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EVALUATION OF GENETIC DIVERSITY OF *ZATARIA MULTIFLORA BOISS* BY APPLYING RAPD & ISSR MARKERS IN THE SOUTH OF IRAN

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ABSTRACT

The plant of Shirazi thyme with the scientific name of Zartaria multiflora Boiss has been recognized. In this research random amplified polymorphic DNA (RAPD) marker due to high speed and low cost and from inter-simple sequence repeat¬(ISSR) because of increasing of correctness were used for surveying genetic variety of 15 masses of thyme from south of Iran. Thyme leaves were collected from 15 different regions of the country. Extraction the DNA from the leaf by cetyltrimethylammonium bromide¬ (CTAB) method with the little change was carried out. The quantity and quality of extracted DNA was measured by electrophoresis equipment & spectrophotometer. From 15markers of RAPD 10 markers and from 13 markers of ISSR 10 markers which had produced the clearer bands in polymerase chain reaction (PCR) were used for analysis. by NTSYS-pc and using of dice similarity coefficient and Unweighted Pair Group Method using arithmetic Averages¬ (UPGMA)related dendrogram was drawn. the cophenetic correlation coefficient was calculated and 2 dimensional graph was shaped based on Principle component analysis¬(PCA). By considering the results, the markers were produced in the total of 207 bands by percent of polymorphism of 86.9. The size range of bands in markers were variable between 120 up to 3000 bp. 15 selected regions in 3 separated group classified, the established cluster had harmony with geographical conditions. The results of Principle

component analysis¬ and the results of cluster analysis were similar. cophenetic correlation coefficient was equal with 74.60%. in this research . the result proved that markers of RAPD & ISSR are useful for specifying the genetic variety and family relationship of this mass.

Keywords: Genetic diversity, Zataria multiflora , Iran, ISSR , RAPD markers

INTRODUCTION

The plant of Shirazi thyme with the scientific name of *Zartaria multiflora Boiss* is one of the famous medicinal plants from Lamiaceae family. A tree-plant – a bush with the 40-80centimeter, perfumed, having twisted stems and full of rolled up and narrow branches .this plant grows in Afghanestan, Pakistan & in the south of Iran's regions such as Isfahan , Fars, Bousher, Yazd, kerman, Sistan & balochestan and Hormozgan (1,2). It's boiled is useful for curing and is used for antiseptic of stomach & urinary system and also to be used as a diuretic medicine in traditional medical(3). Surveying genetic variety and morphologic are important for subgroups because decreasing of varieties occurs in this level of classifying, so classifying systems is useful for evaluation of genetic diversity(4)

.By considering that study of morphological specification is not exacted due to effect of environment factors on these specification , today there are valuable methods for specifying the genetic relationship(5). today many researches use of molecular markers for specifying genetic varieties of the different plants which are based on PCR such as ISSR & RAPD , by using RAPD getting the information is fast and can compete with other markers in analysis at high level of Heterozygosity(6). It is known that a different marker has different classes of varieties (7). Since the RAPD and ISSR are known as molecular marker for estimation genetic varieties such as studying of peach(8), cotton(9) , melon and cantaloupe(10) , genetic varieties the 44 masses of lettuce could be surveyed by markers of RAPD, ISSR and AFLP. The results showed in the total 216 bands from 7 RAPD & 4 ISSR and 5 AFLP were obtained and from these figures, 196 bands were polymorphism and among the masses more than 90% polymorphism was seen (11), in a research by Razavi and colleagues in 2014 on jujube based on quantity specification and the markers of ISSR & RAPD, the results of cluster analysis based on quantity specification of these ecotypes, they classified in 3 till 6 main groups. Totally the variety of jujube of Iran to be found in 3 provinces of Mazandaran, Isfahan and south

Khorasan and south Khorasan nearly have the whole varieties as one central place(12). In another study which carried out in 2006 among genotype of pistachio from 7 countries by using markers AFLP, ISSR & RAPD, the results proved that 3 markers can show the variety among the mentioned masses. Dendrogram arise of composition the 3 markers established 2 main groups which one including Iran's samples and second group will include Mediterranean samples (13). In a study by Farsani and colleagues in 2008 for evaluation genetic variety of 23 genotypes of *Cynodon dactylon*& 4 hybrid ones by using of morphology and 14 markers ISSR was done and they concluded that the samples to be put in 4 groups(14). This research is in order to evaluate 15masses of *Zataria multiflora* located in south of Iran by using molecular markers RAPD & ISSR.

MATERIALS AND METHODS

From 15 growth places in south of Iran including 8 regions in Kerman and 7 regions located in other south provinces like Sistan & Balochestan, Boushehr , khozestan and Fars some samples were collected(table 1*)* *Zataria multiflora* was collected by native people in the spring of 2014.

Table 1-geographical regions which the masses of *Zataria multiflora* was collected

Row	Collection point	Row	Collection point	Row	Collection point
1	Orzoiyeh (Kerman)	6	Kahnuj (Kerman)	11	Darab (Fars)
2	Jiroft (Kerman)	7	Eizeh (Khozestan)	12	Iranshahr (Sistan& Balochestan)
3	Andimeshk (Khozestan)	8	Galehganj (Kerman)	13	Roodbar (Kerman)
4	Lamerd (Fars)	9	Menojan (Kerman)	14	Faryab (Kerman)
5	Firoz abad (Fars)	10	Bluck kerman	15	Kaki (Boushehr)

The leave of thyme has a lot of essence and second metabolits, So for desirable extraction genomic DNA were tried several protocol for extraction till finally CTAB method with some changes in washing of DNA was selected. fresh leaves related to each region were mixed separately and in final at the rate of 0.4 gram from each sample was extracted. In order to measuring the quality of extracted DNA electrophoreses was applied. DNA samples was put on the Agarose gel %1 in the voltage of 75 and for 1.5 hours , before pouring the agarose gel in the case, cyber green in the amount of 2 µl was added, the gel was transferred to GEL Documentation equipment and was observed under UV light. In PCR circle quantity and quality DNA are important and for this reason Spectrophotometer, Perkin-Elmer, model

EZ-201 was used . the samples which had good pureness were selected, therefore the samples which had not high pureness were removed and extraction protocol was done again. The circle of PCR (polymerase chain reaction) was used by Wanntorp method (15). 15 random marker RAPD, 13 ISSR for PCR were used. These marker were supplied from " Pasargard company (table 2)

Table2-Primers used for generating RAPDs and ISSRsin *Zataria multiflora* accessions

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	OPA18
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 ACA	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

The rate of used compositions in PCR for each primer had a bulk of 25 micro liter which includes: 5 µl genomic DNA(25 Nanogram) , 2.5 µl buffer 10X, 0.75 µl dNTPs, 1 µl primer, 0.5 µl enzyme Taq, 2 µl chloride magnesium, 13.25µl deionized water which was supplied from " Neday fan company". In this research thermocyclers were purchased from Peglab, German companies and the model was Mastercycler. The best connection temperature for each marker was obtained by given TM temperature. (table2) .polymerase chain reaction took more than 4 hours. After doing PCR, the productive products of thyme different genotypes for each primer RAPD & ISSR took place. In primary lane the gene ruler or ladder for estimation of established bands' sizes was used and in other lanes PCR productions were poured, then the gel in the equipment of Gel documentation model Ebox vx2, manufactured in France was placed under UV rays and after observing the bands under this light, from the gel with different formats (Ver 6, 08, 09) Genesnap, the photo was taken.

From 15 *Random*markers RAPD, 10 marker and from 13 marker ISSR, 10 marker which had clearer bands were selected for analysis. For statistical analysis of data, first all the bands were specified in all the gels and by considering the size of standard marker 100bp , the size of each clear band in every lane based on the length and molecular

weight were specified by software Genetools, the different samples on the basis of the bands were appeared on the electrophoreses' gel , the number of 1 for the existing of the band and 0 for not existing of the band in order to comparing the similar bands and identify polymorphism were scored and then in Excel software one matrix from the numbers of 0 & 1 for markers were applied then the data was transferred to NTedit , the input section of NTSYS .

Dendrogram was drawn by Dice similarity coefficient and by the UPGMA method, the cophenetic correlation coefficient was calculated for testing the correlation of input data to cluster and output of cluster and also 2 dimensions graph from it was drawn by NTSYS software and by the help of Principle component analysis.

RESULTS

From the total of 28 primary markers, 20 markers of clearer bands and polymorphism were selected. In total of 238 pieces, 207 numbers had polymorphism that signs of high percentage of polymorphism 86.9% in this species. Most numbers of increased pieces were related to ISSR-P8 and the least one was related to D18-RADP, the size of amplified bands in all markers in the limitation had the length of 120-3100bp (figure1). The results obtained from Dice similarity coefficient of NTYSIS software was indicating that the genetic similarity of thyme mass is variable between 43.9% & 84.6 % (table3) that the least similarity was between Jiroft with Iranshahr & the most similarity was between the samples of Andimeshk and Eizeh. 15 samples were collected in the cutting line of 72% which divided into 3 groups , first group including 3 members, Firozabad ,Orzoiyeh, Lamerd, second group including 4 members, Andimeshk, EIzeh, Ghalehganj and Iranshahr, third group including 8 members , Kaki, Jiroft, Roodbar, Faryab, Kahnuj, Bluck, Darab, menojan . The third group had the most members which itself had subgroups. This grouping was accordance with geographical conditions but there were cases that among the groups there was not any accordance, for example two masses of Iranshahr and Eizeh which are not close from the geographical point have been put in one group by considering the molecular point and this shows that the masses which are not next to each other from geographical point could have genetic similarities. In the study of Principle component analysis (PCA) of variables to be converted to lesser numbers and by this way the masses which are under investigation will be drawn into 2 dimensional graph (figure 3). The grouping

arise dendrogram with 2 dimensional graphs were the same. In this research cophenetic correlation coefficient was %74.60 and it showed that cluster analyzing to use well of matrix 0 & 1 data.

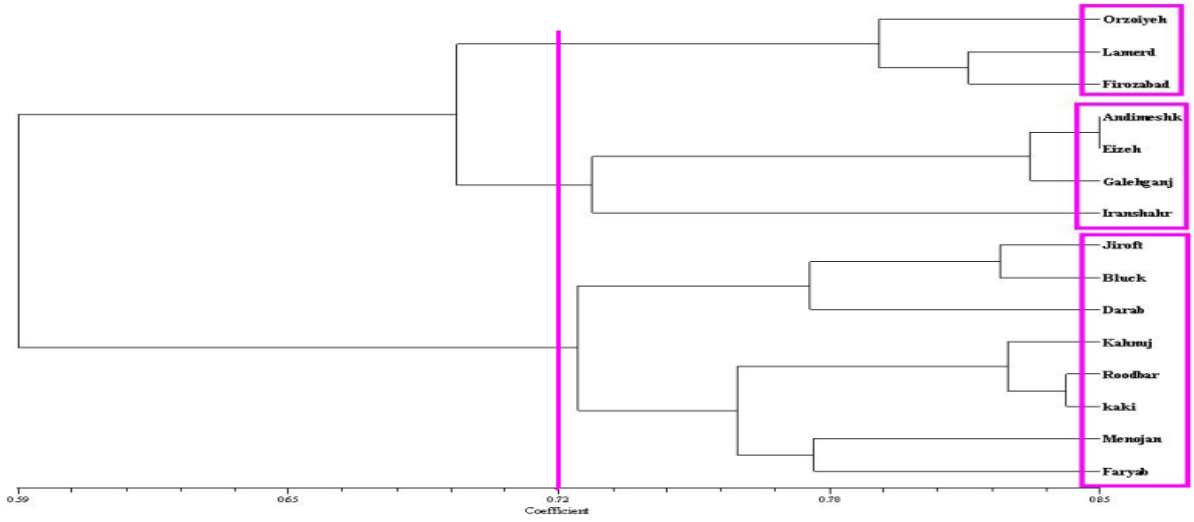

Figure 2-The dendrogram of 15 genotypes of thyme based on UPGMA and on Dice similarity coefficient in RAPD and ISSR data

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

Orzoiyeh	1.0000000													
Jiroft	0.6315789	1.0000000												
Andimeshk	0.7640449	0.4883721	1.0000000											
Lamerd	0.7954545	0.5882353	0.6582278	1.0000000										
Firozabad	0.7912088	0.5909091	0.6585366	0.8148148	1.0000000									
Kahnuj	0.6666667	0.7070707	0.5806452	0.5869565	0.5894737	1.0000000								
Eizeh	0.7356322	0.5476190	0.8461538	0.6233766	0.6750000	0.5274725	1.0000000							
Galehganj	0.7173913	0.6067416	0.8192771	0.6829268	0.7294118	0.6250000	0.8395062	1.0000000						
Menojan	0.6875000	0.6881720	0.5977011	0.5813953	0.6292135	0.7400000	0.5647059	0.6000000	1.0000000					
Bluck	0.6236559	0.8222222	0.5238095	0.5783133	0.5581395	0.7422680	0.4878049	0.5517241	0.6373626	1.0000000				
Darab	0.6516854	0.7441860	0.5500000	0.5316456	0.5365854	0.6881720	0.5384615	0.5783133	0.7126437	0.8095238	1.0000000			
Iranshahr	0.7294118	0.4390244	0.7894737	0.6933333	0.6410256	0.6067416	0.6756757	0.7088608	0.6024096	0.5000000	0.5000000	1.0000000		
Roodbar	0.6185567	0.7234043	0.5227273	0.5517241	0.5777778	0.8118812	0.5581395	0.6153846	0.7578947	0.6521739	0.7045455	0.5476190	1.0000000	
Faryab	0.6304348	0.7415730	0.5542169	0.5853659	0.6117647	0.7500000	0.5679012	0.6279070	0.7777778	0.7356322	0.7710843	0.5316456	0.6813187	1.0000000
Kaki	0.7547170	0.7766990	0.6804124	0.6250000	0.6666667	0.8363636	0.6736842	0.7200000	0.8076923	0.7524752	0.7835052	0.6236559	0.8380952	0.8200000 1.0000000

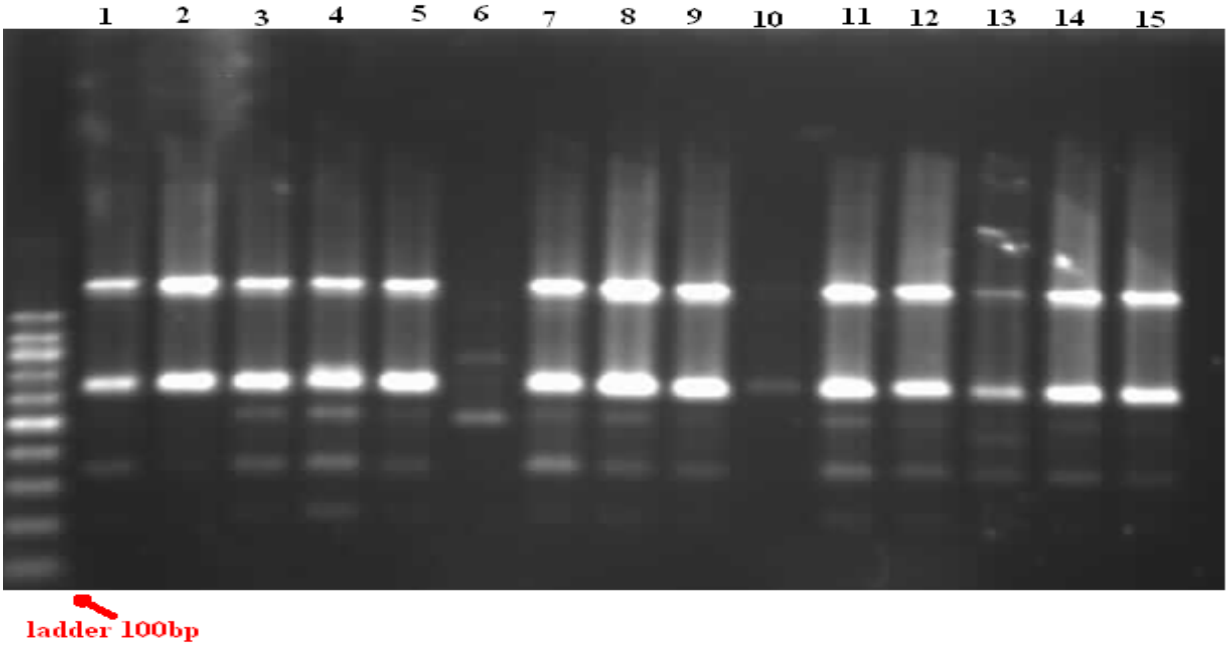

Figure1- polymorphism created bands with marker 394 (RAPD) in Kerman after electrophoresis , lane2 up to 14 based on table 1

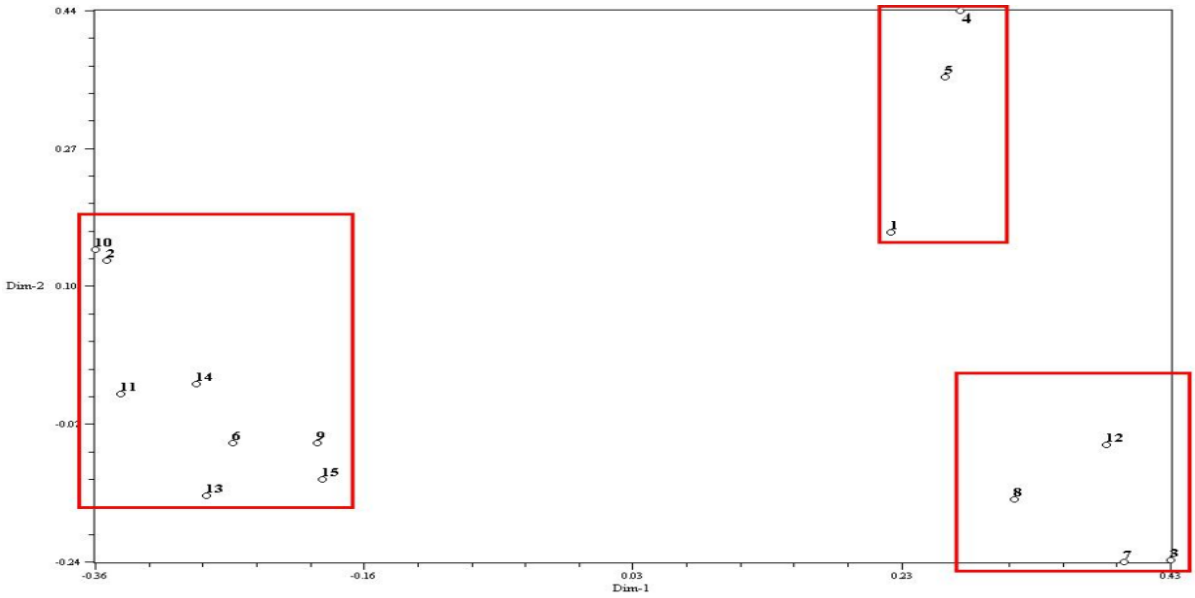

Figure3- two dimensional graph related to principle component analysis for 15 regions by applying RAPD & ISSR markers

DISCUSSION

This research was Investigated for 15 masses of *Zataria multiflora boiss*, the result showed that the variety among the masses of thyme is considerable and the varieties could be used for amending the cultivars and better production of cultivars and protecting seed bank and gene library . Many polymorphism

bands RAPD & ISSR and the range of similarity of mentioned masses proved high genetic varieties among thyme population , as it was specified in dendrogram the second group including the samples of Sistan & Balochestan, Kerman and Khoozestan had the same germplasm. In genetic study of *Thymus pubescens* with 14 different species by the help of RAPD marker, it had the most genetic similarity with *T.Leucotrichus* (16) and in another research by Ismaeili and colleagues in 2013 in order to surveying genetic variety, they concluded that different collected masses from Iran's different regions are divided into 3 groups and also showed that RAPD is a useful marker for studying genetic varieties of *T.Pubescens(17)* . In the research of Shi and the colleagues were done on *Cornus*Spp by using ISSR marker, they concluded that molecular markers ISSR are the most useful tool for finger printing of DNA(18) . By considering to the research and the grouping of *Zataria multiflora* and showing the range of varieties among these masses, it could be concluded that the markers RAPD & ISSR are the right tools for genetic varieties of *Zataria multiflora* which to protect of germplasm well.

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