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Original article

Genetic variability organisation and gene flow in natural populations of *Medicago polymorpha* L. prospected in Tunisia

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Abstract – Sixteen accessions of Tunisian germplasm of *Medicago polymorpha* L. were characterised using enzymatic markers to identify useful phytogenetic resources for their integration in programmes of safeguard and conservation. Protein extracts from populations of this species were analysed by electrophoresis to estimate isoenzyme variation. Two enzymatic systems providing six alleles were chosen from six systems tested. From statistical treatments, it appears that a considerable variation was found between accessions, indicating an interesting genetic potential for selection. Such a result (50 % of the total variation due to population differentiation) is in accordance with the strictly autogamous system of reproduction in this taxon. Isozyme data suggest that the level of gene flow between populations of *M. polymorpha* is very low. Additionally, genetic drift may be responsible for strong among-population differentiation. It seems that the genotypic structure of natural populations, as seen from isoenzymatic markers, is not affected by the geographic parameters (distance and altitude). Thus, these markers are of practical interest in the background selection and improvement programmes. © Inra/Elsevier, Paris

***Medicago* / genetic variability / isoenzyme / gene flow / germplasm conservation**

Résumé – Organisation de la variabilité génétique dans les populations naturelles de *Medicago polymorpha* L. Pour identifier les ressources phytogénétiques utiles et leur intégration dans des programmes de sauvegarde et de conservation, seize accessions de germoplasm Tunisien de *Medicago polymorpha* L., sont caractérisées pour leur diversité enzymatique. Les extraits protéiques des populations de cette espèce sont analysés par électrophorèse pour estimer la variation isoenzymatique. Deux systèmes enzymatiques révélant six allèles ont été retenus, parmi les six

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systèmes testés par électrophorèse sur gel d'amidon, pour leur profil électrophorétique polymorphe. Les analyses statistiques ont montré une importante variabilité entre les populations étudiées indiquant un potentiel génétique intéressant pour la sélection. L'hétérogénéité interpopulation est élevée, un tel résultat est en accord avec le système de reproduction strictement autogame de ce taxon. Les données isoenzymatiques suggèrent que le flux génique entre les populations de cette espèce est faible. La dérive génétique est sans doute responsable de la différenciation entre les populations. Par ailleurs, aucun paramètre éco-géographique (distance et altitude) ne semble influencer leur structuration génotypique. Les marqueurs isoenzymatiques ainsi révélés sont très utiles dans des schémas de sélection pour une meilleure efficacité d'amélioration.

***Medicago* / variabilité génétique / isoenzyme / flux génique / germoplasm / conservation**

1. INTRODUCTION

In Tunisia, in the last 10 years, the preservation of plant genetic resources has been of prime concern for wild forage and pastoral legumes because they have been subject to severe genetic erosion. Indeed, the local genetic resources have been damaged by the reduction of range land and overgrazing; pastoral spaces are relegated to the dry areas of the country dominated by irregular rainfall. The results of the erosion factors have been the degradation and rarefaction of the native flora, and the disruption of equilibrium between ever-increasing livestock requirements and limited resources. Species of *Medicago* and especially the annual species can be used to valorise and enhance resources. The annual species of *Medicago* constitute an extremely diversified phytogenetic patrimony. By their integration into ley-farming, they can play an important role in crop production increase. They are also able to improve pastoral production in semi-arid areas. *Medicago* is recognised as one of the most important genera of pasture plants in the world. The Mediterranean basin is the centre of the origin of *Medicago* species and includes the world's greatest variability (Heyn, 1963).

Conscious of the value of these plants, we initiated a large research programme in order to introduce them to fallow and marginal pastoral zones. The aim of this work is: 1) to build an inventory of local *Medicago* species and to acquire great genetic diversity through a collection of accessions of *Medicago* species and 2) to evaluate genetically the phytogenetic resources using morphological and physiological traits as well as biomolecular tools. Selection and improvement programmes are based on the search for genetic markers. Isoenzymes electrophoresis is a useful tool for this purpose. The genetic resources assessed by molecular techniques must be considered in conservation strategies.

We used enzymatic markers to characterise *Medicago polymorpha*. This approach has been widely used in elucidating the genetic structure of *Medicago* species (Brunel, 1979; McCoy et al., 1991; Kiss et al., 1993; Bullita et al., 1994; Fayed-Lameche et al., 1996; Salhi Hannachi et al., 1997).

Medicago polymorpha L. syn. *Medicago hispida* Gaertn., the annual species, belongs to the section Spirocarpos of the *Medicago* genus. It is autogamous (self-pollinating) and diploid ($2n = 2x = 14$) (Lesins and Lesins, 1979). The species is widely distributed throughout the Mediterranean region and tolerates a great

variety of habitats. The ubiquitous and polyvalent nature of *M. polymorpha* should be noted (Prosperi et al., 1996). Their spiny pods are one of the characteristics favouring their invasion ability; such pods hook onto sheep wool and hay (Coks et al., 1980).

According to Heyn (1963), three botanical varieties should be recognised: *brevispina*, *polymorpha* and *vulgaris*. Several cultivars of this species are commercialised in Australia. The most common are '*circle valley*', '*serena*' and '*santiago*'. These Australian selected varieties are not well adapted to the environmental conditions of North Africa and Europe (Olea et al., 1989; Bullita et al., 1994).

Observations of *M. polymorpha* at natural sites show that it is well adapted to neutral and slightly acid soils (Reid et al., 1989). Its geographical distribution would indicate a preference for the upper semi-arid bioclimatic stage (Abdelkefi et al., 1990, 1992, 1996). *M. polymorpha* is equally adapted to areas in which the altitude ranges from 10 to 900 m and rainfall from 100 to 800 mm.

In the present study, we investigated the genetic diversity by means of isozyme markers.

2. MATERIALS AND METHODS

2.1. Plant material

Isoenzymatic variation was analysed in 16 spontaneous populations of *M. polymorpha*, collected from a wide range of climatic and edaphic conditions in 1990 (Abdelkefi et al., 1990, 1992). The distribution map (*figure 1*) shows that this species covers a range of bioclimatic stages ranging from the humid to the upper arid. Seeds randomly selected from each population were germinated, and the plants were grown under uniform conditions (25 °C and 12-h day length).

2.2. Protein extraction

Electrophoresis procedures have been described by Salanoubat (1983) and Baatout et al. (1990). For every sample, 50 mg of young leaves were ground in 200 µL of extraction sodium ascorbate buffer (pH 8.4) comprising 4.15 mg of sodium ascorbate, 8.35 mg of D sucrose and 35 µL of 2-beta-mercaptoethanol at 4 °C. The homogenate was centrifuged at 13 000 rpm for 2 min.

2.3. Electrophoretic procedures

The six enzymatic systems (*table I*) were electrophoresed in 13 % horizontal starch gels. Twenty-four wicks of Whatman 3 MM chromatography paper were moistened with the protein extracts and inserted into slits in the gel.

Migration was anodic and conducted under 50 mA for 6 h at 4 °C in the extraction buffer and at constant power (16 W). Procedures for staining enzyme activity were as in Brunel (1979), Stuber and Goodman (1980), Cardy et al. (1980) and Goodman et al. (1980) (*table I*).

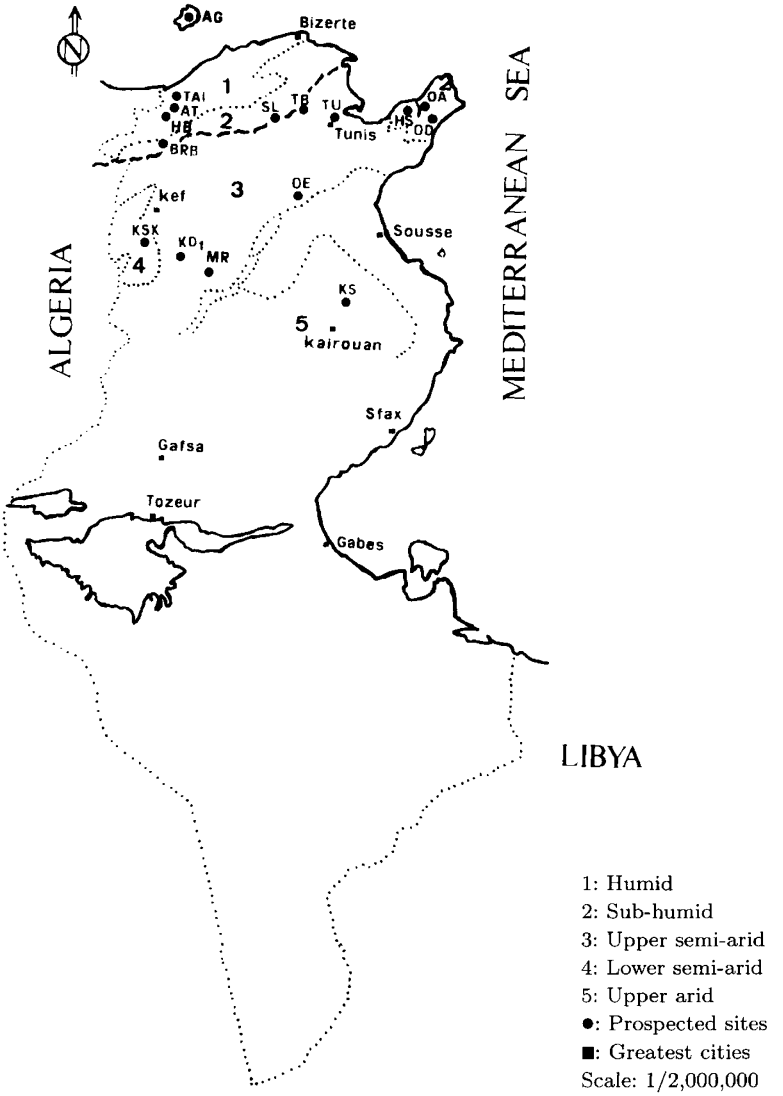

Figure 1. Natural distribution in collection sites of *Medicago polymorpha* in relation to bioclimatic stages in Tunisia.

2.4. Data analysis

The bands of each zymogram were interpreted in terms of loci and alleles. Allelic frequencies were used to describe the distribution of genetic variation within and between populations and to estimate the gene flow among populations. These parameters were estimated from Wright's F-statistics (Wright, 1951, 1969; Weir and Cokerham, 1984).

Table I. Enzyme systems analysed by horizontal starch gel electrophoresis.

Abbreviation	European Community recommended name	EC No.	Subunit structure	References for procedures of staining enzyme activity
GOT	Glutamic-oxaloacetate transaminase	2. 6. 1. 1	Dimer	Goodman et al. (1980)
ICD	Isocitrate dehydrogenase	1. 1. 1. 42	Dimer	Cardy et al. (1980)
LAP	Leucine aminopeptidase	3. 4. 1. 1	Monomer	Brunel (1979)
6-PGD	6-Phosphogluconate dehydrogenase	1. 1. 1. 40	Dimer	Stuber and Goodman (1980)
PGM	Phosphoglucomutase	2. 7. 5. 1	Monomer	Cardy et al. (1980)
PGI	Phosphoglucoisomerase	5. 3. 1. 9	Dimer	Cardy et al. (1980)

Fst measures degree of between-population differentiation; *Fis* measures within-population variability. A positive value of *Fis* indicates a deficit in heterozygotes in comparison with the Hardy-Weinberg equilibrium expectations.

Fst is widely used to estimate the degree of subdivision between populations. Moreover, the strengths of gene flow and random drift can be assessed from the formula $Nm = 1/4\ (1/Fst - 1)$, where N is the effective size of a population and m is the rate of migration (Wright, 1969).

Geographical distances between populations have been measured on a map, except when a barrier to migration was present (e.g. Mediterranean Sea). Isolation by distance was estimated according to Slatkin (1993).

The linkage disequilibrium between pairs of loci was estimated for each population using the correlation coefficient (Weir, 1990). Either selection pressures or genetic drift acting on pairs of loci can produce a linkage disequilibrium among two alleles. To distinguish between the two cases, gametic associations of the whole data set *Dst* were decomposed into four coefficients which show the parts created within populations concerning *Dis* and *D'is* indices and between populations with *Dst* and *D'st* coefficients. According to Ohta (1982), the comparison of *Dis* and *Dst* values, on the one hand, and of *D'is* and *D'st* values, on the other, allows the two situations to be differentiated.

The *F*-statistics were calculated using GENEPOP (version 1.2) software (Raymond and Rousset, 1995). The overall significance of tests for each locus was estimated by Fisher's combined probability test (Fisher, 1970).

3. RESULTS

3.1. Genetic interpretation

Allelic frequencies at the three polymorphic loci analysed are reported in *table II.* A total of six alleles were identified in all loci. Four enzymes appear to be strictly monomorphic: *LAP*, *PGM*, *PGI* and *6-PGD*.

Table II. Allele frequencies for loci *GOT1*, *GOT2* and *ICD1* in populations of *M. polymorpha*.

Populations	*GOT1*			*GOT2*			*ICD1*		
	GOTA	*GOTB*	*N*	*GOTC*	*GOTD*	*N*	*ICDA*	*ICDB*	*N*
OA	0	1.000	3	0.500	0.500	24	0	1.000	24
HS	0	1.000	13	0	1.000	23	0.957	0.043	23
TAI	0	1.000	15	0.708	0.292	24	0.708	0.292	24
OD	0	1.000	24	0.333	0.666	24	0	1.000	24
KS	0.571	0.429	14	0	1.000	24	0.104	0.896	24
HB	0	1.000	16	0.875	0.125	16	0	1.000	16
SL	0.870	0.130	23	0.522	0.478	23	0	1.000	23
KD1	0.727	0.273	11	0.957	0.043	23	0.065	0.935	23
MR	*	*	24	0.053	0.947	19	0.053	0.947	19
TU	0.023	0.977	22	0.682	0.318	22	0.409	0.591	22
TB	*	*	24	0.087	0.913	23	0	1.000	23
AG	0	1.000	23	0.630	0.370	23	0	1.000	23
OE	0	1.000	7	0.458	0.542	24	0	1.000	24
AT	0.042	0.958	24	0.417	0.583	24	0.042	0.958	24
BRB	0	1.000	8	0.833	0.166	24	0	1.000	24
KSK	0	1.000	7	0.667	0.333	24	0.396	0.604	24

* Untested alleles; N = sample size.

3.1.1. Glutamate oxaloacetate transaminase (GOT)

Two staining regions are apparent for this enzyme. Each region exhibits three patterns: a triple-banded pattern corresponding to heterozygotes and two single-banded homozygotes for diallelic loci *GOT2A*, *GOT2B* and *GOT1C*, *GOT1D*. Enzymes *GOT1* and *GOT2* are thus dimeric. Interlocus interaction is not apparent (*figure 2*).

3.1.2. Isocitrate dehydrogenase (ICD)

Only one gene coding for this enzyme has been detected in *M. polymorpha*. This gene appears to be polymorphic and biallelic (*ICD1A*, *ICD1B*). The *ICD* isozymes (*figure 2*) produced zymograms with one or three bands, suggesting that those with one band were homozygote phenotypes, and those with three bands heterozygotes.

Only one monomorphic zone of enzyme activity with a single band for *LAP* (monomeric enzyme) and *PGI* (dimeric enzyme) was stained. Two monomorphic bands were detected in all populations for *PGM* (monomeric system) and *6-PGD* (dimeric protein) (*figure 2*).

The rare heterozygote individuals detected by electrophoresis prompted us to use allelic frequency to test the autogamous reproduction system of *M. polymorpha* using the *Fis* parameter (*table III*). This test showed that the populations studied were not panmictic. The observed heterozygosity deficiency confirms the autogamous nature of the system of reproduction but with the occurrence of a residual allogamy. The average heterozygosity was 0 to 0.055 % of the sampled polymorphic loci.

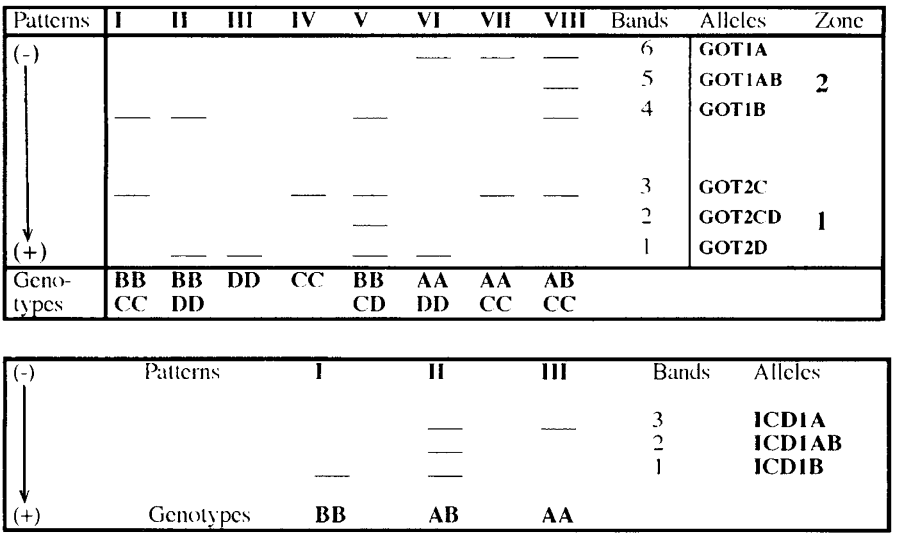

Figure 2. Banding patterns obtained for two polymorphic enzymes (*GOT* and *ICD*) in populations of *Medicago polymorpha* L.

Table III. Departure from Hardy-Weinberg proportions for loci *GOT1*, *GOT2* and *ICD1* in populations of *M. polymorpha* using *Fis* according to Weir and Cokerham (1984) is indicated.

Populations	*GOT1*		*GOT2*		*ICD1*	
	Fis	*P*	*Fis*	*P*	*Fis*	*P*
OA	*	*	**+1**	**0.0000**	*	*
HS	*	*	*	*	**+1**	**0.0222**
TAI	*	*	**+0.806**	**0.0000**	**+0.806**	**0.0000**
OD	*	*	**+0.531**	**0.0153**	*	*
KS	**+1**	**0.0001**	*	*	**+0.785**	**0.0071**
HB	*	*	+0.455	0.1902	*	*
SL	**+1**	**0.0002**	**+1**	**0.0000**	*	*
KD1	**+1**	**0.0022**	**+1**	**0.0222**	+0.656	0.0667
MR	*	*	**+1**	**0.0270**	**+1**	**0.0270**
TU	*	*	**+1**	**0.0000**	**+1**	**0.0000**
TB	*	*	**+1**	**0.0016**	*	*
AG	*	*	**+0.911**	**0.0000**	*	*
OE	*	*	**+1**	**0.0000**	*	*
AT	**+1**	**0.0213**	**+1**	**0.0000**	**+1**	**0.0213**
BRB	*	*	**+1**	**0.0000**	*	*
KSK	*	*	**+1**	**0.0000**	**+0.748**	**0.0003**

Bold type indicates significance ($P < 0.05$); * monomorphic locus; P: the probability of Fisher's exact test on contingency table.

3.2. Population differentiation

The distribution of genetic variation within and among the 16 populations, using F-statistics, showed high genetic interpopulational differentiation. The overall differentiation among populations was highly significant ($P < 10^{-5}$) and corresponded to an *Fst* value of 0.49 (*table IV*), indicating significant genetic heterogeneity between the populations studied.

GOT1, *GOT2* and *ICD1* loci contributed to the distinction between populations by *FST* values of 0.68, 0.34 and 0.56, respectively (*table V*).

Table IV. *Fst* and probability values (P) from contingency tests in *M. polymorpha*.

Locus	*Fst*	*P*
GOT1	0.68483*	$< 10^{-5}$
GOT2	0.34235*	$< 10^{-5}$
ICD1	0.56874*	$< 10^{-5}$
Mean	**0.49803***	

* Highly significant values; bold type indicates the multilocus estimate; P: the probability of Fisher's exact test.

Table V. Summary of Wright's F-statistics at all polymorphic loci in *M. polymorpha*.

Locus	*Fit*	*Fst*	*Fis*	*P*
GOT1	**+0.9848**	**+0.6848**	**+0.9516**	$< 10^{-5}$
GOT2	**+0.9462**	**+0.3423**	**+0.9182**	$< 10^{-5}$
ICD1	**+0.9357**	**+0.5687**	**+0.8509**	$< 10^{-5}$

Bold type indicates significant values.

3.3. Statistical linkage disequilibrium among loci

Genotypic association among pairs of loci was estimated from correlation coefficients. Estimate of linkage disequilibrium was only significant for the association of *GOT2–ICD1*, in the (TAI) Tabarka population ($P < 10^{-5}$) (*table VI*). Linkage disequilibrium between loci for pooled populations (*table VII*) indicated preferential association or linkage between *GOT2–ICD1* ($P < 0.05$) and independence between *GOT1–GOT2* and *GOT1–ICD1* associations (*table VII*).

The analysis of the Ohta indices (*table VIII*) supports the hypothesis that the gametic associations were only due to the action of drift, except for combination of the pair *GOT2–ICD1*. Ohta indices are presented for both populations in *table VIII*. Comparison of D_{IS} and D_{ST}, on the one hand, and of D'_{IS} and D'_{ST}, on the other, shows that in *M. polymorpha* the disequilibrium between the loci pairs was not systematic and created by genetic drift in each site ($D_{IS} < D_{ST}$ and $D'_{IS} > D'_{ST}$).

Table VI. Linkage disequilibrium between pairs of loci in populations of *M. polymorpha*.

Populations	Locus # 1	Locus # 2	P
TAI	***GOT2***	***ICD1***	$\mathbf{< 10^{-5}}$
KS	*GOT1*	*ICD1*	1.00000
SL	*GOT1*	*GOT2*	1.00000
KD1	*GOT1*	*GOT2*	1.00000
KD1	*GOT1*	*ICD1*	1.00000
KD1	*GOT2*	*ICD1*	0.07693
MR	*GOT2*	*ICD1*	1.00000
TU	*GOT1*	*GOT2*	1.00000
TU	*GOT1*	*ICD1*	0.40810
TU	*GOT2*	*ICD1*	0.37593
AT	*GOT1*	*GOT2*	0.41567
AT	*GOT1*	*ICD1*	1.00000
AT	*GOT2*	*ICD1*	0.41243
KSK	*GOT2*	*ICD1*	0.58710

Bold type indicates significant estimate.

Table VII. Linkage disequilibrium between loci for pooled populations of *M. polymorpha*.

Pairs of loci	χ^2	df	P test
GOT1–GOT2	1.756	8	0.98761
GOT1–ICD1	1.792	8	0.98673
GOT2–ICD1	**Infinite**	**12**	**Highly significant**

Estimates of P-values are indicated; bold type indicates significant estimates.

Table VIII. Ohta's indices computed for the 16 populations of *M. polymorpha* for pairs of loci. $D_{IS} < D_{ST}$ and $D'_{IS} > D'_{ST}$.

Pairs of loci association	Within populations		Among populations		Total inbreeding
	D_{IS}	D'_{IS}	D_{ST}	D'_{ST}	D_{IT}
GOT1–GOT2	0.00231	0.93065	0.22128	0.00092	0.93156
GOT1–ICD1	0.00158	1.40489	0.30556	0.00888	1.41377
GOT2–ICD1	0.04454	1.04110	0.24467	0.00057	1.04167

3.4. Population dynamics

3.4.1. Gene flow

Wright (1951) showed that *Fst* is related to the level of gene flow. The *Fst* of 0.498 leads to a low value of $Nm = 0.26$ ($Nm < 1$), suggesting a low level of gene flow among populations.

3.4.2. Isolation by distance

The distinct ecogeographic origins of studied accessions of *M. polymorpha* were used to identify whether environmental factors are responsible for the allozyme variation. Additionally, gene flow was analysed in relation to geographical distances according to the Slatkin method founded on slope sign of regression between Nm and distance of a log. base (Slatkin, 1985, 1993; Chevillon et al., 1995 a, b).

Computing of correlation between Nm and distance shows that the slope of regression was near zero (*figure 3*). Another correlation coefficient was calculated between *Fst* and geographical distance (Pasteur et al., 1995), and again slope was weak (0.069) and regression not significant ($P = 0.313 > 0.05$) (*figure 4*). We can conclude that no isolation by distance was detected.

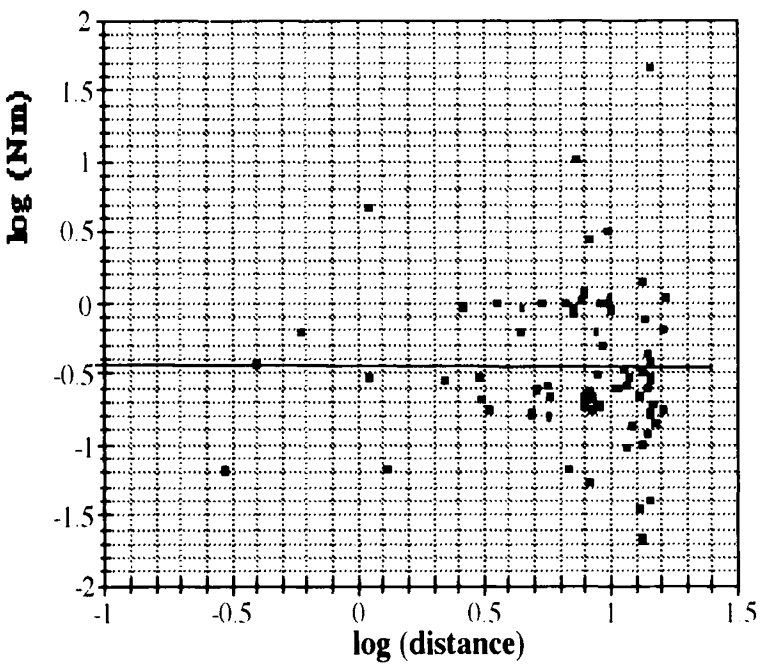

Figure 3. Plot of *Nm* (number of migrants) against geographic distance (km) in populations of *Medicago polymorpha*. Pairwise estimates of *Nm* are calculated using the *Fst* estimate of Weir and Cockerham (1984). Slope = −0.017.

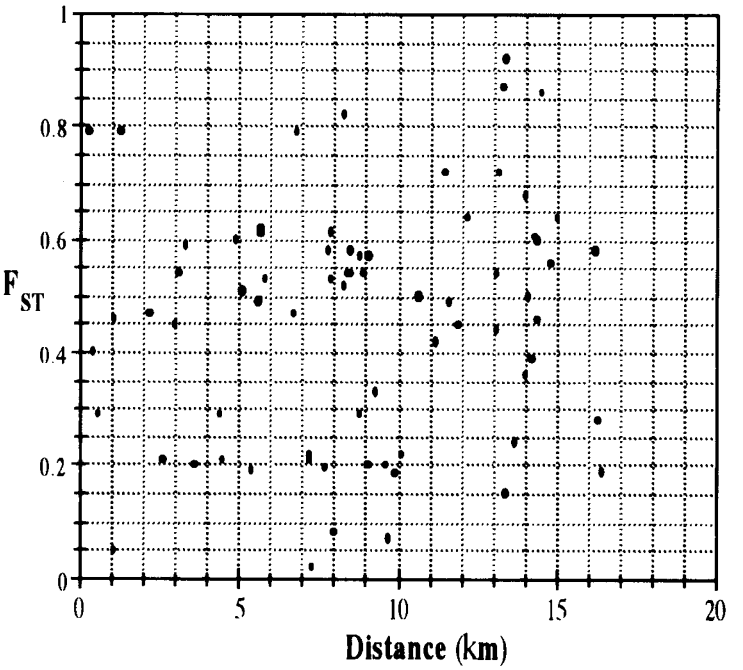

Figure 4. Correlation between *Fst* and geographic distance for populations of *Medicago polymorpha*. Slope = 0.069; $P = 0.313 > 0.05$ not significant.

Structuration of genetic diversity was analysed in relation to the altitude of sites colonised by *M. polymorpha*. Three groups of populations were defined: group I: populations from low altitude (from 10 to 80 m); group II: populations colonising stations from 100 to 500 m; and group III: populations native at high altitude (from 650 to 900 m).

Table IX shows a differentiation among populations, estimated by *Fst*. The differentiation within each group was stronger than the differentiation between groups (*Fst* within groups > *Fst* among groups). Altitude did not affect the structuration of the genotypes studied.

4. DISCUSSION AND CONCLUSION

Previous work showed that in *M. polymorpha* natural accessions a high level of phenotypic diversity exists among populations. The variability range seemed to be continuous over the prospected area. Parameters of precocity, vegetative development and seed production contribute to the distribution of phenotypic diversity (Salhi Hannachi, 1996). Because morphological traits only represent a part of the genetic variability, and because the organisation of this diversity is highly subject to natural selection and environmental factors, knowledge of the evolution and dynamics of populations requires analysis of neutral markers such as isozymes. Neutral markers should help to specify the genetic structure

Table IX. Comparison of *Fst* within groups and between altitude groups in *M. polymorpha.*

		GOT1	*GOT2*	*ICD1*	All
		Within groups			
Group I	*Fst*	**0.6824**	**0.2165**	**0.0689**	**0.4646**
	P	< 0.0001	0.0001	0.052	0.0001
Group II	*Fst*	− 0.0267	**0.4400**	**0.7556**	**0.5788**
	P	1	< 0.0001	< 0.0001	< 0.0001
Group III	*Fst*	**0.6376**	**0.3734**	**0.4140**	**0.4457**
	P	< 0.0001	< 0.0001	0.0001	< 0.0001
		Between groups			
Groups I and II	*Fst*	**0.3799**	**0.00021**	**0.1881**	**0.1900**
	P	< 0.0001	0.25	< 0.0001	< 0.0001
Groups I and III	*Fst*	**0.2109**	**0.1414**	**0.1484**	**0.1675**
	P	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Groups II and III	*Fst*	**0.0709**	**0.0773**	− 0.0044	**0.04718**
	P	0.02	0.0003	0.56	0.0004
Allgroups	*Fst*	**0.2568**	**0.0794**	**0.0898**	**0.1353**
	P	< 0.0001	< 0.0001	< 0.001	< 0.0001

Bold type indicates significant *Fst*; 'All' refers to multilocus estimate; * *Fst* not significant.

of populations and the level of gene flow among them. In this study, we investigated genetic variability using several enzymatic systems. Among the six systems tested, two (*GOT* and *ICD*) were retained. These two systems delivered six alleles and were used to estimate the gene flow between populations, to give information on the organisation of genetic diversity.

The genetic variation occurring within *M. polymorpha* is organised with high levels of differentiation between populations. According to Hamrick and Godt (1990), species that have selfing or mixed mating systems have lower levels of genetic variability than predominantly outcrossed species, and 51 % of their total genetic diversity is apportioned between populations in comparison to 10 % for outcrossed species. Our results on *M. polymorpha* agree with these generalisations (50 % of the total divergence is attributable to the differentiation among populations). Indeed, the level of variability between altitudinal groups is significantly lower than that between populations within groups. The high levels of autogamy in this species result in a strong pattern of among-population genetic differentiation. Distribution of genetic variability and the extent of gene flow between populations are important for the understanding of genetic evolution. The degree of among-population differentiation is affected by the rate at which genes are carried between populations by the migration of pollen or seeds. The *Fst* of 0.498 leads to $Nm = 0.26$, suggesting a low gene flow among populations. This could be due to the high levels of autogamy in *M. polymorpha.* Genetic drift and selection could also result in strong among-population differentiation ($Nm < 1$). That selection occurs is suggested by differentiation, albeit rather weak, between altitudinal groups. The high pod dispersal contributes to the observed organisation of variability; the spiny pods favour the invasion of *M. polymorpha.* The pattern observed is typical of species

with a high dispersal of seeds (Hamrick and Godt, 1990). In *M. polymorpha*, easy seed dispersal does not prevent isolation of populations. This is confirmed by the noncorrelation between Nm and geographic distances separating populations. Genotypic structure is not affected by environmental factors such as distance and altitude. *M. polymorpha* is interesting because of its ability to adapt to difficult soils and climates. The survey and collection of the genetic resources of this species must be incorporated into conservation strategies. To establish a gene bank we need to know how the genetic variability is organised in the species. In-situ gene banks have been used in Syria and in Iran to select the *M. polymorpha* variety (Cocks et al., 1986; Nazari-Dashlibrown and Francis, 1988). The conservation strategies were fundamental to the development of successful mixtures of *M. polymorpha* for ley-farming systems in Tunisia.

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