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Assessment of genetic variability of 36 populations of Sainfoin (*Onobrychis sativa*) based on RAPD markers

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Abstract

Random amplified polymorphic DNA (RAPD) markers have been used to characterize the genetic diversity of 36 Iranian populations of sainfoin (*Onobrychis sativa* Scop.). Five out of 20 tested RAPD primers produced 79 polymorphic bands and the average band produced for each primer was 15.8 polymorphic bands. The highest and lowest percentage of polymorphic loci was obtained for the population Divandara (80%) and the population Aligodarz3 (49%), respectively, with average 66%. Dendrograms were prepared using cluster analysis based on Nei's distance coefficients. Clustering pattern, made on the basis of RAPD data, grouped the accessions differently and gave no clear indication of origin/source. Further examination of the different components of genetic variation by analysis of molecular variance indicated that larger proportions of variability existed within populations (78%). The results also showed that comprehensive germplasm collection in major geographic regions and exploitation of the existing variation are required to widen the genetic base and sample the full extent of the available variation in breeding strategies for sainfoin.

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Introduction

The genus *Onobrychis* Miller belongs to *Fabaceae-Hedysareae* (Polhill, 1981). Many confusions and contradictions are found in the taxonomy of *Onobrychis*. It comprises about 130 species and is mainly distributed in the north temperate regions, but its biodiversity centers are in the eastern Mediterranean area and western Asia especially Iran and Anatolia (Yildiz *et al*., 1999). Sainfoin (*Onobrychis sativa* Scop.) is one of the most important forage legumes which are cultivated by farmers due to high palatability and high nutritional value properties (Delgado *et al*, 2008). Sainfoin sometimes also known as holy clover; is a perennial forage legume with a deep root often grow in conjunction with forage grasses to reduce bloat hazard as well as to improve soil fertility due to its nitrogen fixing ability (Lu *et al*., 2000). It contains condensed tannins which reduce its potential to produce bloat and improve protein digestion by grazing animal (Rumball and Claydon, 2005), While advances in plant breeding have led to improved Lucerne varieties, sainfoin production has remained relying on old cultivars. Characterization of existing germplasm seems to be necessary in order to preserve these genetic resources and provide alternative approaches for developing further breeding programs. While morphological characterization is a necessary step in plant breeding, several molecular tools are being used to complement plant variety characterization and identification (Soltis *et al*., 1992; Kidwell *et al*., 1994; Cruzan, 1998; Gherardi *et al*., 1998) such as RFLPs (Botstein *et al*., 1980; Tavoletti *et al*., 1996; Pupilli *et al*., 2000), RAPDs (Williams *et al*., 1990), AFLPs (Zabeau and Vos, 1993; Julier *et al*., 2003) and SSRs (Tautz, 1989; Morgante and Olivieri, 1993; Provan *et al*., 1999). Their applicability could be extended in determining heterotic groups and identifying parents (Brummer, 1999). However, molecular markers are not successful in predicting heterosis (Riday *et al*., 2003; Riday and Brummer, 2004). As a result, the use of molecular markers to select parents has the dynamics to allow simultaneous maintenance of genetic diversity and performance. RAPD markers, which use one or sometimes two short arbitrary primers (usually 8-10 bases) to amplify anonymous stretches of DNA (Yu & Pauls, 1994), have been applied in gene mapping, population genetics, Molecular evolutionary genetics, and plant animal breeding. This is mainly due to the speed and cost-effectiveness of the RAPD technique in generating many numbers of markers in a short time period (Welsh and McClelland, 1990; Williams *et al*., 1990; McClelland and Welsh, 1994; Skroch and Nienhuis, 1995; Jones *et al*., 1997; Bardakci, 2001). Investigate of the relationship between eco-geographical factors and genetic similarity in different populations of *Onobrychis viciifolia* using RAPDs showed capable to detect ecotypes of Sainfoin, and that they were affected by ecological factors (Nosrati *et al*., 2011).

Emre *et al.* (2007) suggested a classification based on total seed protein profiles. Studied species of sections Lophobrychis, Onobrychis, Hymenobrychis clustered together. Protein profiles were basically similar among *Onobrychis* species and the most similarity occurred between 66.2 and 116 kDa. Some minor differences in protein patterns for *Onobrychis* species were particularly seen among 18.4-66.2 kDa (Arslan and Ertugrul, 2010.). AFLP and SSR fingerprinting were attempted to investigate *O. viciifolia* genetic diversity The potential of these techniques was shown, but the latest improvements needed to obtain solid data were not achieved during this study Still, It was shown that molecular marker assisted breeding programs can be elaborated for *O. viciifolia* (Carbonero, 2011). Sánchez-Yélamo (2006) characterised some species of the genus *Onobrychis* using isozyme methods. *Onobrychis* species section Eubrychis clustered in a main group. Ahangarian *et al.* (2007), clarified the phylogeny of *Onobrychis* genus based on nrDNA ITS sequences. It has been suggested recently that subgenus *Sisyrosemae* was derived from subgenus *Onobrychis* (Ahangarian, 2007).Variations in sainfoin morphological features, distributional patterns, adaptive and agronomic characters, and allozymes are well documented (Carbonero, 2011). DNA profiling techniques that have been successfully used in assessing genetic

diversity and relatedness of sainfoin germplasm include randomly amplified polymorphic DNA (RAPD) markers Williams *et al*., 1990), inter-simple sequence repeat (ISSR) (Gupta *et al*., 1994), Sequence-related amplified polymorphism (SRAP) (Vandemark *et al*., 2005) and AFLPs (Vos *et al*., 1995). However, there is a lack of information on the genetic diversity of Iranian sainfoin wild populations using molecular markers.

The objectives of our study were

1) To estimate the genetic diversity of sainfoin (*O. saiva*) cultivars by RAPD marker technique and the possibility for their use in future breeding programs and make conservation recommendations based on genetic data.

2) To clarify the phylogenetic and genetic relationship between of these cultivars;" Our study would provide useful information for molecular classification, for breeding new cultivars of sainfoin and compare the genotypes with each other.

Materials and methods

Genomic DNA preparation

Seed of 36 populations of sainfoin (*Onobrychis sativa*) that originated from various parts of Iran consisting of different climates (with different features in terms of humidity, temperature, longitude and ...) were provided from Natural Resource Gene Bank, (RIFR-Iran), (Table 1). The seeds were sown in pots in spring 2012, and sampling for DNA extracting was done. All plant materials were frozen in liquid nitrogen immediately after harvesting before transferring into freezer. Total genomic DNA was extracted from frozen leaves (30 mg per ecotype) and (each population 10 samples) following the CTAB procedure (Saghai-Maroof *et al*., 1984) with minor modifications. Their variability was evaluated via RAPD analysis. Plant materials were homogenized in a 300ul DNA extraction buffer (2 %(w/v) CTAB: 5 M NaCl, 2 M Tris-HCl pH= 8, 0.5 M EDTA pH= 8 and pvp-40(w/v) 1%). In a next step, another 300 ul of extraction buffer were added and centrifuged at 10000 g for 10 min at 4 ºC. The supernatant was transferred in a clean tube for isopropanol extraction using centrifugation at 10000 g for 10 min at 4 ºC. The resulted pellets were washed with 70% ethanol, dried, and re-suspended in 50ul sterile fresh TE buffer. The quantity and quality of genomic DNA extracts were determined using spectrophotometrical and electrophoretical (0.8% w/v of Agarose gels) procedures.

In vitro template amplification

RAPD reactions were conducted on a Programmable Thermal Cycler (Bio-Rad, model:Q-Cycler) using the following profile: 4 min at 94ºC for initial denaturation, 40 cycles of 1 min at 93 ºC for denaturation, 1 min at 37ºC and 40ºC for annealing, and 90 sec at 72ºC for extension. Final extension time was 7 min at 72ºC. The total volume for reactions was 15ul. Each reaction contained 2ul of DNA template (25 ng), 1ul of arbitrary primers (at a final concentration of 4 pmol ul^{-1}), 7.5 ul of CinnaGen PCR Master Mix (CinnaGen PCR Master Kit, Cat. No. PR8251C), and 4.5 ul sterile deionized water. Amplified fragments were separated on 0.8% Agarose gel and Cyber green for stained and then documented with a Bio-Doc Analyze System (Biometra) under UV illumination. Twenty Operon primers were screened, and among them, five primers were selected and used further studies tested as single primers for the amplification of profile RAPD sequences obtained from operon:opj_4, opj_9, opj_{13}, opj_{18} and opj_{19} CO.LTD Germany metabion (Table 2 and 3)

Analysis of locus profiles

Marker patterns were scored as presence (1) or absence (0) for each locus separately. Genetic analysis was carried out using the Genalex (ver 6.5) software to compute the number of polymorphic loci per population, Nei's gene diversity (Nei, 1973) and the genetic distances among populations (Nei, 1972, 1978). The hierarchical variance components and the F statistic for the RAPD phenotypes were measured via the AMOVA procedure (Excoffier *et al*., 1992; Huff *et al*., 1993; Huff, 1997) using Arlequin 3.11 (Excoffier *et al*., 2005). The permutational procedure was set at 2000 on the original Inter-individual squared distance matrix to provide significance tests for each

of the hierarchical variance components and related F statistic. To cluster the populations, the matrix of pairwise Nei's measures of genetic distances (Nei, 1978) and was used for the sequential agglomerative hierarchical nested clustering method as available in NTSYS (ver.2.02) based on the UPGMA algorithm (Sneath and Sokal, 1973).

Results

An informative and reproducible amplification of RAPD fragments was established using the arbitrary primers (Fig. 1, Table 2) From the 5 primers used with RAPD-PCR technique suggest that the highest percentage of polymorphic loci (80%) was recorded in Divandara population, which was followed by the Khalkal (78%), Marand (51%) and Aligodarz3 (49%) and the average band produced for each primer was 15.8 polymorphic band. The highest percentage of Heterozygosity (genetic diversity) (0.29) were recorded in populations Divandara, Khalkal the lowest Heterozygosity (0.15) were recorded in population Marand. In total, with an average 66% of the bands were polymorphic. The maximum and minimum numbers of bands observed by primers OPJ4, OPJ19 and OPJ9, OPJ13, OPJ18 were 17 and 15, respectively. Populations Khalkal, Tabriz, Miandoab, Divandara, Arak, Polycross were the most polymorphic baring 69-72 bands, respectively whereas population Marand with 44 bands had the lowest polymorphic (Table 4). Genetic distance among 36 *O. sativa* populations was estimated using Nei's genetic distance (Nei, 1973) ranged from 0.08 (between Khoramabad and Aligodarz2) to 0.43 (between Marand and Khoramabad) with an average value of 0.25 (Table 5). According to the dendrogram resulted from the banding pattern the Genetic distance among populations based on Nei genetic similarity coefficients ranged from 0.08 to 0.43. The highest similarity was found between Khoramabad vs. Aligodarz2 (0.08), whereas the lowest was among Marand vs. Khoramabad, Aligodarz2 and Khonsar2 vs Heris (0.43). Molecular variance analysis showed significant variation among and within populations, with average values of 22% and 78%, respectively. Analysis of population structure based on F-statistics revealed a higher values (Fst=0.22) of variation within populations (Table 6). The molecular data were subjected to unweighted pair group method with arithmetic average (UPGMA) cluster analysis and 36 populations were partitioned into three groups. Cluster I consisted of populations Khoramabad, Aligodarz2 and Aligodarz3; cluster II included populations Ormia, Khonsar2 Faridonshr2, Faridan and Khomin2; cluster III consisted of noise populations (Fig. 2). Results of principal coordinate analysis (PcoA) strongly supported cluster analysis results (Fig. 3). The interpopulation genetic distance showed no association with the geographic distance between the population sites of origin. In general, RAPD marker data proved to be a good method of assessing genetic variation among populations of sainfoin. (Mantel, 1967; Rohlf, 1972; Smouse *et al*, 1986). The AMOVA (Excoffier *et al*, 1992; Huff *et al*, 1993; Huff, 1997) (p<10−5) based on 2000 random permutations.

Table 1. Environmental and Geographic characteristics Profile of 36 populations of *O. sativa*.

Entry	population	Relative Humidity	Annual average Temperature (°C)	Rainfall Annual (mm)	Altitude (m)	Latitude (N)	Longitude (E)
1	Khalkal	65.0	8.6	363.3	1796	37°38'	48°31'
2	Sarab	60.2	9.1	249.8	1682	37°56'	47°32'
3	Ahar	58.6	11.3	278.3	1391	38"26'	47°04'
4	Heris	50.6	14.0	229.3	1361	38°05'	46°17'
5	Asadabad	53.7	12.0	450.7	1742	34°52'	48°32'
6	Arak	45.1	14.4	307.6	1708	34°06'	49°46'
7	Khomin1	45.1	14.4	307.6	1835	33°39'	50°05'
8	Bonab	52.5	13.4	257.5	1290	37°20'	46°04'
9	Marand	52.0	12.1	321.8	1550	38°28'	45°46'
10	Khonsar1	41.0	14.5	251.4	2300	33°14'	50°19'
11	Damavand	52.1	5.0	391.5	1976	35°55'	52°50'
12	Varzaghan	50.6	14.0	229.3	1361	38°05'	46°17'

13	Tabriz	50.6	14.0	229.3	1361	38°05'	46°17'
14	Faridonshr1	42.4	11.2	316.4	2490	32°56'	50°06'
15	Azna1	50.0	12.4	430.5	1872	33°27'	49°25'
16	Kahloran	70.9	9.6	282.4	1332	38°15'	48°17'
17	Khoramabad	45.4	17.1	447.7	1148	33°26'	48°17'
18	Aligodarz2	41.3	12.6	412.7	2022	33°24'	49°42'
19	Faridonshr2	42.4	11.2	316.4	2490	32°56'	50°06'
20	Faridan	42.4	11.2	316.4	2490	32°56'	50°06'
21	Khonsar2	41.0	14.5	251.4	2300	33°14'	50°19'
22	Khomin2	45.1	14.4	307.6	1835	33°39'	50°05'
23	Ormia	54.3	11.9	257.9	1316	37°32'	45°05'
24	Miandoab	51.9	13.4	384.8	1385	36°58'	46°03'
25	Silvana	54.3	11.9	257.9	1316	37°32'	45°05'
26	Polycross	47.7	15.4	239.2	1313	35°55'	51°37'
27	Aligodarz3	41.3	12.6	412.7	2022	33°24'	49°42'
28	Azna2	50.0	12.4	430.5	1872	33°27'	49°25'
29	Kermanshah	15.5	45.0	375.9	1319	34°21'	47°09'
30	Oshnevia	54.3	11.9	257.9	1316	37°03'	45°08'
31	Kbotarabad	37.5	16.6	130.4	1545	32°31'	51°51'
32	Azarshar	50.6	14.0	229.3	1361	38°05'	46°17'
33	Divandara	48.9	14.3	374.5	1373	35°20'	47°00'
34	Songor	15.5	45.0	375.9	1700	34°47'	47°35'
35	Zanjan	54.8	11.4	267.3	1663	36°41'	48°29'
36	Meskinsahre	60.7	10.7	395.9	1569	38°23'	47°40'

Table 2. Characteristics of 5 Random Amplifed Polymorphic DNA Primers.

RAPD	Sequences	primers	Number of bands	Annealing temperature	GC content
1	5_CCGAACACGG_3	Opj4	17	37	70%
2	5_TGAGCCTCAC_3	Opj9	18	40	60%
3	5_CCACACTACC_3	Opj13	19	40	60%
4	5_TGGTCGCAGA_3	Opj18	18	40	60%
5	5_GGACACCACT_3	Opj19	19	37	60%
6	5_CCTGGGCCTA_3	-	-	-	-
7	5_CCTGGGGGTT_3	-	-	-	-
8	5_CCTGGCGGTA_3	-	-	-	-
9	5_CCTGCGCTTA_3	-	-	-	-
10	5_GGGGGGATTA_3	-	-	-	-
11	5_TTTGGGCCCA_3	-	-	-	-
12	5_TTTGGGGGGA_3	-	-	-	-
13	5_CCGGCCTTAA_3	-	-	-	-
14	5_CCGGCCTTAC_3	-	-	-	-
15	5_CCGGCCTTAG_3	-	-	-	-
16	5_CCGGCCTTAA_3	-	-	-	-
17	5_GGGGCCTTAA_3	-	-	-	-
18	5_CCGGCTGGAA_3	-	-	-	-
19	5_CCGGCCCCAA_3	-	-	-	-
2	5_CCGGGGTTAA_3	-	-	-	-

Table 3. Details of the Polymerase Chain Reaction.

Step	Temperature(°C)	Time (min)	Cycle number
Initial denaturing	94	4	1
Denaturing	93	1	40
Annealing	37.4	1	40
Extension	72	1.5	40
Final extension	72	7	1

Table 4. Composition and Percent of Polymorphism in 5 Random Amplified Polymorphic DNA Primers.

Entry	population	OPJ19	OPJ18	OPJ13	OPJ9	OPJ4	No. of bands	Mean Heterozygosity	Polymorphism %
1	Khalkal	14	14	14	14	16	72	0.29	%78
2	Meskinsahre	14	11	11	7	15	58	0.20	%61
3	Kahloran	12	8	13	10	15	58	0.21	%65
4	Sarab	11	15	13	10	17	66	0.27	%72
5	Ahar	13	12	13	12	14	64	0.26	%73
6	Heris	11	14	10	9	16	60	0.22	%64
7	Bonab	13	15	14	13	13	68	0.27	%75
8	Marand	12	7	9	8	8	44	0.15	%51
9	Varzaghan	11	14	9	14	12	60	0.24	%65
10	Tabriz	13	14	15	15	12	69	0.25	%77
11	Azarshar	12	14	11	15	11	63	0.21	%65
12	Ormia	7	16	9	8	12	52	0.20	%58
13	Miandoab	13	15	12	14	16	70	0.26	%77
14	Silvana	14	14	9	12	16	65	0.28	%76
15	Oshnevia	17	9	10	13	11	60	0.19	%53
16	Zanjan	13	12	7	14	12	58	0.21	%67
17	Divandara	16	15	13	12	13	69	0.29	%80
18	Asadabada	16	14	10	12	15	67	0.24	%71
19	Arak	17	11	13	11	17	69	0.27	%77
20	Khomin1	14	12	11	11	14	62	0.24	%71
21	Khomin2	14	12	8	13	10	57	0.19	%55
22	Songor	12	14	11	12	11	60	0.22	%61
23	Kermanshah	16	14	10	15	12	67	0.28	%76
24	Aligodarz2	10	10	12	13	16	61	0.20	%54
25	Aligodarz3	12	10	7	12	16	57	0.19	%49
26	Azna1	15	11	8	13	13	60	0.22	%60
27	Azna2	15	11	13	12	16	67	0.28	%72
28	Khoramabad	14	11	9	14	16	64	0.24	%61
29	Damavand	11	9	11	15	11	57	0.21	%66
30	Polycross	14	13	14	14	14	69	0.28	%76
31	Kbotarabad2	12	9	9	13	12	55	0.17	%48
32	Faridan	15	9	13	9	14	60	0.20	%55
33	Khonsar1	11	12	15	13	14	65	0.27	%76
34	Khonsar2	14	12	11	13	11	61	0.22	%58
35	Faridonshr1	17	14	10	13	14	68	0.25	%71
36	Faridonshr2	14	14	11	13	13	65	0.23	%67
		17	15	15	15	17		0.23	%66

Table 5. Similarity Matrix Based on of Nei's genetic distances of the 36 *O. Sativa* populations Genetic distances.

populations	Khalkhal	Meshkinsahre	Chloran	Sarab	Ahar	Haris	Bonab	Marand	Varzeghan	Tabriz	Azarsahre	Oromie	Meandoab	Silvelna	Oshnaveh	Znjan
Meshkinsahre	0.25															
Chloran	0.21	0.16														
Sarab	0.15	0.26	0.22													
Ahar	0.15	0.20	0.16	0.17												
Haris	0.26	0.19	0.23	0.25	0.14											
Bonab	0.24	0.22	0.22	0.22	0.21	0.21										
Marand	0.32	0.20	0.16	0.28	0.19	0.20	0.25									
Varzeghan	0.32	0.23	0.24	0.30	0.22	0.28	0.29	0.26								
Tabriz	0.27	0.18	0.15	0.24	0.20	0.20	0.23	0.22	0.17							
Azarsahre	0.25	0.23	0.25	0.29	0.20	0.22	0.23	0.27	0.27	0.16						
Oromie	0.24	0.23	0.21	0.24	0.24	0.26	0.28	0.18	0.31	0.20	0.20					
Meandoab	0.24	0.27	0.26	0.29	0.23	0.21	0.21	0.25	0.22	0.24	0.26	0.25				
Silvelna	0.24	0.19	0.22	0.26	0.18	0.20	0.20	0.26	0.22	0.20	0.17	0.25	0.14			
Oshnaveh	0.30	0.27	0.22	0.28	0.22	0.29	0.24	0.31	0.36	0.23	0.18	0.35	0.33	0.24		
Znjan	0.27	0.13	0.13	0.24	0.18	0.17	0.23	0.16	0.22	0.15	0.23	0.21	0.24	0.21	0.26	
Divandarreh	0.18	0.17	0.18	0.23	0.17	0.20	0.20	0.25	0.23	0.21	0.14	0.21	0.28	0.21	0.24	0.21
Asadabad	0.23	0.16	0.19	0.25	0.17	0.16	0.23	0.25	0.30	0.24	0.27	0.28	0.33	0.25	0.30	0.21
Arak	0.22	0.18	0.17	0.19	0.14	0.13	0.20	0.22	0.31	0.22	0.21	0.25	0.22	0.22	0.20	0.15
Khomain1	0.24	0.18	0.19	0.25	0.20	0.20	0.13	0.18	0.31	0.22	0.27	0.23	0.26	0.24	0.34	0.17

Khomain2	0.29	0.23	0.17	0.32	0.30	0.34	0.27	0.26	0.28	0.24	0.29	0.18	0.25	0.25	0.27	0.20
Songhor	0.30	0.27	0.19	0.26	0.19	0.25	0.22	0.24	0.13	0.13	0.26	0.31	0.28	0.28	0.28	0.22
Kermansah	0.25	0.26	0.20	0.25	0.15	0.19	0.22	0.28	0.29	0.21	0.18	0.29	0.25	0.18	0.12	0.21
Aligodarz2	0.27	0.31	0.25	0.25	0.27	0.26	0.31	0.43	0.31	0.26	0.32	0.32	0.29	0.24	0.40	0.21
Aligodarz3	0.28	0.32	0.33	0.31	0.25	0.27	0.29	0.40	0.30	0.32	0.27	0.34	0.26	0.17	0.32	0.28
Azna1	0.28	0.18	0.20	0.22	0.18	0.24	0.26	0.22	0.30	0.23	0.26	0.34	0.33	0.25	0.24	0.17
Azna2	0.23	0.23	0.22	0.26	0.18	0.23	0.25	0.30	0.30	0.22	0.21	0.31	0.20	0.16	0.17	0.23
Khorrmabad	0.26	0.30	0.28	0.28	0.30	0.27	0.28	0.43	0.30	0.24	0.33	0.33	0.30	0.24	0.38	0.21
Damavand	0.28	0.18	0.16	0.26	0.17	0.19	0.17	0.11	0.19	0.17	0.22	0.18	0.26	0.22	0.30	0.16
Polycross	0.11	0.27	0.26	0.14	0.20	0.26	0.26	0.29	0.30	0.31	0.31	0.26	0.21	0.26	0.34	0.25
kabotarabad	0.29	0.30	0.28	0.31	0.27	0.26	0.27	0.34	0.36	0.28	0.19	0.27	0.29	0.20	0.26	0.31
Freidan	0.27	0.30	0.26	0.28	0.29	0.34	0.31	0.33	0.33	0.30	0.32	0.31	0.33	0.24	0.38	0.31
Khansar1	0.24	0.23	0.20	0.23	0.19	0.23	0.15	0.16	0.25	0.21	0.24	0.23	0.25	0.27	0.33	0.19
Khansar2	0.26	0.28	0.22	0.30	0.29	0.43	0.30	0.31	0.33	0.28	0.32	0.25	0.31	0.29	0.29	0.27
Fridonsahre1	0.29	0.15	0.16	0.28	0.22	0.24	0.26	0.27	0.22	0.15	0.23	0.28	0.30	0.21	0.20	0.18
Fridonsahre2	0.26	0.22	0.17	0.21	0.22	0.31	0.25	0.22	0.26	0.23	0.29	0.29	0.28	0.23	0.33	0.22

populations	Divandarreh	Asadabad	Arak	Khomain1	Khomain2	Songhor	Kermansah	Aligodarz2	Aligodarz3	Azna1	Azna2	Khorrmabad	Damavand	Polycross	kabotarabad	Faridan	Khansar1	Khansar2	Fridonsahre1
Asadabad	0.14																		
Arak	0.17	0.11																	
Khomain1	0.20	0.21	0.17																
Khomain2	0.29	0.32	0.27	0.25															
Songhor	0.27	0.30	0.29	0.22	0.28														
Kermansah	0.23	0.27	0.19	0.24	0.28	0.21													
Aligodarz2	0.32	0.28	0.25	0.37	0.34	0.34	0.28												
Aligodarz3	0.30	0.33	0.28	0.38	0.37	0.38	0.24	0.15											
Azna1	0.21	0.20	0.18	0.23	0.32	0.29	0.23	0.35	0.35										
Azna2	0.24	0.25	0.18	0.27	0.25	0.30	0.14	0.29	0.20	0.20									
Khorrmabad	0.33	0.29	0.27	0.34	0.31	0.31	0.28	0.08	0.14	0.31	0.28								
Damavand	0.23	0.22	0.21	0.13	0.23	0.17	0.23	0.37	0.36	0.26	0.26	0.37							
Polycross	0.24	0.28	0.21	0.24	0.30	0.32	0.25	0.33	0.31	0.29	0.23	0.31	0.28						
kabotarabad	0.22	0.30	0.29	0.32	0.34	0.36	0.22	0.23	0.18	0.37	0.30	0.31	0.31	0.31					
Freidan	0.26	0.34	0.33	0.32	0.35	0.30	0.29	0.25	0.27	0.36	0.37	0.23	0.31	0.32	0.18				
Khansar1	0.19	0.22	0.20	0.17	0.28	0.20	0.29	0.35	0.31	0.24	0.25	0.32	0.14	0.24	0.34	0.30			
Khansar2	0.30	0.32	0.28	0.31	0.13	0.32	0.31	0.34	0.39	0.32	0.29	0.29	0.31	0.26	0.37	0.26	0.25		
Fridonsahre1	0.16	0.21	0.21	0.23	0.23	0.24	0.22	0.32	0.34	0.12	0.22	0.29	0.24	0.33	0.30	0.30	0.22	0.23	
Fridonsahre2	0.23	0.31	0.28	0.21	0.22	0.22	0.26	0.31	0.38	0.22	0.28	0.29	0.22	0.24	0.33	0.14	0.25	0.19	0.21

Table 6. Results of Analysis of Molecular Variance.

Source	DF	SS	MS	Var%	Prob
Among Pops	35	1191.53	34.04	22%	0.01
Within Pops	144	2017.20	14.01	78%	0.01
Total	179	3208.73	48.05	Fst=22%	

Discussion

Since only a wide genetic base gives the opportunity to select genotypes with a trait of interest, it is essential to understand the extent and distribution of genetic variation. This information is particularly important in Sainfoin which is an allogamous species susceptible to severe inbreeding depression. Decreased heterozygosity and heterogeneity of populations will decrease vigor and productivity. Evaluation of forage legumes germplasm is essential to ensure its efficient and effective use. In the present investigation the AMOVA revealed a higher distribution of genetic variation within populations (78%). According to Hamrick and Godt (1989), reproductive biology is the most important factor in determining the genetic structure of plant populations. They showed that out-crossing plant species tend to exhibit between 10% and 20% genetic variation among populations, while self-pollinated species exhibit on average 50% variation. However, Hamrick and Godt (1996) pointed out that life history traits alone only explain a relatively low amount of the variation in genetic structure. The high intra-population variability and genetic homogeneity across

populations could have arisen by high levels of gene flow. Variation among and within populations revealed that *O. sativa* populations had more genetic variation within rather than between populations (78% and 22%, respectively). Data on the genetic diversity of sainfoin population with different geographical origin are presented in this study. Based on results of previous investigations on genetic relationships of allogamous forage crop species by RAPD markers *(*Mengoni *et al.* 2000), 5 individuals were sampled per each population. The five primers used in this study amplified 79 polymorphic bands across all sainfoin population, with an average of 15.8 polymorphic bands per primer and, relatively upper level of polymorphism (80%). This is in agreement with the number of RAPD bands used by Musial *et al* (2002) who analyzed genetic diversity within Australian alfalfa cultivars, and slightly higher than the number of RAPD markers used to investigate white clover collection and cultivars (Gustin *et al*., 2002) and *Dactylis glomerata* populations (Tuna *et al*., 2004). High or low frequency loci could negatively affect the actual amount of the heterogeneity as it has been reported by Kidwell *et al*. (1994). This was probably a consequence of geographical isolation which limited the extent of gene flow from cultivated *O. sativa* species. The diverse proportion of polymorphic bands observed across *O. sativa* populations was probably a reflection of differences in the amount and type of germplasm used to develop these populations. In addition, most of the investigated populations are synthetic populations and other factors, such as the number of parental plants included in their development or a number of selected plants or previous selection for a specific trait affected the level of polymorphism. Results indicated that the applied primers may be used in the identification of these materials. According to the dendrogram resulted from the banding pattern the genetic distance among populations based on Nei genetic similarity coefficients ranged from 0.08 to 0.43. The highest similarity (least genetic distances) was found between Khoramabad vs. Aligodarz2 (0.08) populations, whereas the lowest similarity (highest genetic distances) was among Marand vs. Khoramabad, Marand vs. Aligodarz2 and Khonsar2 vs. Heris (0.43) This shows that the diversity ranks of different pairs of genotypes differ when worked out on the basis of different marker systems. Under these circumstances, one may like to select a pair of most diverse genotypes on the basis of consistency in the diversity rank of a particular pair of genotypes selected as fairly diverse with a high level of confidence and used as parents in a hybridization program. The molecular data were subjected to unweighted pair group method with arithmetic average (UPGMA) cluster analysis and populations were partitioned into three groups. Results of principal coordinate analysis strongly supported cluster analysis results. The interpopulation genetic distance showed no association with the geographic distance between the population sites of origin, which is contrast with the results of Musial *et al. (2002)*, Segovia-Lerma *et al.* (2003) and Maureira *et al.* (2004), who used molecular markers to study genetic diversity of *Medicago sativa* germplasm. The results indicated that Maron population is genetically divergent from the investigated alfalfa cultivars, and therefore a potential source of novel, favorable alleles that could be used to improve alfalfa cultivars. The results of this study indicate that RAPD analysis could be successfully used for the estimation of genetic diversity among sainfoin populations. Also, they showed that sainfoin population (*O. sativa*) is genetically diverse from most of the evaluated populations. This genetic distinctiveness suggests that these sources have novel alleles which may, by introducing them to sainfoin (*O. sativa*) gene pools, produce a positive heterotic response in a breeding programs. Molecular markers may did not seem to be useful for selecting genotypes to be used directly as parents for synthetic cultivar development, but they could be used to identify new promising germplasm complementary to the existing. Finally, the heterotic group identification could be efficient by using both classical and molecular methods based on either of the approaches studied.

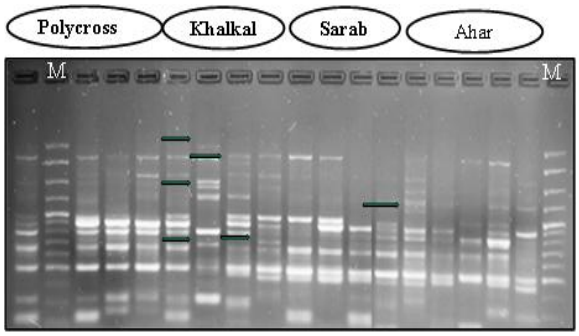

Fig. 1. RAPD patterns of 17 *O. sativa* populations using the primer OPJ4. (M)molecular-size marker(2), Numbers1, 2-5Polycross, 6-9 Khalkal, 10-13Sarab, 14-18 Ahar and 19 molecular-size marker.

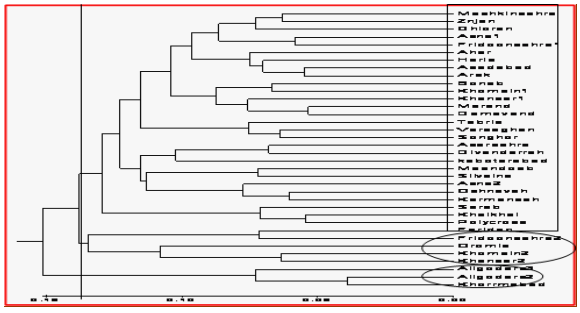

Fig. 2. Clustering of 36 populations of *O. sativa* using UPGMA method based on random amplified polymorphic DNA marker (color figure available online).

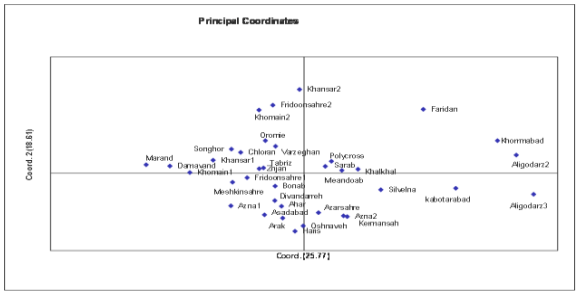

Fig. 3. Grouping of 36 populations of *O. Sativa* by principal component analysis based on RAPD markers.

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