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ORIGINAL ARTICLE

EVALUATION OF GENETIC DIVERSITY OF SAINFOIN (*ONOBRYCHIS VICIIFOLIA* SCOP.) LANDRACES USING RAPD MARKERS

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ABSTRACT: In order to evaluate the diversity within and among sainfoin landraces of Iran,10 landraces of different geographical regions of East Azarbaijan,were studied using RAPD markers.Of the 20 primers under evaluation,10 RAPD primers produced 90 polymorphic bands with the mean of 9 bands per primer.Total number of polymorphic bands within the landraces varied from 71 to 80 bands and genetic distance among them was calculated 0.16 - 0.30 based on Nei's index.The total mean of genetic diversity,the mean within landraces,and gene differentiation coefficient were estimated 0.32, 0.44,and 0.28,respectively.Genetic diversity within landraces was calculated using Nei's genetic standard in the range of 0 – 0.5. Molecular variance analysis based on squared Euclidean distance showed a significant difference within and among landraces of sainfoin, while the variance among landraces (%16.13) was less than the variance within the landraces (%83.87).Based on cluster analysis, 10 sainfoin landraces were placed in three groups.Grouping based on molecular data showed no correspondence with geographical grouping of the landraces.In principle coordinate analysis,the first three coordinates justified 55.81 percent of data variations,which indicates proper genomic distribution of the markers used.According to the results,it can be claimed that sainfoin landraces are potential sources for different genes,and that the diversity within and among these landraces can be utilized in breeding programs.

Keywords*:* Cluster Analysis,Molecular Variance Analysis,RAPD markers,Sainfoin (*Onobrychis viciifolia* Scop.)

INTRODUCTION

Sainfoin*(Onobrychis viciifolia* Scop.)is considered an important forage legume due to its capability for producing high quality fodder which competes well with alfalfa,among crop and pasture plants.It has a high compatibility with different regions particularly cold climates, so that it is widely cultivated for fodder production in those regions.Furthermore,it has properly naturalized in warm climates and yields even higher than alfalfa in some regions. Drought resistance and adaptation to low precipitation conditions have made sainfoin a desirable plant for cultivation in pastures and dry lands.So that it grows well in mountainous areas and ranges,and the soils which are irrigated temporarily.(Heidari Sharif Abad&Dari, 2002;Tourchi et al,2007;Majidi&Arzani,2005).

Landraces are always viewed as important gene pools in crop breeding,and awareness of genetic diversity level of plant resources is a basic factor for effective use of their potentials in plant breeding programs.Because of the impact of environment on gene expression,length of evolutional tests,and restriction of genetic information obtained,phenotypic evaluations have diversity within species level(Dos Santos et al.1994; Lerceteau et al.1997),matches other markers,and due to low costs,it is appropriate for genetic analysis at large scale.RAPD is a dominant marker which has been broadly used for studying genetic diversity,measuring genetic similarity and distance,classification of communities in different plant species.(Sapna et al,2007).

To evaluate genetic diversity in the species T.pratense (red clover),some researchers have used RAPD markers successfully(Kongkiatngam et al.1996;Campos de Quiroz&Ortega-Klose 2001;Gustine et al.2002;Greene et al.2004;Sica et al.2005).Ulloa et al.(2003) have used RAPD markers for genetic diversity analysis of red clover.They stated that RAPD markers were useful devices in clover breeding programs.In order to identify the genetic diversity among modified superior parents in red clover,Alegandro et al.(2000) used RAPD markers.In the study,with 55 primers,132 reliable bands were recognized,and a proper grouping among parents was accomplished.They concluded that RAPD markers can be considered as a valuable genetic marker for red clover breeding programs.

With the use of 8 RAPD primers,Arzani&Samei(2004)studied the genetic diversity of 20 Iranian clover

populations.Totally,83 bands were reproduced,of which 80 percent was polymorphic.Cluster analysis based on UPGMA algorithm was done and populations under study were categorized in six groups.The results showed that the efficiency of RAPD technique in population differentiation on the basis of geographical distribution was very high.Using RAPD markers,Ghanavati et al.(2004)studied the genetic diversity of 54 populations out of 22 alfalfa species collected from the natural habitats of the country.The results indicated that 11 random primers resulted in the production of 195 gradable bands of which,170 bands showed a good polymorphism(85 percent) among all landraces.The number of polymorphism parts produced by each primer and each species population was 5 – 20,and the range of reproduced part size was 100 – 3500 bp.Nosrati et al.(2011) studied the genetic diversity of 5 landraces of sainfoin in East Azarbaijan using 10 RAPD primers.On the whole, five primers produced 12 polymorphic bands in the range of 300 – 2800 base pairs.Cluster analysis placed 5 populations in 3 groups.Noeparvar et al.(2008)studied the genetic diversity of 5 landraces of alfalfa in East Azarbaijan,using 10 RAPD primers.Molecular variance analysis showed that approximately 80 percent of total genetic variance was attributed to inter-population variance.In order to evaluate the diversity within and among sainfoin landraces, the present study was done using molecular RAPD markers and their grouping.

MATERIALS AND METHODS

Plant materials used in this study included 10 sainfoin landraces from different regions of East Azarbaijan province(table 1).Out of each landrace,7 plants were selected randomly after of which a 0.5 gr leaf sample was taken separately from each plant.DNA extraction was done from leaf samples for each selected plant separately using Saghai-Maroof's modified method (Saghai-Maroof et al,1984).The quality and quantity of the extracted genomic DNA was determined using agarose gel electrophoresis and spectrophotometer.

For genomic DNA Amplification,20 random RAPD primers were used.PCR reaction in 25 micro litters volume included 1 micro litter DNA(30ng),4 pico moles random primers(Cina gene),13 micro litters PCR Master Kit 1X(Cina gene no.PR8250C),and 10 micro litters ddH2O.Thermal cycles included 4 minutes in 94°C(denaturation),following 40 cycles including DNA denaturation(1 min in 94°C),primer binding(1 min in 40°C),primer extension(1.5 min in 72°C),and finally,6 minutes in 72°C for final extension.Products reproduced by PCR using agarose gel electrophoresis %1,were separated and detected by staining with Ethidium bromide.Banding patterns obtained were scored as 0 and 1(band absence or presence).The PCR products were measured using generuler 1kb DNA ladder(fermentase no.SM0313),with part size of 250 – 10000 bp.To evaluate the repeatability of banding pattern of PCR products, PCR reaction for each primer was repeated 2 times.The total number of polymorphic bands, number of bands in each landrace,total genetic diversity(H_T),genetic diversity within landraces (H_S),degree of gene differentiation among them(G_{ST}/H_T)(Nei 1973),and rate of genetic diversity within landraces for each marker using Nei's standard of genetic diversity (Nei 1973) were calculated.The genetic distance among landraces was measured on the basis of Nei's standard,and the dendrogram for landrace grouping was drawn with complete linkage (CLINK) method.In order to determine the efficiency of cluster analysis method, cophenetic correlation coefficient was used.For more accurate identification of genetic relations among landraces,the principle coordinate analysis was done based on Nei's genetic distance.Molecular variance analysis based on squared Euclidean distance(Excoffier et al, 1992)was done for separating total molecular variance to variance of within and among landraces.The softwares Popgene3.2(Yeh et al.1997),Ntsys2(Rohlf,1992),and Arleqine3(Excoffier et al.2005)were used for statistical calculations.

RESULTS

10 primers out of 20 RAPD primers evaluated with proper banding patterns for studying all landraces were used (table 1).These primers produced 90 polymorphic bands in the range of 250 – 1500 base pairs with the mean of 9 bands per primer.The band number per primer varied from 3 bands for marker 1 to 12 in regard with marker 4(table 2).Using the primer 6, RAPD banding pattern in two landraces of Ardehal Sarab and Zenouz is illustrated in figure 1.The total number of polymorphic bands within landraces of sainfoin studied using 10 primers varied from 71 to 80 bands(table 2);therefore,the mean of band number in the landraces under study differed from 7.1 to 8 bands per primer(table 2).The landraces of Asnag Mehrban,and Ahar had the minimum and

maximum number of polymorphic bands, respectively.Among the primers used,primer number 4 produced the maximum number of polymorphic markers,so it differed from 9 markers in the landrace of Zenouz to 12 markers in the landrace of Bostanabad and Gharababa,Bostanabad.On the basis of Nei's genetic diversity standard,the genetic diversity within sainfoin's landraces calculated 0 – 0.5 (data is not shown).The maximum and minimum mean of diversity level were observed in the populations number 2 (Bostanabad) and number 8 (Asnag Mehrban),which valued 0.3429 and 0.3022 respectively.The mean number of polymorphic bands for landraces was 75.5,and mean percentage of polymorphism obtained 83.89 percent(table 3).Gherardi et al.(1998) studied genetic diversity in 8 alfalfa populations using 5 RAPD primers. In their study,the mean percentage of polymorphism within population was 70.87 percent.The total genetic diversity mean in the entire population(H_T)and within sainfoin landraces(H_S),based on Nei's genetic diversity standard(Nei, 1973)were calculated 0.32 and 0.44 respectively.The mean degree of gene differentiation(G_{ST})among landraces for all bands was calculated 0.28.The F_{ST} was estimated 0.16 which matched the mean degree of gene differentiation(G_{ST})among landraces.The results of the study showed that diversity within the landraces had the maximum proportion of total diversity.Mengoni et al (2000) also studied 10 alfalfa populations with 41 RAPD markers and obtained high gene diversity within alfalfa ecotypes.

Nei's genetic distance(Nei, 1978)among 10 sainfoin landraces varied from 0.16 between two landraces of Bostanabad and Asnag Mehrban to 0.30 between landraces of Sperakhoun, Lighvan and Guashin Sarab(data is not shown).Totally,the genetic distance among populations was low,which can be attributed to their geographical proximity.

The results of molecular variance analysis showed that genetic diversity within and among sainfoin landraces was significant(table 4).The diversity among and within landraces were 16.13 and 83.87 percent respectively.The F_{ST} was calculated 0.16 which matched the mean degree of gene differentiation(G_{ST}) among landraces(0.28).Therefore,molecular variance analysis confirmed the results obtained from previous analyses,and showed a high diversity within the sainfoin landraces.Using RAPD markers on 5 sainfoin populations,Nosrati et al. (2011)calculated 10.97 and 89.03 percent diversity among and within the populations, respectively.In the study of alfalfa populations,Noeparvar et al.(2008)stated that approximately 80 percent of total genetic variance attributed to the variance within population.

Table 1: Names, collection sites of 10 sainfoin landraces and primers used for RAPD analysis

No.	Sequence	Primer	Altitude	Collection site
1	CAGCACCCAC	E12=(1)	1500	Ahar
2	GGTGACGCAG	E14=(2)	1750	Bostanabad
3	GGCATCGAGG	E16=(3)	1900	Ardehal Sarab
4	GGTGGCGGGA	E18=(4)	1900	Zenouz
5	GGGCCGTTTA	E19=(5)	1800	Guashin Sarab
6	CCGGCCTTAG	E21=(6)	1800	Miab Marand
7	GAGCTCGTGT	E23=(7)	1850	Gharababa Bostanabad
8	GGGTGGTGGC	E25=(8)	1700	Asnag Mehrban
9	GGGGGGTTGG	E26=(9)	1600	Heris
10	GGCGGCATGG	E27=(10)	2300	Sperakhoun Lighvan

Table 2: Number of polymorphic markers generated by 10 RAPD primers in 10 landraces of sainfoin

primer	Sequence	Number of polymorphic bands in all landraces	Number of polymorphic markers in landraces of sainfoin									
			Ahar	Bostanabad	Ardehal Sarab	Zenouz	Guashin Sarab	Miab Marand	Gharababa Bostanabad	Asnag Mehrban	Heris	Sperakhoun Lighvan
E12=(1)	CAGCACCCAC	7	6	4	4	5	5	4	5	5	3	5
E14=(2)	GGTGACGCAG	10	8	10	6	8	8	9	8	9	7	8
E16=(3)	GGCATCGAGG	8	7	8	7	6	7	8	6	7	7	5
E18=(4)	GGTGGCGGGA	12	11	12	11	9	10	10	12	10	11	10
E19=(5)	GGGCCGTTTA	10	9	7	8	8	9	9	8	7	9	9
E21=(6)	CCGGCCTTAG	11	10	10	10	10	9	10	9	8	10	8
E23=(7)	GAGCTCGTGT	9	7	7	9	7	8	7	8	6	6	8
E25=(8)	GGGTGGTGGC	8	7	8	8	7	7	7	6	8	7	7
E26=(9)	GGGGGGTTGG	7	7	7	6	6	6	5	5	6	5	5
E27=(10)	GGCGGCATGG	8	8	6	7	6	6	8	7	5	7	7
Total number of polymorphic bands		90	80	79	77	78	75	77	74	71	72	72
Polymorphic bands average		9	8.0	7.9	7.7	7.8	7.5	7.7	7.4	7.1	7.2	7.2

Table 3: Percentage of polymorphism, and average gene diversity within landraces of sainfoin based on 10 RAPD primers

Landrace	Nei's gene diversity	Polymorphic loci (%)	Number of Polymorphic bands
Ahar	0.3365	88.89	80
Bostanabad	0.3429	87.78	79
Ardehal Sarab	0.3323	85.56	77
Zenouz	0.3247	86.67	78
Guashin Sarab	0.3096	83.33	75
Miab Marand	0.3302	85.56	77
Gharababa Bostanabad	0.3109	82.22	4
Asnag Mehrban	0.3022	78.89	71
Heris	0.3102	80.00	72
Sperakhoun Lighvan	0.3030	80.00	72
mean	0.3202	83.89	75.50

Table 4 - Analysis of molecular variance of 70 plant samples from 10 landraces sainfoin based on 90 RAPD markers

Source of variation	d.f.	Sum of squares	Variation (%)	Prob.
Among landraces	9	361.96	16.13	0.00
Within landraces	60	1027.39	83.87	0.00
F_{ST} :			0.16354	

Non-parametric test of significance using repeated random sampling 1023 times

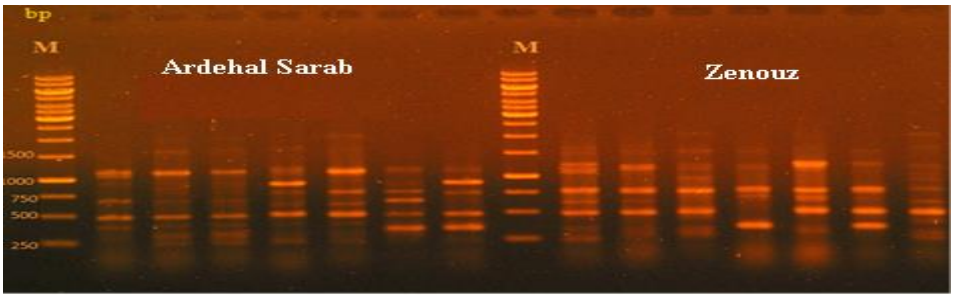

Figure 1: RAPD banding patterns for Ardehal Sarab and Zenouz landraces by using of primer E21

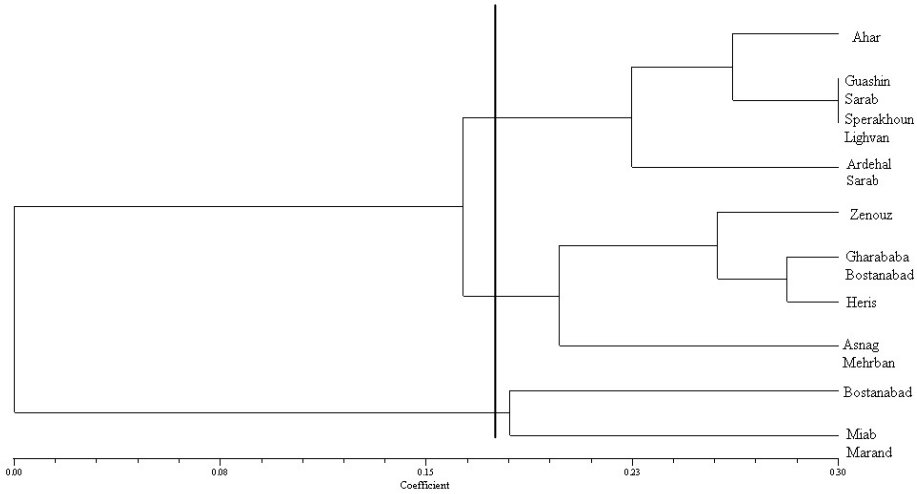

Figure 2 - Sainfoin landraces grouping based on data from RAPD markers using the algorithm CLINK and Nei's genetic distance

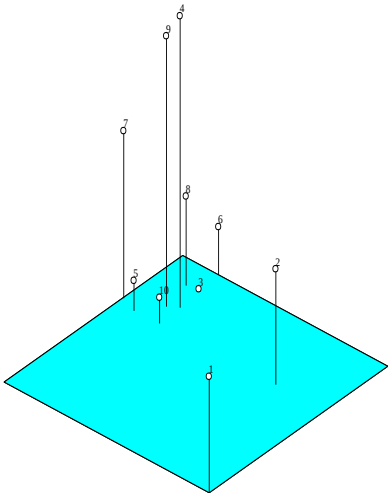

Figure 3 -Three-dimensional representation of landraces sainfoin on the first three coordinates from principal coordinate analysis of RAPD markers

DISCUSSION

According to the results of this research,it can be claimed that sainfoin landraces of Iran are potential resources for different genes.This result is expected in the species with high cross-fertilization.In these species a low level of population differentiation is seen (Hamrick, 1990).Recent studies on genetic diversity patterns among communities indicate the genetic variance correlation among populations with the specific features of regeneration system, particularly cross-fertilization(Hamrick&Godt,1997)and cross-pollination with gene exchange (Sork et al,1998).In a study on the results from molecular variance analysis of data obtained from RAPD markers on genetic structure of 38 plant species,Bussell(1999)showed that genetic variance mean among the populations of 30 cross-pollinated species,was 14.4 percent. Thus,it can be said that there is higher population differentiation in the sainfoin landraces of Iran than other cross-fertilized species with free gene flow.Higher population differentiation in sainfoin landraces can mainly be attributed to their pollination by the insects with short migration intervals,which has resulted in the limitation of gene flow among them.Of course, sampling error due to samples' small size should not be ignored.Several cluster analysis methods have been reported for analyzing molecular data(Gizlice et al.1994;Grabau et al. 1992;Kresovich et al.1994;Thompson & Nelson 1998).In this study,cluster analysis was done based on Nei's genetic distance with two methods:CLINK and UPGMA(Unweighted Pair-Group Method with Arithmetic mean).Cophenetic correlation coefficient was calculated 0.60 and 0.52 in CLINK and UPGMA methods respectively.Because of chain-like dendrogram of UPGMA and low correlation of cophenetic correlation coefficient,and due to higher cophenetic correlation coefficient in CLINK method, cluster analysis was selected based on this method.In this grouping,10 sainfoin landraces were placed in three groups.The first group included landraces of Ahar,Guashin Sarab,Sperakhoun Lighvan and Ardehal Sarab.The second group included landraces of Zenouz,Gharababa Bostanabad, Heris, and Asnag Mehrban.Landraces of Bostanabad and Miab Marand were included in the third group (figure 2).This grouping showed that the genetic diversity pattern was not the same as the geographical distribution pattern,and the landraces attributed to different geographical regions are placed in one group.Several reports on non concurrence of genetic diversity with geographical diversity in legume plants have been presented (Greene et al, 2004;Zhang et al,2010).In order to determine the genetic relations among landraces,and to observe the distances among them in three-dimensional form, principle coordinate analysis was also done as a complementary method for cluster analysis. The results showed that the three coordinates justified totally 55.81 percent of data variations. The first coordinate explained 29.29 percent of the total diversity.The percentage for the second and third coordinate was 19.29 and 14.29 percent respectively.In spite of morphologic data,weak explanation of data by a few coordinates indicates proper distribution of the markers used in the genome and sampling from different parts of the genome(Mohammadi,2006).Also in this analysis good dispersion of the markers used and appropriate sampling from the total genome,considering low justification of variation by the first three coordinates, was observed.Three-dimensional illustration of the landraces based on the first three coordinates,confirmed grouping obtained from cluster analysis,and could separate the landraces into 3 groups.(figure 3).This study showed that RAPD method can be considered as a quick,inexpensive,and effective device for evaluating genetic diversity among sainfoin landraces,and a complement for morphological evaluations.Moreover,in regard with the considerable diversity within sainfoin landraces,breeding programs can be proceeded for these landraces.

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