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

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Abstract: The genetic diversity within 29 populations, when 15 populations are local originating in the Tunisian south and 12 introduced of Italy, Austerely, France and Morocco, was analysed by ISSR markers (Inter Simple Repeat sequence). The genetic similarity between these various populations is estimated by the index of Rogers and Tanimoto. Genetic diversity is analyzed by one statistical procedure: hierarchical classification. The total number of bands varied between the various populations from 9-16. The percentage of total polymorphism is about 60%. Four large groups were obtained.

Key words: Alfalfa (*Medicago sativa* L.), genetic diversity, ISSR, hierarchical classification, Rogers and Tanimoto

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most cultivated forage legume. It is an autotetraploid with 2n = 4x = 32; (Dermaly, 1954) and allogamous species.

Alfalfa (*Medicago sativa* L.) is a cross pollinated forage legume (Julier *et al*., 2000), a traditional forage crop, widely used for animal feeding. Lucerne (alfalfa) is the most important sown perennial fodder in Tunisia. It is widely adapted and can be found throughout the country. Lucerne originates from Asia Minor, Transcaucasia, Iran and Turkmenistan and is diversified in the Mediterranean area.

Variability for agronomic and morphological traits of alfalfa is frequently used in breeding programs for developing cultivars with a high forage production and quality but seed-yielding ability has rarely been an important criterion in the early stages of selection programs (Julier *et al*., 2000). Alfalfa is able to fix atmospheric nitrogen in symbiosis with Rhizobium meliloti, (Kiss *et al*., 1992).

Several methods were used to study the level of differentiation among a set of cultivars or populations using biochemical, isozymes and molecular markers, 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. RFLP are generally detected using low copy nuclear DNA sequences as hybridation probes on Southern blots of restriction genomic DNA (Botstein *et al*., 1980), RAPDs are identified by the Polymerase Chain Reaction (PCR) using arbitrary primers, making them simpler to assay than RFLPs and can detect polymorphism in both low copy and repetitive DNA sequences (William *et al*., 1990). 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*., 1992). 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 (Estoup and Angers, 1998). 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 *Medicago 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.

Inter Simple Sequence Repeat (ISSR) is a dominant molecular marker revealed in mass. Under conditions of adapted amplifications, the DNA fragments were separated in agarose or acrylamide gel.

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The genetic resources presently available are the register varieties and the wild populations and the landraces in which most of the genetic diversity in most of the cultivated species underlines the need to describe the genetic diversity available in the material under collection which could be stored as genetic resources or used in breeding programs.

In the present study, we report the use of molecular markers such as ISSR for the assessment of genetic diversity and relationships among different populations of cultivated alfalfa collected from three different sites of Tunisian south, Gabes, Tozeur and Kebilli; Italy; France; Morocco and Austerely.

MATERIALS AND METHODS

Plant material: Twenty nine populations of the cultivated alfalfa (*Medicago sativa*) were involved in this study, including 15 local originating in the Tunisian South and 12 introduced. They are shown in Table 1. 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 young leaflets were carried two month after this date. There are dried and conserved at-40°C for other use.

Table 1: List of various studied populations of cultivated alfalfa

Name	Origin	Designation
Locales populations		
Chenchou	Gabès (Tunisia)	P2
Cheninni 1	Gabès (Tunisia)	P3
Cheninni 3	Gabès (Tunisia)	P5
Teboulbou	Gabès (Tunisia)	P6
Metwia	Gabès (Tunisia)	P7
Ghannouch	Gabès (Tunisia)	P8
Zerkine	Gabès (Tunisia)	P9
Essdada	Tozeur (Tunisia)	P10
Bouhlel	Tozeur (Tunisia)	P11
Degach	Tozeur (Tunisia)	P12
Hamma jerid	Tozeur (Tunisia)	P13
Zaafarane	Kébili (Tunisia)	P14
Nouael	Kébili (Tunisia)	P15
Jerzinze	Kébili (Tunisia)	P16
El golaa	Kébili (Tunisia)	P17
Limaguess	Kébili (Tunisia)	P18
Douz	Kébili (Tunisia)	P19
Introductive populations		
Sardi	France	P21
Ecotiposiciliano	Italy	P22
ABT	Italy	P23
Ameristand	Italy	P24
Erfoud 1	Marocco	P25
Melissa	France	P26
Siriver	Itay	P27
Rich 2	Marocco	P28
Demnat 203	Marocco	P29
Mamuntanas	Italy	P33
Cossouls	France	P34
Africaine	Australy	P35

DNA extraction

Experimental protocol: Genomic DNA was extracted from young leaves of each plant following the method described by Pallotta *et al.* (2003) 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: OD260 nm: DNA concentration was determined by spectrophotometer at 260 nm. The measurement of the OD 280 nm is used to detect the contaminants. The ratio OD260/OD280 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.

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

Primers and ISSR-PCR assays: The detection of genetic polymorphism inter populations has been performed using 19 primers. These were based on either di- or-multinucleotide repeats that were complementary to microsatellite (Table 2). The dinucléotides repeats were anchored at 3' ends.

DNA samples of the 29 individuals plants were adjusted to 80 ng μL^{-1} and used in the amplification reactions with a final volume of 20 μL containing: 80 ng DNA; 1.6 μL of primer (10 μM); 1.5 μL of dNTP (2 mM); 0.2 μL Taq DNA polymerase (5 u μL^{-1}); 2 μL of PCR Buffer (1 X); 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

Table 2: Various primers tested

Code	Sequence	Tm (°C)
A1	(TGGA)5	55
A2	(ACTG)5	55
A3	(GACA)5	55
A7	(AG)10T	55
A8	(CT)10A	55
A9	(CT)10G	55
A10	(CT)10T	55
A11	(GT)6GG	55
A12	(GA)6CC	55
A13	(GT)6CC	55
A17	(GTG)3GC	55
A21	(CA)6AC	55
A22	(CA)6GT	55
A23	(CA)6AG	55
UBC 818	(CA)7G	52
UBC 812	(GA)8A	50
UBC 849	(GT)8CG	56
primer 2	(CA)6GG	44

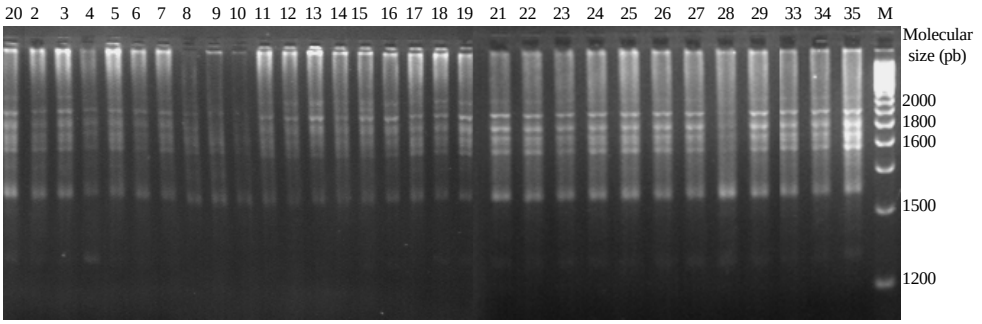

Fig. 1: ISSR PCR fingerprints of 29 populations of cultivated alfalfa (*Medicago sativa* L.) using the 3' anchored primer (GA) 6CC. M: Molecular size (pb); 2-35: DNA templates

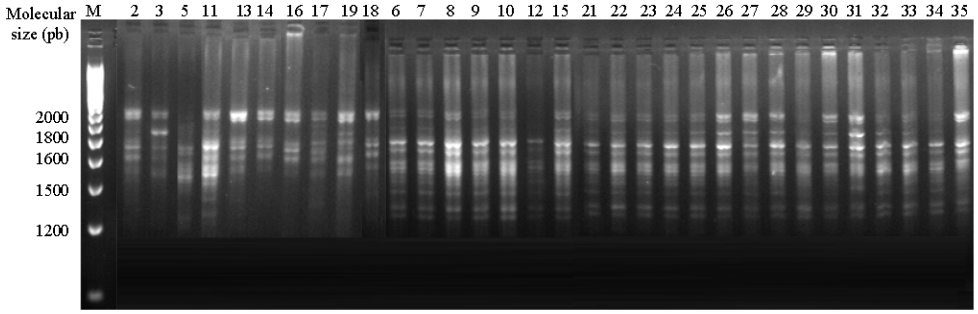

Fig. 2: ISSR PCR fingerprints of some populations of cultivated alfalfa (*Medicago sativa* L.) using the 3' anchored primer (CA) 7G. M: Molecular size (pb); 2-35: DNA templates

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. The gels obtained by the two primers A12 and UBC 818 were represented, respectively by the Fig. 1 and 2.

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 (1960) similarity coefficient. The matrix generated were analysed with SPSS version 12 software to group the different populations by hierarchical classification.

RESULTS

Molecular polymorphism: The 19 primers were screen for their ability to generate ISSR polymorphic DNA bands using the accessions total DNA. The Number of Total Bands and their polymorphism and Percentage of Polymorphism (%) were present in Table 3. According to this table one notes that: The total number of bands varies between the various populations from 9-16; the number of polymorphic bands lies between 4 and 11; the percentage of polymorphic bands is located between 44 and 67% and the percentage of total polymorphism is about 60%.

The data exhibit that the dinucléotides repeat: A12 [(GA) 6CC] and UBC-818 [(CA) 7G] are more informative than tetra nucleotide and three nucleotide ones.

Figure 1 shows typical examples of the amplified ISSR banding patterns with DNA stretches ranging from 1200-1900 pb. In this case A12 oligonucleotid was applied using all the samples DNA.

Figure 2 shows typical examples of the amplified ISSR banding patterns by UBC-818 oligonucleotid.

A total of 355 ISSR bands were amplified using the two available primers with 29 populations of cultivated alfalfa. Among these bands 210 bands are unambiguously reproducible and polymorphic. The percentage of polymorphic bands and the percentage of polymorphism inter populations were estimated.

Table 3: A number of polymorphic bands and their percentage of polymorphism for the different populations, by combining the two starters: A12 and UBC - 818

Populations	No. of total bands	No. of Polymrphics bands	Percentage of Polymorphics bands (%)	Percentage of Polymorphism (%)
Chenchou	10	5	50	60 %
Chenenni 1	11	6	54.54	
Chenenni 3	10	5	50	
Tboulbou	17	12	70	
Metwia	16	11	67	
Ghannouch	14	9	65	
Zerkine	12	7	59	
Essdada	14	9	65	
Bouhlel	12	7	59	
Dgach	12	7	59	
Hammajerid	11	6	54.54	
Zaafarane	13	8	62	
Nouael	16	11	67	
Jersine	12	7	59	
Elgolaa	13	8	62	
Limaguess	9	4	44.44	
Douz	11	6	54.54	
Sardi	14	9	65	
Ecotiposiciliano	16	11	67	
ABT 805	16	11	67	
Ameristand	14	9	65	
Erfouf3	15	10	66.66	
Melissa	13	8	62	
Siriver	12	7	59	
Rich2	14	9	65	
Demnat	12	7	59	
Mamuntanas	13	8	62	
Coussouls	12	7	59	
Africaine	11	6	54.54	
Total	355	210	-	

Relationships among populations of alfalfa: Hierarchical classification: Cluster analysis (Fig. 3) devised the 29 populations into four large groups:

Group 1 (G1): It is formed by the majority of the introduced populations: Melissa, Mamuntanas, Demnat, Rich2, Siriver, Coussouls, Ecotiposiciliano, African ABT805, Ameristand and Erfoud which have got 12 at 16 bands with a molecular weight between 1300 and 1900 pb.

Group 2 (G2): It is included several locals' populations: Tboulbou, Metwia, Essdada, Ghannouch, Zerkine and Nouael and a single introduced population, Sardi, which have got 12 at 17 bands with a molecular weight between 1550 and 1950 pb.

Group 3 (G3): It is included only one local population, Dgach, which have got 12 bands, with molecular weight between 1300 and 1800 pb.

Group 4 (G4): It is included several locals populations: Chenchou, Limaguess, Elgolaa, Douz, Zaafarane, Jersine, Bouhlel, Hammajerid, Chenenni 1 and Chenenni 3, which have got 9 at 13 bands, which a molecular weight between 1500 and 1800 pb.

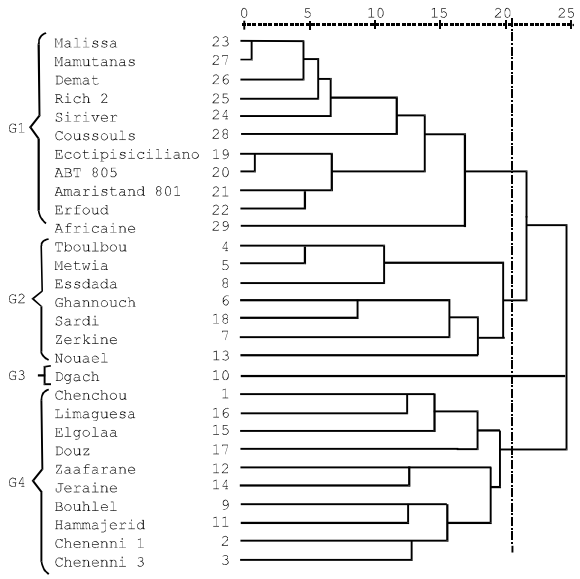

Fig. 3: Dendrogram grouped the various populations after combining the two primers: A12 and UBC-818 by hierarchical classification

DISCUSSION

The cultivated alfalfa (*Medicago sativa* L.) is characterized by a height genetic variability (Mauriès, 2003). The study of genetic diversity, in this research is based on mineral composition, some morphological traits and ISSR markers.

The clustering grouped the studied populations into three clusters with no correlation to geographical origins. In this study we conclude that the dinucléotides repeat: A12 [(GA) 6CC] and UBC-818 [(CA) 7G] are more informative than tetra nucleotide and three nucleotide ones. By combining the two primers, the total number of bands varied between 9-16 and the polymorphic bands from 4-11. These markers were successfully for the polymorphism genetic study of maize, rice, potato, to study the genetic relations between the various coffee species. These markers are also used to identify and analyze genetic variability of date palm. In addition to the ISSR markers, several molecular markers are used to identify and to study of genetic diversity of cultivated alfalfa, as the RAPD markers witch are very much used for the *Medicago* genus, primarily, to estimate the genetic relations of cultivated alfalfa (Yu and Pauls, 1993a) the development of the genetic carts (Echt *et al*., 1993) analysis of the genetic variability of the diploids annual species, for the characterization of the *Medicago* species and to target the genes (Yu and Pauls, 1993b). SSR markers were used by Julier *et al*. (2005) to study the genetic diversity of the cultivated alfalfa (*Medicago sativa* L.).

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