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RAPD Molecular Markers Used to Assess Genetic and Morphological Diversity of some *SaturejaHortensis L.* Masses

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ABSTRACT

In this study, genetic and morphological diversity of 9 garden savories (*saturejahortensis L.*) were collected from different regions of Iran (Estahban, Esfahan, Khuzestan, Shiraz, Talkhunche, Ghorveh, Mobarake, Mashhad and Bahar of Hamadan) and were investigated using RAPD molecular markers. Analysis of morphological features was performed using a randomized complete block design with six replications. The results of variance analysis showed a significant difference ($P<0.05$) in stem length, stem diameter, leaf length, root diameter, number of lateral branches and number of lateral roots. 17 primers out of 40 generated polymorphic bands and the average number of generated bands by each primer and the average number of polymorphic bands were 13.5 and 6.4, respectively. Overall, the average percentage of polymorphism for random primers was 84%. The average polymorphic information for each primer (PIC) was 0.312 and the average marker index (MI) for each primer was 2.07. Cluster analysis based on RAPD data categorized the masses into 5 groups and cluster analysis based on morphologic data divided the masses into three groups but no correspondence between morphologic and molecular data was observed. Regarding the findings of this study, RAPD molecular marker can be an effective tool to assess genetic diversity between *Saturejahortensis L.* masses and masses with greater genetic distance can be used in reforming plans to produce segregating generations and benefit from heterosis phenomenon.

KEYWORDS: garden savory, cluster analysis, genetic diversity, morphologic diversity, RAPD.

1. INTRODUCTION

Saturejahortensis is known as garden savory and belongs to labiates and is an herbaceous plant, a year or more that its organization is the Mediterranean. This plant is scattered all across southern Eroupe and its main distribution is in Iran, Caucause and Turkmenistan, Anatoly and Iraq. There are 12 herbaceous species in Iran that are one or several years and 8 of these species are unique to Iran (Motaghinejad et al., 2009).

Because of the medical value of this plant including anti-microbial properties, high anti-oxidation in masses, determining morphological differences and herbal genetic along with the relationship between them to identify and collect masses, they are scientifically and economically important (Shafizade, 2002).

Knowledge about inheritance resources diversity and genetic relationship between genotypes is a great assist to develop breeding plans and the evaluation of cultivated and wild populations seem necessary in preserving and proper utilization of valuable inheritance resources. The study of genetic diversity is possible with different methods and molecular markers based on Polymerase Chain Reaction (PCR) are a powerful way to identify polymorphic DNA and genetic diversity analysis. These markers are less affected by environmental conditions and a great number of these markers can be searched easily in the entire genome (Asghari et al., 2010).

There are different masses of savory in Iran that their genetic relationship and properties have not been studied using molecular markers. The present study aimed to evaluate genetic diversity between savory masses and grouping them so that they can be used in breeding plans.

2. MATERIALS AND METHODS

Herbal materials used in this study consisted of 9 populations from different regions of Iran (Estahban, Esfahan, Khuzestan, Shiraz, Ghorve, Mobarake, Mashhad, Bahar of Hamadan and Talkunche). Seeds of the study masses were planted in greenhouse of Islamic Azad university of Estahban and 10 genotypes were selected from each mass and at four-leaf stage a sample leaf was taken from each one separately. Extraction of DNA was performed using CTAB method with some modification in Krizman et al. (2006) method. Quality and quantity of

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DNA samples were determined using agarose gel electrophoresis and spectrophotometer. DNA concentration of 25 ng×µl was diluted and used for PCR reaction. In PCR reactions, random primers of RAPD (number 1 to 40) were used (Metabio company in Germany) to amplify genomic DNA. PCR reaction with 25 µl volume contains: 2.5 µl of buffer (10×), 2.5 µl $Mgcl_2$, 0.5 µl dNTPs, 15.5 µl water 1 unit Taq DNA polymerase enzyme, 2 µl DNA (10 ng×µl) and was performed with 1 µl primer.

Thermal cycles included: initial denaturation for 2 minutes at 94°C and then 42 cycles including DNA denaturation (39seconds at 94°C), primer binding (30 seconds at 32°C) and primer extension (1 minute and 30 seconds at 72°C) and finally are cycle for 7 minutes at 72°C as the ultimate binding. Generated products by PCR were detected using agarose gel electrophorsis of 1.2% and coloring with ethidium bromide. Presence or absence of a bar was coded with the numbers zero and one for the study population. Thus, the total number of polymorphic bands was calculated. In this study, cluster analysis was done based on simple matching similarity coefficient (SM) and UPGMA method and with NTSYS and Mega 4 programs. In the present project, 10 traits of stem length, stem diameter, leaf width, major root length, lateral root length, root diameter, number of lateral branches, number of lateral roots and internodes length were measured in a randomized complete block design with six replications. Data analysis was performed using SAS software.

Polymorphism information content (PIC) and marker Index (MI) that are criteria for primers separation strength were calculated using equations 1 and 2:

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

(2)$MI= PIC\times B$

In this equation, Pi is frequency of I band and 13 is the number of polymorphic bands (Namayande et al., 2010).

3. RESULTS & DISCUSSION

Variance analysis of the study traits was done using SAS software and significant differences were observed between the populations. According to variance analysis (Table 1) in 9 masses, 6 traits (out of 10 measured traits) including stem length, stem diameter, leaf length, root diameter, number of lateral branches and number of lateral roots showed significant differences.

The comparison of mean of traits was performed using Duncan's test at 5% probability level (Table 2).As the results of variance analysis indicated, four traits showed significant a difference among the populations under investigation. Farshadfar et al. (2010) stated that there is a possibility that in data structure variation among the masses, an overlap is observed and mass variance decreases, leading to non-significant results of the mass effect while there exists a real variation; therefore, if the mass effect is not significant, it is better to conduct mean comparison so that variation can be determined.

3.1. Genotypes Grouping Based on Data of RAPD Molecular Markers and Morphological Traits

DNA reproduced fragments were between 500-2000. Zero matrixes and a yield for analyzing genotypes variety included 90 rows and 109 columns. The row contained the genotypes and the columns were consisted of polymorphic bans.

In this experiment, 17 primers generated polymorphic among 40 randomly used primers and other primers did not generate any band. The average number of produced bands by each primer was 13.5 bars. P_7, P_6 and P_9 generated most polymorphic bands. Moreover, the average number of polymorphic bars for each primer was 6.4. The percentage of polymorphic random primers ranged between 28.5-80. P_{14} with 80 percent polymorphic and P_1 with 28.5 percent polymorphic generated the highest and lowest percentages of polymorphic, respectively. The average polymorphic for random primers was 84%. Hadian et al. (2008) in an investigation of 28 masses of garden savory *Saturejahortensis L.* observed up to 83% polymorphic using RAPD marker in different parts of Iran (Table 1).

Table 1- Simple variance analysis of the study traits, figures in the Table are the mean of squares

Sources of variation	Degree of freedom	Stem length	Stem diameter	Leaf length	Leaf width	Main root length	Lateral root length	Root diameter	Number of lateral branches	Number of lateral roots	Internodes length
Block	5	10.75	0.003	0.13	0.009	2.15	1.52	0.25	6.65	5.585	0.19
Genotype	8	*50.12	*0.012	*0.72	0.012	1.12	1.2	*1.69	*23.79	*8.005	0.08
Error	40	12.5	0.006	0.32	0.017	1.72	2.07	0.29	10.65	6/18	0.15
CV%		9.37	17.54	18.66	25.72	22.95	10.34	20.17	16.18	11.06	21.4

*Sig. 5%

The average polymorphic information for each of the primers in this study was 0.312. The maximum amount of information of polymorphic pertained to P_7 (0.49) and the minimum amount of information was for P_{12}, P_{16} and P_{17} (0.14). Average marker index for each primer calculated and the maximum and the minimum were for P_5(3.29) and P_{17} (0.56), respectively (Table 3 and 4).

Table 2- comparison of the mean of masses for the study traits using Duncan test with 5% probability

mass	Stem length	Stem diameter	Leaf length	Leaf width	Main root length	Lateral root length	Root diameter	Number of lateral branches	Number of lateral roots	Internodes length
Estahban	42.41a	0.23b	1.83abc	0.25 a	3.58 a	13.33 a	0.74b	23.33 ab	24.33a	1.81 a
Isfahan	41ba	0.23b	1.93abc	0.22 a	4.25 a	14.33 a	0.69b	18.66 c	24 ab	1.81 a
Khuzestan	37.33bcd	0.21b	1.95ab	0.2 a	4.33 a	14.33 a	0.78b	21abc	21.66 ab	1.93 a
Shiraz	38.91abc	0.23b	1.85abc	0.26 a	4.25 a	13.66 a	1.51a	20.83abc	22.33 ab	1.83 a
Talkhunche	33.25de	0.23b	2.32a	0.186a	4.25 a	14.5 a	2.16a	18 c	20.66 b	1.9 a
Kurdistan	32.66e	0.21b	1.18c	0.18 a	4.25 a	13.66 a	2 a	16.66 c	22 ab	1.93 a
Mobarake	41.08ab	0.38a	1.22bc	0.31 a	4.55 a	13.5 a	2.03a	24a	21.33ab	1.91 a
Mashhad	34.83cde	0.2b	1.2bc	0.3a	4.56 a	13.83 a	2.16a	19.66 bc	22.33 ab	1.83 a
Hamadan	38abc	0.26b	1.25bc	0.2 a	4.13 a	14 a	2.08a	19.33 bc	23.66 ab	1.78 a

In each column means with common letters are not meaningful at significant level of 5%

Table 3- List of primers and their features

Marker Index	Amount of polymorphic information	Polymorphism percentage	Number of polymorphic bands	Total number of bands	Primers sequence	Name of primer	primer
1.36	0.34	28.5	4	14	5´ACC GTT CCA G3	S10	P_1
2.4	0.3	40	8	20	5´ TCT CGC CTA C3´	AD3	P_2
1.48	0.37	44.5	4	9	5´ GTG AGG CGT C3´	OPC-2	P_3
2.24	0.28	73	8	11	5´ GAT GAC CGC C3´	OPC-5	P_4
3.29	0.47	47	7	15	5´ CTA CTG CGC T 3´	S1	P5
2.9	0.29	45	10	22	5´ TGG TGC ACT C3´	AL14	P6
4.9	0.49	45	10	22	5´ CTG GGT GAG T3´	S12	P7
0.8	0.16	33	5	15	5´ CTG GCG AAC T3´	S18	P8
4.6	0.46	67	10	15	5´ CAA ACG TCG G3´	0PA-19	P9
1.6	0.4	57	4	7	5´ AGA CAG CCA C3´	OPY-7	P10
2.3	0.46	45	5	11	5´ AGT CGG GTG G3´	S11	P11
0.98	0.14	39	7	18	5´ AGG CCA ACA G3´	BE19	P12
0.84	0.21	57	4	7	5´ CAG GCC CTT C3´	OPA-01	P13
2.03	0.29	80	8	10	5´ TCT GGA CGG A3´	S20	P14
2.22	0.37	46	6	13	5´ CTG CTG GGA C3´	OPB-10	P15
0.7	0.14	33	5	15	5´ GGG AGA CAT C3´	OPH-20	P16
0.56	0.14	67	4	6	5´ TCC GAT GCT G3´	S7	P17

Table 4- Statistical summary of primers

Marker Index(MI)	No. of polymorphic bands	PIC value	
2.07	6.4	0.312	Mean
3.29 (P5)	10 (P6،P7،P9)	0.49 (P7)	Maximum
0.56 (P17)	4 (P1،P3،P10،P13،P16)	0.14 (P12 ، P16، P17)	Minimum

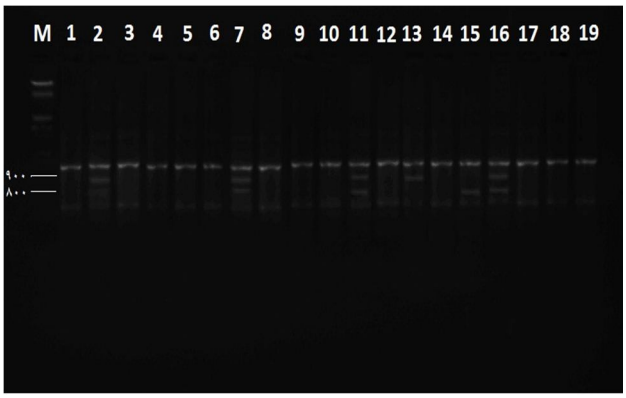

Figure 1- polymorphism resulted from S_{18} in 19 masses of Esfahan (wells 1 to 14) and Estahban (wells 15 to 19)

The obtained tree diagram from RAPD data divided the mass into five groups using NTYSYS software and similarity coefficient of 0.77. The first group included 9 Mashhad genotypes masses and the second group consisted of 7 Shiraz genotype masses. Group 3 included all genotype masses of Talkunche and Group 4 comprised of Khuzestan masses. Group 5, too, included genotype masses of Hamadan, Kurdistan, Estahban, Isfahan and Mobarake. The markers failed to segregate the population of these five masses, which indicated a high genetic relationship of these five masses (Table 2).

DNA markers can indicate genetic similarities at the genomic level parts without password (heterochromatic). Thus, it is possible that the population of five masses of Hamadan, Estahban, Isfahan, Mobarake and Kurdistan may differ in terms of their appearance properties but are similar in terms of the entire genome (Asghari et al., 2010). Moreover, it is possible that it resulted from the transfer of seeds from a region to another one and consequently the germplasm exchange. For instance, despite a long geographical distance between masses of Isfahan and Estahban, these two masses are grouped into one category based on molecular analysis. Isfahan is considered one of the most important centers of cultivating herb seeds (Pure Seeds Institute).

Probably, the seeds of Estahban masses are collected from Isfahan city and were originally from one genotype or they may be derived from a primary population. For example, Isfahan. In addition, regarding Mobarake masses, due to the geographical proximity, the above mentioned probability may be true.

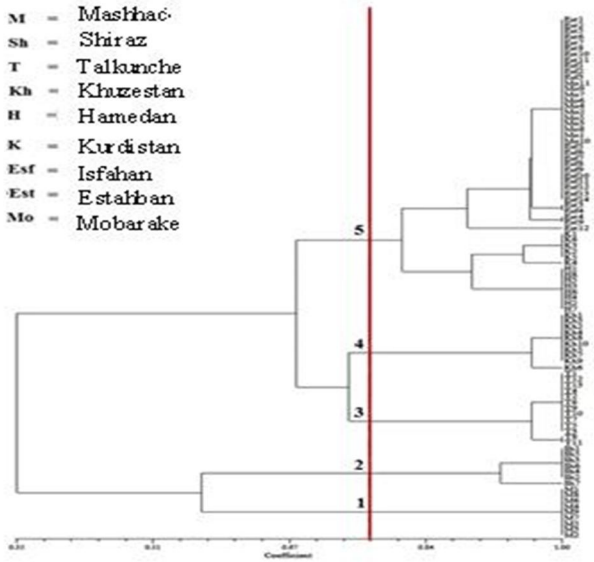

Figure 2- Genotypes grouping based on RAPD Molecular Marker

RAPD statistical analysis was conducted using Mega 4 software (Boostrapping method) and the obtained dendogram was highly in accordance with the resulted dendogram of NTSYS-pc software (Table 3).

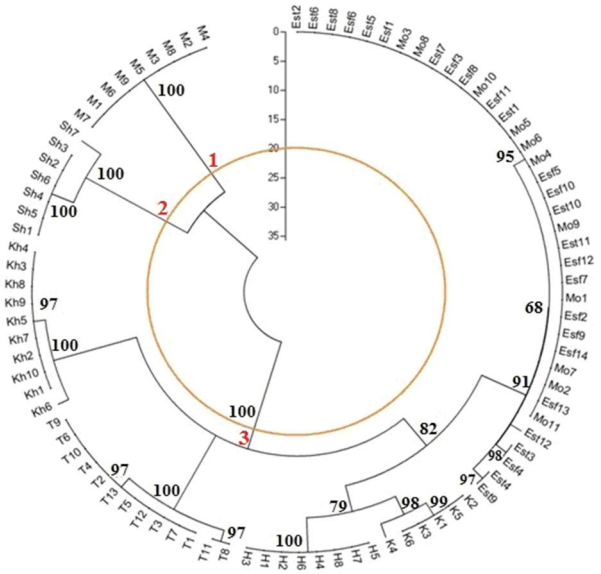

Figure 3- Genotypes grouping based on RAPD Molecular Marker using Mega 4 software

The first group consisted of masses of Mashhad (with a value of 100% Bootstrap). The second group included the masses of Shiraz (with 100100% Bootstrap value). And the third group consisted of masses from Khuzestan, Tlkhunche, Hamedan, Kurdistan, Esfahan, Estahban and Mobarake (with 100% Bootstrap value).

In this grouping as the previous grouping, groups of 1 and 3 showed minimum similarity. The obtained Diagrams from morphologic traits data were not able to clearly distinguish the study populations. The diagram divided the masses into three groups with a similarity coefficient of 3.64 (Table 4). Group 1 consisted of genotype 4 of Mobarake and Group 2 included genotypes 5 of Kurdistan and Talkhunche. Group 3 included other genotypes of Talkhunch, Kurdistan, Shiraz, Mashhad, Hamadan, Khuzestan, Esfahan, Estahban and Mobarake. In this group no significantly meaningful correlation was observed between the patterns of genetic diversity and geography distribution. According to the results, it can be stated that in controlled experimental conditions RAPD marker can be an appropriate and effective tool for evaluating genetic diversity between savory garden masses and completing morphological evaluation. Examining genetic diversity using these markers indicated that the study masses with 84% polymorphic are potential sources for various genes. Thus, studying the above mentioned polymorphic among germplasm of each population provided opportunities for parental choice to cross according to the presented table separately for each primer. It can be expected that the parents generated superior results while conferencing each other and raised the mean of traits among the populations. Moreover, the observed diversity among masses can be applied for breading programs.

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