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Pheno-morphological variation, genetic diversity and population structure of Tunisian Echinus Medic (*Medicago ciliaris* L.)

C. Jabri[1], H. Sbei[2], N. Zitouna[1], N. Trifi-Farah[1] and A. Zoghlami Khelil[3]

[1]Laboratory of Molecular Genetics, Immunology and Biotechnology, Faculty of Sciences, University of Tunis El Manar, Tunis, Tunisia
[2]Laboratory of Plant Breeding, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan
[3]Laboratory of Animal and Forage Production, National Institute for Agricultural Research, University of Tunis Carthage, Ariana, Tunisia

Corresponding author: N. Trifi-Farah
E-mail: neilatrifi@gmail.com

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ABSTRACT. *Medicago ciliaris* L., considered as a valuable genetic resource, is a good candidate for the improvement of marginal or degraded lands with low fertility or high salinity. In this study, the pheno-morphological and genetic diversity were investigated in 14 Tunisian populations of *M. ciliaris* for the first time. Fourteen morphological traits showed significant differentiation between populations and high levels of diversity. Two amplified fragment length polymorphism primer combinations (*E*-AGC/*M*-CAA; *E*-AAG/*M*-CTG) were analyzed using an automated capillary electrophoresis system. A total of 528 loci were

generated, of which 54% were polymorphic. Allelic polymorphism ranged from 0.02 to 0.5. Significant variation between populations was found for gene diversity, mean number of alleles per locus and Shannon index for which mean values were 0.17, 0.26, and 1.57, respectively. Analysis of molecular variance revealed a high rate of genetic variation within populations. Principal component analysis and genotypic clustering discriminated *M. ciliaris* populations according to their geographical origin. *M. ciliaris* clustered into three main groups. The first group was associated with high inland and cold areas, the second was defined by low areas with mild winters while the third described low coastal areas. Similarity of morphological and molecular results indicated that either markers could be used for the study of genetic diversity in this species.

Key words: *Medicago ciliaris* L.; Genetic diversity; AFLP markers; Eco-geographical factors; Genetic differentiation; Bayesian structure

INTRODUCTION

In Tunisia, the genus *Medicago* is well distributed in all bioclimatic regions. Annual medic species represent about 50% of the total spontaneous flora; however, frequencies vary within the region (Seklani et al., 1995). *Medicago ciliaris* L. is a diploid species (2n = 16), predominantly autogamous plant found in the Mediterranean region (Badri et al., 2008). In Tunisia, *M. ciliaris* grows spontaneously in northern areas, restricted to humid, sub-humid, and superior and inferior semi-arid bioclimatic stages (Seklani et al., 1995). Cherifi et al. (2011) reported that in addition to its good forage quality, it has the capacity to improve nitrogenation of salted land. It is a glycophyte plant that can grow in moderately saline soils in association with halophytes, producing up to 40% of the vegetative cover in rainy years (Barrett-Lennard and Setter, 2010). Ben-Salah et al. (2011) reported that *M. ciliaris* is a good candidate for the improvement of marginal or degraded lands with low fertility, and/or high salinity lands such as 'Sabkha'. Despite the potential of this species, little attention was given by scientists to its use in breeding programs. Studies have mainly focused on genetic diversity using morphological traits, enzymatic and molecular markers (Badri et al., 2008), cytological (Sadeghian et al., 2014), and physiological aspects associated with ecological stress (Rabhi et al., 2007; Ben-Salah et al., 2011). Investigation of the genetic diversity of *M. ciliaris* is imperative in order to identify valuable genotypes adapted to local conditions, especially to drought and salinity.

Knowledge on the genetic diversity of *M. ciliaris* populations would be valuable to identify populations adapted to local climatic conditions. Indeed, the assessment of existing genetic diversity at some level is fundamental for the efficient management of crop genetic resources, which is dependent on the available germplasm having great diversity (Mondini et al., 2009). Molecular markers are a good alternative for the assessment of existing genetic diversity, characterization of local germplasm and for establishing breeding programs (Mondini et al., 2009). Amplified fragment length polymorphisms (AFLPs) are the most suitable molecular technique for such a purpose. They produce a large number of markers scattered across the whole genome and able to resolve small genetic differences without prior knowledge of the corresponding DNA sequence (Vos et al., 1995). AFLP markers are

reproducible and are used to identify individuals and for parentage analysis, gene mapping, and to identify quantitative trait loci (Mueller and Wolfenbarger, 1999; Mondini et al., 2009). These markers are mainly used to study genetic variation and determine genetic structure at species and population level. Such analyses are crucial for conservation genetics (Mueller and Wolfenbarger, 1999). Therefore, it is important to evaluate the genetic diversity of *M. ciliaris* for pheno-morphological and molecular variations.

There is currently little information available on genetic variation of Tunisian *M. ciliaris* populations. Nevertheless, an important variability within and between Tunisian populations of *M. ciliaris* was found by Cherifi et al. (1993) in studying four populations of *M. ciliaris* using 20 morphological characters related to vegetative and reproductive stage and for Badri et al. (2008) who analyzed the genetic variability of four populations of *M. ciliaris* using 19 quantitative traits and 12 SSR markers. The latter found high levels of genetic diversity at quantitative trait and microsatellite loci occurred within populations.

The present study investigated pheno-morphological and molecular variation of 14 populations of *M. ciliaris* in Tunisia. It aimed to a) estimate diversity within and between populations for pheno-morphological traits; b) characterize the genetic diversity using AFLP markers, and c) analyze the relationships between populations using Bayesian and phylogenetic methods. The results may provide information for the potential use of this species in breeding programs and for selection of ecotypes suitable for forage and pasture purposes.

MATERIAL AND METHODS

Plant material

Fourteen local populations of *M. ciliaris* L., conserved at the National Gene Bank of Tunisia, were used in this study. Seeds were sampled from different regions in northern Tunisia (from the north-east to the north-west). The eco-geographical characteristics of sampling sites are presented in Table 1.

Table 1. Main characteristics of the collection sites of *Medicago ciliaris* L. populations.

Population code	Site name	Bioclimatic stage	Altitude (m)	Annual rainfall (mm)	Max/Min T (°C)	Tcal	OM	pH	EC	N	P_2O_5
9140	Siliana	SA	575	341.1	38.6/1.6	53.8	2.4	8.1	0.3	1075	2.9
9141	Oued Messouge	SA	500	367.1	38.6/1.6	27.7	1.5	8.0	0.3	1043	3.4
9142	Dougga	SH	320	493.5	21.7/9.9	65.9	2.0	8.0	0.2	765	2.1
9143	Zaghouan	SA	300	496.0	32.6/6.7	19.4	2.0	7.6	2.4	1244	4.2
9144	Ghzala	SH	5	527.1	33.4/4.9	42.3	2.5	7.9	1.0	1044	13.3
9145	Bizerte	SH	5	554.2	31.8/6.7	18.2	2.9	8.2	1.3	1558	11.5
9146	Mateur1	SH	125	581.1	33.4/4.9	22.1	15	7.9	0.7	130	8.3
9147	Aouja	SH	150	507.4	31.1/7.6	45.2	2.5	7.9	0.6	1474	13.5
9148	Frétissa	SH	240	582.0	33.4/4.9	20.2	2.3	8.0	0.5	1273	18.6
9149	Mateur 2	SH	10	527.1	33.4/4.9	18.2	2.4	8.0	0.4	1504	10.2
9150	Zana	SH	10	507.0	31.8/6.7	44.2	1.8	8.0	1.1	1064	23.3
9151	Raoued El Hessiane	SA	5.8	398.5	35.1/5.4	36.2	2.6	7.6	0.4	1460	12.0
9152	Kalaat Andalous	SA	5.9	398.5	35.1/5.4	52.6	2.8	7.6	0.4	430	22.6
9153	Ghar El Melh	SH	6.2	497.8	32.9/6.7	54.3	1.2	7.2	0.2	160	26.6

SA, semi-arid; SH: sub-humid; Max/Min T, maximum temperature/minimum temperature; Tcal, total calcareous (%); OM, organic matter (%); EC, electric conductivity (Mmho/cm); N, nitrogen (ppm); P_2O_5, assimilated phosphorus (ppm).

Pheno-morphological analyses

To estimate the genetic variability within and between populations of *M. ciliaris*, 30 seeds per population were used. They were surface sterilized with 6% sodium hypochloride, scarified using sand paper and sown in Jiffy pots. After the emergence of the second trifoliate leaf, seedlings were transplanted in the experimental field of INRAT on loamy clay soil (32% clay, 38% loamy, 29% sand) with alkaline pH (8.2). The field is located in the semi-arid area in north Tunisia with 426 mm as average annual rainfall and 9.8° and 34.2°C as minimum and maximum temperatures, respectively. Total rainfall during the 2009/2010 growing season was 506.3 mm. A randomized block design was used with three blocks. Each population was sown as one row of 10 plants, each spaced 0.50 m apart with 1 m between rows. During the trial, 50 kg P_2O_5/ha was used for fertilization under rain-fed conditions prior to sowing. Weeds were removed manually. Complementary irrigation was provided during plant establishment (late autumn). Twenty-one quantitative variables were investigated by measuring each individual plant (Table 2).

Table 2. Measured pheno-morphological traits and their abbreviations.

Traits	Names of measured traits	Abbreviations
Growth traits	Number of nodes of the main stem at harvest	NN
1)	Position of the node of the first raceme flowered	PNF
1)	Length of the peduncle of the first raceme of the main stem	LP
1)	Diameter of the collet (mm)	DC
1)	Number of total branches at harvest	NS
1)	Leaf surface (leaf between internodes 5 and 6 of the main stem) (cm^2)	LS
1)	Length of the main stem at the first flower ripening (cm)	LMF
1)	Length of the main stem at maturity of the first pod (cm)	LMS
1)	Growth rate expressed by GR = (LMS - LMF) x 100	GR
Reproductive traits	Number of days from field transplantation to flowering time	NF
1)	Number of days from field transplantation to maturation of the first pod	NDM
1)	Stem dry weight (g)	SDW
1)	Pod dry weight (g)	PDW
1)	Reproductive effort expressed by REP = PDW / (SDW + PDW)	REP
1)	Seed weight of 30 pods (g)	SWTP
1)	Number of racemes with one pod on the main stem	NROP
1)	Number of raceme with two pods on the main stem	NRTP
1)	Number of raceme with three pods on the main stem	NRTHP
1)	Total number of pods on the main stem	NTP
1)	Number of seeds per pod	NSP
1)	Thousand seed weight (g)	TSW

Measures were taken every 10 days between 09/02/2010 and 12/04/2012. GR, growth rate; LMS, length of the main stem at maturity of the first pod (cm); LMF, length of the main stem at the first flower ripening (cm). REP, reproductive effort; PDW, pod dry weight (g); SDW, stem dry weight (g).

Molecular assessment

DNA extraction

Total genomic DNA was extracted from 20 plants per population following the method described by Saghai-Maroof et al. (1984) using 200 mg fresh leaf tissue. After purification, the DNA concentration was estimated using a UV spectrophotometer (Spectro UV-Vis UVD-2950, LABOMED) according to the A_{260}/A_{280} ratio. The DNA quantity was estimated according to the formula DNA (ng/µL) = A_{260} x 50 x DF, where DF is the dilution factor. The DNA samples were stored at -20°C until use.

Amplified fragment length polymorphisms

Genomic DNA (50 ng) was double-digested with the following restriction enzymes: 5 U *Eco*RI (New England Biolabs) in a 20-µL final volume at 37°C for 4 h, and 5 U *Mse*I (New England Biolabs) in a 40-µL volume at 65°C for 3 h. Next, the digested products were incubated for 10 min at 75°C to determine enzymatic activity.

The restricted fragments were ligated to synthetic adapters of *E*coRI and *M*seI using T4 DNA ligase overnight at 4°C. A 25-µL sample was subjected to pre-selective polymerase chain reaction (PCR) in a C1000™ (Bio-Rad) thermal cycler. The thermal cycling conditions were as follows: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C; and a final extension step of 15 min at 72°C. Following pre-selective PCR, the generated pre-amplified DNA fragments were used for a final selective amplification in a second PCR with a 20-µL volume using double-primer combinations, each with three selective nucleotides (*Eco*RIA-AAG, *Mse*IC-CTG) and (*Eco*RIA-AGC, *Mse*IC-CAA) (Vos et al., 1995). The *Eco*RI primer was labeled with the fluorescent dye VIC for the first combination and with the fluorescent dye NED for the second combination. The mixture was loaded onto the capillary system of the Applied Biosystems ABI PRISM 3130 Genetic Analyzer using 3130 POP-7 polymer (Applied Biosystems) and analyzed using the Gene Mapper v4.0 software (Applied Biosystems).

Statistical analysis

For pheno-morphological analyses, data were analyzed using the general linear model procedure (PROC GLM) using the SAS software (SAS, 9.0). The model used included the main effects of populations, individuals, and their interaction and blocks. Populations and blocks were fixed effects, whereas individuals (within population) were a random variable. Differences among means were separated using the Duncan multiple range test at significance level of $P < 0.05$. Correlations were carried out between pheno-morphological traits using the correlation procedure (PROC CORR). Multivariate analyses were performed using means of pheno-morphological traits (only those with significant variation at a level of $P < 0.05$) and eco-geographical factors, and correlations were calculated.

For molecular analyses, the polymorphic markers were compiled into a binary matrix (0/1). Polymorphic information content (PIC) was calculated according to the formula described by Powell et al. (1996). The parameters of genetic diversity between and within populations were estimated according to Nei (1973) using the POPGENE 1.32 software (Yeh and Boyle, 1997) and were listed as: i) within populations - gene diversity for each molecular

marker (*H*); mean number of alleles per locus (N_A), Shannon index (*I*), percent and number of polymorphic loci. All these parameters except for percent and number of polymorphic loci were subjected to analysis of variance and Duncan's means classification with the SAS software version 9.0. ii) between populations - total gene diversity (H_T), genetic diversity within population (H_S), coefficient of gene differentiation (G_{ST}), and gene flow (N_m) according to the method described by Slatkin and Barton (1989). Finally, variation between populations was estimated using analysis of molecular variance (AMOVA), carried out in Genalex ver.6.5. (Peakall and Smouse, 2006).

Genetic distance (G_D) was used as a tool to draw the dendrogram based on the un-weighted pair group method with arithmetic mean (UPGMA) (Nei, 1978). Bayesian structure was estimated using the STRUCTURE 2.3.4 software (Pritchard et al., 2000). The number of groups or subpopulations (k) was set from 1 to 10, and five iterations were performed. An admixture model with a burn-in period of 500,000 and 50,000 Markov Chain Monte Carlo repetitions was adapted. The efficient (k) value was suited with the higher *ad hoc* index (Δk) as used by Evanno et al. (2005).

RESULTS

Pheno-morphological variation

Analysis of variance applied on pheno-morphological traits showed significant differences among populations for 14 traits among the 21 traits recorded. The within-population variation was not significant (Table 3). The most discriminating traits were length of the peduncle of the first raceme of the main stem, thousand seed weight, leaf surface, number of days to flowering, and position of the first flowering node. Considering the classification of populations according to the Duncan test (data not shown), populations from high inland areas (9140 from Siliana and 9141 from Oued Messouge) displayed the highest values for the following traits: number of seeds per pod, number of racemes with one pod, diameter of the collet, and length of the main stem at the first flower ripening (6.07, 14.03, 5.85, and 25.70, respectively). Population 9144 from Ghzala (low altitude and high rainfall area with a mild winter) had the highest number of total branches at harvest, the latest flowering date (5.03 and 97.03, respectively), and the lowest leaf surface, shortest stems, shortest peduncle, and the lowest thousand seed weight (2.54, 14.06, 1.40, and 9.54, respectively). Population 9151 from Raoued El Hessiane (low coastal area) showed the highest values for leaf surface, pod dry weight, and thousand seed weight (5.46, 76.73, and 13.95, respectively) compared to population 9153 from Ghar El Melh (near the lake of Ghar El Melh), which displayed the lowest number of branches and the lowest stem dry weight (4.24 and 38.49, respectively). Finally, plants with a large collet had the highest leaf surface, the longest main stem at maturity, the highest flowered node position, and produced numerous branches at harvest.

Significant correlations were found between growth traits ($r \geq 0.30$; $P \leq 0.001$); indeed, number of days to flowering time was correlated with length of the main stem at the first flower ripening, at maturity of the first pod, and growth rate ($r = -0.49$; $r = -0.34$, $r = 0.39$; respectively). Diameter of the collet was correlated to the dry weight of stems and pods ($r = 0.70$; $r = 0.60$, respectively); total pod number on the main stem was correlated to the number of nodes on the main stem at harvest, the diameter of the collet, and the length of the main stem at the first flower ripening ($r = 0.66$, $r = 0.30$, $r = 0.44$, respectively); number of seeds per pod was correlated to the length of the main stem at the first flower ripening ($r = 0.33$).

Table 3. Analysis of variance for the 21 pheno-morphological traits recorded in *Medicago ciliaris* L. populations.

Parameters	DF_{pop}	Mean	R^2	MSE	F_{ind} d.f. = 9	$Pr > F_{ind}$	SS_{pop}	MS_{pop}	F_{pop}	$Pr > F_{pop}$
NN	13	25.74	0.24	3.64	1.01	0.4307	239.09	18.39	1.39	0.1615
PNF	13	8.83	0.31	1.90	1.03	0.4120	230.19	17.71	4.90	<0.0001
LP	13	1.83	0.41	0.38	1.59	0.1176	15.15	1.17	8.19	<0.0001
DC	13	5.30	0.29	1.13	0.60	0.7934	40.18	3.09	2.44	0.0037
NS	13	4.64	0.31	1.15	1.25	0.2654	39.65	3.05	2.29	0.0067
LS	13	3.84	0.35	1.55	0.94	0.4937	214.68	16.51	6.84	<0.0001
LMS	13	129.40	0.33	27.91	0.95	0.4812	34459.81	2650.75	3.40	<0.0001
LMF	13	18.50	0.32	10.70	1.29	0.2401	4086.98	314.38	2.75	0.0010
GR	13	9.05	0.22	6.56	1.01	0.4292	909.42	69.96	1.63	0.0759
NF	13	91.84	0.40	8.61	0.51	0.8639	4773.35	367.20	4.95	<0.0001
NDM	13	155.09	0.14	12.32	0.31	0.9702	2557.29	196.71	1.30	0.2133
SDW	13	48.76	0.33	27.96	1.40	0.1860	21537.39	1656.72	2.12	0.0128
PDW	13	60.44	0.20	34.51	0.91	0.5123	27453.66	2111.82	1.77	0.0461
REP	13	0.58	0.15	0.31	0.80	0.6147	1.33	0.10	1.09	0.3670
SWTP	13	1.64	0.30	0.67	1.77	0.0725	21.76	1.67	3.77	<0.0001
NROP	13	12.65	0.32	3.06	0.52	0.8614	268.01	20.62	2.20	0.0093
NRTP	13	3.68	0.24	1.87	0.57	0.8185	40.38	3.11	0.89	0.5648
NRTHP	13	1.91	0.44	1.08	1.15	0.3357	21.16	1.63	1.40	0.1666
NTP	13	16.66	0.20	3.41	0.81	0.6066	199.20	15.32	1.32	0.1995
NSP	13	5.03	0.21	1.48	1.46	0.1624	74.53	5.73	2.61	0.0018
TSW	13	11.92	0.38	2.42	2.10	0.0294	538.12	41.39	7.09	<0.0001

DF_{pop}, degrees of freedom (d.f.) between populations; Mean, means of all scores for phenotypic traits; R^2, squared error; MSE, mean squared error; F_{ind}, F ratio within populations, $Pr > F_{ind}$, significant level of probability of variance within populations; SS_{pop}, sum of squares between populations; MS_{pop}, mean square between populations; F_{pop}, F ratio among populations; $Pr > F_{pop}$, significant level of probability of variance among populations. For other abbreviations, see Table 2.

Significant correlations were also found between eco-geographical and pheno-morphological factors. The most influential factors on plant growth were organic matter, which enhanced the number of total branches at harvest (r = 0.70; n = 14), and stem dry weight (r = 0.55; n = 14) and nitrogen content, which enhanced the diameter of the collet and pod dry weight (r = 0.79 and r = 0.6, respectively; n = 14). Concerning reproductive traits, the flowering date was mainly affected by rainfall (r = 0.53; n = 14) while the number of racemes with one pod was influenced by min and max temperatures (r = 0.80 and r = -0.68, respectively; n = 14).

PCA applied to both pheno-morphological and eco-geographical data clearly discriminated the studied populations of *M. ciliaris*. The first three components explained 61.7% of the total variation in plants traits. PC1 explained 23.3% of the total variation and was negatively correlated to flowering date and rainfall, and was positively correlated to length of the main stem and min temperature. PC2 accounted for 20.1% of the total variation and was positively correlated to the diameter of the collet, pod dry weight, peduncle length, main stem length at flowering, organic matter, pH, and nitrogen content. PC3 explained 18.3% of the total variation and was defined positively by thousand seed weight, leaf surface, assimilated

phosphorus, and max temperature and was negatively defined by min temperature.

Four groups of populations were revealed along with PC1 and PC2 (Figure 1). The first component opposed population 9141 from Oued Messouge with population 9144 from Ghzala. While the second component opposed populations 9146 of Mateur and 9153 of Ghar El Melh with the rest of the sampled populations.

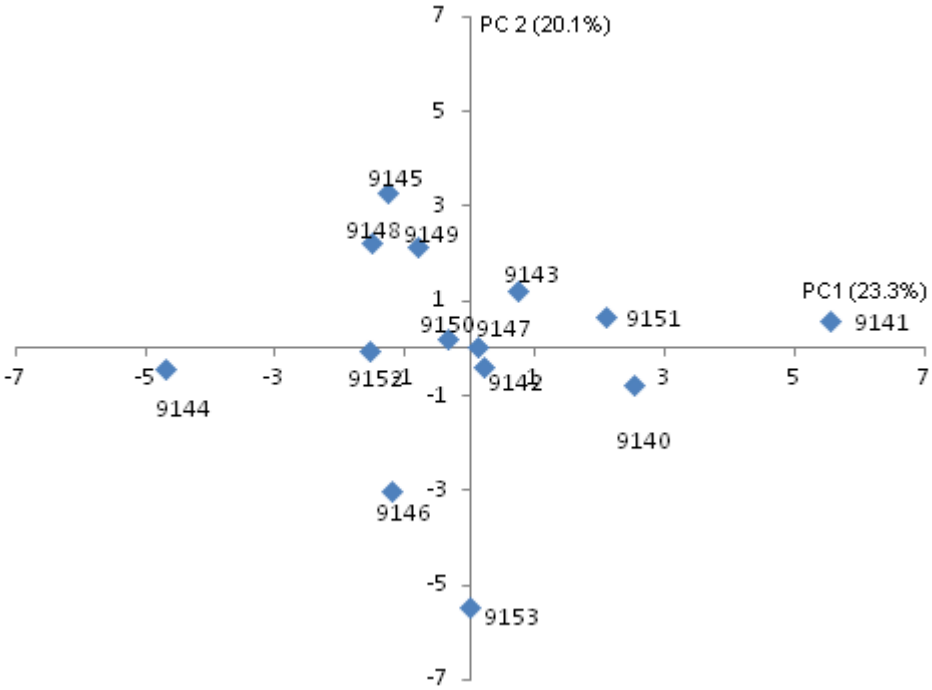

Figure 1. Plot (1-2) of the principal component analysis (PCA) of the 14 populations of *Medicago ciliaris* L. for measured pheno-morphological traits and eco-geographical factors. Along PC1, group 1 corresponds to populations from Oued Messouge (9141), which oppose group 2 containing the Ghzala (9144) population. Along PC2, populations from group 3 from Mateur and of Ghar El Melh (9146 and 9153) oppose group 4, which contains the rest of the populations.

AFLP marker polymorphisms

In addition to the pheno-morphological variation found for *M. ciliaris* populations, polymorphisms at the molecular level were also investigated using AFLP markers. For this purpose, two AFLP primer combinations were used to examine the genetic diversity, genetic relationships, and genetic structure of the 14 Tunisian populations of *M. ciliaris* collected in different northern regions. A total of 260 samples were assayed and 612 alleles were generated. Only 528 well-resolved peaks were considered for the subsequent analysis.

A large range of variation was obtained using the two AFLP primer pair combinations (Table 4). The number of polymorphic bands was 284 over 528 scorable patterns (53.8%). The primer combination *E*-AGC/*M*-CAA produced 120 bands while *E*-AAG/*M*-CTG revealed 164 bands. For the two primer combinations, AFLP markers varied from 52 to 491 bp for *E*-AAG/*M*-CTG and from 52 to 494 bp for *E*-AGC/*M*-CAA.

Table 4. Polymorphism indices generated by the AFLP primer combinations.

Primer combination	Number of bands	Number of polymorphic bands	Polymorphism rate (%)	PIC
E-AAG/*M*-CTG	286	164	57.34	0.29
E-AGC/*M*-CAA	242	120	49.59	0.32
Total	528	284	53.78	-
Mean	*264*	*142*	*53.47*	*0.30*

PIC: polymorphic information content.

The *PIC* for all polymorphic markers ranged from 0.02 to 0.5 (data not shown) with an average of 0.29 for the primer combination *E*-AAG/*M*-CTG and 0.32 for the primer combination *E*-AGC/*M*-CAA. The primer combination *E*-AAG/*M*-CTG showed the highest average number of polymorphic bands per population at 92.57, with a maximum of 146 (89.02%) and a minimum of 66 (40.24%) for the population from Oued Messouge (9141) and from Siliana (9140), respectively. The combination *E*-AGC/*M*-CAA revealed fewer polymorphic bands with an average of 69.21. The number of polymorphic bands ranged from a maximum of 93 (77.50%) for the population from Siliana (9140) to a minimum of 49 (40.83%) for the population from Bizerte (9145) (Table 5).

Table 5. Number and rate (%) of polymorphic AFLP markers generated for Tunisian populations of *Medicago ciliaris.*

	E-AAG/*M*-CTG		*E*-AGC/*M*-CAA	
Population	Number	Frequency (%)	Number	Frequency (%)
9140	66	40.24	52	43.33
9141	146	89.02	93	77.50
9142	102	62.20	65	54.17
9143	87	53.05	63	52.50
9144	99	60.37	89	74.17
9145	69	42.07	51	42.50
9146	88	53.66	65	54.17
9147	91	55.49	49	40.83
9148	90	54.88	70	58.33
9149	115	70.12	74	61.67
9150	90	54.88	86	71.67
9151	72	43.90	64	53.33
9152	83	50.61	61	50.83
9153	98	59.76	87	72.50
Mean	92.57	56.45	69.21	57.68

Population genetic diversity

From the analyzed polymorphism obtained by the primer combinations used in the AFLP assay, we estimated the parameters of genetic diversity among and within populations. The genetic diversity revealed by the AFLP markers showed large variation between the studied populations. This finding was supported by the results of the *H*, mean N_A, and *I* assessments

(Table 6); the mean values were 0.17, 0.26, and 1.57, respectively. The number and frequency of polymorphic loci also varied markedly between the populations and their mean values were 161.79 and 56.97%, respectively. Analyses within the population scale predicted that the Oued Messouge population had the highest level of *H* (0.29), the highest mean N_A (1.84), the highest *I* (0.42) and the highest number and ratio of polymorphic loci (239; 84.15%, respectively). The lowest level of genetic variation was observed for the population from Bizerte with an *H* of 0.12, a mean N_A of 1.42 and an *I* of 0.19. The lowest number and percentage of polymorphic loci were attributed to the population from Siliana (239 and 84.15%, respectively). In addition, the calculated genetic diversity (H_T) was 0.26 for the locus *E*-AAG/*M*-CTG, 0.29 for *E*-AGC/*M*-CAA and a total diversity of 0.27 (Table 7).

Table 6. Partitioning of genetic diversity within populations of *Medicago ciliaris.*

Population	*H*	*I*	N_A	Number of polymorphic loci	Frequency of polymorphic loci
9140	0.14^{fge}	0.20^{gf}	1.42^{g}	118	41.55
9141	0.29^{a}	0.42^{a}	1.84^{a}	239	84.15
9142	0.17^{dc}	0.27^{dcf}	1.59^{cbd}	167	58.8
9143	0.15^{dfge}	0.22^{gfe}	1.53^{fed}	150	52.82
9144	0.19^{c}	0.30^{c}	1.66^{b}	188	66.2
9145	0.12^{g}	0.19^{g}	1.42^{g}	120	42.25
9146	0.16^{dfce}	0.25^{de}	1.54^{fed}	153	53.87
9147	0.15^{dfe}	0.23^{fe}	1.49^{feg}	140	49.3
9148	0.15^{dfge}	0.23^{fe}	1.56^{ced}	160	56.34
9149	0.23^{b}	0.34^{b}	1.67^{b}	189	66.55
9150	0.17^{dce}	0.26^{dce}	1.62^{cb}	176	61.97
9151	0.13^{fg}	0.20^{gf}	1.48^{fg}	136	47.89
9152	0.16^{dfce}	0.24^{fe}	1.51^{fe}	144	50.7
9153	0.19^{c}	0.29^{dc}	1.65^{b}	185	65.14
Mean	0.17***	0.26***	1.57***	161.79	56.97

***Highly significant at the $Pr < 0.001$ level. Values with the same superscript letters are not significantly different at the 5% level of probability; *H*: gene diversity, *I*: Shannon Index; N_A mean number of alleles per locus.

Table 7. Genetic diversity estimates for all populations of *Medicago ciliaris*: total gene diversity (H_T), gene diversity within a population (H_S), gene differentiation among populations (G_{ST}) and gene flux (N_m).

Loci	SS	H_T	H_S	G_{ST}	N_m
E-AAG/*M*-CTG	260	0.27	0.17	0.33	0.99
E-AGC/*M*-CAA	260	0.29	0.17	0.37	0.85
Mean	260	0.28	0.17	0.38	0.83
SD		0.0166	0.0076		

SS, sample size; SD, standard deviation.

AMOVA revealed that there was a higher rate of genetic variation within populations (57% of variation). Indeed, the mean genetic differentiation among populations (G_{ST}) was 0.38, which means that 62% of the total genetic variation occurred within populations of *M. ciliaris* and that only 38% of the total genetic diversity was obtained from differentiation

among populations. The two primer combinations revealed almost the same amount of genetic differentiation between the 14 studied populations with a slight advantage for *E*-AGC/*M*-CAA (G_{ST} = 0.37). The primer combination *E*-AGC/*M*-CAA implied less genetic diversity among populations (37% portioned between populations; 63% portioned within populations) than the other primer combination (G_{ST} = 0.33; 33% portioned between populations; 67% portioned within populations). The mean value for gene flow was N_m = 0.83, which is relatively high if we consider the self reproductive system of *M. ciliaris*.

Genetic identity and population structure

From the extent of the genetic diversity observed between the studied populations, Nei's genetic distances (Table 8) computed among *M. ciliaris* populations showed that the smallest distance between pairs of populations was G_D = 0.034; this was also found between populations from Ghar El Melh (9153) and Kaalat Andalous (9152). These populations were considered to be closely related with a similar genetic diversity and exhibited strong similarity (G_I = 0.967). Conversely, the most distant populations were those from Siliana (9140) and Raoued El Hessiane (9151) with G_D = 0.480 and G_I = 0.619, respectively.

Table 8. Nei's (1978) unbiased measures of genetic identity (G_I) (matrix above the diagonal) and genetic distance (G_D) between 14 populations of *Medicago ciliaris* (matrix below the diagonal).

	9140	9141	9142	9143	9144	9145	9146	9147	9148	9149	9150	9151	9152	9153
9140	****	0.870	0.685	0.701	0.668	0.693	0.676	0.651	0.666	0.898	0.652	0.619	0.625	0.664
9141	0.139	****	0.897	0.891	0.890	0.888	0.900	0.857	0.884	0.945	0.860	0.822	0.823	0.870
9142	0.378	0.109	****	0.924	0.947	0.939	0.936	0.908	0.930	0.841	0.920	0.884	0.891	0.915
9143	0.355	0.115	0.079	****	0.925	0.955	0.912	0.887	0.918	0.873	0.906	0.883	0.875	0.902
9144	0.404	0.117	0.055	0.078	****	0.915	0.957	0.960	0.966	0.829	0.957	0.926	0.929	0.952
9145	0.368	0.118	0.063	0.046	0.089	****	0.897	0.871	0.894	0.864	0.895	0.869	0.874	0.898
9146	0.392	0.106	0.067	0.093	0.044	0.109	****	0.947	0.962	0.837	0.923	0.898	0.899	0.927
9147	0.429	0.154	0.097	0.120	0.041	0.138	0.055	****	0.954	0.801	0.924	0.904	0.908	0.927
9148	0.407	0.123	0.073	0.086	0.035	0.112	0.039	0.047	****	0.825	0.939	0.917	0.906	0.925
9149	0.108	0.056	0.173	0.136	0.188	0.147	0.178	0.222	0.193	****	0.813	0.773	0.781	0.827
9150	0.428	0.151	0.083	0.099	0.044	0.112	0.080	0.079	0.063	0.207	****	0.965	0.957	0.963
9151	0.480	0.196	0.123	0.125	0.077	0.141	0.108	0.101	0.087	0.258	0.035	****	0.957	0.942
9152	0.470	0.195	0.116	0.134	0.074	0.134	0.107	0.096	0.098	0.247	0.044	0.044	****	0.967
9153	0.410	0.140	0.089	0.103	0.050	0.107	0.075	0.076	0.079	0.190	0.038	0.059	0.034	****

Based on the molecular profiles obtained using AFLP markers and structure analysis, the 14 *M. ciliaris* populations were clustered into two main groups. Δk (Figure 2) showed a clear peak at k = 2, such that Δk was equal to 700. This was used to generate the Q matrix. Group 1 and group 2 (Figure 2) comprised 43 and 217 individuals, respectively. Group 1 included individuals of populations 9140, 9141, and 9149 originating from Siliana, Oued Messouge, and Mateur, respectively (Figure 3). These populations seem to be the most diverged and were from the most geographically distant regions. Group 2 is the largest group and contained all samples from 11 populations. Based on UPGMA clustering (Figure 4), group 2 contained two sub-clusters: The first sub-cluster (Sub1) contained three populations 9142, 9143, and

9145 from Dougga, Zaghouan, and Bizerte, respectively. The second sub-cluster grouped populations that were located closer to each other, and stronger genetic identity was observed within this sub-cluster. The population clustering showed a clear dependence of the population on geographical origin. This implies that populations sharing the same geographical area have closer origin sites and might express high genetic similarities on their molecular profiles.

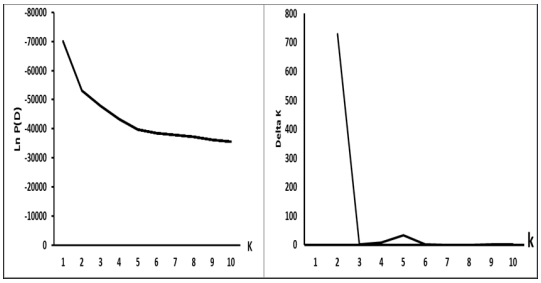

Figure 2. Two methods for determining the optimal K value from STRUCTURE analysis. **A.** Rate of change in mean log probability of the data LnP (D) between successive K (K averaged over the three runs), K ranges from 1 to 10. **B.** *Ad hoc* procedure described by Pritchard et al. (2000).

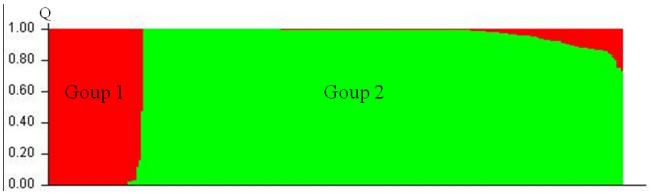

Figure 3. Plots of Q estimates for k = 2 in 14 populations of *Medicago ciliaris* using the STRUCTURE software. Each individual is represented by a single vertical divided in K-colored segments, where K is the number of clusters assumed with the length proportional to each of the K inferred clusters. The vertical axis represents the Q estimate and the horizontal axis represents the samples. The red portion of the plot corresponds to group 1 of the studied populations while the green portion of the plot contains populations from group 2.

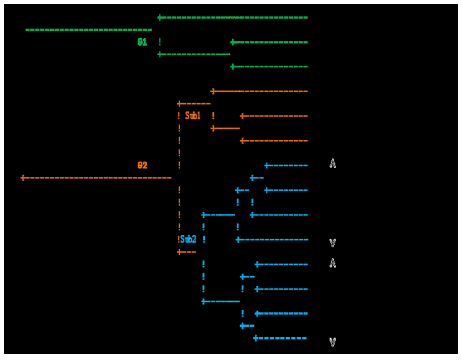

Figure 4. Dendrogram illustrating the relatedness of 14 *Medicago ciliaris* populations collected in Tunisia with clustering algorithm of unweighted pair group method with arithmetic mean (UPGMA) based on Nei's (1978) genetic distance generated by two AFLP primer combinations, *E*-AAG/*M*-CTG and *E*-AGC/*M*-CAA. The first group (G1) contained populations from Siliana, Oued Messouge, and Mateur: 9140, 9141, and 9149, respectively. The second group (G2) is the largest and contained all other populations with two sub-clusters: sub-cluster 1 (Sub1) contained three populations: 9142, 9143, and 9145, which were from Dougga, Zaghouan, and Bizerte, respectively. The second sub-cluster (Sub 2) grouped the rest of the populations from more closely located geographical area (sub 2a and sub 2b).

DISCUSSION

Pheno-morphological variation

The results of this study revealed considerable variation among the studied populations of *M. ciliaris* for the majority of recorded traits. The within-population variation was less significant. The same results were found for Sicilian populations of burr medic (Graziano et al., 2010) and for four Tunisian populations of echinus medic (Badri et al., 2008). This is likely to be associated with the high selfing rate of the latter species. Our results showed that vigorous populations with high pod production are late flowering, while those with short vegetative phase and less biomass and high seed and pod weight are the early flowering ones. These finding are consistent with those of Berger et al. (2002) who demonstrated that seed size and phenology are substitutable traits. Mean flowering time varied between 83 days for population 9141 from Oued Messouge and 97 days after sowing for populations 9144 from Ghzala and population 9148 from Fretissa, which represented a difference of 14 days, close to that found by Graziano et al. (2010). The difference in flowering time is mainly attributed to the origin site characteristics. Variation among populations was also found for reproductive traits, showing that variation is useful for the self-regeneration and survival of annual medics in dry environments. Correlations between eco-geographical factors and pheno-morphological traits are related to the weather conditions, particularly temperature and insulation, but also to the sum and distribution of rainfall during the flowering, pod setting, and seed ripening periods (Bodzon, 2004). In addition, soil factors, such as organic matter and nitrogen content, strongly influence plant vigor, and consequently, pod production and biomass. Finally, according to Taleisnik et al. (2009), plant organs tend to show small variations in size under the same genetic background and identical growth conditions, which implies that final organ size is determined by intrinsic mechanisms. Plasticity in plant morphogenesis is a response strategy to variation in environmental conditions (temperature, light, water, and nutrient availability). In addition, as noted by Cruse-Sanders and Hamrick (2004), environmental variation in terms of temperature, precipitation, length of the growing season, and day length during the latitudinal range, may affect the establishment of plant seedlings and consequently, the spatial distribution of the variation within population.

Molecular characterization

The aim of this study was to assess the genetic diversity and genetic structure of 14 *M. ciliaris* populations collected from northern Tunisia. For that purpose, we used the AFLP technique, which has been applied to a wide range of topics in biotechnological research and has been used extensively to assess genetic diversity and characterize germplasm collections (Badr et al., 2008).

The AFLP data obtained in the present study using the two primer combinations and an automated electrophoresis system were effective at revealing polymorphisms in the 260 samples. Indeed, in this assay the number of AFLP fragments was 528 (well-resolved peaks), which is high and confirmed previous findings using AFLP analysis to identify *Hedysarum* varieties as reported by Karudapuram and Larson (2005). In fact, according to those authors, AFLP technique can be performed on variety of systems, including gel-based systems and automated systems, for these systems the successful AFLP assay depends on the availability of optimized reagents and a robust electrophoresis platform, as well as analytics software that can correctly score AFLP sample data.

Moreover, results from the AFLP analysis revealed the existence of high levels of polymorphism; 284 polymorphic AFLP markers were defined among 528 scorable markers with a rate of 53.8% polymorphism. Thus, the molecular technique and the number of primer combinations used in this study were sufficient and reliable for revealing genetic polymorphisms. These results are consistent with findings from studies that used the AFLP technique for a variety of applications, such as genetic diversity assessment, phylogenetic studies, and genetic mapping in several crops (Badr et al., 2008; Elazreg et al., 2011; Marghali et al., 2014).

In the present study, use of the primer combination *E*-AAG/*M*-CTG for the AFLP assay revealed a higher average number of polymorphic bands per population (92.57) compared to the *E*-AGC/*M*-CAA primers (69.21). The latter primer was previously used in studies assessing genetic diversity of alfalfa populations and clover species. For alfalfa populations, this primer combination produced the highest mean number of polymorphic fragments (29.9) per population, which was considered relatively low compared to the results of other studies in the same or related species according to Dobrzycka et al. (2009). In addition, for clover species, this primer combination amplified 52 bands across four studied populations, which was the lowest number among the four primers used (Bennett and Mathews, 2003). In other studies, an average 25.2 markers generated per pairwise combinations (10 primers combinations) with a highest number of 28 markers was sufficient for analyses of genetic structure and diversity in eight populations of *Parapiptadenia rigida* (De Souza et al., 2013). Chennaoui-Kourda et al. (2012) obtained 24-51 scorable bands using the AFLP technique to study the genetic diversity of *Sulla*, with an average of 36.8 bands per combination, and Marghali et al. (2014) detected a total of 295 polymorphic bands. Herein, the two primer combinations used revealed a high number of AFLP fragments per primer combination and per population; which is superior to the results previously cited and to those reported by Elazreg et al. (2011). This permitted us to assess the level of genetic polymorphism and to analyze the genetic structure of these *M. ciliaris* populations, consistent with previous studies of genetic diversity in closely related species carried out over different geographic ranges using AFLP molecular markers (Skrede et al., 2009; Keivani et al., 2011).

The PIC scores for all the 284 polymorphic AFLP markers showed a moderate mean value of 0.3, varying from 0.02 to 0.5 over all polymorphic markers. Landraces of the Chinese traditional cabbage were investigated using the same AFLP markers, and the PIC was 0.31, suggesting that narrow genetic diversity characterizes the corresponding plant material (Kang et al., 2011). PIC has also been used to test the efficiency of the AFLP primer combinations to characterize alfalfa (PIC = 0.26) (Keivani et al., 2011), soybean (PIC = 0.32) (Roldan-Ruiz et al., 2000), wheat (PIC = 0.32) (Rosales-Serna et al., 2005), corn salad (PIC = 0.25) (Laurentin and Karlovsky, 2007), and pongamia (PIC = 0.22) (Thudi et al., 2010). These scores were relatively high and can be compared to the PIC value obtained for *M. ciliaris* populations. Indeed, according to Thudi et al. (2010), PIC values from 0.18 to 0.26, with an average of 0.22 found in *Pongamia pinnata* using five primer combinations, was considered to be moderate, and this can be attributed to either the diverse nature of the germplasm or to the highly informative AFLP primer combinations used.

In general, the PIC value of dominant markers ranges from zero for monomorphic markers to 0.5 for markers that are present in 50% of the plants and absent in the other 50% as described by Anderson et al. (1993). This supports a high degree of allelic polymorphism characterizing the studied population of *M. ciliaris*, which seems to be dependent on the primer combinations and their ability of generating different polymorphic profiles among and within populations.

Regarding the amount of genetic diversity among and within populations revealed by the AFLP markers, high levels were demonstrated by several indices. Mean total genetic diversity was H_T (0.27) and most of the genetic variation was found to occur within populations. Indeed, only 38% of the total genetic diversity was obtained among populations, and 62% of the total genetic variation was found within populations of *M. ciliaris*. Badri et al. (2008) used SSRs to characterize Tunisian populations of *M. ciliaris* and reported that 80.94% of the total diversity occurs at the intra-population level. Similar studies on the genetic diversity of various land plants reported the same results, including *Trifolium glomeratum* and *Trifolium nigrescens* (Bennett and Mathews, 2003), *Hedysarum coronarium* (Marghali et al., 2003), Alfalfa (*Medicago sativa* L.) (Keivani et al., 2011), perennial ryegrass (*Lolium perenne* L.), and tall fescue (*Festuca arundinacea* Schreb.) (Elazreg et al., 2011).

The degree of genetic population differentiation based on AFLP markers was equal to G_{ST} 0.38, which fits with the G_{ST} for widespread selfers (0.446) reported by Hamrick and Godt (1996). This value is higher than the mean genetic differentiation F_{ST} value (0.18) obtained via SSR markers in *M. ciliaris* (Badri et al., 2008). *M. ciliaris* is predominantly a selfing species (Badri et al., 2008) with a low rate of observed heterozygosity (mean 1.3%) and a mating system in which a large proportion of the total genetic variation is expected to be represented among populations (Steinger et al., 2002; Charlesworth, 2003). Our estimate of the level of differentiation (G_{ST}) based on AFLP markers seems to be inconsistent with the above results. The degree of genetic differentiation observed for our study is higher than the values of the molecular differentiation (F_{ST}) reported for SSR markers (0.08 to 0.30) between pairwise populations of *M. ciliaris* (Badri et al., 2008). This inconsistency may be related to the geographic origin of the analyzed sites. Indeed, AFLP markers are probably better able to estimate genetic diversity than a few microsatellites, due to their broader coverage of the genome. Furthermore, AFLP diversity increased significantly with population size (Gaudeul et al., 2004). Otherwise, the genetic structure of populations and their corresponding high intra-population variability are mainly affected by evolutionary factors, including the breeding system, gene flow, and seed dispersal, as well as by natural selection and ecological and geographical factors (Laurentin and Karlovsky, 2007; Elazreg et al., 2011). The low population differentiation at SSR markers (F_{ST}) found in *M. ciliaris* cannot be predicted by the model of isolation by distance, since the evolutionary mechanism that affects the genetic diversity of the studied populations of *M. ciliaris* may be explained by the restricted geographical distribution of this species in Tunisia (Badri et al., 2008). Geographic distances have little predictive power on the genetic differentiation among populations of *S. vulgaris*, using 0.49 as a degree of population differentiation. This finding was supported by similar results using random-amplified polymorphic DNA markers (Steinger et al., 2002).

The inference drawn from the UPGMA dendrogram based on Nei's genetic distance showed a clear dependence on the geographic origin, with the exception of two populations (Mateur2 and Dougga, 9146 and 9142, respectively). Indeed, populations from the north-west area clustered together. The first main group linked populations from Siliana, Oued Messouge, and Mateur2 (9140, 9141 and 9149, respectively). It is worth noting that the Mateur2 (9149) sub-cluster (north east) seems to share similar gene pools to the population from Oued Messouge (9141), which may have occurred by animal seed dispersal. Moreover, the second major cluster yielded two sub-clusters, which almost exhibited dependence on their corresponding geographic origin and which enclosed all populations from north-east areas except for the Dougga population (9142), which is located in the north-west area. Indeed,

the first sub-cluster (Sub1) contained three populations from Dougga, Zaghouan, and Bizerte (9142, 9143 and 9145 respectively). From this group the population from Dougga (9142), sampled from the north-west area, seems to be genetically distant from populations sharing the same area, and a more likely explanation may be the effect of local adaptation, which may cause genetic divergence of populations via genotype-environment interactions (Badri et al., 2008). The second sub-cluster (Sub2) grouped the populations of greater genetic similarity which are from more closely located geographic areas: population from Mateur1 (9146) and those from coastal areas: from Aouja, Raoued El Hessine, Ghar El Melh and Kaalat El Andalous (9147, 9151, 9153 and 9152, respectively). According to Badri et al. (2008), these populations might not have been isolated for long period of time based on their low genetic differentiation. Therefore, clustering of *M. ciliaris* populations based on the geographic origin was also reported for *Solanum tuberosum* varieties (Esfahani et al., 2009).

In conclusion, variation among populations of *M. ciliaris* was observed for several pheno-morphological traits as well as for AFLP molecular markers. Two gene pools can be distinguished: one pool form north-west and another from north-east area of Tunisia. The association between the genetic structure of *M. ciliaris* populations and the pheno-morphological diversity would be of great interest when looking for valuable genotypes with high agronomic potential. For arid and semi-arid areas with a dry spring, early populations are recommended for rotation with cereals. Thus, identifying the degree and distribution of genetic diversity in the studied populations could be the first step for developing effective conservation and management strategies for this species. In addition, the high level of genetic variability could provide a large base for future breeding programs in order to improve this forage legume species and release cultivars for agronomic purposes.

Conflicts of interest

The authors declare no conflict of interest.

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