. One of the reasons for this issue is the use of short primers and low temperature to connect primer to the DNA pattern. This causes non-specific and random amplification in some areas that are only slightly similar. Two solutions are proposed for this problem:a) Replication of the experiment in the same situation and removal of

unrepeatable bands. b) Conducting only one test and accepting a percentage of error (Anju et al., 2006). However, since the main part of genome consisted non-coding parts, another reason for the lack of correlation between the two dendrograms was distribution of selected RAPD primers at all levels of genomes (Mc Clean et al., 2009).

Results of one study showed that the RAPD method along with horticultural analysis seems appropriate in long-term. This method with high accuracy is suitable for fingerprinting and assessment of genetic relationships in *Satureja* Spp species(Hadian et al., 2008).

Another study on the relationship between *Satureja* Spp species show that the cluster analysis is somewhat consistent with the geographic distribution (Hadian et al., 2010). According to results obtained in this study, molecular RAPD marker is a useful tool in the evaluation of the molecular genetic relationships of *Satureja* Spp species.

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