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Utility of ISSR molecule marker in examine of genetic diversity 17 genotypes of perennial alfalfa (*Medicago sativa*)

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ABSTRACT: The genetic diversity of 17 genotypes of alfalfa was studied using eight ISSR primers. A total of ISSR primers were able to detect 44 loci. The primers IS3, IS14 and IS11 showed highest number of bands (7) and the primer IS16 showed the lowest bands(3). The average polymorphism percent in primers used was 90%. The lowest polymorphism percent was for primers IS16, IS14, and IS5 and it was100% for the other primers. The average of PIC and MI for the studied primers was respectively, 0.36 and 1.88 that the maximum PIC and MI belonged to primers IS11, IS3, IS13 and IS9, and primer IS16 had the lowest PIC and MI. Cluster analysis by UPGMA method based on Dice coefficient for reviewing genotypes, classified them in 4 groups. Cluster analysis and Scatter plot based on first and second axis from principal coordinate analysis for genotypes, showed that genetic variation did not agreement with the geographical distribution.

Keywords: alfalfa, genetic diversity, molecular marker, ISSR.

INTRODUCTION

Alfalfa is a forage plant that among the forage plants Due to the high palatability adaptation to different environmental conditions and terrestrial plants has been dubbed as the Queen (Fazli and Yazdisamadi, 1992). The genus *Medicago* is distributed worldwide and consists of approximately 83 species (Small and Jomphe, 1989). The cultivated alfalfa (*Medicago sativa* L.) is an autotetraploid, (2n = 4x = 32), cross-pollinated (allogamous) and seed – propagated species (McCoy and Bingham, 1988). Medicago sativa is a leguminous plant species that originated in Asia and Iran. Alfalfa is the world's main forage legume and, at the same time, it is a model culture for numerous genetic engineering studies. This plant species has been grown for a variety of purposes such as soil improvement, animal feed, medicinal uses and suitable foliage (Steppler, 1987). One of the markers that using to study genetic diversity is Inter Simple Sequence Repeats or ISSR that is based on PCR method and it is also semi-random marker. This marker is used to amplification DNA fragment between two micro sattellites which is placed inversely in genotype of a species. This marker is much more efficient than RAPD (Sicard et al*.,* 2005). Tools for determining genetic variation identification and resolution figures, heritability reserve collections management, genetic mapping and marker assisted selection are considered (Gupta and Varshney, 2000). Several microsatellite loci introduced in Medicago sativa and thus used in genetic mapping (Diwan, 2000). In survey genetic diversity within and between 19 variety and line Medicago sativa genotypes based on cluster analysis divided to different groups and showed that genetic diversity within variety and lines difference Medicago sativa more of between diversity (Musial et al, 2002). The purpose of this study was to evaluate the genetic diversity of 17 genotypes of Medicago sativa using ISSR molecular markers.

MATERIALS AND METHODS

In this study, 17 genotypes of species Medicago sativa that obtain from natural resources gene bank were then planted (Table1).

Table 1. Gen bank cod and Origin of genotypes of *Medicago sativa*

Number	Origin	Gene bank Code
1	Sirjan1	Es-026
2	Kazakh1	KR-2197
3	Jiroft	Es-035
4	Damghan	Es-023
5	Kermanshah	Es-049
6	Atalia	Es-074
7	Khansar	Es-046
8	Gorgan	Es-009
9	Hamand	Es-030
10	Varamin	Es-030
11	Sabzevar	Es-025
12	Italy2	Es-075
13	Exotic	Es-012
14	Kebrit	Es-088
15	France	KR-3002
16	Unknown	G16
17	Unknown	G17

Total genomic DNA was extracted for young leaves of greenhouse-grown plants using a modified CTAB (Murry and Tompson, 1980) with modification described by De la Rosa et al. (2002). Quality and quantity of extracted DNA were examined using 0.8% agarose gel. The compounds of polymerase chain reaction were carried out according to table 2.

Table 2. compounds of optimized ISSR reaction

To provide 20 µl	compounds of a sample
12.6 µl	Water distillated twice
2 µl	Buffer PCR (X10)
1.5 µl	$MgCl_2$ (50 mmol)
0.4 µl	Nucleotides mixture (10 mmol)
1.2 µl	Primer (10 µmol)
0.3 µl	Tag polymerase
2 µl	DNA (10 ng)
20 µl	total

Template DNA was initially denatured at 92ºC for 5 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 30 seconds at 95ºC, primer annealing for 30 seconds at the temperature based on primer temperature (temperatures of annealing in this study was 50, 55 and 60 ºC) and primer extension for 1 min at 72ºC. A final incubation for 5 min at 72ºC was performed to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 1.5% agarose gels using TBE buffer. The gels were put in the Ethidium bromide for 30-45 min and visualized by gel document. ISSR bands were treated as binary characters and coded accordingly (presence =1, absence = 0). Number of bands scored, Number of polymorphic bands, Percentage of polymorphic bands were calculated for each primers and each genotypes. Marker index (MI) and Polymorphism information content was measured for each primer (Anderson *et al*, 1993). Cluster analysis, similarity matrix and principal coordinate analysis axis were carried out for 17 genotypes using Darwin and NTSYS.

$$PIC = 1 - \sum_{i=1}^{n} PI^{2}$$

MI = PIC.N.β

RESULTS AND DISCUSSION

***Results*:**

Primers sequences, code, number of bands scored, number of polymorphic bands, percent of polymorphic

bands (PPB), marker index (MI) and polymorphism information content (PIC) were showed for ISSR primers in table 3. In this study the genetic diversity among 17 genotypes of *medicago sativa* was investigated using 8 ISSR primers. For all primers, the number of 44 bands was scored that polymorphism was observed for 40 of them. The average number of bands produced by each primer for genotype 17 versus 5.5, genotype Es-026.

Was the most common genotype Es-049 had the lowest number of bands. Primers IS3, IS14 and IS11 with 7 bands had the highest and primer IS16 with 3 bands had the lowest number of bands. Band pattern of 17 genotypes for IS3 showed in Fig 1.

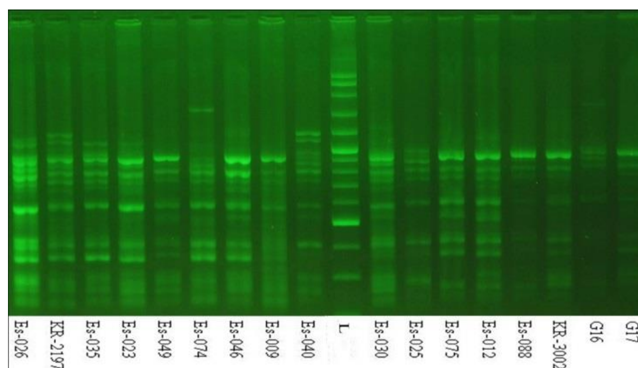

Figure 1. banding patterns of 17 alfalfa genotypes using primers IS3

The average percent of polymorphic primers used in the 90.18%, the lowest percentage of polymorphic primers IS16 (66.66%), IS14 (71.14%) and IS5 (83.33%) and the percentage of polymorphic the other primers were 100%. Polymorphic information content (PIC) ranged from zero to one and the same figure is larger than the frequency for status polymorphism genotype is under investigation. The highest value of PIC related to primers IS11, IS3, IS13 and IS9, Also the highest marker index (MI) of the primers IS11, IS3, IS13, and IS9.

However all used primers were useful for determination of genetic diversity based on PPB in alfalfa genotypes, but considering polymorphism information content and marker index the primers of IS11, IS3, IS13 and IS9 were useful for polymorphism study and can be used to analysis of genome of other genotypes of alfalfa in the future researches.

Primer IS16 with the lowest PIC and MI did not a good ability to separate genotypes.

Table 3. ISSR primers used in this study and some summary results

ISSR code	Sequencing primer	No. of bands scored	No. of polymorphic bands	Percentage of polymorphic bands(PPB)	MI	PIC
IS_1	5'- ACACACACACACAC ACYA-3'	4	4	100%	1.3	0.32
IS_3	5'- GAGAGAGAGAGAGA GAYC-3'	7	7	100%	2.69	0.38
IS_5	5'- AGAGAGAGAGAGAGAGC-3'	6	5	83.33%	1.88	0.37
IS_9	5'- CTCTCTCTCTCTCTCTG-3'	5	5	100%	2.06	0.41
IS_{11}	5'-ACACACACACACACACC-3'	7	7	100%	2.75	0.39
IS_{13}	5'- AGAGAGAGAGAGAGAGYT-3'	5	5	100%	2.26	0.45
IS_{14}	5'- GACAGACAGACAGACA -3'	7	5	71.42%	1.54	0.30
IS_{16}	5'-DBDACACACACACACACA-3'	3	2	66.66%	0.57	0.28
Average		5.5	5	90.18%	1.88	0.26

Similarity matrix

Similarity matrix based on Dice's coefficient for genotypes showed that (Table 4) the average of Similarity between accessions was 0.65 and the range of similarity was 0.48 [Between the (KR-2197 with Es-040), (Es-023 and Es-025), (Es-040 and Es-012) and (Es-088 with G-16)] to 0.80 [Between the (Es-074 and Es-025), (Es-026 and Es-049), (Es-049 and KR-3002) and (Es-074 and Es-046)].

Table 4. Similarity matrix for studying genotypes based on Dice's coefficient

genotype	Es-026	KR-2197	Es-035	Es-023	Es-049	Es-074	Es-046	Es-009	Es-040	Es-030	Es-025	Es-075	Es-012	Es-088	KR-3002	G16
KR-2197	0.760															
Es-035	0.593	0.696														
Es-023	0.679	0.667	0.714													
Es-049	0.788	0.593	0.643	0.733												
Es-074	0.742	0.741	0.667	0.640	0.667											
Es-046	0.724	0.760	0.727	0.739	0.667	0.769										
Es-009	0.694	0.651	0.629	0.718	0.741	0.698	0.634									
Es-040	0.586	0.480	0.591	0.565	0.552	0.577	0.583	0.579								
Es-030	0.702	0.735	0.605	0.711	0.621	0.667	0.681	0.732	0.638							
Es-025	0.679	0.542	0.619	0.500	0.741	0.800	0.652	0.722	0.652	0.578						
Es-075	0.644	0.667	0.533	0.681	0.714	0.717	0.612	0.667	0.694	0.750	0.596					
Es-012	0.678	0.667	0.667	0.681	0.741	0.755	0.694	0.650	0.490	0.625	0.681	0.640				
Es-088	0.737	0.735	0.512	0.622	0.640	0.667	0.681	0.750	0.553	0.609	0.533	0.750	0.583			
KR-3002	0.607	0.583	0.667	0.591	0.769	0.640	0.696	0.703	0.565	0.578	0.591	0.596	0.723	0.622		
G16	0.600	0.698	0.632	0.585	0.727	0.667	0.619	0.629	0.636	0.634	0.585	0.681	0.698	0.500	0.714	
G17	0.702	0.694	0.558	0.667	0.720	0.667	0.681	0.718	0.596	0.739	0.533	0.625	0.667	0.609	0.711	0.744

Cluster analysis

UPGMA hierarchical clustering for grouping genotypes based on Dice and classification of genetic distance from 0.66 the Group genotypes classified in four groups (Figure 3). The first group consists of genotypes Es-026, Es-049, Es-074, Es-025, Es-009, Es-088, Es-030 and Es-075, respectively, which the average similarity was 0.67 for this group. The second group of genotypes Es-023, Es-035, Es-046 and KR-2197 was used, which the average similarity coefficient was 0.71 for this group. The third group consists of genotype Es-012, KR-3002, G-16 and G-17 respectively. The average of Dice's coefficient was 0.70 for this group. Es-040 genotype with the largest gap was in a group with other genotypes.

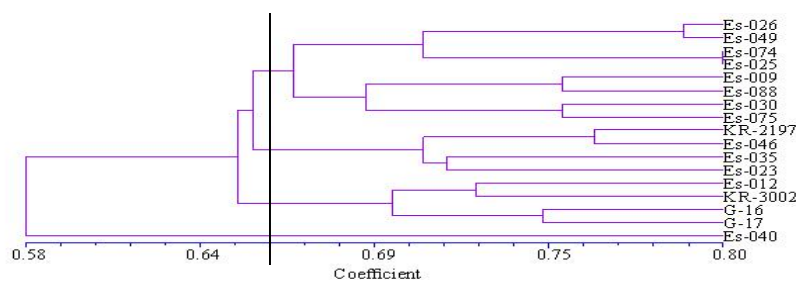

Figure 2. Dendrogram of cluster analysis for genotypes based Dice's coefficient by UPGMA

Principal coordinate analysis

The results of the analysis based on the coordinates of the first and second coordinates of 39.37% of the variation explained by the first two coordinates and accordingly, the genotype distribution was plotted diagram, the diagram with the coordinates of the second match of Cluster analysis (Figure 4).

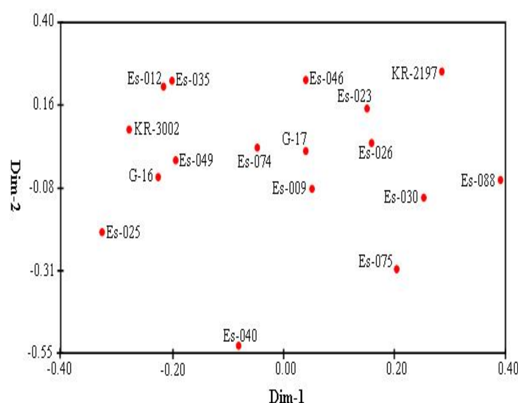

Figure 3. Scatter plot for genotypes based on two first axes from principal coordinate analysis.

Discussion:

In this study it was shown that using techniques (ISSR) at least one model of each identified genetic alfalfa genotypes. This technique has been used to study a variety of plants. The results of this study Lee and colleagues, (2009) were consistent. Four primers IS11, IS3, IS13 and IS9 could be better than other primers showed polymorphism among the genotypes. Genetic diversity in Alfalfa genotypes were studied by Habibi et al, (2012) based on ISSR marker and they reported a different PIC for used primers. Primers based on the study of genotype (Es-074 and Es-025), (Es-026 and Es-049), (Es-049 and KR-3002) and (Es-074 and Es-046) had the highest genetic similarity and genotype of (KR-2197 with Es-040), (Es-023 and Es-025), (Es-040 and Es-012) and (Es-088 with G-16) had the highest genetic distance. Grouping of genotypes based on cluster analysis and principal coordinate analysis indicated that genetic variations do not agreement with the geographical distribution of genotypes. Finally, the results of the genetic diversity of alfalfa germplasm management planning would be useful to develop a breeding program. Sandrin et al (2008) using microsatellite markers to assess genetic diversity among seven alfalfa cultivars with high diversity were found in them.

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