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SURVEY OF GENETIC DIVERSITY IN ZATARIA MULTIFLORA POPULATION BY RAPD & ISSR MARKER IN KERMAN PROVINCE

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ABSTRACT

Recognizing genetic diversity and classifying the Germplasm is one of important activities in race improvement and management of genetic herbaceous sources. In this research random amplified polymorphic DNA (RAPD) &inter-simple sequence repeat (ISSR) to be used for surveying genetic varieties of 13 masses of *Zataria multiflora*. The leaf of thyme from 13 different regions in Kerman was collected. The extraction of DNA from the leaf was done by cetyltrimethylammonium bromide(CTAB) method with a little change. The quantity and quality of extracted DNA was measured by electrophoresis equipment & spectrophotometer. 10 markers of RAPD and 10 markers of ISSR which had produced the clearer bands in polymerase chain reaction (PCR) were used for analysis. by considering of the results, the markers were produced in the total of 179 polymorphism of 212 bands by percent of polymorphism of 84.4. 13selected regions in 3 separated groups that the most genetic similarity due to the similarity the coefficient of Dice by NTSYS software among the Bluck masses and Jiroft was 89.20% and the less similarity was considered among Bluck masses with Bam (52.5%). created cluster was had harmony with geographical conditions. 2dimensional graph resulting from the Principle component analysis(PCA) nearly was accordance with dendrogram due to Unweighted Pair Group Method using arithmetic Averages(UPGMA) method and cophenetic correlation coefficient was 77% in this research. the results of this research showed that markers RAPD &

ISSR could be a useful tool for surveying genetic varieties of herbaceous samples and help for amendments of future researches & management of *Zatraia multiflora* germplasm.

Keywords: Genetic diversity, Zataria multiflora, Kerman Province, ISSR, RAPD markers

INTRODUCTION

Zataria multiflora is from Spearmint family (Lamiaceae) one tree's plant- a bush in Afghanistan, Pakistan and south regions of Iran (7), with small leaves, nearly round, meaty, having ribs with 4 stamens and having style with two short & thin embranchments (6,7), the most important existed material in this plant are Timol and Carvacrol which have anti oxidation(13), anti microbial and anti fungous effects(11). Knowing about genetic diversity of plants help to save herbal genetic resources, ability of using them to help for supplying of race improvement, also knowing about genetic distance among the populations creates the possibility of finding populations for drawing genetic varieties and the places genes (17), since the studies of morphologic specifications are not exacted due to environment factors on these specification alone, today there are valuable methods for evaluations and molecular researches to define genetic relationship among the masses and different figures(9). Many researches for defining decorative plant's genetic varieties by using molecular markers especially DNA markers on the basis of PCR has been carried out which are RAPD markers. This marker has some advantages like requirement of low amount DNA genomic in each evaluation, non-requiring of Sequence previous information, facility of evaluation and measuring and the ability of generalizing the results to other studies(18), also it does not need any expensive equipments and does not have any radioactive materials(8). ISSR markers are on the basis of PCR which has the advantages of RAPD and any defects such as lack of repetition and has lower cost in ratio to other molecular markers (12, 19). This technique is fast and can make clear the difference among the people possessing the high nearness (1). Furthermore, the marker based on DNA has removed the problems related to morphological and biochemical of markers through establishing of unlimited markers and deleting the effects arise of environmental factors (14).

Pejman Mehr and colleagues in 2008 surveyed genetic varieties of 20 ecotype *Bunium persicum* by molecular markers of RAPD and AFLP. The results of research showed that there is high variety for native *B.persicum* in Iran even in the ecotypes next

to each other (geographical point)(10). Trindate in 2008 studied genetic varieties by RAPD and chemical polymorphism of 31 single bush in species of thyme (*Thymus caespititus*). The Principle component analysis of essence chemical composition of 31 single bush was done and caused to recognizing 4 chemical type in them, applying markers of RAPD in the study of 31 single bush was divided to 6 groups, the result showed there is no special relationship among the place of collecting the samples, chemical polymorphism and molecular evaluating of RAPD (15). Fazeli and his colleagues in 2011 surveyed genetic diversity the masses of pea by using 21 molecular markers of ISSR, from 361pieces of total masses and the cultivars, 257 pieces were polymorphism that was the sign of high percentage of polymorphism, also 104 masses of pea by the help of similar coefficient of Jakard and the method of UPGMA were divided into 15 groups (5). In this research for more understanding and protecting the Germplasm of thyme, the study of *Zatarin multiflora* was carried out in Kerman and the molecular markers of RAPD & ISSR were applied.

MATERIAL & METHODS

13 kinds of *Zatarin multiflora* from different regions in the season of spring in 2014 were collected in Kerman (table1) .the samples on the ice were transferred to the laboratory of agricultural University of Jiroft and were kept in the temperature of -80 till extraction time. Several protocol for desirable extraction of DNA were tested till finally the method of CTAB was selected with some changes in the process of washing of DNA, fresh leaves related to each region were mixed separately and in final at the rate of 0.4 gram from each sample were extracted, for specifying of quantity and the quality of extracted DNA, two methods of electrophoresis DNA in Agazor gel with the concentration of 1 percent & the method of spectrophotometer by the equipment of Perkin-Elmer , model Ez-201 in the wavelength of 280 nanometer were used, the existence of clear bands and not establishing the smear on the gel shows correct extraction of DNA from the tissue.

Table 1-The geographical regions of the collected *Zataria multiflora* masses across Kerman province for DNA extraction

Row	Collection point	Row	Collection point	Row	Collection point
1	Jroft	6	Esfandegheh	11	Orzoiyeh
2	Bam	7	Faryab	12	Menojan
3	Bluck	8	Galehganj	13	Rabor
4	Kahnoj	9	Roodbar	14	
5	Anbar abad	10	Baft	15	

By considering that the circle of the polymerase chain reaction (PCR) needs a certain amount of DNA concentration, diluting the DNA was done. In this order after calculating the DNA concentration the formula: 100*50(the amount of absorption in 320 nanometer- the amount of absorption in 260 nanometer) was equal with: DNA µg/ml of concentration.

By applying of formula C1V1=C272, the bulk amount which to be decreased from DNA stock, then reached to 100 micro liter by sterile water , in this formula the amount were calculated as 5=C2nanogram ,100=v2 µl & C1 , the volume of concentration of DNA from the first formula and in this formula V1 was calculated. The rate of required compositions in PCR for both markers in the bulk of 25 µl was carried out. The special compositions of PCR such as Buffer 10X, dNTPs, Taq enzyme, Magnizium chloride, were supplied from the “Neday Fan" company. Accidental markers, RAPD & ISSR were purchased from “macro gene/ Korea Company” and dilution was occurred in markers RAPD & ISSR by ordinary method. In this research termocyclers were in the model of "Masercycler" in simple manufacturing of Peglab/German Company. The best connection temperature for each marker was given by using of TM temperature (table2) .after processing of PCR of the samples, immediately were drawn out from termocycler and were kept in the temperature of -20 till the time of electrophoresis. In order to surveying repetition of ISSR & RAPD bands, PCR were selected in primers by accident till the clearance of the bands was confirmed. The products of PCR for each primers of RAPD on the 1% agarose gel & buffer TBE with the voltage of 75 was took in 90 minutes and the products of polymerase chain reaction related to ISSR primers on the 1.5% Agarose gel and buffer TBE with the voltage of 80 was took step in 120 minutes.

In primary lane, the 100 bp Ladder was applied for estimation of the size of bands and in other lanes the products of PCR were poured then the gel was put inside the equipment of 'GEL Documentation ,model Ebox vx2 , made by France and was put under the violet ray. Subsequent observation of bands under this ray was prepared the different formats from gel and the photos were taken by software of Genesnap (Ver 6.08.09), the number of 10 RAPD primers which had clearer bands together with 10 ISSR was selected for analysis. The size of

each of clear bands in each lane was defined on the basis of the length and the molecular weight which were specified by software Gene tool. The presence & non-presence of each of observed bands for each 20 marker were scored with the figures of 1 and 0, the places which possessed a collection of 0 &1, were introduced as polymorphism places, then a matrix was prepared from the figures 0 &1 for each marker in the Excel software, after that the data was transferred to NTedit, the input section of NTSYS software, the analysis of cluster occurred by Unweighted Pair Group Method using arithmetic Averages (UPGMA) and the similarity coefficient of Dice by help the NTSYS –pc software and the related dendrogram was drawn. In addition to cluster analysis, the genotypes could be grouping by applying of Principle component analysis that was done by NTSYS software and in the menu of" Eigen". 2 dimensional graph was drawn as well. for surveying the correlation between dendrogram and matrix of similarity, cophenetic correlation coefficient was calculated.

Table 2 - markers used in this study

Melting temperature ° c	Sequence	ISSR	Melting temperature° c	Sequence	RAPD
52.4	CTC TCT CTC TCT CTC TG	ISSR-8	34	CAG GCC CTT C	OPA01
60	AGC AGC AGC AGC AGC AGC	UCB862	32	AGG TGA CCG T	OPA18AGC
52.4	ACA CAC ACA CAC ACA CC	826	32	AGC GCC ATT G	OPD11
50	AGA GAG AGA GAG AGA GT	807	32	CAG GAC ATC G	376
50	ACA CAC ACA CAC ACA CT	ISSR-7	32	CCT CAC CTG T	J
59.4	ACT TCC CCA CAG GTT AAC A	ISSR-25	32	TAG CCC GCT T	UBC110
48.2	CTA GCT AGC TAG CTA G	ISSR-19	34	CCT GGG CTT C	UCB1
50	TGT GTG TGT GTG TGT GA	828	34	TGC CGA GCT G	A02
47	ATG ATG ATG ATG ATG ATG	UBC864	32	GAG AGC CAA C	D18
52.4	ACA CAC ACA CAC ACA CG	P8-ISSR	30	TCA CGC AGT T	394

RESULTS

On the basis of obtained information from 20 markers and by using Dice coefficient, dendrogram was drawn by UPGMA method, the range of genetic similarity is between 66% and 89%. Cutting line in the genetic distance was considered 71%. On this basis 13 genotype was studied in 3 main groups (figure 1). The first group including 4 members in the names of Jiroft. Anbar abad. Bluck and Roodbar, second group including 2 members, Bam & Kahnooj and third group consist of 7 members as Esfandegheh, Baft, Orzoiyeh, Rabor, Faryab, Galehganj and menojan .

The first and third groups have sub groups as well, the third group had the most herbaceous masses related to 13 regions that genetic variety of some of these masses were accordance with geographical conditions. The least similarity between Bluck & Bam is 52.5% and the most similarity between masses of Jiroft with Bluck 89.20% (table3). The range of established similarity is the sign of efficiency of RAPD & ISSR in evaluation of genetic diversity, the difference among masses and groupings are obvious. In the total collection of markers 212 pieces, of 179 numbers had polymorphism that signs of high percentage of polymorphism 84.4% in this species (figure2), Principle component analysis also was carried out for completion of cluster analysis method. From analysis to component on the data arise of RAPD & ISSR, 2dimentional and 3 dimensional graphs were created. 3 Principle components which have had the most share for creation the variety, by using the obtained information from 2 & 3 dimensional graphs, the genotypes could be grouping in 4 different groups that this grouping has accordance with grouping due to dendrogram (figure3). Principally, the calculation of correlation coefficient is the last cluster analysis process. This coefficient shows that how much is dendrogram accordance with similarity matrix or distancematrix. In this research calculation of cophenetic correlation coefficient is 77% that the logarithm UPGMA has high efficiency in grouping of genotypes and has used well from given primary matrix.

Table3- the calculation of the genetic similarity among different samples obtained from similarity coefficient Dice

Jiroft	1.0000000											
Bam	0.5409836	1.0000000										
Bluck	0.8923077	0.5254237	1.0000000									
Kahnuj	0.6000000	0.8305085	0.5714286	1.0000000								
Anbarabad	0.8345324	0.6456693	0.8148148	0.6814815	1.0000000							
Esfandegheh	0.7517730	0.6666667	0.7153285	0.7153285	0.7671233	1.0000000						
Faryab	0.6456693	0.6434783	0.6341463	0.7154472	0.6060606	0.7611940	1.0000000					
Galehganj	0.6720000	0.6017699	0.6446281	0.6942149	0.6000000	0.7575758	0.8813559	1.0000000				
Roodbar	0.7692308	0.6101695	0.6825397	0.6349206	0.8740741	0.7153285	0.5691057	0.5454545	1.0000000			
Baft	0.6865672	0.6721311	0.6461538	0.6769231	0.6906475	0.8368794	0.6614173	0.6560000	0.6461538	1.0000000		
Orzoiyeh	0.7164179	0.7049180	0.6923077	0.7384615	0.7769784	0.8368794	0.6929134	0.6720000	0.6615385	0.7462687	1.0000000	
Menojan	0.6666667	0.6333333	0.6718750	0.7031250	0.6861314	0.8201439	0.8640000	0.8455285	0.6250000	0.7121212	0.7272727	1.0000000
Rabor	0.7619048	0.7407407	0.7272727	0.7552448	0.8157895	0.8831169	0.71428570	0.7101449	0.7412587	0.8571429	0.8299320	0.7586207	1.0000000

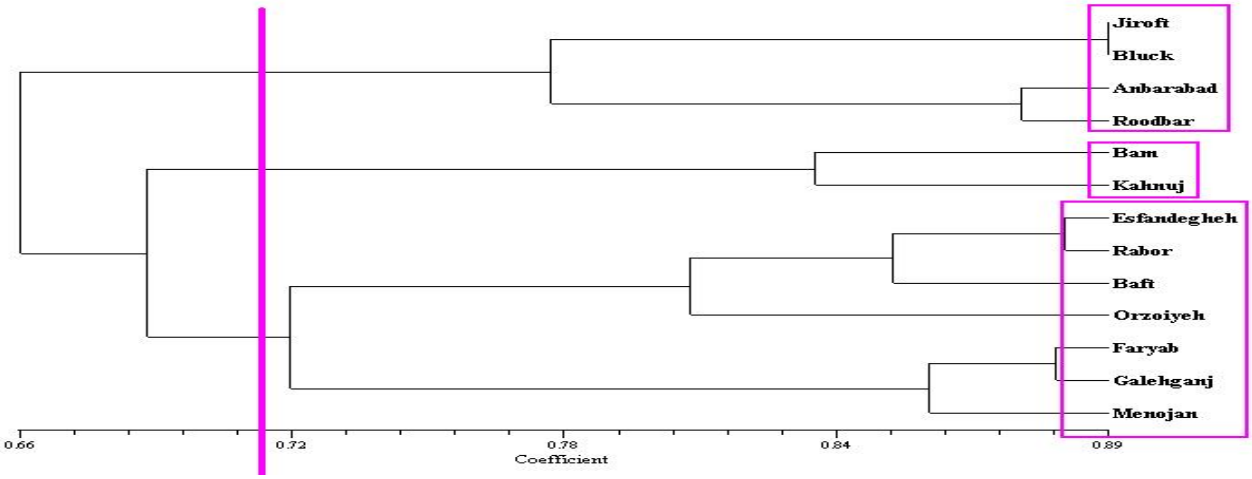

Figure 1- dandrogram (UPGMA) on the basis of dice similarity coefficient illustrating the relationship among 13 masses.

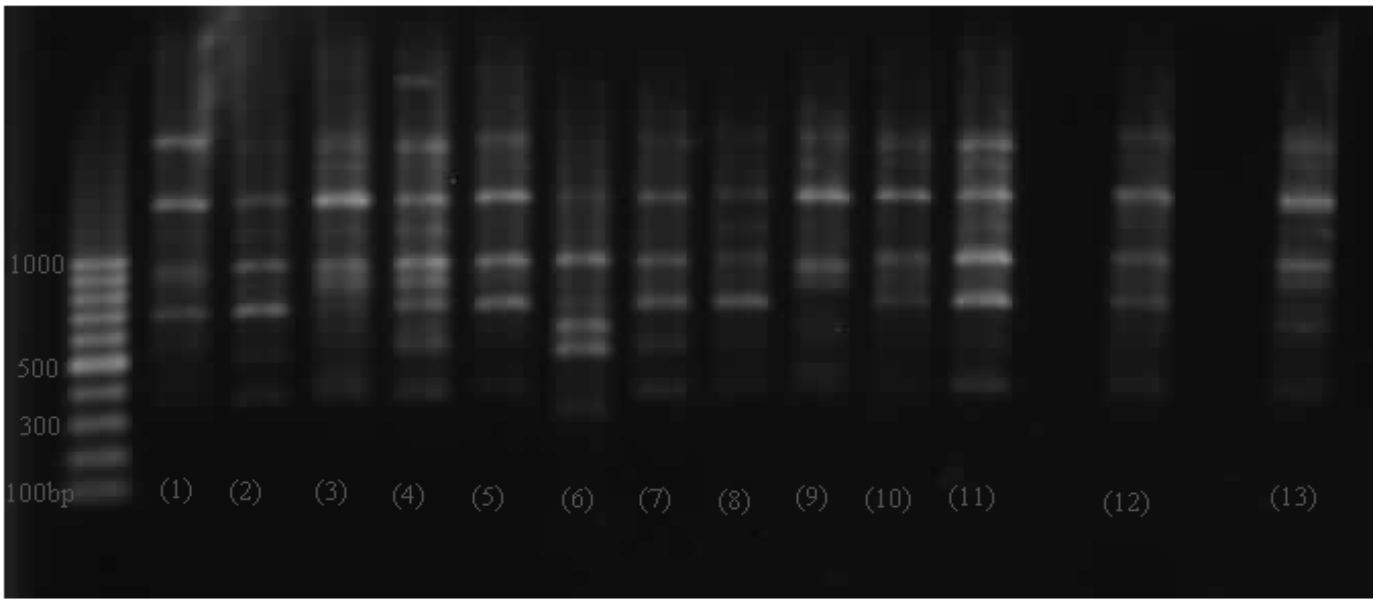

Figure2- polymorphism created bands with marker 376-RAPD in Kerman after electrophoresis, lane2 up to 14 based on table 1

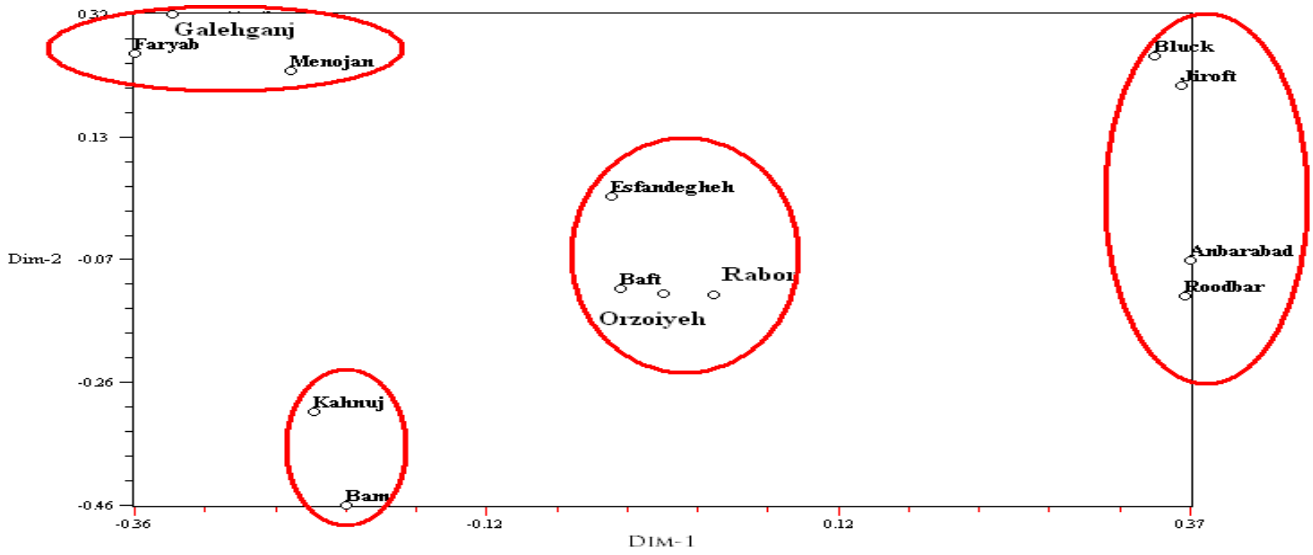

Figure3- two dimensional graph related to principle component analysis for 13 regions by applying RAPD & ISSR markers

DISCUSSION

in this article the genetic relationship among the samples surveyed in two markers with Dice coefficient and UPGMA method, on the basis of the obtained results of grouping genotypes of *Zataria multiflora* , it was evident that the used samples with ISSR marker in 3 groups were divided, genetic variation of many of these masses are according to geographical conditions but there are cases which do not have harmony for example Anbarabad & Kahnoj that are next to each other from geographical point but they have put in different group from molecular point . the molecular markers of RAPD in addition to objections, for evaluation and genetic variation study of herbal resources which there is no information of genomic and variation among DNA, us a simple tool and efficient . In the research of Echeverrigaray (2001) on cultivars of thyme *(thymus vulgaris L.)*Showed that there is high correlation between matrix of genetic distance due to molecular markers of RAPD and the matrix distances of phytochemical markers (4). Molecular marker ISSR to be used widely in the researches of genetic diversity, philogentic, genetic maps and biology for a wide range of herbal species (3).

Researchers by surveying the genetic diversity of box-tree in the north of Iran with ISSR markers concluded that ISSR is trustable (2), in another study by Ven Katachalam, (2008) for clarifying of polymorphism DNA , recognizing Genotype and the philogenetic relations among 21 type of Indian banana from 50 RDAP and 12 ISSR primers were used . these marker between the figures 60.15 & 56.73 respectably clarified the percentage of polymorphism for RAPD & ISSR that shows these two markers are similar and have high ability for distinguish (16). By considering that established polymorphism bands in markers and the similarity range among the masses of *Zataria multiflora* in Kerman, genetic variation among of native populations of kerman, even among the masses close to each other from the point of geographical conditions was shown, in the research on *Zataria multiflora* with the markers of RAPD & ISSR proved that the grouping has been well and also indicating that the range of variety in this masses could be prove that the markers ISSR and RAPD is desirable for genetic variety 's study and genetic survey on *Zataria multiflora* in Kerman.

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