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Research Article

DETECTING ALFALFA (*MEDICAGO SATIVA* L.) POPULATIONS USING MORPHOLOGICAL TRAITS AND RAPD MARKERS

Hassan Monirifar [1]*, Sajjad Moharramnejad [2]

[1] *Crop and Horticultural Science Research Department, East Azerbaijan Agricultural and Natural Resources Research and Education Center, AREEO, Tabriz, Iran*
[2] *Crop and Horticultural Science Research Department, Ardabil Agricultural and Natural Resources Research and Education Center, AREEO, Moghan, Iran*

**Corresponding Author:*
E-mail: monirifar@yahoo.com

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ABSTRACT. In order to evaluate several alfalfa populations from East Azerbaijan of Iran, an experiment based on randomized complete block design was performed under field conditions during three appropriate growing seasons (2010-2013) at the East Azarbaijan Agricultural and Natural Resources Research and Education Center, Tabriz, Iran. Thirty alfalfa populations were selected from East Azerbaijan, Iran. The morphological traits and molecular marker (RAPD) were evaluated in all alfalfa populations. The result showed that the most morphological attributes had a significant difference between alfalfa populations, and it was indicated that each alfalfa population is related to various locations of East Azerbaijan. Correlation drawn between dry weight and agronomic attributes in the alfalfa populations showed that dry weight was strongly correlated with plant height. Cluster analysis, using UPGMA procedure, based on RAPD banding pattern in 30 alfalfa populations formed four groups. Overall, all alfalfa populations had more genetic diversity and can be used in a breeding program that can be made synthetic cultivars.

Key words: *Alfalfa, cluster, diversity, environment, germplasm, morphological*

INTRODUCTION

Alfalfa (*Medicago sativa* L.), a fundamental forage crop grown in the temperate regions, is cultivated over 32 million hectares worldwide [15] and about 680 thousand hectares in the northwest of Iran. In Iran, cultivated alfalfa mostly contains various adaptive landraces consisting of Gharayonja and Hamadani as well as occasionally introduced cultivars such as Ranger or Maopa [16].
Alfalfa cultivars (2n = 4x = 32), with allogamous fertilization, are autotetraploid which show high variability [4]. The intra-varietal variability, environment impact, and life cycle make it hard to apply morphological or agronomic traits to explain the variations in alfalfa [5].

The selection for breeding improvement is experienced by genetic diversity**.** To raise population varieties, the selection range becomes more extended. Morphological attributes of alfalfa are being used to categorize and assess its genetic diversity in the germplasm collections [5]. Further, the molecular markers including RAPD [3], AFLP [5], RFLP [9], and SRAP [12] are documented to detect the genetic diversity of alfalfa. RAPD was used by Wang et al. [17] to evaluate genetic

variability of 7 alfalfa cultivars in Northwest China, a total of 132 discernible loci were obtained for all populations using 10 primers, and 88.64% of these loci were polymorphic, which indicated that a high diversity existed in the cultivars from Northwest China. The six RAPD primers generated 90 polymorphic bands across the 300 individual plants analyzed, and also genetic distance among cultivars/population ranged from 0.28 to 0.40 [13].

The common alfalfa cultivars are the synthetic ones generated by randomly crossing selected parents [16]. Consequently, each genotype selection could be used as a good parent to combine in improving programs, and this is a serious goal for breeding programs of forage plants [8]. The aim of the current research was to assess the alfalfa populations' response to the East Azerbaijan region in Iran and to group them by morphological traits and molecular markers.

MATERIALS AND METHODS

Plant source

The plant source of the current study included 30 alfalfa populations that were acquired from 16 states of East Azarbaijan, Iran (Fig. 1 and Table 1). The alfalfa populations were sowed in single pots including a blend of sandy-loam soil. The alfalfa populations were transplanted to field conditions at the East Azarbaijan Agricultural and Natural Resources Research and Education Center, Tabriz, Iran, and evaluated them during growing seasons (2010-2013). In each plot, 24 plants were transplanted in the 4-row plot (6 plants in each row) 0.6 m apart and 2.4 m long. The experiment was conducted based on a randomized complete block design in four replications. The measurements of plant height, fresh as well as dry weight, ratios of leaf fresh and dry weight to stem fresh and dry weight were performed from the two central rows.

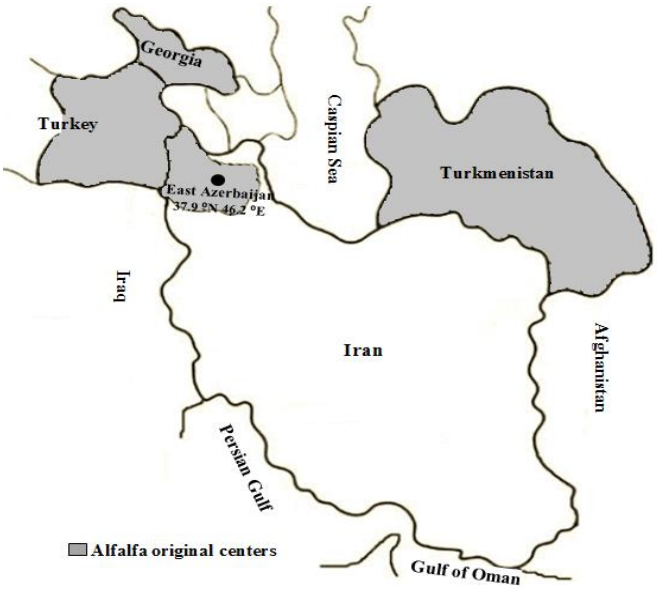

Fig. 1. *East Azerbaijan, Iranian alfalfa populations and alfalfa original centers*

Table 1. *List of alfalfa populations from East Azerbaijan, Iran*

No.	Locality	District	County
1	Marazad	Siah-Rud	Jolfa
2	Qeran-Chay	Mulan	Kaleybar
3	Lighan	Dodangeh	Hurand
4	Zonuzaq	Zonuz	Marand
5	Siran	Zonuz	Marand
6	Khvor-Khvor	Ilkhchi	Osku
7	Satellu	Khosrowshah	Tabriz
8	Ismailabad	-	Malekan
9	Gol-Tappeh	-	Maragheh
10	Almalu	-	Ajab Shir
11	Kordeh-Deh	-	Maragheh
12	SeparoKhun	Liqvan	Tabriz
13	Qareh-Baba	Qareh-Chay	Bostanabad
14	Zu-ol-Bin	-	Hashtrud
15	Zaviyeh	-	Hashtrud
16	Siviyar	-	Hashtrud
17	Akramabad	-	Hashtrud
18	Balesin	Tark	Mianeh
19	Bashkand	Tekmeh-Dash	Bostanabad
20	Eyn-ol-Din	Tekmeh-Dash	Bostanabad
21	Baftan	Bahreman	Sarab
22	Yurchi-Gharbi	-	Sarab
23	Khajeh	-	Heris
24	Gowaravan	-	Heris
25	Dizaj	-	Varzaqan
26	Kordlar		Ahar
27	Jushin	Kharvana	Varzaqan
28	Chelanab	-	Varzaqan
29	Alharod	-	Varzaqan
30	Ghara-yonjeh*	-	-

*A majority cultivar in Iran

RAPD markers

DNA genomes from each alfalfa populations were extracted [5]. The value and sincerity of DNA extractions were measured by spectrophotometry and 1% agarose gel electrophoresis. The RAPD primers were 10 including OPJ_4 (5'CCGAACACGG3'), B_1 (5'GGTTCGCTCC3'), B_6 (5'TGCTCTGCCC3'), B_7 (5'GGTGACGCAG3'), B_8 (5'GTCCACACGG3'), OPJ_{13} (5'CCACACTACC3'), B_{10} (5'CTGCTGGGAC3'), OPA_1 (5'CAGGCCCTTC3'), OPJ_{19} (5'GGACACCACT3') and OPJ_{20} (5'AAGCGGCCTC3'). RAPD reactions were carried out using the following program: initial denaturation at 94ºC for 4 min, 40 cycles of denaturation at 93ºC for 1 min, annealing at 40ºC for1 min, and extension at 72ºC for 100 secs with a final extension at 72ºC for 6 min. The reactions were set in the total volume of 25 µl containing 12.5 µl PCR Master Mix (CinnaGen PCR Master Kit, Cat. No. PR8250C), 1 µl of DNA template (25 ng), 1 µl of arbitrary primers

(4 pmol μl^{-1}). Amplified fragments were visualized on 1.5% agarose gel stained by 0.1 g ml^{-1} ethidium bromide (EtBr) [8].

Statistical analysis

The alfalfa populations were detected RAPD bands as presence and absence, and the data was obtained in the binary matrix including variables of discrete which was 1 for presence and 0 for absence. Pairwise F_{ST} matrix and molecular variance using the hierarchical analysis of molecular variance were investigated by ARLEQUIN 2.0 software [17]. Cluster analysis was done by a pairwise F_{ST} matrix. Cluster analysis and genetic homology computing were performed using NTSYS-pc 2.02. Allelic polymorphic information content (PIC) was determined using the formula below [5]:

PIC = $1 - \Sigma(Pi\ j)^2$, $Pi\ j$: the ith allele frequency in the jth population.
The mean comparisons were performed using LSD test ($p \leq 0.05$). Analysis of data used SPSS 16.0 to morphological traits.

RESULTS AND DISCUSSION

Combined analyses showed a highly significant effect of the alfalfa population on the height, fresh as well as dry weight, and the ratios of leaf fresh and dry weight to stem fresh and dry weight (variance analysis not shown). Monirifar [10] observed significant differences between 13 alfalfa ecotypes in terms of plant height and the ratios of biomass and stem fresh and dry weight. Alfalfa forage yield correlates with four factors containing plant number per unit area, plant height, stem number per plant, and single-stem yield [15], which are used in breeding programs. Tucak et al. [13] indicated that the plant height of alfalfa is one of the forage yield components, and is frequently applied as a standard in the early stage of selection. The output of current research is in a good settlement with the detections [10].

The mean comparisons of plant height, biomass, rations of leaf fresh and dry weight to stem fresh and dry weight are shown in Table 2. It seems that the alfalfa populations have a high genetic diversity. In general, the coefficient of variation shows the degree of difference and power of trait variation. The results indicated a broad amount range of wide diversity among morphological traits which were plant height and fresh as well as dry weight by studying alfalfa populations. Plant fresh and dry weight had the highest variation coefficient (9.89 and 10.09%) while the average weight per plant from 15.177 to 24.822 g (Table 2). The Satellu population had more plant weight than other alfalfa populations (Table 2). The plant height average was between 69.106 and 80.589 cm, and also the Satellu population had maximum plant height (Table 2). Arab et al. [1] obtained similar results in trait variation. Mohammadzadeh Jalaly et al. [6] reported that forage yield and quality are intricate attributes that are affected by plant genetic structure as well as surrounding elements. Because of the above-mentioned reasons, specifying the genetic profile of the alfalfa landraces and establishing the interconnection between their characteristics, are of great importance [10]. As a result, we could be introduced to the Satellu population for breeding program East Azarbaijan, Iran.

Correlation drawn between dry weight and agronomic attributes in the alfalfa populations showed that dry weight was strongly correlated with plant height (Fig. 2).

Correlations between agronomic traits in the 12 alfalfa half-sib families indicated that dry weight had a positive correlation with plant height [15].

Table 2. *Mean comparisons of morphological traits in alfalfa populations*

Population	Plant height (cm)	Fresh weight (g/ plant)	Dry weight (g/ plant)	Fresh weight of leaf/stem	Dry weight of leaf/stem
Marazad	71.148	17.006	8.449	0.493	0.430
Qeran-Chay	71.111	17.450	8.669	0.496	0.435
Lighan	76.371	21.606	10.726	0.493	0.432
Zonuzaq	70.889	15.569	7.737	0.484	0.418
Siran	75.511	20.850	10.352	0.495	0.433
Khvor-Khvor	71.398	16.378	8.138	0.496	0.434
Satellu	80.589	24.822	12.426	0.511	0.460
Ismailabad	72.478	17.498	8.664	0.496	0.433
Gol-Tappeh	72.388	17.100	8.459	0.493	0.431
Almalu	72.911	17.864	8.874	0.498	0.435
Kordeh-Deh	71.944	16.729	8.312	0.498	0.435
SeparoKhun	74.067	18.559	9.083	0.494	0.431
Qareh-Baba	74.300	19.239	9.554	0.502	0.439
Zu-ol-Bin	70.467	15.177	7.543	0.491	0.429
Zaviyeh	71.000	15.783	7.844	0.493	0.428
Siviyar	72.567	16.944	8.418	0.498	0.435
Akramabad	69.106	14.289	7.104	0.494	0.434
Balesin	71.111	18.828	7.866	0.493	0.427
Bashkand	69.811	15.828	7.866	0.493	0.431
Eyn-ol-Din	71.411	16.259	8.079	0.498	0.435
Baftan	75.700	21.294	10.57	0.516	0.459
Yurchi-Gharbi	75.786	20.704	10.279	0.512	0.455
Khajeh	75.880	21.074	10.463	0.495	0.432
Gowaravan	70.419	15.400	7.654	0.491	0.430
Dizaj	74.933	19.922	9.892	0.497	0.434
Kordlar	70.252	15.433	7.671	0.494	0.432
Jushin	74.000	19.809	9.837	0.495	0.432
Chelanab	70.144	15.300	7.605	0.486	0.424
Alharod	76.833	22.020	10.931	0.493	0.431
Ghara-yonjeh*	77.222	22.647	11.241	0.495	0.432
$LSD_{5\%}$	2.001	2.002	0.991	0.008	0.007

*A majority cultivar in Iran

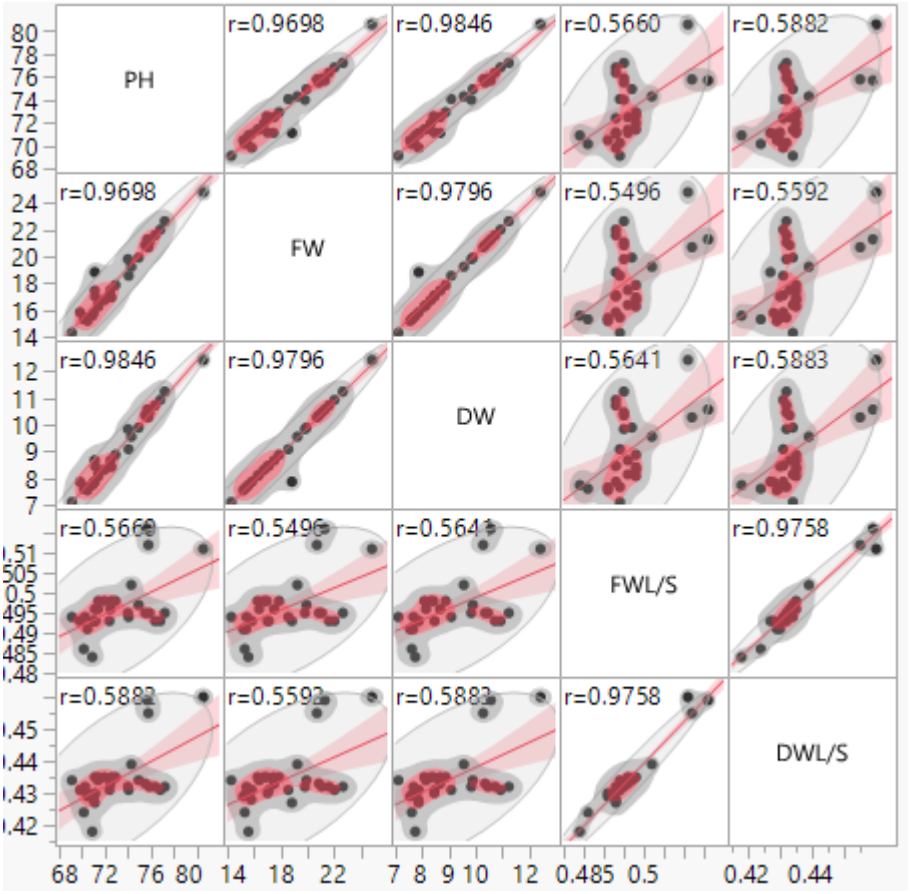

Fig. 2. *Pearson's correlation coefficients between plant height (PH), fresh weight (FW), dry weight (DW), fresh weight leaf/stem (FWL/S) and dry weight leaf/stem in the 30 alfalfa populations.*

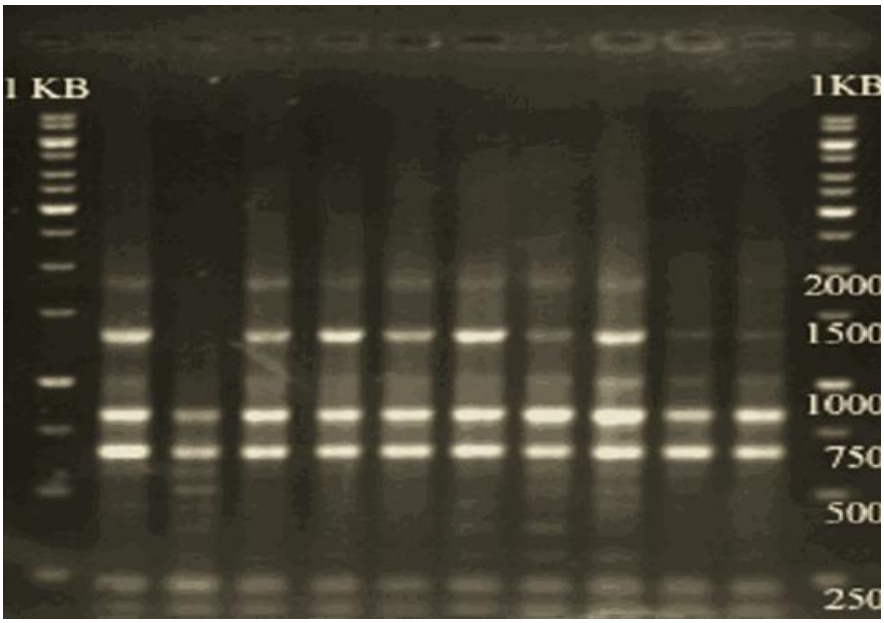

Fig. 3. *RAPD fragments in alfalfa populations using the primer B_6 in bulk analysis*

Among 30 RAPD primers used in current research, 10 primers made reproducible bands. RAPD pieces showed a high variation in alfalfa populations (Fig. 3). The polymorphic bands which were associated with RAPD markers were 78 in the alfalfa populations. SeparoKhun and Jushin had minimum and maximum polymorphism in all alfalfa populations, respectively (Table 3). In this study, the mean percentage of polymorphism in alfalfa populations was determined as 63.25%. Jushin population had the highest, and also SeparoKhun and Bashkand populations had the lowest genetic variation (Table 3).

AMOVA was also carried out to measure within and between genetic diversity in the alfalfa populations (F_{ST} = 0.346; P = 0.01). The diversity between populations was considerable, and genetic variation within populations was measured as 81.37%. The genetic differentiation degree between populations (G_{ST}) was calculated as 0.3335. As a result, it can be inferred that diversity between populations was lower than within populations.

Cluster analysis using UPGMA method which used genetic distance amounts RAPD indicated a remarkable sharpness dendrogram form alfalfa populations. In this research, thirty alfalfa populations were placed into four major groups (Fig. 4). The matrix correlation was calculated as 0.731.

Reproducibility is the main factor in RAPD researches [14], just reproducible bands were applied in the current study. Mohammadzadeh et al. [5] reported respectively 0.2349 and 0.1892 as the whole genetic diversity (H_T) and within-population genetic diversity (H_s). A high degree of genetic diversity is shown in alfalfa populations [4]. Northwestern of Iran is the main area covering diverse alfalfa plants, thus there is a high potential of genetic within and among the populations employed in this work as a fraction of Iranian alfalfa populations [5]. The within-population variation based on Nei's gene diversity was higher to all alfalfa populations, as found in recent studies, and the results of the current study are in accordance with these findings [2, 5, 16].

AMOVA analysis revealed that the highest ratio of genetic diversity was ascribed to diversity within alfalfa populations. The results were in agreement with previous studies [3, 6, 15, 18]. Genetic interval amounts were applied to create a UPGMA dendrogram in which alfalfa genotypes were divided in four groups. Mohammadzadeh, et al. [7] was done cluster analysis by Nei's for alfalfa populations and showed that genetic distances were grouped in 3.

Table 3. *Population polymorphism and gene diversity*

Population	Number of polymorphic bands	Percentage of polymorphic bands	*He*	*I*
Marazad	45	57.69	0.2049	0.3064
Qeran-Chay	45	57.69	0.2081	0.3102
Lighan	39	50.00	0.1823	0.2690
Zonuzaq	42	53.85	0.1847	0.2768
Siran	47	60.26	0.2089	0.3132
Khvor-Khvor	44	56.41	0.1969	0.2953
Satellu	54	69.23	0.2083	0.3187
Ismailabad	45	57.69	0.2001	0.3013
Gol-Tappeh	50	64.10	0.2098	0.3165
Almalu	52	66.67	0.2271	0.3428

Table 3. *(Continuation of the table)*

Population	Number of polymorphic bands	Percentage of polymorphic bands	*He*	*I*
Kordeh-Deh	62	79.49	0.2571	0.3891
SeparoKhun	36	46.15	0.1633	0.2434
Qareh-Baba	52	66.67	0.1912	0.2972
Zu-ol-Bin	46	58.97	0.2037	0.3043
Zaviyeh	42	53.85	0.1891	0.2816
Siviyar	51	65.38	0.2243	0.3377
Akramabad	48	61.54	0.2205	0.3294
Balesin	47	60.26	0.2039	0.3076
Bashkand	52	66.67	0.1567	0.2542
Eyn-ol-Din	41	52.56	0.1744	0.2632
Baftan	51	65.38	0.2201	0.3299
Yurchi-Gharbi	49	62.82	0.2162	0.3248
Khajeh	59	75.64	0.2299	0.3517
Gowaravan	40	51.28	0.1756	0.2631
Dizaj	63	80.77	0.2464	0.3723
Kordlar	53	67.95	0.2242	0.3383
Jushin	68	87.18	0.2813	0.4205
Chelanab	41	52.56	0.1744	0.2613
Alharod	66	84.62	0.2812	0.4185
Ghara-yonjeh	50	64.10	0.2001	0.3082
Mean	49.33	63.25	0.2088	0.3149

H_e: Nei'gene diversity
I: Shannons information index

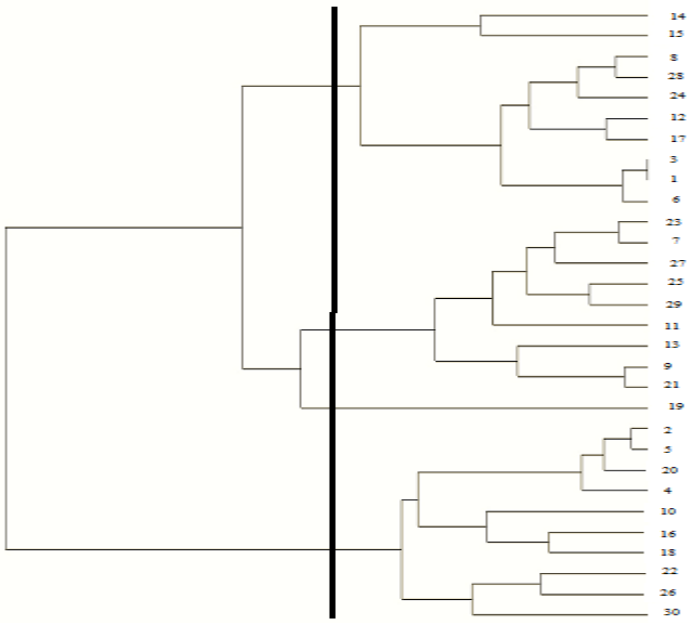

Fig. 4. *UPGMA dendrogram for alfalfa populations*

CONCLUSION

The current study showed that most morphological attributes had a significant difference between alfalfa populations. Heatmap correlation between agronomic attributes indicated a significant correlation of fresh and dry weight with plant height. Among 30 RAPD primers used in current research, 10 primers made reproducible bands. RAPD pieces showed a high variation in alfalfa populations. The polymorphic bands which were associated with RAPD markers were 78 in the alfalfa populations. The results showed that variation within and between populations (F_{ST} = 0.346) and the genetic differentiation degree between populations (G_{ST}) was calculated as 0.3335. The cluster analysis based on RAPD markers in the 30 alfalfa populations showed four groups. These alfalfa populations had more genetic diversity and can be used in breeding programs which can be made synthetic cultivars.

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