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Genetic Diversity of Some Mediterranean Populations of the Cultivated Alfalfa (*Medicago sativa* L.) Usmg SSR Markers

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Abstract: This species study was to investigate the differentiation level among 26 populations in which 12 are locals originating from the Tunisian South and 14 introduced from Italy, Austerely, France and Morocco with two SSR markers. These highly polymorphic and co dominant markers, together with recent population genetic statistic extended to autotetraploids, offer tools to analyse genetic diversity in alfalfa. The number of alleles per locus varied between 8 and 9. The genetic similarity between these various populations is estimated by the index of Rogers and Tanimoto. Genetic diversity is analysed by two statistical procedures: Hierarchical classification and Correspondence Factorial Analysis (CFA). Four large groups were obtained.

Key words: CFA, hierarchical classification, genetic diversity, SSR, Alfalfa (*Medicago sativa* L.)

INTRODUCTION

Alfalfa has been proven to be one of the most productive forage crops and also one of the most effective man-planted vegetations to conserve soil and water and to fix soil nitrogen.

Belong its trade value, alfalfa is able to fix atmospheric nitrogen in symbiosis with *Rhizobium meliloti* and therefore it is tremendous importance in safe, energy-efficient and pollution free agriculture. The various possible uses of lucerne forage are direct animal feeding and also industrial treatments: dehydratation and now protein extraction, perhaps fibber extraction. The improvement of these characters is necessary for the feeding of highly productive animals. The *M. sativa* complex shows a large genetic variability due to both natural and human selection under various climates and locations. Its main 2 sub-species, sativa and falcate show very different morphological traits. This *Sativa* sp. has purple flowers, a tap root, an erect growth habit, coiled pods and no inter dormancy. It's an autotetraploid, with 2n = 4x = 32 and allogame species. The genetic progress in this species is slow because of its autotetraploidy, allogamy synthetic structure of the varieties. Through, the use of methods to DNA polymorphisms, such as Restriction Fragment Length Polymorphisms (RFLPs) or Random Amplified Polymorphic DNAs (RAPDs), Simple Sequence Repeat (SSR), it is possible to efficiently mark any portion of the genome for inheritance studies. While most RFLP markers are codominants and may detect many alleles at a locus, most RAPD markers are dominants and can detect only two alleles for a locus (presence or absence of the marker). Dominant RAPD markers thus provide less genetic information than RFLP markers in certain mating and may segregate in only a limited number of populations (Echt *et al*., 1993). The use of SSR loci as polymorphic DNA markers has expanded considerably over the past decade both in the number of studies and in the number of organisms, primarily due to their facility and power for population genetic analyses. To date, few studies have been conducted using SSR markers to assess the level of variation among perennial *Medicago* species and populations. Diwan *et al*. (1997) have been the first to develop SSR markers in Medicago. They have shown how SSR can be used to describe genetic diversity and to analyze the genetic relationships among genotypes in alfalfa. Recently a set of 107 SSRs identified in the EST data base of *Medicago truncatula* was mapped in *M. sativa* (Julier *et al*., 2003) and can be used to perform genetic diversity analysis. Among these markers, some were easy to score allele doses with tetraploid genotypic information.

The objective of this study was to provide evidence that the SSR procedure is an suitable approach to the examination of molecular polymorphism and the phylogenic relationships in the cultivated alfalfa (*Medicago sativa* L.).

MATERIALS AND METHODS

Plant material: Twenty six populations of the cultivated alfalfa (*Medicago sativa* L.) were involved in the study, including 12 local originating in the Tunisian South and 14 introduced. They are shown in Table 1.

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This study was carried out in experiment field of Institute Arid Area of Medenine. Tunisia. Seeds of different genotype were sowed in April 2005. The measurements were carried two month after this date. The collect of vegetable material was realised in July 2005. The young leaflet were extracted, dried and conserved at -40°C for other use.

DNA extraction

Experimental protocol: Genomic DNA was extracted from young leaves of each plant following the method described by with minor modifications relating to the treatment of the AND with two enzymes: proteinase K and RNase.

Quality and quantity of the DNA

Optical density: OD_{260} nm: DNA concentration was determined by spectrophotometers at 260 nm. The measurement of the OD_{280} nm is used to detect the contaminants. The ratio OD_{260}/OD_{280} must situate between 1.8 and 2. A value lower than 1.8 testifies a contaminations by proteins, whereas a value higher than 2 testifies to a contamination by salts.

Table 1: List of various studied populations of alfalfa

Name	Origin	Code
Locals populations		
Kattana	Gabès (Tunisie)	P1
Chenchou	Gabès (Tunisie)	P2
Cheninni1	Gabès (Tunisie)	P3
Cheninni3	Gabès (Tunisie)	P5
Teboulbou	Gabès (Tunisie)	P6
Ghannouch	Gabès (Tunisie)	P8
Zerkine	Gabès (Tunisie)	P9
Essdada	Tozeur (Tunisie)	P10
Bouhlel	Tozeur (Tunisie)	P11
Hamma jerid	Tozeur (Tunisie)	P13
Jerzinze	Kébili (Tunisie)	P16
El golaa	Kébili (Tunisie)	P17
Introduced populations		
Sardi	France	P21
Ecotiposiciliano	Italie	P22
ABT805	Italie	P23
Erfoud3	Maroc	P25
Melissa	France	P26
Siriver	Italie	P27
Rich2	Maroc	P28
Demnat203	Maroc	P29
Tamantit	Italie	P30
Magali	France	P31
Prosemet	Italie	P32
Mamunts	Italie	P33
Cossouls	France	P34
Africaine	Australie	P35

Electrophoresis on agarose gel: DNA concentration was determined by 2% agarose gel. The absence of smear on the level of freezing testifies the purity to the AND.

Primers and ISSR-PCR assays: The detection of populations' polymorphism has been performed using a total of 4 primers (Table 2).

DNA samples of the 26 individuals plants were adjusted to 50 ng μL^{-1} and used in the amplification reactions with a final volume of 20 μL containing: 50 ng DNA; 1.6 μL of primers (10 μM) (0.8 μL of each primer); 0.4 μL of dNTP (10 mM); 0.2 μL Taq DNA polymerase (5 U μL^{-1}); 2 μL of PCR Buffer (1X); 1.2 μL of $MgCl_2$ (25 mM), adjusted at 20 μL by deionised water. DNA amplification was carried out using a Gene Amp PCR System 9700 thermal cycler programmed with 6 min at 94°C for initial denaturation, followed by 35 cycles of 30 sec at 94°C; 45 sec at 72°C. After DNA amplification, the DNA fragments were separated by electrophoresis for about 2 h under constant voltage (60 V) in 3% agarose gel submersed in 1X TBE buffer. The gels were stained with ethidium bromide solution and observed under ultraviolet light. Each gel was photo documented using the image capturing system bio print. The Jules DNA ladder (Q Bio gene) was used standard molecular weight marker.

Data analysis: The amplification bands were scored as 1 and 0 based on band (allele) presence and absence, respectively. Sizes amplification bands were estimated using Gel Pro analyser software. The similarity of all samples for at scored bands was assessed using Rogers and Tanimoto's (1960) similarity coefficient. The matrix generated were analysed with SPSS version 12 software to group the different populations by hierarchical classification. This grouping was performed by CFA with Stat Box version 3.4 software.

Table 2: Various primers tested for SSR markers

Code	Séquence	Température d'hybridation	Motif amplifié	Taille désirée (pb)
MTIC 297	*CTAAGCTTTGGCCATGTATC *TGAAATGAGTTTGACTGAGG	50	[TAC]5	115
MTIC249	*TAGGTCATGGCTATTGCTTC *GTGGGTGAGGATGTGTGTAT	55	[TCA]5	105-115
MTIC 234	*GGATGATTCCCTAAATTCAA *AAAAGGAGAATTTATTCATTTCA	55	[ATT]7	100
MTIC 430	*GCGTCTTTTCTTCATTTCAC *TGATAGCCATAACTCCGAAT	55	[AG]5	140

RESULTS

Molecular polymorphism: The 4 primers were screened for their ability to generate SSR polymorphic DNA bands using the accessions total cellular DNAs. The resultant bands patterns and their size shown in Table 3.

According to the Table 3, one notes that the only one SSR 1oci was amplified; the size of the SSR 1oci obtained by MTIC 297 varied between 127 and 140 pb; the size of the SSR 1oci obtained by MTIC 430 varied between 187 and 201 pb and the number of allele per locus ranged from 8 for MTIC297 to 9 for MTIC430.

The data exhibit that the two primers: MTIC 197 and MTIC 430 are more informative than MTIC249 and MTIC 234.

Figure 1 shows typical examples of the amplified SSR banding patterns with DNA stretches ranging from 127 to 140 pb. In this case MTIC297 oligonucleotid was applied using all the samples DNAs.

Figure 2 shows typical examples of the amplified SSR banding patterns by MTIC430.

Relationships among populations of alfalfa : hierarchical classification: Cluster analysis (Fig. 3) divided the 26 populations into four 1arge groups:

Table 3: A number of polymorphic bands and percentage of polymorphism at the studied populations, by combining the two primers: MTIC297 and MTIC430

	MTIC 297		MTIC 430	
Populations	No. of bands	Size of bands	No. of bands	Size of bands
Kattana	1	137	1	188
Chenchou	1	138	1	190
Chenennil	1	135	1	188
Chenennil3	1	133	1	187
Tboulbou	1	129	1	187
Gharmouch	1	138	1	191
Zerkine	1	127	1	196
Essdada	1	140	1	193
Bouhlel	1	140	1	193
Hammajerid	1	138	1	196
Jersine	1	138	1	200
Elgolaa	1	131	1	201
Africaine	1	133	1	194
Coussouk	1	131	1	194
Mamamtanas	1	131	1	193
Prosementi	1	129	1	191
Magali	1	129	1	191
Tamentit	1	129	1	190
Demnat	1	133	1	191
Rich2	1	135	1	190
Siriver	1	129	1	191
Melissa	1	129	1	190
Erfoud3	1	127	1	190
ABT805	1	127	1	190
Ecotiposiciliano	1	129	1	193
Sardi	1	133	1	191

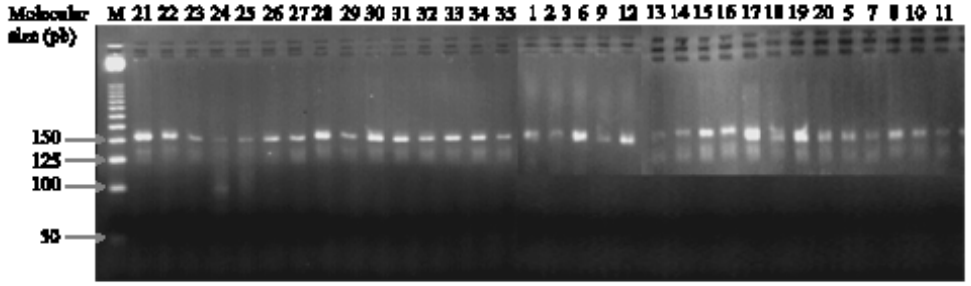

Fig. 1: Electrophoresis pattern obtained in the SSR Primer MTIC 297 in 26 populations of cultivated alfalfa (*Medicago sativa* L.) [M: Molecular size; 1….35: different populations]

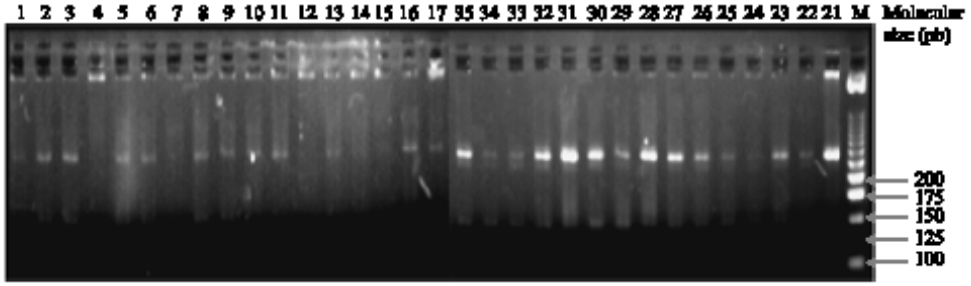

Fig. 2: Electrophoresis pattern obtained in the SSR Primer MTIC 430 in 26 populations of cultivated alfalfa (*Medicago sativa* L.) [M: Molecular size; 1….35: different populations]

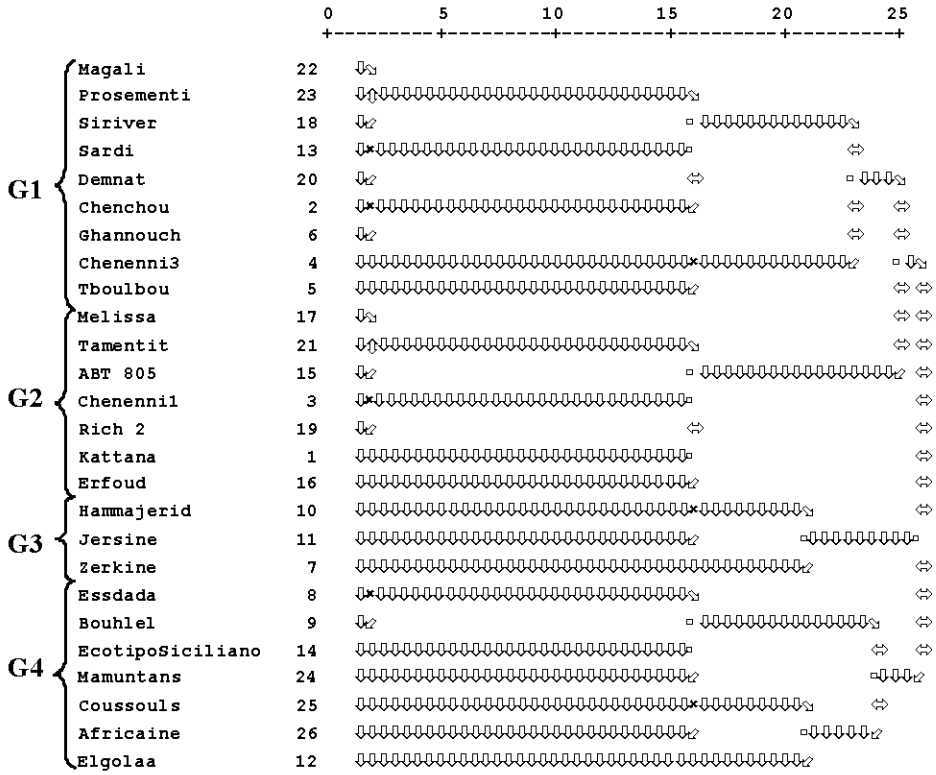

Fig. 3: Dendrogram grouped the various populations after combination the tow SSR markers: MTIC 297 and MTIC430 by hierarchical classification

Group 1: (G1) formed by: Magali, Prosementi, Siriver, Sardi, Demnat, Chenchou, Ghannouch, Chenenni3 and Tboulbou which have got only one SSR loci for each primer, MTIC297 and MTIC430, with a molecular weight ranged between 129-138 and 187-191 pb, respectively.

Group 2: (G2) included several populations: Melissa, Tamentit, ABT 805, Chenennil, Rich2, Kattana and Erfoud which have got only one SSR loci for each primer, MTIC297 and MTIC430, with a molecular weight ranged between 127-137 and 188-191 pb, respectively.

Group 3: (G3) formed by: Hammajerid, Jersine and Zerkine which have got only one SSR loci for each primer, MTIC297 and MTIC430, with a molecular weight ranged between 127-138 and 188-191 pb, respectively.

Group 4: (G4) included several locals' populations: Essdada, Bouhlel, Ecotiposiciliano, Mamuntanas, Coussoulous, Africaine and Elgolaa, which have got only one SSR loci for each primer, MTIC297 and MTIC430, with a molecular weight ranged between 131-140 pb and 183-201 pb, respectively.

Table 4: Percentage of inertia of the factorial analysis of correspondence by combining the two primers: MTIC297 and MTIC430

	F_1	F_2	F_3
Valeur propre	0.13	0.12	0.11
Variance (%)	14.03	12.80	11.62
Cumulé (%)	14.03	26.82	38.45

Correspondence factorial analysis: The CFA permit to group the various populations by projected them in a plan formed by two axes (F_1, F_2) or (F_1, F_3) or (F_2, F_3). Percentage of inertia by combining the two primers: MTIC297 and MTIC430 are carried by Table 4.

The examination of the axes F_1, F_2 and F3 shows that they cumulate 39% of inertia. The projection of various populations on the plan defined by two axes F_1 and F_2 (27% of inertia) shows existence of 4 groups (Fig. 4).

Figure 4 shows groups which are similar with those obtained by hierarchical classification.

The first group contain by Melissa, Tamantit, ABT 805, Chenennil, Rich2, Kattana and Erfoud is dispersed around the positive part of the axis F_1. For these populations, the molecular weight for the motif amplified by MTIC297 varied between 127 and 137 pb, this by MTIC430 is situate between 188 and 191 pb. The second

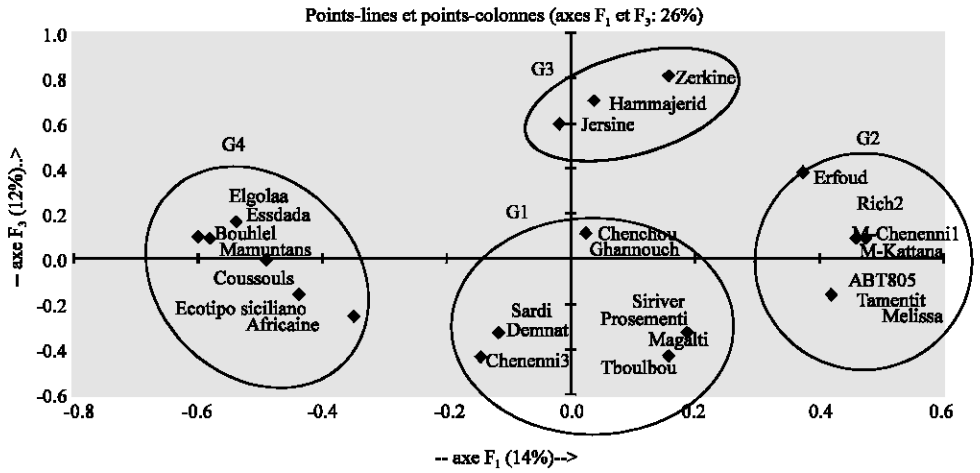

Fig. 4: Provision of the various populations studied according to F_1 axes' and F_2 of the correspondence factorial analysis by combining the two SSR primers: MTIC297 and MTIC430

group constitute by Hammajerid, Jersine and Zerekine is dispersed around the positive part of the axis F_2. For these populations, the molecular weight for the motif amplified by MTIC297 varied between 127 et 138 pb, this by MTIC430 is situate between 196 and 200 pb. Other population: Essdada, Elgolaa, bouhlel, Mamuntanas, Africaine, Coussouls et Ecotiposiciliano are dispersed around the negative part of the axis F_2. For these populations, the molecular weight for the motif amplified by MTIC297 varied between 129 and 140 pb, this by MTIC430 is situate between 193 and 201 pb.

DISCUSSION

The cultivated alfalfa (*Medicago sativa* L.) is characterized by a great genetic variability which makes it able to adapt to the very contrast mediums of hottest to cold. The study of molecular variability, in this study is based primarily on SSR marker. The first used primer is MTIC297 with 8 alleles; the second is MTIC430 with 9 alleles. The different cluster obtained was formed by some local and some cultivated populations of alfalfa. Results showed that population clustering was not correlated with geographic origin. The amplification of the SSR by each one of these two primers required to beguine with a development of technique PCR with this intention experimentation based on the variation of some parameters during the realization of the reactions of PCR was performed by varying the quantity of DNA, dNTP, primer and the selected program: time, temperature of primer hybridisation and genomic DNA denaturation temperature.

This molecular marker is very used to study the genetic diversity of *Medicago* genus. SSR markers was used by Julier *et al.* (2005) to investigate the differentiation level among seven cultivars originating from one breeding program and between these cultivars and the breeding pool, with eight SSR markers. The number of alleles per locus varied between 3 and 24. Diwan *et al.* (1997) have been the first to develop SSR markers in Medicago. They have shown how SSR can be used to describe genetic diversity and to analyse the genetic relationships among genotype in alfalfa. Recently a set of 107 SSRs identified in the EST database of Medicago truncatula was mapped in *M. sativa* (Julier *et al.*, 2003) and can be used to perform genetic diversity analysis.

In addition to the SSR markers, several molecular markers are used for the identification and the study of the genetic diversity of the alfalfa, as the RAPD markers witch are very much used for the *Medicago* genus, primarily, to estimate the genetic relations of cultivated alfalfa the development of the genetic charts (Echt *et al.*, 1992) analysis of the genetic variability of the diploïdes annual species (Burmmer *et al.*, 1995 ; Bonnin *et al.*, 1996), for the characterization of the Medicago species and to target the genes. These RAPD markers are used to differentiate between two populations from leguminous natural plants and cultivated *Medicago sativa* in Spain, by comparing them with allozymic markers.

This study provides evidence that the SSR procedure is an informative and suitable approach to the examination of the molecular polymorphism and the phylogenic relationships in the cultivated alfalfa (*Medicago sativa* L.). Work is currently in progress to

exchange the number of markers by the use of other molecular technologies in order to have a deeper insight into the molecular polymorphisms and to establish a varietals identification key in this crop.

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