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GENETIC DIVERSITY OF ALFALFA DOMESTICATED VARIETAL POPULATIONS FROM LIBYAN GENBANK REVEALED BY RAPD MARKERS

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Abstract: Alfalfa (*Medicago sativa* L.) is an important forage legume in Libya. The genetic diversity of nine alfalfa domesticated varietal populations was studied using thirteen RAPD primer combinations. The number of polymorphic fragments detected per primer combination ranged from 8 to 46 bands with an average of 24 bands. The number of polymorphic bands detected was from 6 (Atalia population) to 37 (Gabsia population). The lowest genetic distance was 0.058 and the highest was 0.655. The average genetic distance was (0.356). The dendrogram based on Ward's minimum variance clustering method grouped the nine populations into the two main clusters. The first group included Fazania, Atalia, Masratia, Zawia, Denamo Ferade and Arezona. The second group was composed of Tagoria, Gabsia and Wade Alrabeh. The simplicity of RAPD assays for detection of genetic polymorphisms is confirmed in our study, and results can be utilized in breeding practice.

Key words: Alfalfa, genetic diversity, RAPD

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most important forage crop with high biomass yield, whose excellent nutritive value makes it ideal for dairy and livestock production (Veronesi et al., 2010). Michaud et al. (1988) reported that alfalfa originated in the Caucasus. It is grown in large areas between 55˚ northern and 50˚ southern latitude and at 2500 m above sea level (Ivanov, 1988) due to its good adaptability to different environment conditions, such as high water salinity and drought (Garnett et al., 2004). The major alfalfa producing regions are North America with 11.9 million ha (41%), Europe with 7.12 million ha (25%), South America with 7 million ha (23%), Asia 2.23 million ha (8%), Africa (2%) and Oceania (1%) (Yuegao and Cash, 2009).

Cultivated alfalfa is a tetraploid ($2n = 4x = 32$), (Brummer, 2004) open pollinated, perennial species that is usually grown on cultivated land for four to five years and it could be used as green mass, hay, silage, and pellets. In Libya, it is the most important forage legume, cultivated primarily in the oases and widely adapted in farming regions with an important role in providing cheap forage of high nutrition value.

The diverse genetic accessions of arid regions are considered as an important genetic source for

drought and salinity resistance for future breeding (Le Berre and Ramousse, 2003; Loumerem et al., 2004). The analysis of genetic diversity in outbreeding forage species such as alfalfa is an important step for cultivar identification, seed purity analysis and germplasm management (Falahati et al., 2007). Alfalfa infers polysomic inheritance and is characterized by high levels of polymorphism within and among populations (Veronesi et al., 2003). Therefore, most of the new populations represent synthetics generated by the intra-crossing of numerous superior selected individual plants in all combinations and they constitute heterogeneous populations of heterozygotic individuals (Tucak et al., 2010). The genetic complexity of alfalfa at both individual and population levels makes the breeding improvement of alfalfa rather complex.

Recent developments in plant molecular biology (Baquerizo-Audiot et al., 2001) and emerging molecular markers have allowed for the detection of diversity and differences among and within cultivars at the DNA level (Noeparvar et al., 2008). Molecular markers are independent of environmental factors, and they enable a more precise determination of genetic relatedness (Tucak et al., 2008). DNA marker technology provides a new dimension, accuracy and perfection in the screening of germplasm (Tar'an et al., 2005). Different molecular marker types have been used to assess genetic diversity in alfalfa: random amplified polymorphic DNA (RAPD) (Barcaccia, 1994; Crochemore et al., 1996; Gherardi et al., 1998; Mengoni et al., 2000; Musial et al., 2002; Tucak et al., 2008), amplified fragment length polymorphism (AFLP) (Zaccardelli et al., 2003; Segovia-Lerma et al., 2003), restriction fragment length polymorphism (RFLP) (Kidwell et al., 1994; Pupilli et al., 2000; Maureira et al., 2004), simple sequence repeat (SSR) (Flajoulot et al., 2005; Touil et al., 2008; Julier, 2009) and sequence related amplified polymorphisms (SRAP) (Vandemark et al., 2005; Ariss and Vandemark, 2007).

Random amplified polymorphic DNAs (RAPD) is a rapid, simple and inexpensive method. The basis of RAPD technique is differential PCR amplification of genomic DNA. It deduces DNA polymorphisms produced by rearrangements or deletions at or between oligonucleotide primer binding sites in the genome using short random oligonucleotide sequences mostly ten bases long (Agarwal et al., 2008). RAPD markers have been applied in different species, as well as in alfalfa, in gene mapping, population genetics, molecular evolutionary genetics, and plant breeding.

Despite limitations such as dependence on confounding environmental factors to some stages of plant growth and control by epistatic and pleiotropic gene effects, morphological traits have been used for genetic diversity analyses and cultivar development (Dodig et al., 2010). In that respect, alfalfa varietal populations that are widely used in Libya are routinely differentiated using morphological descriptors, but no attempts have been made so far to characterize them by the use of RAPD markers. The objectives of this study were to: (i) screen the germplasm of nine alfalfa domesticated varietal populations acquired from the Libyan GenBank with a set of thirteen RAPD primers; (ii) study the genetic diversity among the alfalfa germplasms at molecular levels; (iii) identify polymorphic RAPD markers with high information value for the chosen alfalfa populations.

MATERIALS AND METHODS

Plant material

Nine different alfalfa domesticated varietal populations, cultivated at different locations of west Libya (Table 1), were used for genetic diversity assessment by RAPD analysis. The seed material was obtained from the GenBank of the Agriculture Research Centre in Libya.

DNA extraction and PCR amplification

Green, healthy, young leaves from ten randomly selected individual plants from each of nine varietal populations were collected, and total genomic DNA was extracted following the method of Pallotta et al.

Table 1. The names of alfalfa domesticated varietal populations and geographical locations of cultivation.

Names	Location-cultivation regions	Longitude North	Latitude East	Elevation (m)
Gabsia	Gabes-Tunisia	33˚ 53’	10˚ 06	6
Atalia	Al Jafara-Libya	32˚ 26’	12˚ 52	137
Denamo Ferade	Wade al Haya-Libya	26˚ 09’	12˚ 26	613
Arezona	Sabha-Libya	27˚ 03’	14˚ 23	1365
Tagoria	Tagora-Libya	32° 49’	13° 28’	135
Masratia	Misrata-Libya	32° 20’	15° 04’	70
Wade Alrabeh	Tripoli-Libya	32° 56’	13° 08’	110
Fazania	Sabha-Libya	27° 03’	14° 23’	1365
Zawia	Zawia-Libya	32° 32’	12° 31’	310

Table 2. List of primers and the number of amplified DNA bands per primer.

Primer	Sequence 5’-3’	Number of polymorphic bands
A9	GGGTAACGCC	20
A12	TCGGCGATAG	29
A13	CAGCACCCAC	8
B10	CTGCTGGGAC	37
B19	ACCCCCGAAG	6
B20	GGACCCTTAC	13
C8	TGGACCGGTG	15
C11	AAAGCTGCGG	15
C12	TGTCATCCCC	30
E3	CCAGATGCAC	8
E4	GTGACATGCC	7
E7	AGATGCAGCC	14
Q7	CCCCGATGGT	14
Total		216

(2003). DNA quantity and quality were determined by spectrophotometrical procedure at a wavelength of 260 nm and 280 nm and by 1.2% agarose gel electrophoresis. In order to obtain RAPD fragments, an initial selection of primers was performed. Of the seventeen arbitrary primers, thirteen were selected to quantify genetic diversity among alfalfa accessions (Table 2)

Polymerase chain reaction (PCR) was performed in a final reaction as follows: the final reaction volume was 12 µl containing 2 µl (20-30 ng) of genomic DNA, 2 µl of each primer (5 pmol), 0.9 µl (1mM) of each dNTP, 1.5 µl (10 x) of the buffer solution PCR Buffer (1 x) with $MgCl_2$, 0.15 µl (0.54 µl) Taq Polymerase and 5.35 µl distilled water twice ddH_2O. RAPD reactions were conducted in a thermal cycler programmed for 40 cycles using the following profile: 5 min at 94°C, 1min at 94 °C, 1 min at 37°C, 2 min at 72°C and a final extension step of 7 min at 72°C. DNA amplification products were separated by electrophoresis on 1.2% agarose gels. The gels were soaked in 150 ml of a buff-

Table 3. The number of polymorphic RAPD bands observed within each varietal population using the 13 primers.

Population	Number of polymorphic bands
Gabsia	46
Zawia	25
Tagoria	37
Arezona	25
Atalia	8
Masratia	25
Denamo Ferade	12
Fazania	9
Wade Alrabeh	29
Average	24

Table 4. Genetic distances among alfalfa accessions based on Jaccard's method and RAPD data.

	Gabsia	Zawia	Tagoria	Arezona	Atalia	Masratia	Denamo Ferade	Fazania
Zawia	0.535							
Tagoria	0.457	0.580						
Arezona	0.450	0.440	0.645					
Atalia	0.222	0.121	0.222	0.222				
Masratia	0.591	0.280	0.354	0.160	0.242			
Denamo Ferade	0.655	0.540	0.367	0.270	0.300	0.378		
Fazania	0.290	0.235	0.347	0.294	0.117	0.058	0.285	
Wade Alrabeh	0.613	0.592	0.818	0.629	0.270	0.296	0.536	0.473

er solution amid deportation 1 x Tris-EDTA buffer at 80Volt for 45minutes. Gel is placed under UV light to study the output format.

Data analysis

Electropherograms were evaluated visually to distinguish polymorphic fragments (Fig. 1). Only sharp and precise bands were scored as (1) present and (0) absent for each primer combination and entered into a binary matrix.

Genetic distances (GD) based on pairwise comparisons were calculated by means of Jaccard's formula (Jaccard, 1908), $GD = 1 - N_{xy}/ (N_x + N_y - N_{xy})$, where

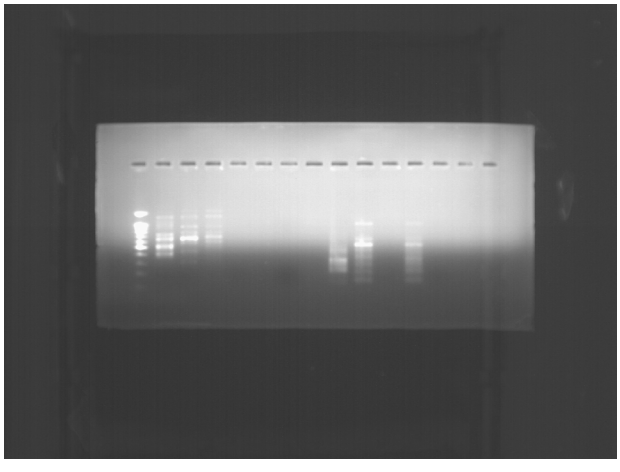

Fig. 1. Electrophoretic patterns for the B10 primer obtained by the alfalfa RAPD analysis.

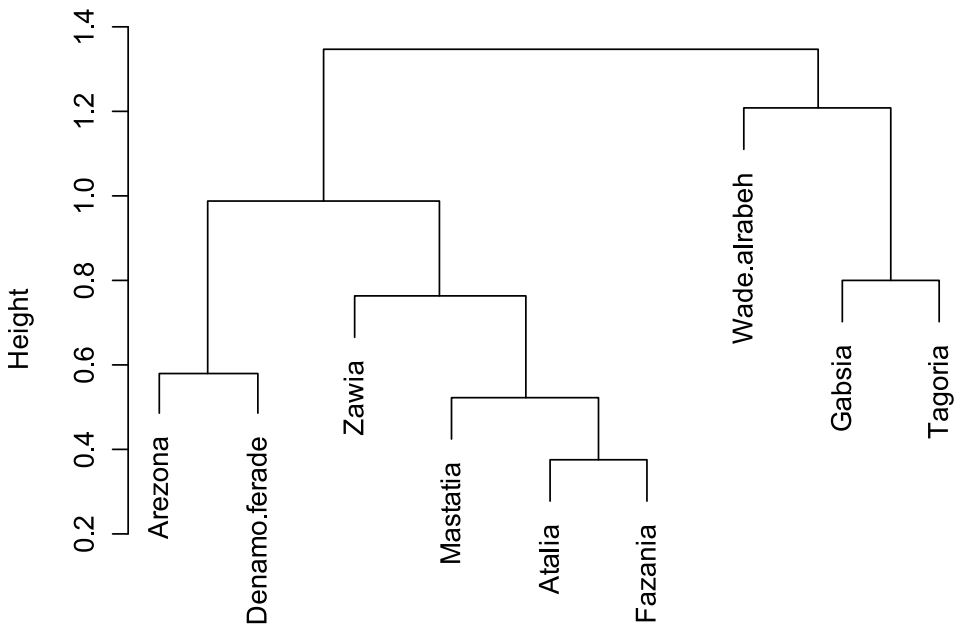

Fig. 2. UPGMA dendrogram based on the Ward's minimum variance method for RAPD data, showing the genetic relationship among nine alfalfa varietal populations.

N_{xy} is the number of bands common to accession x and accession y, N_x is the total number of bands in the accession x and N_y is the total number of bands in the accession y. Genetic analysis was carried out using the software NTSYSpc v.2.02e (Rohlf, 2005).

A matrix of pairwise genetic distances was employed to cluster the populations by applying Ward's Minimum Variance method (Ward, 1963) and a dendrogram was produced using UPGMA (unweighted pair group method using arithmetic averages).

RESULTS

Out of the 17 primers tested, we selected 13 primers based on the quality and reliability of their amplification products. A total of 216 polymorphic bands from 90 individuals of nine alfalfa varietal populations were generated by these primers. The highest number (37) of polymorphic bands was achieved with primer B10 and the lowest number of polymorphic bands was achieved with the primer B19 (6) (Table 2). The number of polymorphic bands identified within each domesticated varietal population ranged from 8 in the Atalia population to 46 in the Gabsia population, with an average of 24 bands (Table 3).

Genetic distances among pairs of varietal populations ranged from 0.058 between populations Fazania and Masratia to 0.655 between populations Denamo Ferade and Gabsia. The average distance among populations was 0.356 (Table 4).

The dendrogram based on Ward's minimum variance clustering method (Fig. 2) grouped the nine domesticated varietal populations into two main clusters. The first group included the following populations: Fazania, Atalia, Masratia, Zawia, Denamo Ferade and Arezona. The second group was composed of Tagoria, Gabsia and Wade Alrabeh alfalfa populations.

DISCUSSION

In the present study, the efficiency of RAPD markers in assessing the genetic diversity of nine alfalfa

accessions (domesticated varietal populations) from the Libyan GenBank was analyzed. RAPD amplification of alfalfa DNA revealed a rather high degree of genetic variability among populations because most of the primers produced a different banding pattern for each alfalfa varietal population.

Alfalfa is a difficult crop to breed because of its complicated genetics and self-breeding restrictions, and the development of new cultivars is usually undertaken at the population level (Sengupta-Gopalan et al., 2007). For this reason, it is especially important to understand the extent and distribution of its genetic variation. Genetic distances calculated on the basis of RAPD data show that there are certain relationships between the pairs of populations (the lowest genetic distance is 0.058 between varietal populations Fazania and Masratia, and the largest genetic distance is 0.655 between populations Denamo Ferade and Gabsia) which could be further visualized by Ward's minimum variance clustering method. Our variation range of genetic distances was higher than previously reported by Mohammadzadeh et al. (2011), Hu et al. (2009), Tucak et al. (2008), Tucak et al. (2010), and Wang et al. (2011), and was most similar to the variation range of Noeparvar et al. (2008).

Cluster analysis showed that the nine domesticated varietal populations were grouped into the two main clusters. The Tagoria, Gabsia and Wade Alrabeh alfalfa populations are clustered in the same group and they are genetically distanced from the other populations that comprise another group (Fazania, Atalia, Masratia, Zawia, Denamo Ferade and Arezona).

Genetic diversity studies could be useful in furthering breeding programs in the processes of improving and creating varieties. Identifying complementary sources of germplasm and marker-assisted heterotic group identification is still a challenging assignment in alfalfa breeding that should be accomplished on the basis of DNA fingerprinting. Molecular phenotyping and molecular methods, when supported with morphological data, are very important and represent essential phases in this process. The polymorphism detected among varietal populations in our study indicates that RAPD analysis could be successfully applied for estimating genetic diversity among alfalfa accessions in order to maximize the use of genetic resources and for the efficient organization of breeding material.

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