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Genetic Relationships Among *Medicago sativa* L. Clones Commonly Grown in Central Anatolia

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Abstract: Amplified fragment length polymorphism (AFLP) markers were used to investigate genotypic variability among 34 alfalfa clones (*M. sativa*) using 15 primer combinations with restriction enzymes *Eco*RI, *Pst*I and *Mse*I. 34 unique AFLP fragments were observed. The 15 primer pairs produced a total of 1002 fragments of which 460 were polymorphic. The number of polymorphic fragments detected per primer combination ranged 7 to 67. Furthermore, 22 clone-specific markers were also detected in the 13 clones. Data analysis was performed with NTSYSpc version 2.1 software. Genetic distance values ranged 5.9374 to 1.1453. Fifteen clones which showed the highest genetic variation were selected for producing synthetic variety of Alfalfa.

Key Words: *Medicago sativa*, amplified fragment length polymorphism, synthetic variety improvement

Orta Anadoluda Yaygın Olarak Ekilen *Medicago sativa* L. Klonları Arasında Genetik İlişkiler

Öz: Çoğaltılmış parçacık uzunluğu polimorfizmi (AFLP) markörleri, *Eco*RI, *Pst*I ve *Mse*I restriksiyon enzimleriyle birlikte 15 primer kombinasyonu kullanılarak, 34 adet yonca (*M. sativa*) klonu arasındaki genotipik varyasyonu araştırmak için kullanılmıştır. 34 adet klona özgü AFLP bandı gözlenmiştir. 15 primer çifti 460'ı polimorfik olan toplam 1002 bant üretmiştir. Her primer kombinasyonu için tespit edilen polimorfik bantların sayısı 7 ile 67 arasında değişmiştir. Bunlara ilaveten 13 adet klonda da 22 adet klona özgü markör tespit edilmiştir. Veri analizi NTSYSpc version 2.1 yazılımı ile gerçekleştirilmiştir. Genetik uzaklık değerleri 5.9374 ile 1.1453 arasında sıralanmıştır. En yüksek genetik çeşitlilik gösteren onbeş klon, yoncanın sentetik varyetelerini üretmek amacıyla seçilmiştir.

Anahtar Kelimeler: *Medicago sativa*, çoğaltılmış parçacık uzunluğu polimorfizmi, sentetik varyete ıslahı

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important high feeding value leguminous forage crops in the world (Sumberg et al. 1983; Rumbaugh et al. 1988). It is an autotetraploid (2n = 4x = 32) outcrossing and seed-propagated species (Labombarda et al. 2000).

Alfalfa is distributed worldwide and grows in highly contrasting environments. This extensive geographical adaptation promotes genetic variation and give breeders possibility of using highly diverse genotypes in breeding programs (Maureira et al. 2004). Synthetic variety breeding is most effective and intensive method to improve perennial forage crops like alfalfa through polycross. Classical breeding studies require long time to select individual clones for synthetic variety production (Moreno-Gonzales and Cubero 1994).

Knowledge about genetic variability in species is important for optimal use of genetic resources in plant breeding programs. The use of molecular markers especially AFLP (Amplified fragment length polymorphism) markers help to select genetic dissimilarity potential parents for production of synthetics (Kidwell et al. 1994a). Some studies in alfalfa have detected positive associations between DNA marker diversity and hybrid yield (Kidwell et al. 1994a, Kidwell et al. 1994b, Osborn et al. 1998, Segovia-Lerma et al. 2003). AFLP is frequently used for the identification of molecular markers because of certain advantages over other techniques, such as high level of identified polymorphism, high reproducibility, and relative technical simplicity (Vos et al. 1995).

The aim of the study was to genetically evaluate alfalfa plants, collected from different regions of Turkey

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and select them on the basis o genotypes using AFLP molecular markers techniques for synthetic variety production.

Material and Methods

Plant material: Thirty four individual plants were collected from different parts of Central Anatolia to breed a new synthetic alfalfa variety compatible with climatic conditions of Central Anatolia (Table 1). Initially, the plants were phenotypically evaluated in terms of important agronomic characteristics. Thereafter, they were multiplied using shoot tip cuttings using perlite and vermiculite (3:1) as rooting medium.

DNA extraction and AFLP analysis: DNA was isolated from leaf tissue of two weeks old seedling as described by Doyle and Doyle (1987).

AFLP analysis was done following Vos et al. (1995) with minor modifications. Thirty four alfalfa clones were analyzed with seventeen primer combinations. Two primer pairs (P55 + CGA/ M55+ CGA and P55 + CGA/ M60+ CTC) were not included in the final analysis because the amplification profile was consistently too faint to score accurately. The Fifteen informative primer pairs used in the final analysis are listed in Table 2. Four *Pst*I/*Mse*I and eleven *EcoR*I/*Mse*I primer combinations were tested using identical procedures. Total genomic DNA was restricted with the restriction enzymes *Pst*I or *EcoR*I and *Mse*I (Promega) along with double-stranded adaptors (Promega, Table 2) ligated at 37 °C.

Amplification of the generated fragments was performed in two consecutive amplification cycles with primers containing one, or three selective nucleotide extensions (Table 2). First, preamplification was performed using two primers P00/M00 or E00+C/ M00 with following PCR conditions: 60 s at 94 ºC; 30 s at 60 ºC; 60 s at 72 ºC; this was followed by 7 min at 72 ºC extension, for 26 cycles. Selective amplification was conducted using three *Pst*I, five *EcoR*I and nine *Mse*I primers. Each primer contained three selective nucleotide extensions at the 3′ end (Table 2). *Pst*I/*EcoR*I primer was labeled by phosphorylating the 5' end with [γ^{33}P]ATP.

The selective amplification was performed for 36 cycles with the following cycle profile: a 30s DNA denaturation step at 94 ºC, a 30s annealing step at 65 ºC and a 1 min extension step at 72 ºC. The annealing temperature of 65 ºC in the first cycle was subsequently reduced in each cycle by 0.7 ºC for next 12 cycles, and was continued at 56 ºC for remaining 23 cycles. All amplification reactions were performed in a Biometra T-Gradient thermocycler.

Table 1. The locations of the clones used in AFLP analysis.

Clone no.	Locations
1	Altinova State Farm- Konya province
2	Experimental Fields of Field Crops Dept. Agriculture Faculty, Ankara University
3	Experimental Fields of Field Crops Dept. Agriculture Faculty, Ankara University
4	Saraykoy – Yozgat province
5	Afyon province
6	Ceylanpinar State Farm- Urfa province
7	Ceylanpinar State Farm- Urfa province
8	Ceylanpinar State Farm- Urfa province
9	Malya State Farm – Kirsehir province
10	Malya State Farm – Kirsehir province
11	Cicekdagi State Farm – Kirsehir province
12	Cicekdagi State Farm – Kirsehir province
13	Bulbul oten - Saraykoy – Yozgat province
14	Cesme basi village – Sivas province
15	Kocas State Farm – Aksaray province
16	Kulu - Konya
17	Golbasi - Ankara
18	Golbasi - Ankara
19	Golbasi - Ankara
20	Golbasi - Ankara
21	Ogulbey village - Ankara
22	Yunak-Konya
23	Aksehir - Konya
24	Sarkikaraagac-Isparta
25	Karacal village - Burdur
26	Emirdag - Afyon
27	Ilgin-Konya
28	Cay-Afyon
29	Cay-Afyon
30	Bolvadin-Afyon
31	Bolvadin-Afyon
32	Burdur
33	Emirdag-Afyon
34	Emirdag - Afyon

Table 2. Sequences of adapters, preamplification and selective amplification primers employed.

Adapters/Primers	Sequences (5'-3')
*Pst*I-adapter	5'-CTC GTA GAC TGC GTA CAT GCA-3'
	3'-CAT CTG ACG CAT GT -5'
EcoR I- adapter	5'- CTCGTAGACTGCGTACC -3'
	3'- AATTGGTACGCAGTC -5'
*Mse*I- adapter	5'-GAC GAT GAG TCC TGA G-3'
	3'-TAC TCA GGA CTC AT -5
P00 (universal primer)	5'-GAC TGC GTA CAT GCA G -3'
E00+C (universal primer)	5'-GACTGCGTACCAATTC-3'
M00 (universal primer)	5'-GAT GAG TCC TGA GTA A -3'
P55 + CGA	GAC TGC GTA CAT GCA G + **CGA**
P56 + CGC	GAC TGC GTA CAT GCA G + **CGC**
P57 + CGG	GAC TGC GTA CAT GCA G + **CGG**
M49+ CAG	GAT GAG TCC TGA GTA A + **CAG**
M50+ CAT	GAT GAG TCC TGA GTA A + **CAT**
M51+ CCA	GAT GAG TCC TGA GTA A + **CCA**
M52+ CCC	GAT GAG TCC TGA GTA A + **CCC**
M53+ CCG	GAT GAG TCC TGA GTA A + **CCG**
M56+ CGC	GAT GAG TCC TGA GTA A + **CGC**
M60+ CTC	GAT GAG TCC TGA GTA A + **CTC**
M61+ CTG	GAT GAG TCC TGA GTA A + **CTG**
M62+ CTT	GAT GAG TCC TGA GTA A + **CTT**
E17+ CCG	GACTGCGTACCAATT + **CCG**
E25+ CTG	GACTGCGTACCAATT **+ CTG**
E26**+** CTT	GACTGCGTACCAATT **+ CTT**
E38**+** ACT	GACTGCGTACCAATTC **+ ACT**
E36**+** ACC	GACTGCGTACCAATTC **+ ACC**

* E indicates *Eco*RI adapter sequences; P indicates *Pst*I adapter sequences ; M indicates *Mse*RI adapter sequences.

Page analysis: Following amplification, reaction products were mixed with an equal volume of formamide dye (98% [v/v] formamide, 100 mM EDTA, pH: 8.0, 0.025 % [v /v] bromo phenol blue and 0.025 % [v/v] xylene cyanol) which served as a tracking dye. The resulting mixture was heated for 3 min at 94ºC, and then quickly cooled on ice. Each sample was loaded on ice. After electrophoresis for 2 h at 80 watts (constant power), the gel was dried and exposed to X-ray film for 1-4 days depending on the signal intensity.

Data analysis: All genotypes were scored for presence or absence of polymorphic AFLP fragments and the data were entered into a binary matrix as discrete variables ("1" for presence and "0" for absence of a homologous fragment). Only distinct, reproducible, well-resolved fragments were scored. Data were analyzed with NTSYSpc version 2.1 (Numerical Taxonomy and Multivariate Analysis System, Version 2.1) (Rohlf 2000).

Results and Discussion

A large range of variation was obtained using different AFLP primer combinations. Fifteen AFLP primer pairs revealed 460 polymorphic bands among 1002 scorable bands (45.90 % polymorphism). The minimum number of polymorphic bands were produced by E26+M62 and P57+M50 (7 bands), whereas the maximum number of polymorphic bands were produced by E17+M61 and E25+M53 (67 bands) (Table 3).

The best primer combinations detected in this research, considering their ability to produce polymorphic bands are E25+M53 (%73.62), E17+M61 (%69.79), E38+M62 (%58.33), E36+M62 (%56.62), P55+M49 (%54.09), E17+M52 (%50.82), P56+M62 (%50), E25+M49 (%45.05), E38+M56 (%43.9), E36+M56 (%37.5) (Table 3). Defining the primer combinations showing high number of polymorphic bands will be advantageous for the future studies in this species to speed up the analysis and minimize expenses.

There was a marked difference between EcoRI/MseI primers and PstI/MseI primers in the number of the visible bands observed. EcoRI/MseI primers produced a slightly larger number of fragment compared to PstI/MseI primers. No significant differences were recorded in percentage of polymorphic bands produced by both of EcoRI/MseI (21.86-54.09%) and PstI/MseI primers ((16-73.62%) Each clone presented a unique AFLP pattern. There were clone-specific markers (present in one clone but absent in the others). Twenty two (22) clone specific bands for 13 clones were detected (Table 4). This revealed that for identification of a given clone, specific amplification profiles obtained with single primer/clone combination can be used. Distribution of the clone-specific markers according to primer combinations is shown in Table 4.

Table 3. AFLP primer combinations generating polymorphic products after *Pst* I/*Mse* I and *EcoR* I/*Mse* I enzyme digestion, and distribution of AFLP markers.

Primer combinations	Polymorphic bands (no)	Visible band (no)	Polymorphism (%)
E17+M61	67	96	69.79
E17+M52	31	61	50.82
E17+M49	13	50	26
E38+M62	56	96	58.33
E38+M56	18	41	43.9
E26+M50	12	75	16
E26+M62	7	67	10.44
E25+M53	67	91	73.62
E25+M49	41	91	45.05
E36+M62	47	83	56.62
E36+M56	18	48	37.5
P56+M51	17	58	29.31
P56+M62	26	52	50
P55+M49	33	61	54.09
P57+M50	7	32	21.86
Total	460	1002	45.90

Table 4. Distribution of the clone-specific markers obtained from AFLP reactions according to primer combinations.

Primer combinations	Clone no.	Number of clone-specific marker
E17+M61	22	1
	34	1
E38+M62	2	1
	10	1
	29	1
E25+M49	28	1
	31	1
E25+M53	2	1
	7	1
	23	1
E36+M62	23	1
E36+M56	23	1
P56+M51	31	1
P56+M62	2	1
	7	1
	25	1
	30	1
	31	1
	33	1
P55+M49	10	1
	13	1
	22	1
Total		13

Genetic similarity and diversity analysis among thirty four alfalfa clones was performed using the data analysis software, NTSYSpc version 2.1 software (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 2000). The genetic distance matrix was obtained using 'Nei72'algorithm (Table 5). A dendrogram was constructed using the unweighted pair group method average (UPGMA) clustering (Figure 1).

Table 5. Genetic distance matrix based on Nei 72.

Clone no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	****																
2	3.5470	*****															
3	3.7975	4.9318	*****														
4	3.7288	4.4046	4.9522	*****													
5	4.1831	4.6410	4.9958	5.2924	*****												
6	3.0055	3.6452	3.7557	3.5728	3.1209	*****											
7	3.0038	3.9932	3.6895	3.9582	3.3402	2.4589	*****										
8	3.0265	3.7528	4.2926	3.8717	3.1849	2.1652	2.2207	*****									
9	2.6718	3.5948	3.7986	4.1889	2.0986	2.1169	2.2489	2.0531	*****								
10	2.1912	3.6123	3.7834	3.7987	3.6546	2.4672	2.7015	2.4828	2.3221	*****							
11	3.0718	4.3860	4.3991	4.4515	4.4229	3.4424	3.2965	3.0781	2.3197	2.6996	*****						
12	2.7572	3.3484	3.6145	3.8131	4.2680	2.6540	2.8139	2.7715	2.8955	2.6608	2.8405	*****					
13	2.2412	3.8683	4.0038	3.5081	3.9693	2.5490	2.8366	2.5317	2.4149	2.4025	2.7515	2.3812	*****				
14	2.3804	3.4288	3.8013	3.8184	3.6911	2.7591	2.6614	2.4553	2.2910	2.3961	2.5426	2.3471	1.9643	*****			
15	2.8648	3.8457	3.9854	3.7465	4.0069	2.6406	2.6832	2.4148	3.1031	2.4568	3.4319	2.8034	2.8666	1.7235	*****		
16	3.6106	4.2002	4.8545	4.3416	4.5895	3.3174	3.8671	3.4270	3.5258	3.0630	4.2167	2.8829	2.3980	2.9193	3.3344	*****	
17	2.5340	3.5795	3.7543	3.8727	3.7322	2.8009	2.9625	2.2950	2.2858	2.2311	2.8774	2.4509	2.2694	2.2599	2.4003	2.8310	*****
18	2.9243	3.8818	3.9923	4.4638	2.6090	2.6122	2.6067	2.4018	1.9155	2.3938	3.0836	2.6696	2.3900	2.3051	2.8772	3.0464	2.0109
19	4.1831	5.3755	5.2298	4.8159	5.6895	4.7491	4.5868	4.5445	4.4416	4.0908	4.3430	4.2575	4.1179	3.8289	4.3986	4.3883	4.2096
20	2.7583	3.7234	3.8759	4.0776	3.8095	2.6664	2.5025	2.5134	2.6964	2.7639	3.1339	2.3548	2.3543	2.4183	2.4774	3.4441	2.3237
21	2.5848	3.8344	3.7362	3.7720	4.3475	3.0046	3.0801	3.0899	3.0193	2.4808	3.4206	2.9368	2.4691	2.5970	2.7828	3.7091	2.4417
22	4.3773	4.7881	4.5338	5.1641	5.3565	4.0492	4.2446	4.2985	4.2208	3.7782	4.5135	3.5464	3.8667	3.9438	4.2927	4.5974	4.0152
23	4.1197	4.5894	5.1098	4.6991	5.7032	3.9886	4.1385	3.9663	4.1274	3.8368	4.1850	4.2014	3.7238	3.5868	3.9871	4.6737	3.8024
24	4.0655	4.7670	4.8822	4.8133	5.5424	4.1236	4.1650	4.7792	4.3282	3.8894	4.6783	3.8712	3.8077	4.0273	4.5591	4.5217	4.2586
25	3.5974	4.8567	4.4796	4.8172	4.8828	4.0255	3.8515	3.7876	3.8688	3.2907	4.0323	3.5242	3.4346	3.3419	3.5717	3.7584	3.4275
26	3.4586	3.9568	4.6801	1.1453	4.8672	3.6998	3.8792	3.8572	3.9082	3.4178	3.9699	3.3900	3.0369	3.2185	3.2866	3.8020	3.4632
27	3.6242	4.5733	4.4106	4.6371	4.9945	4.1078	4.2778	3.8244	3.9144	3.2626	4.5486	3.5349	3.8008	3.6848	3.9985	4.0299	3.8833
28	3.2500	4.3862	4.4266	4.2398	5.1229	3.8514	4.0669	3.9586	3.7350	3.3376	3.5287	3.3651	3.6403	3.3827	4.4027	3.7195	3.5992
29	3.5983	4.3682	4.1076	3.9972	5.0424	3.5559	3.5133	3.4075	3.4468	2.8539	3.1766	3.3246	3.5368	2.9979	3.2277	4.0783	3.3756
30	3.9449	4.3592	4.7255	5.3938	4.4501	4.2020	3.8672	3.4533	3.3960	3.6233	3.6145	3.6327	3.5399	3.2729	3.5539	4.6098	3.1469
31	3.1507	4.5183	3.6761	4.7035	4.6547	3.2905	3.6228	3.9328	3.6294	3.0285	3.9808	3.6492	3.6951	3.2713	3.1892	3.5724	3.2166
32	3.5595	4.6745	4.3548	4.4698	4.8081	3.8110	3.1791	3.6497	3.5519	3.6263	3.6069	3.4313	3.3314	2.9496	3.3426	3.8828	3.3504
33	4.0262	4.0757	4.4525	4.3482	4.6368	3.6590	3.6736	3.8569	3.9925	3.7337	4.0009	3.7163	3.6568	3.5157	3.0315	3.7535	3.5872
34	3.3112	4.2820	4.0759	4.1565	5.5110	3.4902	3.9711	3.4708	3.8210	3.0239	3.9037	3.3185	3.5426	3.4024	3.3285	3.6013	3.0283

Clone no.	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
18	*****																
19	4.2929	*****															
20	2.2611	4.3044	*****														
21	2 .7934	3.7381	2.6113	*****													
22	4.0370	5.9374	4.1382	2.8479	*****												
23	3.7749	5.7189	4.1307	3.1600	3.9334	*****											
24	3.9604	5.7454	4.1772	3.4203	3.8834	3.8496	*****										
25	3.4818	4.7926	3.4664	3.3713	4.4837	3.8560	4.8441	*****									
26	3.7415	4.8987	3.6265	3.4833	4.3089	3.8378	4.4885	4.0061	*****								
27	3.8210	4.8218	3.5941	3.6411	4.3290	4.8406	4.8250	4.3088	3.9284	*****							
28	3.8265	4.7223	3.6744	3.7976	4.4475	4.7296	4.3145	4.4401	3.6061	4.1207	*****						
29	3.7273	5.0802	3.1423	3.1764	3.7642	3.9338	3.9793	3.9074	3.4138	4.0290	3.7306	*****					
30	3.2300	4.4571	3.3576	3.7652	4.8362	4.9201	5.2535	4.1332	4.3654	4.3638	4.3662	3.8227	*****				
31	3.2426	4.2782	3.2036	3.1735	3.9646	3.7402	3.4573	3.8715	4.2311	3.7918	4.1766	3.9653	4.5629	*****			
32	3.4533	4.7583	3.3655	3.5474	4.4053	4.2056	4.4643	4.3585	3.5190	4.0300	4.2902	3.4900	3.8986	3.6664	*****		
33	3.5394	4.9762	3.7082	3.9110	5.0799	4.5533	5.0307	3.9672	3.6708	4.7100	4.8576	3.8790	4.0802	4.2533	3.7072	*****	
34	3.3174	4.4522	3.6686	3.4087	4.9640	4.1714	4.9461	4.0821	3.7749	3.9512	4.1289	3.8828	4.2356	3.5100	4.3085	4.3924	****

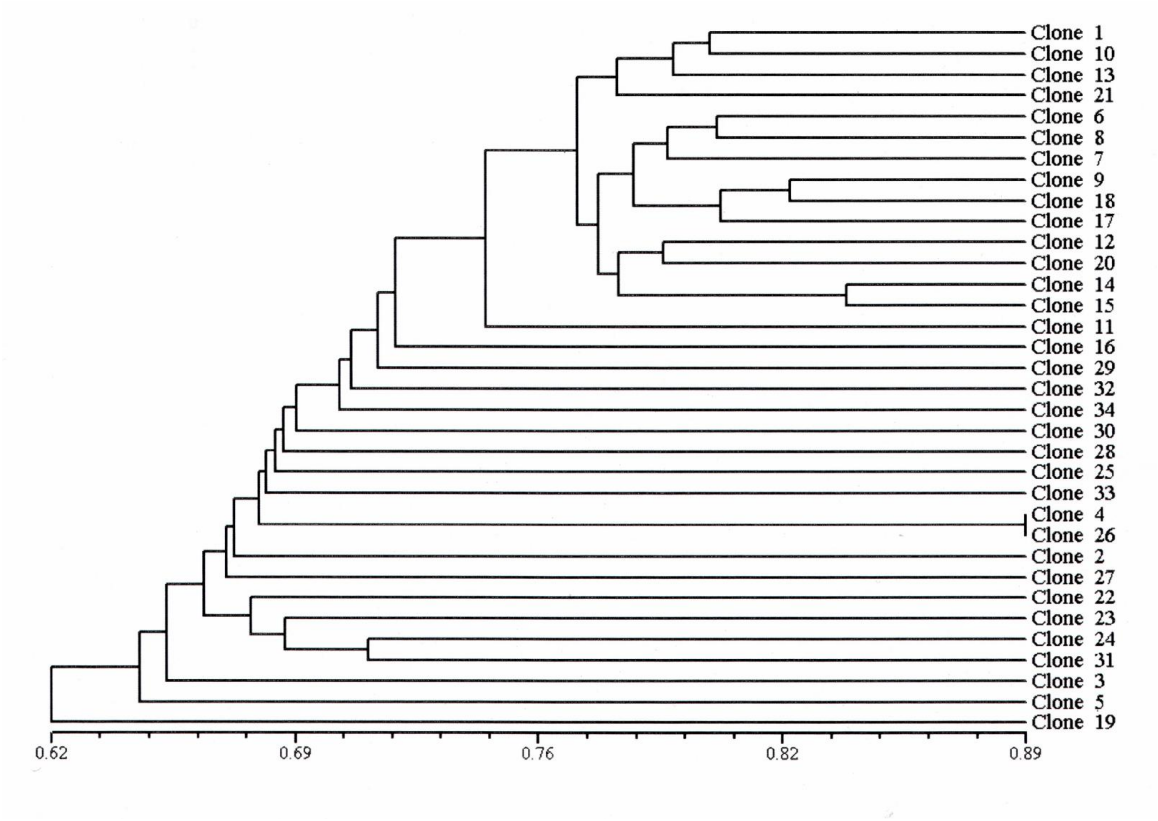

Figure 1. Phenogram constructed using the UPGMA method.

According to the genetic distance matrix presented in Table 5, genetic distance values ranged 5.9374 to 1.1453. The most closely related pairs were clone number 4 & 26, 14 & 15 and clone 9 & 18, with genetic distance of 1.1453, 1.7235 and 1.9155, respectively. These relationships are also reflected in Figure 1**.** The clone pairs (4 & 26, 14 &15 and 9 & 18) were collected from areas showing similar ecogeographic characteristic in Central Anatolia.

Selection of highly variable approximately 10-15 individuals is necessary to obtain a polycross in synthetic variety breeding. The clones with the highest genetic distance values in genetic distance matrix were selected (Table 5). Their genetic distance values ranged 5.9374 to 5.1229 and are listed in Table 6. The highest genetic distance value (5.9374) was found between clone 19 and 22. Related clone pairs showing high genetic distance were clone 19 & 24, 19 & 23, 5 & 23 and 5 & 19 with 5.7454, 5.7189, 5.7032 and 5.6895 respectively) followed closely by genetic distance matrix value of clone pair 19 & 22. It was seen that clone 4, 5 and 19 had the high genetic distance compared to other clones. However, there are no meaningful relations concerning locations of the clones showing high genetic distance.

Table 6. Selected clones for synthetic variety breeding

Clone	The highest genetic distance values according to Nei72 algorithm.
19-22	5.9374
19-24	5.7454
19-23	5.7189
5-23	5.7032
5-19	5.6895
5-24	5.5424
5-34	5.5110
4-30	5.3938
2-19	5.3755
5-21	5.3565
4-5	5.2924
24-34	5.2535
3-19	5.2298
4-22	5.1641
5-29	5.1229

The locations of the clone pairs showing high genetic distance indicate implications of different ecogeoprachic characteristics on development of plants.

A synthetic variety is developed by intercrossing a number of genotypes known for superior combining ability with high genetic distance. Therefore, synthetic variety is made up of genotypes previously tested for their ability to produce a superior progeny when crossed in all combinations in agreement with Ferreira et al. (1995) who emphasised that heterosis and the combining ability of parents depend directly on the genetic diversity between them and the chance of finding promising combinations is better when more divergent material is used.

The feasibility of using AFLP DNA markers in future marker-based assessments of genetic diversity in alfalfa was supported by the observation that hierarchical patterns of diversity among the germplasms were associated with their geographic, origins. Use of these primers for automated AFLP analysis could be used as a high-throughput system for accurately characterizing genetic diversity among large numbers of alfalfa populations for breeding purpose. This information should also prove useful in designing strategies to more efficiently manipulate heterosis in alfalfa. This approach could subsequently be refined to include individual genotype analysis for more detailed characterization of specific populations of interest.

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References

Bingham, E.T. 1980. Maximizing heterozygosity in autopolyploid. In: Polyploidy: Biological Relevance. (Eds. Lewis, W.H.) Plenum, New York, pp. 471–491.

Doyle, J.J. and J.L. Doyle.1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemcal Bulletin 19: 11-15.

Ferreira, D.F., A.C. Oliveira, M.X. Dos Santos, and M.A.P. Ramalho. 1995. Métodos de avaliação da divergência genética em milho e suas relações com os cruzamentos dialélicos. Pesquisa Agropecuária Brasileira 30: 1189-1194.

Moreno-Gonzales, J. and J.I. Cubero. 1994. Selection strategies and choise of breeding methods. In: Plant Breeding. (Eds. Hayward, M.D., Bosemark, N.O. and Omagosa, I.), Chapman and Hall, London, UK.

Kidwell, K.K., E.T. Bingham, D.R. Woodfield and T.C. Osborn. 1994a. Relationships among genetic distance, forage yield and heterozygosity in isogenic diploid and tetraploid alfalfa populations. Theoretical and Applied Genetics 89: 323-328.

Kidwell, K.K., D.R. Woodfield, E.T. Bingham and T.C. Osborn. 1994b. Molecular marker diversity and yield of isogenic 2x and 4x single-crosses of alfalfa. Crop Science 34: 784-788.

Labombarda, P., F. Pupilli and S. Arcioni. 2000. Optimal population size for RFLP-assisted cultivar identification in alfalfa (*Medicago sativa* L.). Agronomie 20: 233–240.

Maureira, I.J., F. Ortega, H. Campos, and T.C. Osborn. 2004. Population structure and combining ability of diverse *Medicago sativa* germplasms. Theoretical and Applied Genetics 109: 775–782.

Osborn, T.C., D.J. Brouwer and K.K. Kidwell. 1998. Exploiting genome differences for higher alfalfa forage yield. Proceedings of the North American Alfalfa Improvement Conference, Bozeman, MT. 95.

Rohlf, J. 2000. Numerical taxonomy and multivariate analysis system. Version 2.1. Exeter Software, Applied Biostatistics Inc, New York, USA.

Rumbaugh, M.D., J.L. Caddel and D.E. Rowe. 1988. Breeding and Quantitative genetics. In: Alfalfa and alfalfa improvement (Eds. Hanson, A.A., Barnes, D.K. and Hill, R.R.). Agronomy monograph no. 29. ASA, CSSA, and SSSA, Madison, Wis. 777–807.

Segovia-Lerma, A., R.G. Cantrell, J.M. Conway and I.M. Ray. 2003. AFLP-based assessment of genetic diversity among nine alfalfa germplasms using bulk DNA templates. Genome 46: 51-58.

Sumberg, J.E., R.P. Murphy and C.C. Lowe. 1983. Selection for fiber and protein concentration in a diverse alfalfa population. Crop Science 23: 11–14.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Fritjers, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP - a new technique for DNA-fingerprinting. Nucleic Acids Research 23: 4407–4.

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