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Comparative Assessment of IRAP, REMAP, ISSR, and SSR Markers for Evaluation of Genetic Diversity of Alfalfa (*Medicago sativa* L.)

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ABSTRACT

The effectiveness of IRAP, REMAP, SSR, and ISSR markers were investigated to assess genetic diversity among and within eight *Medicago sativa* L. populations. A total of 101, 119, 117 loci and 31 alleles were amplified using 10 IRAP, 14 REMAP, 16 ISSR and eight SSR primers, respectively. IRAP markers generated the maximum proportion of polymorphic loci per primer (PPLP) while the maximum value of percentage of polymorphic loci (PPL) was observed for SSR markers. ISSR markers showed the highest value of marker index (MI). The maximum amount of expected heterozygosity (He), effective number of alleles (Ne), and Shannon's information index was produced by SSR markers. UPGMA cluster using Nei's genetic distance coefficients and combined data of four markers separated the populations into three major groups. Correlation coefficients among pairwise genetic and geographic distance matrices, made on the basis of all studied markers, were calculated using Mantel's test. Regression and correlation analysis between genetic distance and geographic distance showed no significant correlations (p>0.05).

Keywords: Cluster analysis, Marker index, *Medicago sativa,* Retrotransposon-based markers.

INTRODUCTION

Alfalfa (*Medicago sativa* L.), which is the most cultivated forage legume, originated from Caucasus region, northeastern Turkey, Turkmenistan, and northwestern Iran. Cultivated alfalfa is autotetraploid (2n=4x=32), cross pollinated, and seed propagated (Michaud *et al*., 1988). The use of heterosis in alfalfa as a means to improve forage yield and other important traits has been demonstrated. Therefore, it is necessary to identify genetically distinct plants for breeding purposes to produce superior synthetic or hybrid varieties in alfalfa (Bourguiba *et al*., 2010; Mardi *et al*., 2011).

In the past two decades, the use of molecular markers for predicting hybrid performance, gene discovery, genetic diversity, molecular breeding and population genetics has become routine, and has revolutionized biology. A variety of different techniques have emerged to study DNA polymorphisms for the selection of the desired parents in breeding programs, and also vastly used for the selection of superior hybrid from a cross population (Mondini *et al*., 2009; Mardi *et al*., 2011).

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Molecular markers may differ with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements, and financial investment (Mondini *et al*., 2009). A better understanding of the effectiveness of the different DNA markers is a prerequisite for more effective application of marker techniques in breeding programs (Scariot *et al*., 2007; Sharma *et al*., 2014). No marker is superior to others for a wide range of applications. Inter simple sequence repeats (ISSRs) are popular for their simplicity as well as quick and easy assay. Moreover, no sequence information is required for primer design; they have high genomic abundance and are randomly distributed throughout the genome. The simplicity of the banding pattern and the multi-allelic nature of simple sequence repeat (SSR) loci may be extremely useful in the interpretation of segregation data in an autotetraploid crop such as alfalfa (Diwan *et al*., 2000). Inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon microsatellite amplified polymorphism (REMAP) are potential markers due to the abundance of retrotransposons (RTNs) in eukaryotic genomes and their ability to create new copies. They are easy to assess and could be established at low cost. A drawback of the IRAP and REMAP is that sequence of the RTNs is required for primer design (Kalendar *et al*., 2011).

However, the utility of all these markers have been proved in both animal and plant genetic research. A variety of molecular marker systems have been used to assess genetic diversity and hybrid performance in alfalfa, including restriction fragment length polymorphism (RFLP) (Kidwell *et al*., 1994b), random amplified polymorphic DNA (RAPD) (Mengoni *et al*., 2000), SSR (Falahati-Anbaran *et al*., 2007), amplified fragment length polymorphism (AFLP) (Zaccardelli *et al*., 2003) and IRAP and REMAP (Abdollahi Mandoulakani *et al*., 2012). To date, there has been no report of direct comparison of RTN-based markers with SSR and ISSRs in genetic diversity analysis of alfalfa. Therefore, the objectives of the present research were to investigate performance difference, discriminating capacity, and the effectiveness of IRAP, REMAP, ISSR, and SSR marker techniques in assessing genetic diversity of cultivated alfalfa populations.

MATERIALS AND METHODS

Plant Material and DNA Isolation

Plant materials consisted of 80 genotypes from eight alfalfa populations (10 plants from each population) (Table 1), kindly provided by the Agricultural and Natural Resources Research Center of West Azarbayejan province, Urmia, Iran. Seeds were planted in small pots with 10 cm diameter containing mixture of soil and vermiculite in the greenhouse with an ambient temperature of 25°C. Genomic DNA was extracted from young leaves of 25-day seedlings using the method described by Ausubel *et al*. (1995) with minor modifications. The quality and concentration of the DNA were measured using a spectrophotometer and electrophoresis in a

Table 1. Studied alfalfa populations and their origin.

Populations	Population abbreviation	Number of plants	Origin
Gharayonjeh-Malekkandi	GhM	10	Iran-Khoy
Gharayonjeh-Urmia	GhO	10	Iran-Urmia
Mahalie-Esfahan	MEs	10	Iran-Esfahan
Baghdadi	Bagh	10	Iran-Kerman
Hamedani	Ham	10	Iran-Hamedan
Azarbayejan-Ordubar	AOrd	10	Azarbayjan
Turkey1	Tu1	10	Turkey
Turkey-Sakuel	TuS	10	Turkey

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0.8% (w/v) agarose gel.

IRAP Reactions

Five single and 10 IRAP primer combinations (Table 2 and S1) were used to study genetic diversity in 80 alfalfa genotypes. Primers had been designed based on RTN families: Tms1Ret1 (Porceddu *et al*., 2002) from *M. sativa*, LORE1 (Madsen *et al*., 2005) and LORE2 (Fukai *et al*., 2008) from *Lotus japonicus* and Tps12a and Tps19 (Pearce *et al*., 2000) from *Pisum sativum*. PCR amplifications were carried out in a Bio-Rad thermocycler (Bio-Rad, Hercules, CA, USA) in a total volume of 20 µL as described by Abdollahi-Mandoulakani *et al*. (2012). PCR products were separated by electrophoresis (Bio-Rad) using 1.8%

Table 2. Used IRAP primers, annealing temperature, total loci, polymorphic loci, polymorphic information content and size range of amplified loci.

Primer	Ta [a]	Total loci	Polymorphic loci	PIC [b]	Band size (bp)
Tms1Ret1	63	12	9	0.21	1000-3000
LORE1	58	11	8	0.17	700-1800
LORE2	55	9	6	0.13	1200-2500
Tps12a	55	4	0	0.00	900-2000
Tms1Ret1-LORE1	60	15	12	0.23	250-2800
Tms1Ret1-LORE2	60	13	8	0.15	400-3000
Tms1Ret1-Tps12a	57	11	10	0.25	700-2500
LORE1-LORE2	58	13	8	0.12	300-1600
LORE1-Tps12a	58	6	5	0.14	850-2500
LORE2-Tps12a	57	7	0	0.00	420-1500
Mean		10.1	6.6	0.14	
Total		101	66		

[a] Annealing temperature, [b] Polymorphic information content

Table S1. Sequences and numbers of the used primers. [a]

Primer	Sequence (5´-3´)	Primer	Sequence (5´-3´)
425 (ISSR)	cacacacacacacacacag	UBC808 (ISSR)	cacacacacacacag
426 (ISSR)	caccaccaccaccaccaccact	UBC812 (ISSR)	gagagagagagagagaa
438 (ISSR)	acacacacacacacacacg	UBC849 (ISSR)	gtgtgtgtgtgtgtgtcg
440 (ISSR)	acacacacacacacacacc	A7 (ISSR)	agagagagagagagagagagt
443 (ISSR)	acacacacacacacacact	A12 (ISSR)	gagagagagagacc
456 (ISSR)	accaccaccaccaccaccc	A13 (ISSR)	gtgtgtgtgtgtcc
459 (ISSR)	tgctgctgctgctgctgcc	B1 (ISSR)	tctctctctctctctctcc
825 (ISSR)	acacacacacacact	Tms1Ret1 (RTN)	cggttttgtggggttgtgttaggccca
840 (ISSR)	gagagagagagagagaYt	LORE1 (RTN)	gagtcctgagtaaccaactaac
848 (ISSR)	cacacacacacacacag	LORE2 (RTN)	cagcttgaggacaagctgagtc
857 (ISSR)	acacacacacacacacYg	Tps12a (RTN)	gggctttgactaatggacctc
AFca11 (SSR)	aacgtttcccaaaacatactt (F) cttgagggaactattgttgagt (R)	AFca1 (SSR)	tgttatcagagagagaaagcg (F) cgtatcaatatcgggcag (R)
AFctt1 (SSR)	ttgtggattggaacgagt (F) cccatcatcaacattttca (R)	AFct45 (SSR)	gccatcttttcttttgcttc (F) taaaaaacggaaagagttggttag (R)
AFat15 (SSR)	caaaatgagtatagggagtgg (F) ttacgggtctagattagagagtatag (R)	B14B03 (SSR)	acctgacttgtgttttatgc (F) gcttgttcttcttcaagctc (R)
AFct60 (SSR)	tggatcaacgtgtctttca (F) cctccctaactttccaaca (R)	MTLEC2A (SSR)	tggttcgctgttctcatg (F) cggaaagattcttgaatagatg (R)

[a] Y: C/T, F: Forward primer, R: Reverse primer

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Resolute™ line Biozyme agarose gel in 0.5X TBE buffer with constant voltage of 70 V for 3 h. Gels were stained by ethidium bromide. DNA fragments were then visualized under UV light and photographed using a gel documentation system.

REMAP Reactions

Forty-eight REMAP primer combinations (Table 3 and S1), derived from four single IRAP primers (Tms1Ret1, LORE2, LORE1, and Tps12a) with 12 ISSR primers A7, A12, B1, 438, 443, 459, 818, 825, 840, 848, 849 and 857, were tested on 6 alfalfa genotypes to select primer combinations producing scorable and discernible banding patterns. PCR amplification reactions and temperature profile, electrophoresis, and visualization of REMAP markers were the same as IRAP.

ISSR Reactions

Sixteen ISSR primers (Table 4 and S1) were screened for detection of the genetic polymorphism among 80 alfalfa genotypes. PCR amplifications, electrophoresis and visualization of ISSR markers were the same as for IRAP and REMAP. Annealing temperature of ISSR primers depended on the primers (Table 4).

SSR Reactions

Eight SSR primer pairs (Table 5 and S1) were tested on DNA samples. PCR reactions were performed with AFct60, MTLEC2A and AFca1 primers in a mixture containing 45 ng of genomic DNA, 1X PCR buffer (10 mMTris–HCl, 50 mMKCl, pH=8.3), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 0.5 U of Taq DNA polymerase (Cinagen, Tehran, Iran), and 10 pmol of each primer using the following PCR profile: 94 °C for 4 min; 10 initial cycles as touchdown PCR; 35 subsequent cycles at 94°C for 30 s, 55 °C for 25 s, 72 °C for 25 s, and a final extension at 72 °C for 10 min. PCR reactions of the remaining SSR primers were carried out in the same mixture but using the following PCR profile: a denaturation period of 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 25 s at Tm (Table 5) for annealing, and 25 s at 68 °C and then 10 min at 72 °C for final extension. PCR products were separated by electrophoresis using 3%

Table 3. Used REMAP primer combinations, annealing temperature, total loci, polymorphic loci, polymorphic information content and size range of amplified loci.

Primer	Ta [a]	Total loci	Polymorphic loci	PIC [b]	Band size (bp)
Tms1Ret1-459	52	14	11	0.28	500-2500
Tms1Ret1-A7	52	5	0	0.00	350-850
Tms1Ret1-A12	52	12	5	0.07	250-1300
Tms1Ret1-B1	52	1	0	0.00	700
Tms1Ret1-438	52	8	2	0.06	200-1000
Tms1Ret1-443	52	5	0	0.00	500-1000
Tms1Ret1-UBC808	52	9	3	0.08	750-2000
Tms1Ret1-825	52	5	3	0.20	150-700
LORE1-840	52	7	3	0.09	200-1000
LORE1-848	52	11	7	0.16	100-1000
LORE2-UBC849	52	7	6	0.31	350-1000
LORE2-857	52	6	3	0.10	200-850
Tps12a-459	52	16	14	0.20	200-1700
Tps12a-438	52	13	5	0.10	200-1400
Mean		8.5	4.42	0.12	
Total		119	62		

[a] Annealing temperature, [b] Polymorphic information content

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Table 4. Used ISSR primers, annealing temperature, total loci, polymorphic loci, polymorphic information content and size range of amplified loci.

Primer	Ta [a]	Total Loci	Polymorphic Loci	PIC [b]	Band Size (bp)
425	54	7	6	0.33	800-3000
426	56	3	0	0.00	250-2500
438	53	3	5	0.41	800-4000
440	53	4	3	0.29	1500-3000
443	52	3	3	0.34	1500-4500
456	50	7	5	0.22	800-1500
459	51	3	2	0.28	800-1300
825	55	12	11	0.33	500-2500
848	55	10	9	0.35	1500-3000
A7	49	6	2	0.16	600-2200
A12	50	7	4	0.24	500-2500
A13	49	9	6	0.25	700-2000
B1	53	10	9	0.33	1300-3500
UBC808	54	12	10	0.29	1000-3000
UBC812	53	10	8	0.32	600-3000
UBC849	55	9	8	0.30	220-600
Mean		7.31	5.6	0.28	
Total		117	91		

[a] Annealing temperature, [b] Polymorphic information content

Table 5. Used SSR Loci, annealing temperature, number of alleles, polymorphic alleles, polymorphic information content and size range of amplified loci.

SSR locus	Ta [a]	Number of alleles	Polymorphic alleles	PIC [b]	Band Size (bp)
AFca11	52	3	3	0.54	130-160
AFctt1	52	7	7	0.78	100-150
AFat15	52	3	3	0.59	150-250
B14Bo3	54	4	4	0.61	150-180
AFct60	Touch Down	5	5	0.69	130-170
MTLEC2A	Touch Down	3	3	0.60	180-190
AFca1	Touch Down	3	3	0.63	110-130
AFct45	50	3	3	0.53	120-150
Mean		3.9	3.9	0.62	
Total		31	31		

[a] Annealing temperature, [b] Polymorphic information content

Resolute™ line Biozyme agarose gel in 0.5X TBE buffer with constant voltage of 70 V for 2-3 h. Gel staining and visualization were the same as for other marker systems used.

Data Scoring and Analysis

The amplified fragments for IRAP, REMAP and ISSR markers were scored as 1 or 0 for their presence or absence at each position, and the obtained binary matrices were used for analysis. SSR markers were scored as co-dominant nature. Five genetic distance matrices for IRAP, REMAP, ISSR, SSR and combined data (IRAP+REMAP+ISSR+SSR) were established between populations using Nei's coefficients in GenAlEx 6.4 (Peakall and Smouse, 2006) and subsequently used to construct dendrograms based on UPGMA clustering algorithm implemented in NTSYSpc 2.2 (Rohlf, 2000). To verify the goodness of fit for dendrogram of each marker system, the cophenetic correlation coefficient

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was estimated. All distance and cophenetic matrices were tested for pair-wise correlation using Mantel's non-parametric test (Mantel, 1967) with 1000 permutations using NTSYSpc 2.2 software. Correlation coefficients among pairwise genetic and geographic distance matrices were also calculated based on all studied markers using Mantel's test with the same software. Number of loci, percentage of polymorphic loci (PPL), number of loci with frequency higher or equal to 5%, number of private loci or alleles, number of less common loci with frequency lower or equal to 25% and 50%, mean of expected heterozygosity (He) and standard error (SE) of He were also calculated for each population using GenAlEx 6.4 based on IRAP, REMAP, ISSR, SSR and combined data. The polymorphic information content (PIC) (Castro *et al*., 2012) was calculated to measure the discriminating power of each primer. To compare the effectiveness of the four marker systems assayed, number of loci per primer (NLP), PPL, proportion of polymorphic loci per primer (PPLP), He, effective number of alleles (Ne), Shannon's information index (I) and marker index (MI) (Powell *et al*., 1996) were calculated in GenAlEx 6.4. To partition the total genetic variation within and among populations, analysis of molecular variance (AMOVA) was carried out using each marker and combined data with this software as well.

RESULTS

Levels of Polymorphism

A total of 101, 119, 117 loci and 31 alleles were amplified using 10 IRAP, 14 REMAP, 16 ISSR and eight SSR primers, respectively. The PPL for each assay was not in correlation with the total number of loci. Only eight loci were scored for SSRs, but all were polymorphic. In contrast, 119 REMAP loci were scored, only 52% of which were polymorphic. IRAP markers generated the maximum value of PPLP (6.6) while the minimum value of that (3.9) was observed for SSR markers (Table 6). Two population-specific fragments were produced by IRAP markers. The mean of percentage of polymorphic loci in population for ISSR and SSR markers were more than those of RTN-based markers (Table 7). PIC values varied from 0.12 for REMAP markers to 0.62 for SSRs. Ne values of IRAP and REMAP markers were almost similar. This parameter for ISSR markers was slightly more than that of IRAP and REMAPs and was the highest for SSRs (2.89). MI ranged from 6.96 for SSR markers to 27.3 for ISSRs (Table 6).

Genetic Diversity

A summary of the Nei's genetic distance estimates between pairs of populations, calculated for each marker system, is shown in Table 8. The average of genetic distances between populations using IRAP and REMAP markers were lower than those of ISSR and SSR markers. Estimates of genetic distances of REMAP markers varied from 0.011 to 0.026, averaging 0.02. The estimates revealed by SSR markers ranged from 0.069 to 0.372, with a mean value of 0.2. Genetic distance between populations using combined data of four markers ranged from 0.051 (Bagh and TuS) to 0.075 (GhO and GhM) with a mean value of 0.065. Across all populations, He, Ne and I values were high for SSRs (He=0.62, Ne=2.8 and I= 1.09), but low for REMAPs (He=0.15, Ne=1.23 and I= 0.21). These parameters for IRAP and REMAP markers were similar (Table 6).

Genetic Differentiation

Cophenetic correlation coefficients of each markers and pair-wise correlation between genetic distance and cophenetic matrices of markers examined are shown in Table 9. No significant correlation was found between the distance and cophenetic matrices of the four markers. The correlation of ISSR with

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Table 6. Comparison of IRAP, REMAP, ISSR and SSR markers in studied alfalfa populations.

Marker system	NPA [a]	TNL [b]	NLP [c]	PPL [d]	PPLP [e]	He [f]	Ne [g]	I [h]	MI [i]
IRAP	10	101	10.1	65	6.6	0.165±0.007	1.24±0.011	0.243±0.009	10.89
REMAP	14	119	8.5	52	4.4	0.147±0.006	1.23±0.011	0.213±0.009	9.11
ISSR	16	117	7.3	78	5.7	0.300±0.007	1.52±0.014	0.409±0.10	27.3
SSR	8	31	3.9	100	3.9	0.625±0.013	2.89±0.115	1.09±0.036	6.96

[a] Number of primers assayed, [b] Total number of loci, [c] Number of loci per primer, [d] Percentage of polymorphic loci, [e] Proportion of polymorphic loci per primer, [f] Expected heterozigosity, [g] Number of effective alleles, [h] Shannon's information index, [i] Marker index

Table 7. Characteristics of amplified IRAP, REMAP, ISSR and SSR loci in the studied alfalfa populations.

Populations		GhM [a]	TuS [b]	Mes [c]	Bagh [d]	Ham [e]	GhO [f]	Azord [g]	Tu1 [h]
Number of loci/alleles	IRAP	96	92	91	95	86	88	91	87
	REMAP	117	117	116	117	117	117	117	117
	ISSR	117	117	116	117	117	117	117	117
	SSR	27	30	30	27	30	25	30	29
Percentage of polymorphic loci	IRAP	57.43	51.49	55.45	58.42	48.51	52.48	54.46	50.50
	REMAP	42.86	44.54	41.18	42.02	44.54	46.22	42.86	44.54
	ISSR	63.25	69.23	69.23	63.25	65.81	67.52	65.81	62.39
	SSR	100	100	100	100	100	100	100	100
Number of loci with frequency ≥5%	IRAP	96	92	91	95	86	88	91	87
	REMAP	108	111	107	107	111	113	109	111
	ISSR	117	117	116	117	117	117	117	117
	SSR	27	30	30	27	30	25	30	29
Number of private loci/alleles	IRAP	1	0	0	0	0	0	1	0
	REMAP	0	0	0	0	0	0	0	0
	ISSR	0	0	0	0	0	0	0	0
	SSR	0	0	0	0	0	0	0	0
Number of less common loci (≤25%)	IRAP	0	1	0	1	0	0	0	0
	REMAP	0	0	0	0	0	0	0	0
	ISSR	0	0	0	0	0	0	0	0
	SSR	0	0	0	0	0	0	0	0
Number of less common loci (≤50%)	IRAP	5	5	3	3	0	3	1	1
	REMAP	0	0	0	0	0	0	0	0
	ISSR	0	0	0	0	0	0	0	0
	SSR	0	0	0	0	0	0	0	0
Mean of heterozygosity (He)	IRAP	0.180	0.157	0.176	0.176	0.144	0.151	0.169	0.164
	REMAP	0.142	0.137	0.142	0.141	0.151	0.159	0.148	0.154
	ISSR	0.286	0.325	0.325	0.282	0.297	0.303	0.299	0.284
	SSR	0.644	0.659	0.632	0.611	0.634	0.584	0.645	0.590
Standard error of He	IRAP	0.019	0.018	0.018	0.019	0.018	0.019	0.019	0.019
	REMAP	0.017	0.016	0.018	0.017	0.018	0.018	0.018	0.018
	ISSR	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021
	SSR	0.022	0.038	0.029	0.037	0.030	0.041	0.031	0.062

[a] Gharayonjeh-Malekkandi, [b] Turkey-Sakuel, [c] Mahalie-Esfahan, [d] Baghdadi, [e] Hamedani, [f] Gharayonjeh-Orumieh, [g] Azarbayejan-Ordubar, [h] Turkey1.

Table 8. Comparison of Nei's genetic distance estimates obtained from four PCR derived techniques.

Marker system	Minimum	Maximum	Mean
IRAPs	0.024	0.044	0.037
REMAPs	0.011	0.026	0.020
ISSRs	0.083	0.18	0.136
SSRs	0.069	0.372	0.2
IRAP+REMAP+ISSR+SSR	0.051	0.075	0.071

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combined data were high and significant (P<0.01.). This correlation was not significant for IRAP and REMAP but significant for SSR (P<0.05). SSR-based UPGMA dendrogram could not separate foreign populations from the Iranian ones (Figure 1D). IRAP, REMAP and ISSR-based AMOVA revealed high level of genetic variation within populations compared to among populations. In contrast, SSR markers revealed high level of genetic variation (33%) between populations. AMOVA using combined data revealed higher level of genetic variation within populations (89%) compared to among populations (11%). Combined data of IRAP, REMAP, ISSR and SSR markers were used to build a dendrogram to evaluate the power of all techniques when accumulated. Populations were grouped into three main clusters (Figure S1). Population GhM was located in cluster 1. Populations TuS, Bagh and Tu1 constituted cluster II. The rest of the populations were in cluster III. Correlation coefficients among pairwise genetic and geographic distance matrices, made on the basis of all studied markers, were also calculated using Mantel's test. Regression and correlation analysis between genetic distance and geographic distance showed no significant correlations (r=0.08, p>0.05)

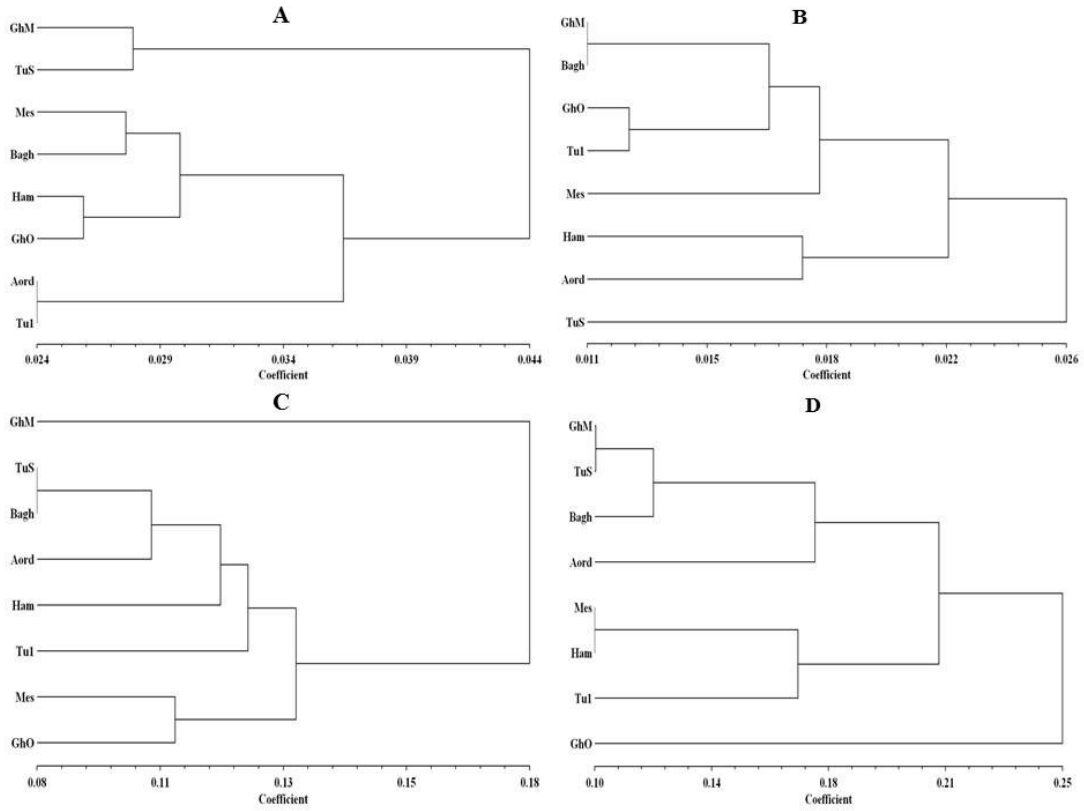

Figure 1. UPGMA dendrogram of eight alfalfa populations based on Nei's genetic distance coefficients using **A**: 101 IRAP loci, **B**: 119 REMAP loci, **C**: 117 ISSR loci, and **D**: eight SSR loci. Populations GhO: Gharayonjeh-Orumieh, GhM: Gharayonjeh-Malekkandi, MEs: Mahalie-Esfahan, Bagh: Baghdadi, Ham: Hamedani, AOrd: Azarbayejan-Ordubar, Tu1: Turkey1, TuS: Turkey-Sakuel.

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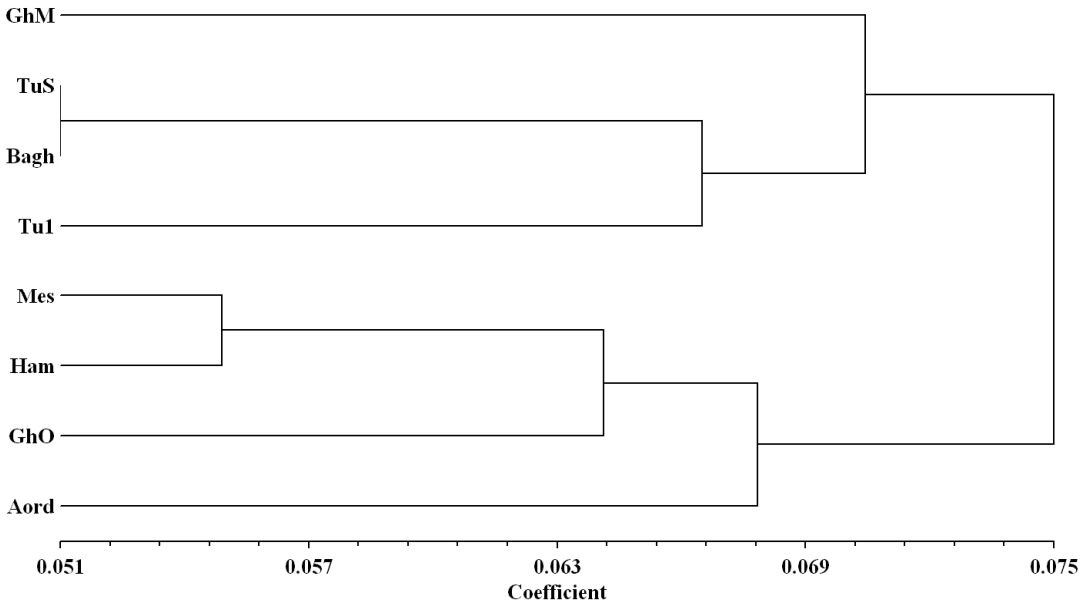

Figure S1. UPGMA dendrogram of eight alfalfa populations using IRAP+REMAP+ISSR+SSR loci based on Nei's genetic distance coefficient, GhO: Gharayonjeh-Orumieh, GhM: Gharayonjeh-Malekkandi, MEs: Mahalie-Esfahan, Bagh: Baghdadi, Ham: Hamedani, AOrd: Azarbayejan-Ordubar, Tu1: Turkey1, TuS: Turkey-Sakuel.

DISCUSSION

Since a wide genetic base gives the opportunity to select genotypes with a trait of interest, it is essential to measure the extent of genetic variation in a plant germplasm. This information is particularly important in alfalfa which is an allogamous and self-incompatible species with remarkable amount of hetrozygosity (Tucak *et al*., 2008). Molecular markers provide a good estimate of genetic diversity since they are independent of confounding effects by environmental factors. Each marker system has its own properties and combined data of different marker systems could provide comprehensive and reliable information in a germplasm (Powell *et al*., 1996). Moreover, comparative analysis of different markers is needed in order to decide which technique is most appropriate for the issue being examined (Scariot *et al*., 2007).

In this study, four PCR-based markers were employed to assess genetic diversity among eight alfalfa populations. Each technique not only differed in principle, but also in the type and extent of polymorphism detected. The NLP and PPLP were the highest for IRAP markers, probably reflecting the high copy number of the studied RTNs in alfalfa genome (Abdollahi Mandoulakani *et al*., 2012). The levels of polymorphism among the four techniques varied widely, from a maximum of 100% for SSRs to 52% for REMAPs. The high level of polymorphism of SSRs was to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz, 1989). Similar results have been already observed in alfalfa (Flajoulot *et al*., 2005; Touli *et al*., 2008; Naghavi *et al*., 2009). The averages of He, Ne, and I values of SSRs were greater than those of ISSRs, IRAPs, and REMAPs. Low polymorphism detected

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by RTN-based markers in our study might be attributed to the used IRAP and REMAP primers, since most of the RTN primers applied in the current work had been designed based on the non-native RTNs and the level of polymorphism obtained by non-native RTN-based markers in comparison to the native ones has been reported as low (Kalendar *et al*., 2011; Abdollahi Mandoulakani *et al*., 2012). The highest value of MI was obtained with ISSR markers, revealing the much higher relative information content of ISSRs. The low amount of MI for SSRs could be due to a few number of the SSR loci tested.

In our study, no significant associations were observed between molecular markers for both cophenetic and similarity matrices (Table 9). This might be due to the type of the genetic polymorphism detected and the number of primers used in different marker systems. Also, poor correlation between the used markers most likely indicates that they refer to different subsets of loci in genome (Biswas *et al*., 2010). As previously reported for *M. sativa* (Falahati-Anbaran *et al*., 2007; Abdollahi Mandoulakani *et al*., 2012), as well as in this study, it becomes evident that the measured relative genetic distances among the studied populations were not correlated with geographical distances of their origins, although RTN-based markers were able, to some extent, to separate foreign populations from the Iranian ones. We would expect that a greater sampling and use of high numbers of primers for each marker would be valuable to conclude more precisely the relationship between diversity and geographical origin.

Overall, the assessment of genetic diversity estimated by different marker systems should provide different levels of information important in the management of germplasm resources. Also, the IRAP and REMAP techniques can be used with ISSR and SSR markers for a more complete genome survey. The ubiquitous presence of RTNs in plant genomes suggests that the use of these techniques would allow breeders to obtain markers close to virtually any important agronomical trait and that the hypervariable nature of these elements should make them excellent sources of polymorphic markers. In conclusion, combined data from different marker systems were polymorphic enough to allow the detection of populations and genotypes with enough genetic distance in *M. sativa*. This information can be more useful to select populations or genotypes with higher genetic distance to potentially reach more heterosis. Work is currently in progress to increase the primer combinations and number of populations in order to have a deeper insight into the genetic diversity and establish varietal identification key in this crop.

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Table 9. Correlation (estimated by mantel test) between cophenetic matrices (above diagonal) and similarity matrices (below diagonal) obtained with four different marker types.

	IRAPs	REMAPs	ISSRs	SSRs	IRAP+REMAP+ISSR+SSR
IRAPs	0.72**	0.08	0.23	0.04	0.12
REMAPs	0.10	0.76**	0.03	0.01	0.10
ISSRs	0.03	0.04	0.79**	0.04	0.33*
SSRs	0.08	0.01	0.02	0.78**	0.35*
IRAP+REMAP+ISSR+SSR	0.25	0.20	0.80**	0.38*	0.68**

*and **: significant at P<0.01 and P<0.05, respectively. Diagonal values are the cophenetic correlation coefficient for each marker system.

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بررسی مقایسه‌ای نشانگرهای IRAP، REMAP، ISSR و SSR برای ارزیابی تنوع ژنتیکی یونجه

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چکیده

در این تحقیق کارایی نشانگرهای IRAP، REMAP، ISSR و SSR برای ارزیابی تنوع ژنتیکی بین و درون ۸ جمعیت یونجه بررسی شد. ده آغازگر IRAP، ۱۴ آغازگر REMAP، ۱۶ آغازگر ISSR و ۸ مکان SSR به ترتیب ۱۰۱، ۱۱۹، ۱۱۷ مکان و ۳۱ الل تولید نمودند. بیشترین تعداد مکان‌های چندشکل به ازای هر آغازگر مربوط به نشانگرهای IRAP بود در حالیکه بیشترین تعداد مکان چندشکل توسط نشانگرهای SSR تولید شد. بیشترین میزان شاخص نشانگری متعلق به نشانگرهای ISSR بود. بیشترین مقدار میانگین هتروزیگوسیتی، تعداد الل‌های موثر و شاخص اطلاعاتی شانون در جمعیت‌های مورد مطالعه توسط نشانگرهای SSR تولید شد. تجزیه کلاستر به روش UPGMA و ضرایب فاصله ژنتیکی نی بر اساس داده‌های ترکیبی ۴ نشانگر، جمعیت‌های مورد مطالعه را در ۳ گروه عمده قرار داد. همبستگی بین ماتریس فواصل جغرافیایی و ژنتیکی بر اساس داده‌های ترکیبی همه نشانگرها، با استفاده از آزمون مانتل محاسبه شد. نتایج حاصل از این تجزیه نشان داد که همبستگی معنی‌داری ($p>0.05$) بین فواصل جغرافیایی و ژنتیکی وجود ندارد.

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