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Genetic Diversity of Alfalfa Grown in Northern Turkey by Random Amplified Polymorphic DNA and Relationship with Morphological Traits

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In this research, genetic diversity among alfalfa ecotypes grown Northern part of Turkey by random amplified polymorphic DNA (RAPD) markers and morphological traits to analyze differences among alfalfa ecotypes were studied and usability of RAPD markers for estimation of genetic diversity among alfalfa ecotypes in comparison with morphological traits were evaluated. Seven RAPD markers generated polymorphic patterns, yielding a polymorphism rate of 70 %. The average genetic similarity among the alfalfa ecotypes/cultivars was 0.51 with values ranging from 0.343 between Ladak and Adiguzel and 0.88 between Kayseri and Mollakasir ecotypes, having the highest genetic similarity. It was followed by similarities between Arrow and L1312; Savas and Dilburnu; Savas and Çayirbasi; Kayseri and Alaköy genotypes with 0.80 similarity. In the principal component analysis (PCA), the first two principal components explained about 69 % of the variation in morphological traits. A Mantel's test showed low correlation between RAPD and morphological data distance matrices.

Key Words: *Medicago sativa*, Genetic diversity, Morphological traits, Ecotypes, RAPD.

INTRODUCTION

It is an important step to measure genetic diversity in crops for successful breeding and creation of new cultivars. The study of phenotypic and genetic diversity to identify groups with similar genotypes is important for conserving, evaluating and utilizing genetic resources; for studying the diversity of pre-breeding and breeding germplasm; and for determining the uniqueness and distinctness of the phenotypic and genetic constitution of genotypes with the purpose of protecting a breeder's intellectual property rights[1].

Alfalfa (lucerne), *Medicago sativa* L., a major forage crop throughout the world and the most important forage legume in Turkey (288 thousand ha), is an outcrossing seed-propagated species whose populations have a

genetic structure complicated by the tetrasomic inheritance and by a rate of selfing which varies according to the environmental conditions[2].

Ecotypes (local landraces) represents a significant share of alfalfa cultivated in Turkey Zaccardelli *et al.*[3] defines an ecotype as a population growing in a specific geographical environment, whose individuals share a common gene pool. The adaptation zone of an ecotype is the region where it has evolved under local climatic, pedological and biotic conditions and in the case of cultivated ecotypes, also under anthropic pressure.

Molecular markers detect variation of the DNA sequences among cultivars and therefore directly bypass problems connected with environmental effects. The random amplified polymorphic DNA (RAPD) technique, regardless of its sensitivity to reaction conditions and problems with repeatability and amplifying of non-homologous sequences[4] is a quick and inexpensive non-radioactive method and has also been widely used in plant population studies[5]. In alfalfa, RAPDs have been evaluated for segregation analyses[6,7], for assessing germplasm introgression[8], constructing genetic maps[9,10], as well as assessing intra- and inter- species variation among annual medics[11] and estimating relationships among alfalfa populations[12,13].

The aims of this research were to study genetic diversity in alfalfa ecotypes grown Northern part of Turkey using RAPD markers, morphological traits to analyze differences among in alfalfa ecotypes and to compare results based on RAPD markers and morphological traits.

EXPERIMENTAL

Random amplified polymorphic DNA (RAPD): Bulk immature unifoliate leaves was used for extracting DNA as described by Dellaporta *et al.*[14]. The reactions were performed in 0.2 µL tubes in Mastercycler personel apparatus (Eppendorf, Germany) programmed to cycle 45 times under the following conditions: for the first two cycles, denaturation for 30 s at 94 ºC, annealing for 60 s at 37 ºC and elongation for 2 min at 72 ºC; second two cycles, denaturation for 30 s at 94 ºC, annealing for 60 s at 35 ºC and elongation for 2 min at 72 ºC; the subsequent 41 cycles were run with the denaturation temperature reduced to 93 ºC, followed by a 4-min hold at 72 ºC. After amplification, the reaction products were separated by electrophoresis in 1.4 % agarose gels, stained with ethidium bromide and photographed under ultraviolet light with Nikon Coolpix5000. A total of 10 primers were used based on the band resolution and polymorphism they provided.

Data analysis: DNA bands were scored as 0 (absence) or 1 (presence). Genetic similartiy between two cultivars i and j was estimated following the formula of Nei and Li[15]. Based on the genetic similarity matrix (denoted GS), UPGMA cluster analysis were used to assess pattern of diversity among the barley entries. Dendrograms were created with the TREE program of

NTSYS. All calculations were performed using the NTSYS-pc version 2.1 software[16].

A principal component analysis (PCA) was performed on observed morphological traits after standardization. Based on standardized trait values, euclidian distances (mdij) between the lines were calculated. Morphological similarities (msij) were also calculated as (1-mdij). Matrix of these values is denoted MS. Using the matrix (denoted MD) of euclidian distances, an UPGMA cluster analysis was performed producing a second dendrogram depicting relationships among cultivars relative to their morphological characteristics. As for genetic similarity, the cophenetic correlation was calculated to measure the quality of the clustering with regard to the original data.

Morphological and agronomic traits: Eight traits (Table-2) were scored on the 15 alfalfa cultivar/ecotypes. All traits were evaluated on ten plants taken from a row plot of 2 m and two replicates. Traits were morphological trait (PH) and yield components (FHY1, FHY2, FHY3, DHY1, DHY2, DHY3, NS). All traits were standardized before analysis by subtracting the mean value and dividing by the standard deviation; this allows removing scale effects before calculating Euclidian distances.

Comparison between RAPD markers and morphological traits: Simple (r) coefficients between the 105 values of genetic similarities (gsij) and morphological similarities (msij) were calculated. P-values for these coefficients were calculated based on their respective asymptotic distributions[17]. Correspondence between the two similarity matrices GS and MS (matrix of msij values) was tested with the Mantel Z statistic[18]. Significance of Z was determined by comparing the observed Z values with a critical Z value obtained by calculating Z for one matrix with 500 permuted variants of the second matrix. All computations were performed with appropriate procedures of the NTSYS-pc version 2.1 software[16]. The purpose of these comparisons was to evaluate the usefulness of RAPD markers as predictors of morphological variability in the genotypes studied.

RESULTS AND DISCUSSION

RAPD polymorphism and genetic distance: Ten RAPD markers dispersed across the genome were used to test the genetic diversity of 15 ecotypes/cultivars. Seven RAPD markers generated polymorphic patterns and three did not give any bands, yielding a polymorphism rate of 70 %. The 7 primers used generated 35 amplification products (bands), with an average of 5 bands per primer. Of these, 34 were polymorphic (4.86 bands per primer) and 1 was monomorphic (0.14 bands per primer). The number of polymorphic bands varied from one for the primers B-10 to eight for the primer B-1 (Table-1).

TABLE-1
NUCLEOTIDE SEQUENCES OF 7 POLYMORPHIC PRIMERS AND TOTAL NUMBER OF BANDS PER PRIMER

Primers	Nucleotide sequences	Total number of RAPD bands
B-8	5'-GTCCACACGG-3'	2
B-10	5'-CTGCTGGGAC-3'	1
B-1	5'-GTTTCGCTCC-3'	8
SD-1	5'-GGTACTCCAG-3'	7
RF-1	5'-GTAGCTGACG-3'	7
A-11	5'-CAATCGCCGT-3'	6
A-19	5'-CAAACGTCGG-3'	4

TABLE-2
EIGHT MORPHOLOGICAL TRAITS USED TO CALCULATE MORPHOLOGICAL DISTANCES

Code	Traits
FHY1	Fresh hay yield at first cutting
FHY2	Fresh hay yield at second cutting
FHY3	Fresh hay yield at third cutting
DHY1	Dry hay yield at first cutting
DHY2	Dry hay yield at second cutting
DHY3	Dry hay yield at third cutting
PH	Plant height
NS	Number of stem

The average genetic similarity among the alfalfa ecotypes/cultivars was 0.51 with values ranging from 0.343 between Ladak and Adiguzel and 0.88 between Kayseri and Mollakasir ecotypes, having the highest genetic similarity. It was followed by similarities between Arrow and L1312; Savas and Dilburnu; Savas and Çayirbasi; Kayseri and Alaköy genotypes with 0.80 similarity.

UPGMA dendrogram (Fig. 1) was drawn to show a visual picture of the relationships among the ecotypes/cultivars. The dendrogram based on the RAPD analysis (Fig. 1) showed that there are two major clusters originated from a common branch, but are distinct from each other. First cluster includes Ladak and Ercis genotypes. In addition; L1312, Arrow, Otluca, Köprüler, Burcu 24, Adigüzel, Alaköy, Kayseri and Mollakasir genotypes fall into same sub-group of second cluster. Meantime, Gülsinberk, Çayirbasi, Savas and Dilburnu genotypes were sub-grouped in the second cluster.

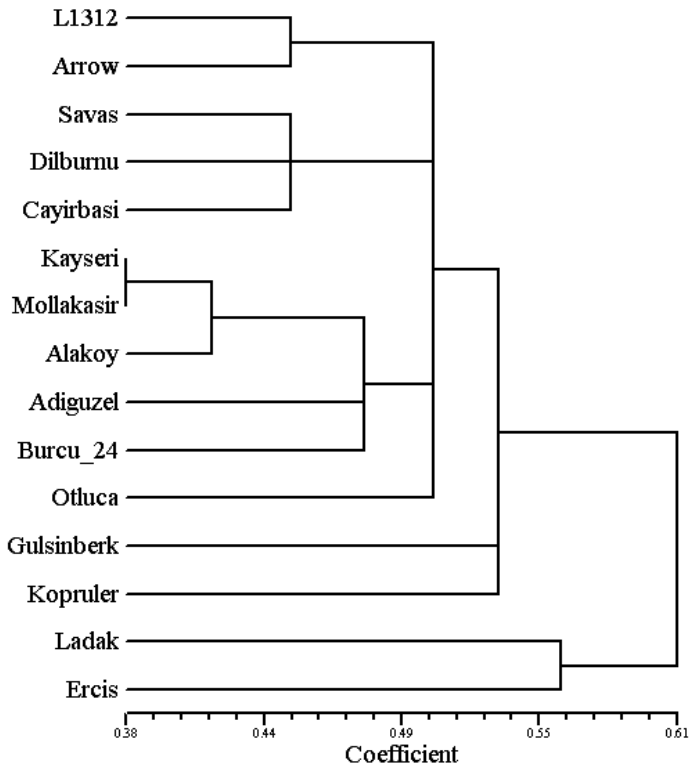

Fig. 1. Dendogram of alfalfa genotypes based on RAPD data using UPGMA

Morphological analyses: In the principal component analysis (PCA), the first two principal components (having eigen values > 1) explained about 69 % of the variation. The first axis indicated about 51.6 % of the variation. It was linked to variable related to dry hay yield at second cut (DHY2) which was positively correlated to FHY2 (r = 0.89), FHY3 (r = 0.71), DHY1 (r = 0.53) and DHY2 (r = 0.69). The second axis, explaining 17.5 % of the variation was fresh hay yield at first cutting. It was only correlated positively with DHYI (r = 0.87). Based on the projection of varieties in the principal plan (Fig. 2), there are main one branch and one group. Branch was cultivar Savas which was distinct than other cultivar/ecotypes based on morphological data. Interestingly, main group was also subdivided into one branch *cv. Ercis* and one subgroup, which was cascaded seven subgroups. Otluca and Ladak was the closest and followed by pair of Dilburnu and Burcu. It can be noted that these cascaded branch and groups are clearly distinguished in the principal plan. The morphological-based dendrogram (Fig. 3) has a good fit to the MD matrix (rcs = 0.79) and gave results in good agreement with the variety grouping obtained from the principal components analysis.

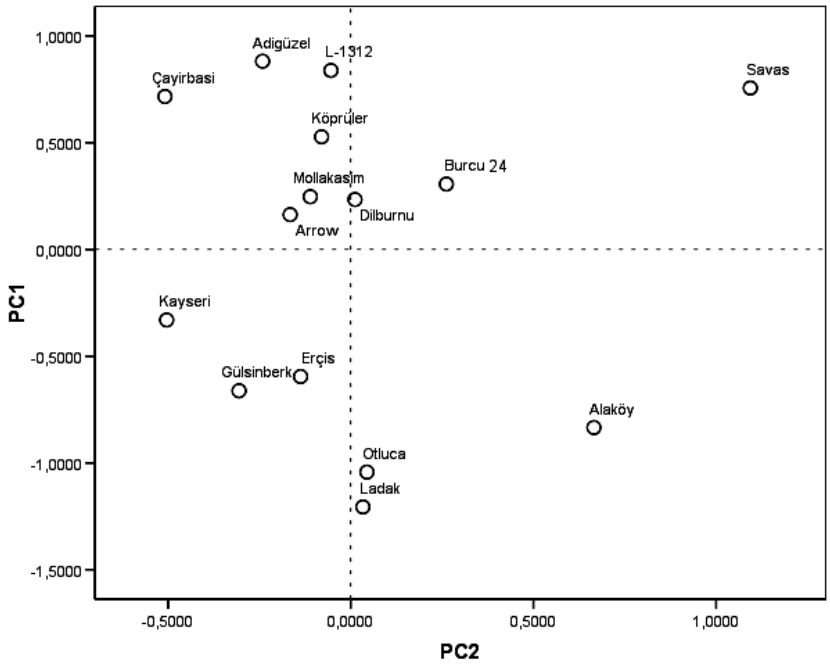

Fig. 2. Principle component analysis two-dimensional plots of 15 alfalfa genotypes based on morphological trait values

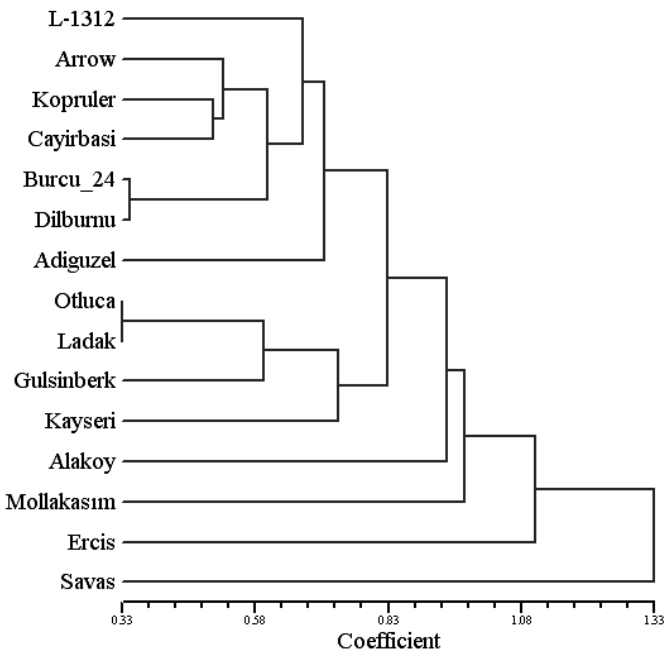

Fig. 3. Dendogram of alfalfa genotypes based on morphological data using UPGMA

A Mantel's test showed low correlation (r = 0.0013) between RAPD and morphological data distance matrices.

In this study, the utility of RAPD and morphological characters in the analysis of alfalfa germplasm were compared. Both RAPD and morphological characters were sufficient to assess the variability in alfalfa genotypes.

The level of polymorphism was superior or equal to previous reports for the alfalfa[3,19]. The elevated level of polymorphism may have been influenced because the primers used were strictly pre-selected with regard to the number and quality of the amplification products.

The UPGMA dendongrams confirmed the differences RAPD and morphological traits to find out genetic variability of alfalfa. No correlation was found between the GD matrices obtained by molecular and morphological data. Similar result was also obtained from other studies in literature[20]. This could be either related with the RAPD technique or some with the analysis of morphological traits. There is always influence of environment in morphological traits and they can show considerable variation. Also, some traits can be incorrectly measured and so cause problems in the estimation of genetic diversity. Finally, the number and choice of morphological traits and sample size can also affect the correlation. In this study, 8 morphological traits were examined. It is possible that if more morphological traits had been used a better correlation with RAPDs would have been obtained. The results of this study indicate that RAPD analysis could be successfully used for the estimation of genetic diversity among alfalfa genotypes.

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