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Genetic Diversity of Natural Populations of *Medicago truncatula* in Morocco Using Isozyme Polymorphism

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Abstract: Nine natural populations of Moroccan *Medicago truncatula* were analysed using enzymatic markers to identify useful phytogenetic resources for their integration in programmes of safeguard and conservation. Starch gel electrophoresis was used to examine genetic variation within and among natural populations. According to our estimates of genetic variation analysed by five loci detected for three enzyme system, the species showed a high genetic diversity. 46% of the total variation is due to the diversity between populations. It appears that a considerable variation was found among populations, indicating an interesting genetic potential for selection. This result is in accordance with the strictly autogamous system of reproduction in this species. The dendrogram generated from pairwise genetic distance among all populations showed three distinct clusters independently from the geographic origin. Conservation programs should take into account the level of genetic diversity within and between populations revealed by isozyme markers.

Key words: *Medicago truncatula* · Isozyme · Genetic diversity · Morocco

INTRODUCTION

Among crops reliable to promote pastoral zones that produce forage and restore destroyed pasture land especially in arid and semi-arid areas, the genus *Medicago* L. (Fabaceae) constitutes an important genetic resource composed of various annual herb or herbaceous perennial species, mostly native to the Mediterranean regions. Due to their capacity to fix atmospheric nitrogen and improve soil fertility in symbiosis with soil bacteria collectively known as 'rhizobia', *Medicago* species do not need costly and polluting chemical nitrogen fertilizer [1]. The genus *Medicago* is distributed worldwide and consists of approx. 83 species [2]. The annuals species collectively known as "medics" are naturally distributed over a very wide range of environmental conditions in the Mediterranean basin. Some medics have been introduced to regions of Australia, Chile, South Africa and United States with Mediterranean-type climate. Most of the selection programs of medics have been conducted in Australia where they are extensively grown in a cereal/pasture legume rotation (ley-farming system). In North Africa, medics can be found in wide-ranging habitats, varying in water availability, temperature and geographical location (longitude, latitude and altitude). The ability of these species to establish a nitrogen-fixing symbiosis with *Sinorhizobium* sp. makes them excellent candidates for use in sustainable agricultural systems as forage and cover crops. Medics, as well as other annual pasture legumes, have a high feeding quality, determined by higher protein, mineral and vitamin contents, low proportion of cell wall and particularly higher level of intake than grasses [3].

Among annuals medics, *M. truncatula* Gaertn is widely distributed throughout the Mediterranean region and has also become naturalized in other regions of the world [4], especially in Maghreb in which *M. truncatula* is the most widespread medic species after *M. polymorpha*. It is a diploid (2n = 16), self-pollinating species with a small genome and the resources to create transgenic plants [4, 5]. The species *M. truncatula* has been split into three subspecies mainly on the basis of

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pod characteristics: ssp *truncatula*, ssp *tricycla* and ssp *longeaculata*. In Morocco, *M. truncatula* is adapted to a diverse range of habitats located in different geographical regions [6], suggesting that genotypes adapted to local biotic and abiotic stresses could be identified in natural populations and integrated into breeding programs. In order to identify these genotypes, a basic knowledge of the level of genetic diversity within and among natural populations is necessary. Germplasm characterization is important for evaluation of genetic resources and utilization of valuable genotypes for conservation programs and breeding purposes [7-9].

In this work, we used enzymatic markers to characterize Moroccan *M. truncatula* populations. This approach has been widely used in elucidating the genetic structure of *Medicago* species [10-16]. This study complements to our previous study reported on Morroccan *M. truncatula* structure based on morphological traits [17]. The aim of the present work is firstly concentrate in estimating the extent of *M. truncatula* genetic diversity and then examine the relation of genetic diversity with geographic distance within this species in Morocco. This could be useful for the development of strategies and methodologies for conservation of plant genetic resources and improvement of valuable *M. truncatula* germplasm utilization for breeding purposes.

MATERIALS AND METHODS

Plant Materials: The accessions as used in this study consisted of nine spontaneous populations of *M. truncatula* collected from the central area of Morocco. The principal characteristics of all sites are summarized in Table (1). Seeds randomly collected from each population were germinated and the plants were grown under uniform conditions.

Enzymes Extraction and Electrophoresis: After two month of culture, young leaves from each sample were used for enzymes extraction. Twenty five plants per population were used. Horizontal starch gel electrophoresis (13%) was used to analyze the variation in three enzymes; 6-phosphogluconate dehydrogenase (6-PGD), phospho-glucomutase (PGM) and leucine amonipeptidase (LAP).

Electrophresis was carried out at 4°C for 6 h (50 mA). Isozyme staining protocols followed standard methods as reported by Brunel [10], Cardy [18] and Stuber and Goodman [19].

Data Analysis: Zymograms obtained for each enzyme system were interpreted considering all populations studied and data were analyzed using the PopGene (ver. 1.31) program. Allelic frequencies were used to describe the distribution of genetic variation within and between populations.

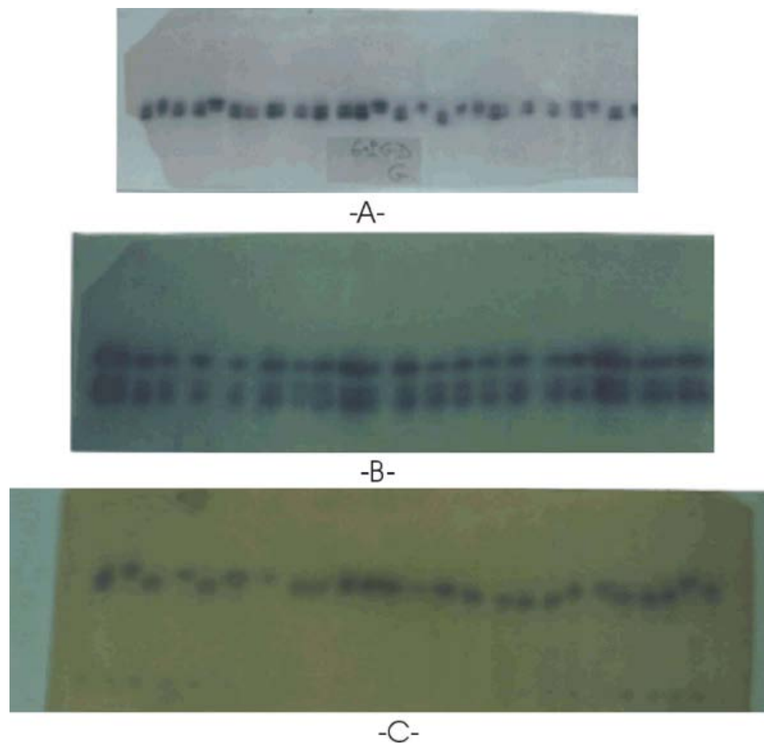
Genetic variation within population was assessed using the number of alleles per locus (A_P), the percentage of polymorphic loci (*P*) and expected heterozygosity (*He*). Differentiation among populations was estimated by Wright's F-statistics [20] and Nei's genetic distance [21].

RESULTS

Among many enzyme systems assayed on *M. truncatula*, three with reliable and interpretable zymograms were selected (6-PGD, PGM and LAP) as shown in Fig. (1). For all populations, the three analysed enzymes were encoded by five polymorphic loci (one locus for 6Pgd (6Pgd-1) and two loci for Pgm (Pgm-1, Pgm-2) and Lap (Lap-1, Lap-2). Further crossing experiments are needed to confirm our interpretations. 6Pgd Zymograms include one active zone with one or three bands suggesting that only one gene coding for

Table 1: Population of *M. truncatula* used in this study and their principal geographical and ecological characteristics.

Population	Pop. code	Geographic origin	Latitude North	Longitude West	Altitude (m)	Rainfall (mm/year)
Casablanca	CA	10 km S E of Casablanca	33°34'	7°/40'	60	440
Rabat	RA	20 km S of Rabat	33°51'	7°/02'	60	500
Benslimane	BS	2 km N E of Benslimane	33°62'	7°07'	280	440
Rommani	RO	3 km N of Rommani	33°52'	6°33'	370	430
EL Kelâa des Sraghna	KS	El Kelaâ desSraghna city	32°03'	7°24'	465	250
El Borouj	BR	El Borouj city 32°31'	7°11'	405	305	
Kasba Tadla	KT	5 km S of Kasba Tadla	32°57'	6°26'	495	380
Oued Zem	OZ	5 km N Wof Oued Zem	32°68'	6°50'	780	370
Sidi Kacem	SK	15 Km S of Sidi Kacem	34°10'	5°48'	160	480



Zymograms of 6-phosphogluconate dehydrogenase (6-PGD), phospho-glucomutase (PGM) and leucine amonipeptidase (LAP) revealed in leaf extracts of *M. truncatula*.

Fig. 1: Diagrammatic representation of allozyme products at the loci 6Pgd-1, Pgm-1, Pgm-2, Lap-1 and Lap-2 revealed in leaf extracts of *M. truncatula*

this dimeric enzyme. The Pgm system showed two zones of activity with two or three bands each suggesting the presence of two loci with two or three alleles that encode a monomeric enzyme. Two zones of activity were observed for the Lap system corresponding to two loci with two or three alleles encode a monomeric enzyme.

Data in Table (2) show the alleles frequencies at each locus for the nine different populations. A total of 12 alleles has been found in all populations. The number of alleles of different loci varies from two (6-Pgd-1, Pgm-2 and Lap-2) to three (Pgm-1 and Lap-1). The largest number of alleles (11) was observed the population Benslimane (BS). In contrast, the populations Rabat (RA) and Oued Zem (OZ) had the fewest number of alleles (7). The alleles absent in some populations are shown in Table 2. The 6Pgd-1b, Pgm-2b and Lap-1c alleles were present in all populations.

Table 2: Allelic frequencies at 5 enzyme loci determined for 9 populations of *M.truncatula*

Population loci	CA	RA	BS	RO	KS	KT	BR	0Z	SK
6Pgd-1a	0.48	0.00	0.44	0.00	0.56	0.00	0.00	0.00	0.00
6Pgd-1b	0.52	1	0.56	1	0.44	1	1	1	1
Pgm-1a	0.36	0.00	0.00	1	0.44	0.44	0.00	0.24	0.00
Pgm-1b	0.54	1	1	0.00	0.36	0.20	0.80	0.44	0.72
Pgm-1c	0.10	0.00	0.00	0.00	0.20	0.36	0.20	0.32	0.28
Pgm-2a	0.46	0.00	0.48	0.66	0.40	0.00	0.00	0.00	0.00
Pgm-2b	0.54	1	0.52	0.34	0.60	1	1	1	1
Lap-1a	0.00	0.00	0.40	0.68	0.00	0.00	0.40	0.00	0.00
Lap-1b	0.00	0.12	0.52	0	0.76	0.92	0.00	0.00	0.12
Lap-1c	1	0.88	0.08	0.32	0.24	0.08	0.60	1	0.88
Lap-2a	0.00	0.88	0.52	0.08	1	0.56	0.90	1	0.40
Lap-2b	1	0.12	0.48	0.92	0.00	0.44	0.10	0.00	0.60
No of alleles	9	7	11	8	10	9	8	7	8

Three parameters describing the genetic variability within populations were estimated in the nine populations. For each population, the mean number of alleles per polymorphic locus (Ap), the percentage of polymorphic loci (P) and the mean expected hetrozygoty (He) are given in Table 3. Levels of genetic diversity varied according to populations. The number of alleles per polymorphic locus (Ap) ranged from 1.4 (populations Rabat (RA) and Oued Zem (OZ)) to 2 (populations Benslimane (BS) and El Kelâa des Sraghna (KS)). The proportion of polymorphic loci (P) varied from 20% in population Oued Zem (OZ) to 80% in populations Benslimane (BS) and El Kelâa des Sraghna (KS). The expected heterozygoty differed from one population to another. The highest genetic diversity (He = 0.419) was observed in population Benslimane (BS), whereas the population Rabat (RA) presented the lowest diversity (He = 0.086).

A high differentiation among the populations, estimated by Wright's F_{ST} index, was observed F_{ST} = O.46 (Table 4). F_{IS} and F_{IT} values were high for all loci which illustrate their deficits in heterozygoty. F_{ST} values for individual loci ranged from 0.381 (Pgm-2) to 0.538 (Lap-1). The hight values of F_{ST} observed for Lap-1 and Lap-2 (0.537-0.538) lead to relatively high differentiation of all *M. truncatula* populations (Table 4).

A genetic distance matrix based on isozymes data was estimated according to the Nei formula (Table 5). The smallest distance value of 0.036 is observed between Rabat (RA) and El Borouj (BR) accessions suggesting a high degree of similarities between these populations. The higher genetic distance value of 0.859 is obtained between populations Rabat (RA) and Rommani (RO). Thus it may be assumed that these last mentioned populations represent the maximum of divergence between the studied populations. All the remaining populations displayed intermediate levels of similarity.

Table 3: Genetic diversity estimates for *M. truncatula* populations

Populations	Ap	P	He
CA	1.8	60	0.319
RA	1.4	40	0.086
BS	2.0	80	0.419
RO	1.6	60	0.210
KS	2.0	80	0.402
KT	1.8	60	0.261
BR	1.6	60	0.200
OZ	1.4	20	0.132
SK	1.6	60	0.223

Table 4: Wright's F-statistics (F_{IS}, F_{IT}, F_{ST}) for allozyme-based genetic diversity of *M. truncatula*

Locus	F_{IS}	F_{IT}	F_{ST}
6Pgd-1	0.892	0.935	0.400
Pgm-1	0.963	0.977	0.386
Pgm-2	0.875	0.923	0.381
Lap-1	0.801	0.908	0.538
Lap-2	0.582	0.807	0.537
Mean	0.833	0.910	0.458

The constructed dendrogram using these distances showed three distinct groups (Fig. 2). One includes two populations Casablanca (CA) and Rommani (RO). The second group composed the four populations Rabat (RA), El Borouj (BR), Oued Zem (OZ) and Sidi Kacem (SK). The Populations Benslimane (BS), EL Kelâa des Sraghna (KS) and Kasba Tadla (KT) clustered together in the third group. Populations seem to be clustered independently from their geographical origin. This is well exemplified in the case of the CA and RA; OZ and KT populations that are significantly divergent in spite of their geographical proximity.

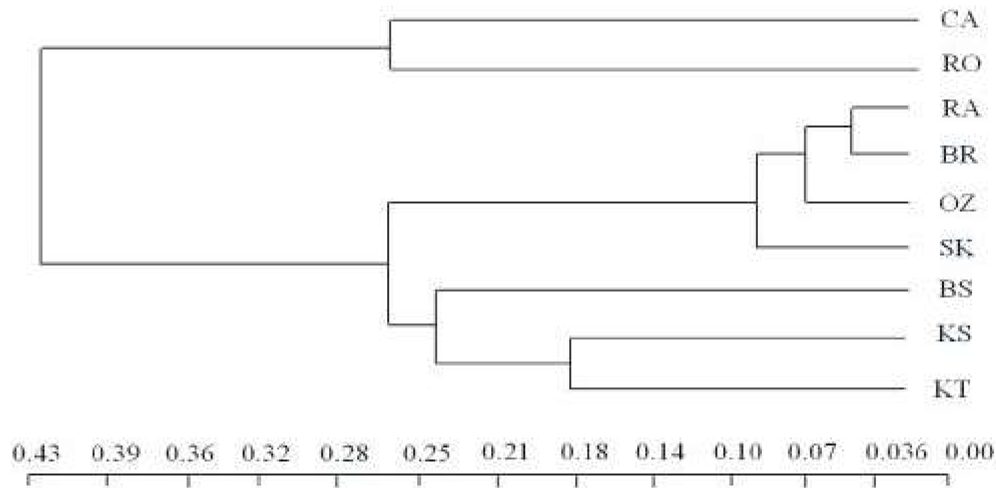


Fig. 2: Dendrogram of Moroccan *M. truncatula* populations based on the Nei's genetic distance

Table 5: Genetic distances among pairs of populations of *M. Truncatula*

Populations	*CA*	*RA*	*BS*	*RO*	*KS*	*KT*	*BR*	*OZ*
CA								
RA	0.419							
BS	0.407	0.293						
RO	0.344	0.859	0.618					
KS	0.683	0.362	0.258	0.830				
KT	0.638	0.342	0.374	0.530	0.229			
BR	0.515	0.036	0.274	0.705	0.364	0.310		
OZ	0.467	0.060	0.520	0.740	0.326	0.309	0.064	
SK	0.210	0.071	0.329	0.577	0.266	0.266	0.099	0.110

DISCUSSION

Our previous work implied morphological traits on Moroccan *M. truncatula* populations have shown a great phenotypic diversity related to vegetative development [17]. The diversity was found to be structured on the basis of ecological factors as rainfall and altitude. In the present study, we have examined the genetic diversity in *M. truncatula* accessions with the help of neutral markers as isoenzymes.

As result of our investigation, the genetic variation occurring within *M. truncatula* is organised with high levels of differentiation between populations. A large degree of genetic diversity among the tested accessions as revealed by the mean F_{ST} = 0.458. This value gives an estimate of 46% of the genetic diversity, accounted for by among population differentiation. A high genetic diversity between populations could be explained by the high levels of autogamy in *M. truncatula*. According to Hamrick and Godt [22], species that have selfing or mixed mating systems have lower levels of genetic variability then predominantly outcrossed species and 51% of their total genetic diversity is apportioned between populations in comparison to 10% for outcrossed species.

Besides, our study indicated a low level of heterozygosity (He = 0.086-0.419). The substantially higher selfing rate in *M. truncatula* could have contributed to a lower overall level of estimated heterozygosity. A low level of heterozygosity (He = 0.348-0.476) was also found for *M. truncatula* in the French Mediterranean region [23, 24]. Our results are generally consistent with the trends of genetic variation observed in many flowering plants groups based on microsatellite data, with average. He values being 0.41 for inbreeding populations versus 0.65 for outcrossing populations.

The dendrogram constructed using Nei's genetic distances confirmed the high degree of diversity between the *M. truncatula* accessions and showed that these accessions can be classified in three distinct groups. However, populations did not cluster on the basis of eco-geographic location. The estimates of genetic distance (D) show low (D)'s between geographically distant populations and large (D)'s between short

geographic distances among populations. This result is similar to that obtained by Akritidis [25] in Greek *M. truncatula* genotypes using microsatellite markers. They reported that the distance dendrogram based on microsatellite markers revealed no characteristic pattern of organization in relation to the geographic location of the accessions.

For conservation purposes, it's essential to safeguard the largest possible collection of alleles. A practical consequence of the high interpopulation variability for genetic resources management could be that a maximum number of populations would be necessary to preserve most of genetic diversity. Another approach to preserve populations of this species is to consider their cultivation after selection. For this purpose, analyses combining genetic diversity and agronomic traits may be useful for the select interesting varieties.

In conclusion, this study reflects high level of genetic diversity in the local germplasm and a moderate level of geographic structure of *M. truncatula* populations in Morocco using enzymes markers. Future study of additional populations and the use of molecular markers such as RAPD, AFLP and SSR should be carried out in order to further clarify the phylogenetic relationship between Moroccan *M. truncatula* populations.

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