RESEARCH ARTICLE

Morphological and microsatellite diversity associated with ecological factors in natural populations of *Medicago laciniata* Mill. (Fabaceae)

MOUNAWER BADRI[1]*, ADEL ZITOUN[1], HOUCINE ILAHI[2], THIERRY HUGUET[3] and MOHAMED ELARBI AOUANI[1,4]

[1]*Laboratoire Interactions Légumineuses Microorganismes, Centre de Biotechnologie, Technopole de Borj-Cédria, B.P. 901, 2050 Hammam-Lif, Tunisia*
[2]*Université 7 Novembre à Carthage, Institut Préparatoire aux Etudes d'Ingénieurs de Bizerte, 7021 Zarzouna, Tunisia*
[3]*Laboratoire de Symbiose et Pathologie des Plantes, INP-ENSAT, B.P. 107, 31326 Castanet Tolosan Cedex, France*
[4]*Present address: NEPAD/North Africa Biosciences Network, National Research Center, El Buhouth St, Cairo, 12311, Egypt*

Abstract

Genetic variability in 10 natural Tunisian populations of *Medicago laciniata* were analysed using 19 quantitative traits and 12 polymorphic microsatellite loci. A large degree of genetic variability within-populations and among-populations was detected for both quantitative characters and molecular markers. High genetic differentiation among populations for quantitative traits was seen, with Q_{ST} = 0.47, and F_{ST} = 0.47 for microsatellite markers. Several quantitative traits displayed no statistical difference in the levels of Q_{ST} and F_{ST}. Further, significant correlations between quantitative traits and eco-geographical factors suggest that divergence in the traits among populations may track environmental differences. There was no significant correlation between genetic variability at quantitative traits and microsatellite markers within populations. The site-of-origin of eco-geographical factors explain between 18.13% and 23.40% of genetic variance among populations at quantitative traits and microsatellite markers, respectively. The environmental factors that most influence variation in measured traits among populations are assimilated phosphorus (P_20_5) and mean annual rainfall, followed by climate and soil texture, altitude and organic matter. Significant associations between eco-geographical factors and gene diversity, H_e, were established in five-microsatellite loci suggesting that these simple sequence repeats (SSRs) are not necessarily biologically neutral.

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Introduction

Diversity in natural populations remains a central theme in evolutionary and ecological work both for fundamental and applied research (Hughes and Boomsma 2004), because future evolutionary adaptation depends on the existence of genetic variation (Shrestha *et al*. 2002; Zhan *et al*. 2005). Thus, to preserve genetic diversity in a species is to maintain its evolutionary potential. Theory suggests that heterogeneous distribution of genetic variation within-populations and among-populations is due to mutation, genetic drift due to finite population size, and heterogeneous natural selection, whereas gene flow and homogeneous directional selection tend to produce genetic homogeneity (Slatkin 1987; Chan and Arcese 2003). On a broad scale, the factors affecting diversity at the ecological level also affect or shape genetic differentiation at the molecular level (Tilman 1999). Comparing patterns of population genetic differentiation at quantitative traits (Q_{ST}) and molecular markers (F_{ST}) permits inferences about the relative role of selection in population divergence, by contrasting the degree of adaptive change with that of differentiation due solely to drift (Spitze 1993; Merilä and Crnokrak 2001; Zhan *et al*. 2005). There are three possible outcomes from the comparison of molecular F_{ST} and quantitative Q_{ST} differentiation. If molecular differentiation in neutral molecular markers among populations F_{ST} is of the same magnitude as that for Q_{ST} or is significantly more than

*For correspondence. E-mail: mounawer_badri@yahoo.fr.

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Q_{ST}, then the hypothesis that among-population variance in quantitative traits is due to random drift cannot be rejected, or uniform selection may be involved as a cause for the reduced differentiation. The third case where $Q_{ST} > F_{ST}$, suggests a prominent role for natural selection in accounting for patterns of quantitative traits differentiation among populations.

Neutral evolutionary theory predicts that within-population genetic variation at molecular markers will be linearly correlated to the within-population additive genetic variation at quantitative traits, if markers and traits share a genetic basis (Falconer and Mackay 1996). However, the validity of this expectation has been questioned by recent theoretical considerations and empirical data (Latta 1998). There has been little concordance between quantitative traits and molecular markers in most empirical studies to date (Merilä and Crnokrak 2001; Stenoien *et al*. 2005; Zhan *et al*. 2005).

Local adaptation of populations to environmental variation is a classic finding in plant ecology (Prosperi *et al*. 2006). In natural environments, the spatial distribution of individual plants within populations often depends on environmental factors that affect seedling establishment, such as temperature, precipitation, growing-season length and changes in day length during latitudinal range changes (Cruse-Sanders and Hamrick 2004).

The genus *Medicago* L. (Fabaceae) consists of large number of species of annual herbs, herbaceous perennials and rare shrubs, mostly native to the Mediterranean regions (Lesins and Lesins 1979). Annual species, collectively known as 'medics', are naturally distributed over a wide range of environmental conditions in the Mediterranean basin (Bena *et al*. 1998). Different species have distinct patterns of regional distribution, and show clear ecological preferences in relation to soil, climate and biotic factors (Badri *et al*. 2007). The ability of these species to establish nitrogen-fixing symbiosis with *Sinorhizobium* spp. makes them an excellent candidates for use in sustainable agricultural systems as forage and cover crops (Garau *et al*. 2005). However, many studies have focussed only on the genetics and genomics of the rhizobial partner, while few studies focused on the host plant. There is very little information about the genetic diversity available within-populations and among-populations of *Medicago* annual and perennial species; only a few species have been studied (Diwan *et al*. 1994; Bonnin *et al*. 1996; Juan *et al*. 2004; Ellwood *et al*. 2006). In Tunisia, annual species of genus *Medicago* can be found in wide range of habitats, varying in water availability, temperature and geographical location (longitude, latitude and altitude). Nevertheless, they are not uniformly distributed throughout the bio-climatic stages. The self-pollinating diploid ($2n$ = 16) annual forage species *M. laciniata* (cut-leaf medic), is a widespread species in the Tunisian Saharan, arid and semi-arid bioclimatic regions. About 95% of the Tunisian range is situated in these bio-climatic stages. *M. laciniata* is the only species of *Medicago* which is restricted to southern Mediterranean regions (Heyn 1963). It is nodulated efficiently by a restricted group of *Sinorhizobium meliloti* (Villegas *et al*. 2006). The latter two properties make *M. laciniata* an excellent tool for understanding the molecular mechanisms of tolerance to drought, as well as symbiotic specificity. Moreover, *M. laciniata* has the advantage of belonging to a genus with a model legume, *M. truncatula* (Cook 1999). Species closely related to model organisms present the opportunity to efficiently apply molecular and functional tools developed by a larger research community (Eujayl *et al*. 2004; Gutierrez *et al*. 2005). One such tool is provided by microsatellite loci or SSRs, hypervariable DNA elements which consist of tandemly repeated mononucleotide, dinucleotide, trinucleotide, tetranucleotide or pentanucleotide motifs, and occasionally complexes of different types of motifs, such as $(AC)_9(AG)_7$ (Gutierrez *et al*. 2005).

Here, we report a study, in which we analysed the genetic variability in 10-natural populations of *M. laciniata* using 19 quantitative traits and 12 polymorphic SSR markers. Association between quantitative traits, SSR markers and eco-geographical factors were studied in these populations. The goals of this study were: (i) to assess the genetic variability levels available within-populations and among-populations of *M. laciniata*, for quantitative traits and SSR markers, (ii) to compare the differentiation among populations at Q_{ST} and molecular markers F_{ST} and to determine if the genetic variability as estimated by SSR markers is a good indicator for that at quantitative traits, and (iii) to estimate associations of quantitative traits and SSR markers with eco-geographical factors.

Material and methods

Plant material

Ten-natural-Tunisian populations of *M. laciniata* covering different climatic regions of the species distribution were used (figure 1; table 1). The populations were sampled in July 1999 (Jelma, Amra, Deguache and Majel Bel Abbes) and July 2003 (El Ghouilet, Khmouda, Kairouan, Djerba, Medenine and Tataouine). These populations were selected on the basis of their belonging to some different edaphic and climatic regions (table 1) and of being accessible and abundant under natural conditions. Populations from El Ghouilet and Khmouda represent the superior limit distribution of *M. laciniata* in Tunisian area. A collection site of mature pods, as defined here, is an ecologically homogenous area of about 500 m^2 that contains only one population. To minimize the chances of sampling the same individual more than once, the minimum distance between sampling pods was 4 m. Based on Heyn's (1963) classification, constitutive lines of studied populations belong to: (i) var. *laciniata* only (El Ghouilet, Jelma, Amra, Khmouda and Kairouan), (ii) both var. *brachyacantha* Boiss. and var. *laciniata* (Majel

Table 1. Eco-geographical data of collection sites of 10-natural populations of *M. laciniata.*

	Texture	O. M (%)	[a]P_2O_5	Climate	Ann. rain (mm)	Altit. (m)	Latitude (N)	Longitude (E)
TNL1	Clay	1.6	26	Inferior semi arid	350	30	36° 03′	10° 19′
TNL2	Sandy clay loam	1.8	18	Superior arid	250	300	35° 14′	09° 30′
TNL3	Sandy loam	1.4	26	Inferior arid	150	400	34° 35′	09° 09′
TNL4	Sandy loam	0.9	22	Superior Saharan	50	25	33° 59′	08° 12′
TNL5	Sand	4.9	24	Superior arid	250	500	34° 19′	08° 41′
TNL6	Sand	2.0	26	Superior semi arid	350	740	35° 21′	8° 47′
TNL7	Sandy loam	1.6	24	Superior arid	300	300	35° 26′	09° 52′
TNL8	Loamy sand	0.7	20	Inferior arid	150	18	33° 46′	10° 57′
TNL9	Sandy loam	0.7	18	Inferior arid	175	119	33° 15′	10° 32′
TNL10	Loamy sand	0.1	15	Superior Saharan	138	137	32° 47′	10° 22′

[a]Assimilated P_2O_5 (ppm); O. M, organic matter; ann. rain, mean annual rainfall; altit., altitude; TNL1, El Ghouilet; TNL2, Jelma; TNL3, Amra; TNL4, Deguache; TNL5, Majel Bel Abbes; TNL6, Khmouda; TNL7, Kairouan; TNL8, Djerba; TNL9, Medenine; TNL10, Tataouine.

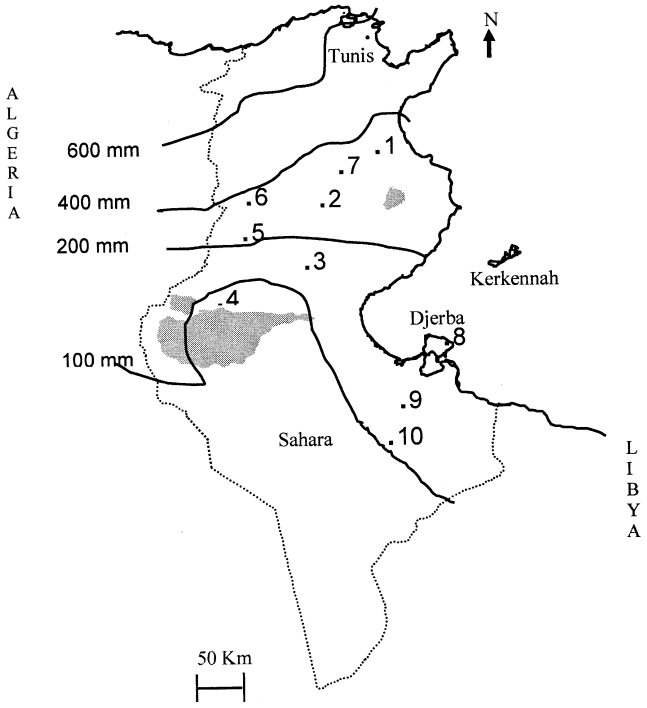

Figure 1. Map of Tunisia with the location of *M. laciniata* sampling sites. 1, El Ghouilet (TNL1); 2, Jelma (TNL2); 3, Amra (TNL3); 4, Deguache (TNL4); 5, Majel Bel Abbes (TNL5); 6, Khmouda (TNL6); 7, Kairouan (TNL7); 8, Djerba (TNL8); 9, Medenine (TNL9); 10, Tataouine (TNL10).

Bel Abbes, Djerba and Medenine), and (iii) var. *brachyacantha* Boiss. only (Deguache and Tataouine).

Lines were created for the four populations (Jelma, Amra, Deguache and Majel Bel Abbes) sampled in 1999 by two generations of spontaneous selfing in the greenhouse. For the rest of the populations (El Ghouilet, Khmouda, Kairouan, Djerba, Medenine and Tataouine), collected in 2003, the five seeds assumed to be representative of one genotype were taken from a single pod. Indeed, previous studies carried out on *M. truncatula* and *M. laciniata*, revealed that all the seeds coming from the same pod have only one genotype (Badri *et al*. 2002; M. Badri, T. Huguet and M. E. Aouani, unpublished data). Each population was represented by 10 lines. We assumed that offspring should be genetically identical in each presumed line. Consequently, the within-line variance can be considered as environmental, while the among-lines variance component is assumed to be solely genetic (Bonnin *et al*. 1997; Badri *et al*. 2007).

Quantitative traits

Hundred genotypes (original plants) of *M. laciniata* were analysed in this trial. To account for the environmental variance within a genotype (i.e. the within-line variance) (Falconer and Mackay 1996), five seeds per genotype were used. A total of 500 plants were studied. Seeds were surface sterilized and scarified with concentrated H_2SO_4 for 13 min, and rinsed 10 times with sterile-distilled water. Soaked seeds were sown in petri dishes on agar agar 0.9% medium before being vernalized at 4°C for 72 h. Seedlings were transplanted in plastic hampers (30/50 cm) filled with a mixture of sand/peat (1:3/2:3) with five plants, representative of one genotype per hamper. Hampers were placed in greenhouse at the Centre de Biotechnologie à la Technopole de Borj-Cédria (CBBC) in October 2003. A completely randomized design was used.

Nineteen quantitative traits related to the different developmental phases of plants were measured (table 2). Each specific assessment was conducted by the same person to minimize human error. Some traits were used by Bonnin *et al*. (1997) and they were also used by Badri *et al*. (2007). All traits showed high-heritability levels. For weight determination, plant organs were dried at 70°C for 48 h.

Molecular markers

Extraction of DNA from leaves was carried out according to the Rogers and Bendish (1988) protocol, modified by Gherardi *et al*. (1998). A total of 12 polymorphic SSR loci with *M. laciniata* were amplified for this study (table 3). The majority (8/12) of these SSR markers were identified in EST

Table 2. List of measured quantitative traits and their abbreviations.

Seedling traits	Emergence date of first leaf (days)	D1F
	Emergence date of sixth leaf (days)	D6F
	Area of the first leaf (cm^2)	SFT6
Growth traits	Length of the main stem at the first flower bud stage (cm)	LP1BF
	Length of the main stem 20 days after the first flower bud stage (cm)	LP20
	Daily growth of the main stem (cm)	CP/J = (LP20 – LP1BF)/20
	Length of secondary stems at the first flower bud stage (cm)	LS1BF
	Length of secondary stems 20 days after the first flower bud stage (cm)	LS20
	Daily growth of secondary stems (cm)	CS/J = (LS1BF – LS20)/20
	Length of orthotropic axis at harvest (cm)	LOR
	Length of plagiotropic axes at harvest (cm)	LPLAR
	Length of orthotropic and plagiotropic stems at harvest	LTOTR
	Weight of dried stems (g)	PTIG
Reproductive traits	Date of the first stem flower bud stage (days)	FLOR
	Total number of pods	NGOU
	Total weight of pods (g)	PGOU
	Total weight (g)	PTOT = PTIG + PGOU
	Weight of 100 pods (g)	P100G
	Reproductive effort	REP = PGOU/PTOT

databases. These markers have been mapped on the genome of the model legume *M. truncatula* using LR4 framework genetic map. This map was performed based on F6 recombinant inbred lines (RILs) population resulting from a single cross between Jemalong A17 line and DZA315.16 Algerian line (P. Thoquet, M. Gherardi, G. Cardinet, F. Chardon and T. Huguet, unpublished data). The use of mapped markers is considered more desirable for diversity analysis than the use of unmapped markers, because the marker loci can be chosen to represent all parts of the genome.

Final concentrations of the PCR cocktail included 11.4 μl of distilled water, 2×10 *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 50 ng of each primer, 0.16 U *Taq* polymerase (Uptima) per 20 μl reaction, and 30 ng of genomic DNA. Each reaction was overlaid with 25 μl of mineral oil to prevent evaporation. Amplifications were carried out in a thermal cycler (Biometra) for 4 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, followed by a final extension for 6 min at 72°C, and an indefinite hold at 4°C. Fragments generated by amplification were separated according to size on 3.5% agarose gels run in 0.5 × TBE (Tris 45 mmol/l, boric acid 45 mmol/l, EDTA 0.02 mmol/l, pH = 8) using 110 V for about 3 h, stained with ethidium bromide and visualized by illumination with ultraviolet light (312 nm). Microsatellite allele sizes were estimated using the Marcel ladder (Bio-Rad, California, USA) as standard size.

Statistical analysis

Quantitative genetic variation: Genetic variance in quantitative traits was partitioned into within-population and among-population components by using SAS Proc GLM (type III) (SAS 1998). The model included population and genotype within population. To estimate variance components, population and genotype were considered as random effects. Mean phenotypic values of each population was compared for all measured traits with Duncan multiple range test.

Total phenotypic variance among populations (σ_T^2) is the sum of three components of variance: (i) arising among populations (σ_P^2), (ii) between genotypes within populations (σ_g^2) and (iii) due to error arising between individuals of the same genotype (σ_e^2). Broad-sense heritability (H^2) was calculated for each trait as the ratio of the variance arising between genotypes (σ_g^2) divided by the sum of (σ_g^2) and (σ_e^2) (Bonnin *et al*. 1997). The level of population differentiation for quantitative traits, Q_{ST}, was computed as described in Stenoien *et al*. (2005) for a predominantly selfing species as the following:

$$Q_{ST} = \frac{\sigma_p^2}{\sigma_p^2 + \sigma_g^2}.$$

The calculation of 95% confidence intervals (CIs) for Q_{ST} was performed by 5000-bootstrap replicates in SAS interactive matrix language (IML). The coefficient of genetic variation (CV_g) was obtained as follows (Bonnin *et al*. 1997).

$$CV_g = 100 \times (\sigma_g^2)^{1/2}/m,$$

where m is the population phenotypic mean. Phenotypic correlations between measured traits were estimated by the SAS CORR procedure (SAS 1998). A standardized principal component analysis (PCA) (PRINCOMP procedure–SAS software 1998, Cary, USA) was performed on the correlation matrix of the synthetic variable based on the mean-line values (average of the five replications). To represent the relationships between studied lines at quantitative traits, hierarchical clustering (UPGMA) was carried out using Euclidean distances of dissimilarity estimated from the coordinates of every line on all PCA scores.

Table 3. Name, linkage group, position, core sequence, primers and origin of 12 used SSR loci.

Name	Linkage group	[a]Position (cM)	Core sequence	Primer left sequence (5′-3′)	Primer right sequence (5′-3′)	Origin
ATPase456	4	0.0	$(TTC)_8$	AAGGTGGTCATACGAGCTCC	GGGTTTTTGATCCAGATCTT	B
B21E13	2	0.0	$(GA)_{13}$	GCCGATGGTACTAATGTAGG	AAATCTTGCTTGCTTCTCAG	C
MTIC80 (= A03E09F1)	8	120.5	$(TC)_8$, $(TC)_{12}$	TGTAGATTTTCAACAGACAAAGCA	GCCAAGACTGTGTTTGGTTTC	B
MTIC451	2	135.1	$(TC)_{11}$	GGACAAAATTGGAAGAAAAA	AATTACGTTTGTTTGGATGC	B
MtN25	3	50.5	$(GGA)_6$	TTGGGAGGAGGACTGAATA	TCCCAAACCAGGTTCTTCAT	B
MTSA5	7	106.6	$(TC)_9$	ACTGTTCCGTCCTTTCAATC	TGAGTTCTTGTTCCTTGTTA	A
TP36B (= FMT11)	1	96.0	$(GA)_{16}$	GGCCCAACCACACAATTTC	CATAACTTCCAATAACTGCCA	A
TPG20C (= FMT08)	7	6.5	$(CT)_{16}$, $(CA)_7$	CAATCACTGGAAGCAAGGT	AGCCTGCTCATTTGTATTGC	A
MTIC452	2	27.3	$(TC)_{15}$	CTAGTGCCAACACACAAAACA	TCACAAAAACTGCATAAAGC	B
MTIC35 (= 004a12)	7	130.1	$(AAG)_8$	GAAGAAGAAAAAGAGATAGATCTGTGG	GGCAGGAACAGATCCTTGAA	B
MTIC153	6	81.4	$(AG)_5$	TCACAACTATGCAACAAAAGTGG	TGGGTCGGTGAATTTTCTGT	B
MTIC169	3	3.0	$(TC)_7$	TCAAAACCCTAAAACCCTTTCTC	GCGTGCTAGGTTTGAGAGGA	B

[a]Genetic distance from the upper telomere estimated according to LR4 framework genetic map (Jemalong A17 × DZA315.16) (P. Thoquet, M. Gherardi, F. Chardon and T. Huguet, unpublished data). A, microsatellite-enriched genomic library; B, expressed sequence tags (ESTs); C, bacterial artificial chromosome (BAC).

Molecular genetic variation

Genetic diversity was estimated for each population using five indices: (i) the number of multilocus genotypes (*Geno*), (ii) the mean observed heterozygosity (H_o), (iii) the gene diversity (H_e) i.e. expected heterozygosity of Nei (1978), (iv) the number of unique alleles (U_a), and (v) linkage disequilibrium (LD), using Genetix software version 4.02 (Belkhir 2001). Linkage disequilibrium was estimated as described in Volis *et al*. (2001).

Molecular differentiation among populations (F_{ST}) was used as a measure of population differentiation for the polymorphic loci. It is defined as a standardized genetic variance among populations and it was estimated according to Weir and Cockerham's (1984) method. Overall F_{ST} among populations and pair-wise estimates for all pairs of populations were computed with FSTAT program (Goudet 1995). The CIs based on resampling techniques, are provided with the software and involve jackknifing over all loci to obtain the mean and the standard error of the overall F_{ST}, and bootstrapping over loci to obtain 95% CIs. Isolation by distance was analysed as described in Rousset (1997) through Mantel tests (Mantel 1967) carried out between matrices of log-transformed geographic distances and odds-transformed genetic distances $F_{ST}/(1 - F_{ST})$. Relationships among lines were studied by the constructed neighbour-joining (NJ) tree. Support for clustering was determined by a bootstrap procedure applied on SSR alleles (5000 replications). The NJ analysis and bootstrapping were performed with DARwin software (Perrier and Jacquemoud-Collet 2006).

Quantitative traits versus SSR markers

Sampling variances on estimates of variance components can be obtained using bootstrap or jackknife procedures. As suggested by Jaramillo-Correa *et al*. (2001), we used bootstrapping over loci and lines (5000 bootstraps) to estimate 95% CIs, respectively, of F_{ST} and Q_{ST} from the observed distribution. Here, we compared Q_{ST} values to F_{ST} level estimated using only the seven SSR markers showing no significant correlation with eco-geographical factors. Associations between CV_g, at both the trait-specific and multi-trait levels, and gene diversity of each locus (H_e) and over all loci (H_e all) were estimated by computing the Pearson correlation coefficient 'r' (Volis *et al*. 2005).

Associations of quantitative traits and SSR markers with environmental factors

Thirteen eco-geographical factors of sampling sites were scored: soil texture, pH, saturation (ml/100g), electroconductivity (mmho/cm), total calcareous (%), active calcareous (%), organic matter (%), carbon (%), assimilated P_2O_5 and K_2O, climate, mean annual rainfall (mm) and altitude (m). Only six (soil texture, organic matter, assimilated P_2O_5, climate, mean annual rainfall, and altitude) (table 1)

out of these 13 parameters, showing the maximum of difference between analysed sites, were retained for the final analyses. To assess the influence of environmental factors in the variation of measured traits and SSRs allelic frequencies between populations, two analyses were performed. First, estimates of variance components within and among populations were computed from quantitative and molecular data using the following mixed model:

$$y_{ijklmn} = \mu + P_i + L_{j(i)} + T_k + A_l + R_m + e_{ijklmn},$$

where y_{ijklmn} is the observations for all quantitative traits or SSR markers; μ, the population mean; P_i, the random effect of population; $L_{j(i)}$, the random effect of genotype nested within population; T_k, the fixed effect of texture; A_l, the fixed effect of altitude; R_m, the effect of mean annual rainfall considered as covariate, and e_{ijklmn} a random residual effect. Second, associations of quantitative traits and SSR markers with eco-geographical factors were performed. Correlations of quantitative traits with environmental factors were estimated using Pearson correlation coefficients (r) (Jaramillo-Correa *et al*. 2001). Significance level was set to 0.05, and adjusted for multiple comparisons by Bonferroni corrections. Associations of gene diversity (H_e) of each locus and mean-gene diversity for over all loci (H_e all) with eco-geographical factors were computed using the Spearman rank correlation analysis (Turpeinen *et al*. 2001).

Results

Quantitative traits

A variance analysis of population effect showed significant differences between analysed populations of *M. laciniata* for all measured traits (table 4). The population from Khmouda was the latest flowering. For the majority of studied characters, the El Ghouilet and Medenine populations showed a contrasting behaviour between them, as well as with other populations. In contrast to El Ghouilet, which showed the largest levels for weight of dried stems trait (PTIG), for length of orthotropic and plagiotropic stems at harvest (LTOTR) and for total weight (PTOT), Medenine allocated the most effort for reproduction (REP). The highest number of pods production (NGOU) was found in populations from Deguache and Tataouine, which are constituted by lines belonging only to *brachyacantha* variety. Overall, all analysed populations of *M. laciniata* allocated high REP ranging from 0.74 for El Ghouilet to 0.83 for Medenine with an average of 0.78.

Of the overall quantitative genetic variability, 54.02% was found between populations. Table 4 gives the coefficients of genetic variation (CV_g) of each trait and for each population in *M. laciniata*. We will arbitrarily consider coefficients above 25% as large. Twelve traits of 19 measured traits in El Ghouilet and Medenine, 3 of 19 in Jelma, 10 of 19 in Amra and Kairouan, 13 of 19 in Deguache and Khmouda, 7 of 19 in Majel Bel Abbes, 14 of 19 in Djerba, and 9 of 19 in Tataouine, revealed high CV_g. The highest within-population genetic variation (CV_g) (14/19) was found in Djerba. Heritability (H^2) of measured traits ranges from 0.10 for PTIG to 0.68 for weight of 100 pods (P100G) with an average of 0.37 (table 5).

Estimates of population differentiation at quantitative traits (Q_{ST}) varied widely among the 19 measured traits (table 5). It ranged from 0.24 for emergence date of first leaf trait (D1F) to 0.78 for PTIG, with an average of 0.47. The bootstrap CI showed that this overall Q_{ST} value is significantly greater than zero. For 171 possible correlations between measured traits, 142 were significant (table 6). The majority of observed correlations were positive. REP was positively correlated with emergence date of sixth leaf (D6F) and negatively associated with flowering time (FLOR).

Six principal components, whose eigenvalues were higher than 0.80, were found to account for 88.11% of the total variation. Using these six components, studied lines of *M. laciniata* were clustered based on Euclidean distances of dissimilarity. For a Euclidean distance of 3.75, studied lines of *M. laciniata* were clustered into five groups (figure 2). First group formed by one line of *laciniata* variety from Djerba population, second group by lines of *brachyacantha* variety from Deguache and Majel Bel Abbes, third group by lines of *brachyacantha* variety from Deguache and two lines of *laciniata* variety form El Ghouilet and Jelma, fourth group by lines of *brachyacantha* variety from Djerba, Medenine and Tataouine, and fifth one by those of *brachyacantha* variety from Deguache, Djerba, Medenine and Tataouine and lines of *laciniata* variety from all studied populations of *M. laciniata*.

Molecular markers

Using the 12 SSR loci, a total of 70 alleles were detected. Among the 100 analysed lines of *M. laciniata*, 72 multilocus genotypes (*Geno*) were distinguished. The number of *Geno* varied between 10 of 10 in Deguache and 5 of 10 in El Ghouilet and Tataouine (table 7). Unique number of allele (U_a), present in only one population, ranged from 0 to 4 with a mean value of 1.6. The largest number of unique alleles was identified in Deguache and Tataouine ($U_a = 4$), which were constituted by lines belonging to variety *brachyacantha* Boiss. only, whereas the lowest value ($U_a = 0$) was found in Majel Bel Abbes and Medenine. Based on this latter parameter (U_a), the two constitutive varieties of *M. laciniata*, var. *laciniata* and var. *brachyacantha* Boiss., are different enough to be considered as distinct varieties.

Among populations and across loci, the mean expected heterozygosity (H_e) ranged from 0.21 to 0.46, with an average of 0.32. The Deguache population displayed the highest level of gene diversity ($H_e = 0.46$), while the lowest levels were found in Khmouda and Kairouan (table 7). The percentage of linkage disequilibrium (*LD*%) between SSR markers varied between 3.21% and 66.65%, with an average of 37.90%.

Table 4. Population means and coefficients of genetic variation (CV_g) (in parentheses) of 19 measured traits for analysed populations of *M. laciniata*.

	TNL1	TNL2	TNL3	TNL4	TNL5	TNL6	TNL7	TNL8	TNL9	TNL10
D1F	10.12^{de}(4.30)	9.30^{e}(4.98)	9.64^{e}(20.29)	12.10^{ab}(40.94)	10.94^{cd}(2.87)	12.76^{a}(30.85)	12.36^{a}(20.85)	11.76^{abc}(11.85)	11.92^{abc}(12.67)	11.20^{bc}(11.69)
D6F	27.64^{cd}(26.69)	25.94^{d}(21.95)	26.36^{d}(18.91)	29.22^{c}(14.80)	26.90^{cd}(11.07)	33.56^{b}(19.13)	33.74^{b}(21.30)	34.76^{b}(25.40)	37.84^{a}(20.39)	38.26^{a}(21.53)
SFT6	76.95^{bc}(27.05)	85.15^{a}(22.71)	82.73^{ab}(20.70)	79.74^{ab}(22.16)	80.37^{ab}(23.18)	54.87^{d}(25.04)	69.60^{c}(22.49)	75.96^{bc}(30.72)	76.62^{bc}(19.62)	69.44^{c}(23.50)
FLOR	62.72^{bcd}(16.40)	63.10^{bc}(10.00)	65.32^{b}(13.67)	59.06^{ef}(12.73)	56.30^{fg}(11.91)	69.68^{a}(14.51)	59.40^{def}(14.20)	60.20^{cde}(11.50)	53.86^{g}(19.96)	57.08^{efg}(11.17)
LP1BF	20.72^{a}(25.98)	21.92^{a}(23.26)	20.18^{a}(22.51)	21.34^{a}(22.17)	21.84^{a}(19.15)	14.18^{c}(28.56)	14.18^{c}(32.57)	18.04^{b}(33.15)	15.48^{c}(34.66)	21.40^{a}(22.83)
LP20	32.28^{b}(19.73)	32.18^{b}(17.71)	30.20^{b}(16.76)	30.26^{b}(27.52)	31.44^{b}(18.05)	26.44^{cd}(17.79)	24.16^{d}(17.06)	30.08^{b}(27.47)	29.14^{bc}(27.91)	39.64^{a}(17.52)
CP/J	0.58^{cd}(20.54)	0.51^{de}(21.82)	0.50^{e}(30.07)	0.45^{e}(62.51)	0.48^{e}(27.03)	0.61^{c}(25.04)	0.50^{e}(20.99)	0.60^{c}(34.71)	0.68^{b}(29.68)	0.85^{a}(20.08)
LS1BF	27.78^{bc}(77.02)	34.24^{a}(58.38)	28.68^{b}(53.33)	18.04^{de}(82.83)	17.66^{de}(56.84)	22.72^{cd}(78.84)	13.76^{ef}(66.42)	9.60^{fg}(86.77)	2.28^{h}(176.56)	5.44^{gh}(107.33)
LS20	123.90^{a}(47.56)	112.20^{ab}(34.45)	107.44^{b}(39.62)	82.96^{cd}(62.27)	76.42^{d}(37.70)	96.36^{bc}(45.72)	82.38^{cd}(35.96)	70.68^{d}(43.64)	41.76^{e}(65.52)	67.18^{d}(51.72)
CS/J	4.81^{a}(44.62)	3.90^{b}(31.14)	3.94^{b}(40.27)	3.25^{cd}(62.92)	2.94^{d}(37.35)	3.68^{bc}(40.04)	3.43^{bcd}(33.54)	3.05^{cd}(40.06)	1.97^{e}(61.09)	3.08^{cd}(48.89)
LOR	68.24^{cd}(13.13)	67.64^{cd}(11.28)	65.62^{cd}(16.53)	67.48^{cd}(19.34)	69.10^{c}(19.04)	59.12^{e}(17.37)	63.44^{de}(15.35)	69.32^{c}(16.85)	76.68^{b}(16.15)	92.34^{a}(12.74)
LPLAR	636.20^{a}(36.41)	579.44^{ab}(24.68)	543.82^{bc}(36.56)	453.08^{d}(39.37)	427.20^{d}(26.21)	496.22^{cd}(39.87)	485.26^{cd}(34.39)	452.76^{d}(33.41)	347.94^{e}(28.98)	572.02^{ab}(33.42)
LTOTR	704.44^{a}(33.32)	647.08^{ab}(22.73)	609.44^{bc}(33.32)	520.56^{d}(34.54)	496.30^{d}(24.35)	555.34^{cd}(36.98)	548.70^{cd}(31.05)	522.08^{d}(29.72)	424.62^{e}(25.36)	664.36^{ab}(29.25)
PTIG	2.59^{a}(28.78)	2.23^{b}(24.18)	2.18^{b}(34.16)	1.58^{d}(35.42)	1.77^{cd}(23.64)	1.94^{c}(33.88)	1.91^{c}(31.19)	1.64^{d}(30.12)	1.33^{e}(33.12)	1.89^{c}(34.43)
NGOU	116.44^{cd}(28.57)	126.98^{c}(21.25)	102.08^{de}(32.98)	190.54^{a}(37.85)	148.30^{b}(54.52)	110.54^{cde}(39.05)	95.02^{e}(30.77)	129.88^{bc}(30.75)	120.44^{cd}(33.36)	198.76^{a}(30.22)
PGOU	7.43^{ab}(28.71)	7.62^{a}(20.16)	7.00^{abcd}(30.24)	5.98^{e}(33.17)	7.22^{abc}(24.65)	6.33^{de}(39.13)	6.57^{bcde}(27.41)	6.96^{abcd}(25.84)	6.46^{cde}(29.18)	7.15^{abcd}(26.45)
PTOT	9.99^{a}(27.71)	9.85^{a}(19.70)	9.18^{ab}(30.15)	7.57^{c}(31.66)	8.99^{ab}(22.74)	8.27^{bc}(36.08)	8.49^{bc}(26.71)	8.60^{bc}(25.46)	7.79^{c}(27.21)	9.04^{ab}(27.17)
P100G	6.43^{b}(9.96)	6.05^{bc}(11.31)	6.97^{a}(10.64)	3.42^{e}(36.38)	5.55^{d}(31.74)	5.81^{cd}(14.93)	7.01^{a}(9.34)	5.45^{d}(10.90)	5.57^{d}(21.09)	3.69^{e}(19.57)
REP	0.74^{f}(5.02)	0.77^{de}(3.97)	0.76^{e}(4.27)	0.79^{cd}(5.99)	0.80^{bc}(4.20)	0.76^{ef}(7.41)	0.77^{de}(5.28)	0.81^{b}(4.13)	0.83^{a}(6.15)	0.79^{bc}(4.06)
CV_g±SD	27.45 ± 16.76	21.35 ± 11.98	26.58 ± 12.07	36.03 ± 19.73	25.07 ± 14.27	31.06 ± 15.87	26.15 ± 13.14	29.08 ± 17.45	36.25 ± 36.88	29.14 ± 22.40

Means followed by the same letters are not significantly different at $P = 0.05$ based on Duncan's multiple range test. TNL1–TNL10 natural populations (refer footnote of table 1) SD, standard deviation.

Table 5. Heritabilities (H^2) of measured traits and population differentiation for quantitative traits (Q_{ST}), and SSR loci (F_{ST}) among *M. laciniata* populations.

	H^2	Q_{ST}	[a]Lower	[a]Upper
D1F	0.56	0.24^{*}	0.204	0.276
D6F	0.37	0.55^{**}	0.495	0.605
SFT6	0.27	0.41^{**}	0.363	0.457
FLOR	0.60	0.27^{*}	0.232	0.308
LP1BF	0.54	0.39^{**}	0.344	0.436
LP20	0.54	0.32^{*}	0.278	0.362
CP/J	0.42	0.50^{**}	0.448	0.552
LS1BF	0.48	0.51^{**}	0.457	0.563
LS20	0.40	0.44^{**}	0.391	0.489
CS/J	0.33	0.37^{**}	0.325	0.415
LOR	0.45	0.56^{**}	0.505	0.615
LPLAR	0.17	0.55^{**}	0.495	0.605
LTOTR	0.17	0.55^{**}	0.495	0.605
PTIG	0.10	0.78^{***}	0.715	0.845
NGOU	0.42	0.52^{**}	0.467	0.573
PGOU	0.12	0.25^{*}	0.213	0.287
PTOT	0.10	0.45^{**}	0.401	0.499
P100G	0.68	0.67^{***}	0.610	0.730
REP	0.26	0.58^{**}	0.524	0.636
Average		0.47^{**}	0.390	0.550
F_{ST}		0.477	0.378	0.608

[a]The lower and upper confidence limits for a 95% CI for Q_{ST} and E_{ST} based on 5000-bootstrap samples (bootstrapping over lines/loci). $^{*}Q_{ST}$ was significantly smaller than F_{ST} at $P = 0.05$, $^{**}Q_{ST}$ was not significantly different to F_{ST} at $P = 0.05$, $^{***}Q_{ST}$ was significantly larger than F_{ST} at $P = 0.05$.

Of the overall molecular genetic variability, 53.56% was found to occur between populations. Substantial molecular differentiation among populations (F_{ST}) was found in *M. laciniata* (table 5), as the bootstrap CI revealed F_{ST} value is significantly greater than zero. It ranged from 0.11 between Djerba and Medenine to 0.66 between Kairouan and Tataouine (table 8). There was a strong positive correlation ($r = 0.48; P = 0.006$) between F_{ST} and geographical distance.

Hierarchical classification of analysed lines of *M. laciniata* based on molecular data revealed that lines belonging to *laciniata* and *brachyacantha* varieties form two distinguishable groups (figure 3). The lines of *brachyacantha* variety form three groups. The first group formed by the lines of Deguache population and some lines of Majel Bel Abbes, second group by Tataouine lines, and third group by those of Majel Bel Abbes, Djerba and Medenine populations. Further, the lines of *laciniata* variety form three groups. The first group constituted by lines of Jelma, Amra and Majel Bel Abbes populations, second group by lines of El Ghouilet, Jelma and Khmouda, and third group by those of Khmouda, Kairouan, Djerba and Medenine populations.

Table 6. Matrix of correlations between 19 measured traits for analysed populations of *M. laciniata*.

	D1F	D6F	SFT6	FLOR	LP1BF	LP20	CP/J	LS1BF	LS20	CS/J	LOR	LPLAR	LTOTR	PTIG	NGOU	PGOU	PTOT	P100G	REP
D1F	1.00																		
D6F	0.43^{***}	1.00																	
SFT6	-0.17^{***}	-0.40^{***}	1.00																
FLOR	-0.01^{ns}	0.15^{***}	-0.29^{***}	1.00															
LP1BF	-0.38^{***}	-0.45^{***}	0.41^{***}	-0.00^{ns}	1.00														
LP20	-0.21^{***}	-0.17^{***}	0.29^{***}	-0.04^{ns}	0.77^{***}	1.00													
CP/J	0.12^{**}	0.29^{***}	-0.03^{ns}	-0.04^{ns}	0.09^{*}	0.59^{***}	1.00												
LS1BF	-0.32^{***}	-0.55^{***}	0.26^{***}	0.33^{***}	0.46^{***}	0.25^{***}	-0.12^{**}	1.00											
LS20	-0.28^{***}	-0.43^{***}	0.14^{***}	0.39^{***}	0.47^{***}	0.36^{***}	0.08^{ns}	0.83^{***}	1.00										
CS/J	-0.22^{***}	-0.30^{***}	0.06^{ns}	0.36^{***}	0.41^{***}	0.37^{***}	0.18^{***}	0.63^{***}	0.96^{***}	1.00									
LOR	-0.07^{ns}	0.07^{ns}	0.10^{*}	-0.28^{***}	0.40^{***}	0.59^{***}	0.50^{***}	-0.13^{**}	-0.03^{ns}	0.02^{ns}	1.00								
LPLAR	-0.25^{***}	-0.37^{***}	0.11^{*}	0.10^{*}	0.40^{***}	0.33^{***}	0.08^{ns}	0.48^{***}	0.57^{***}	0.54^{***}	0.27^{***}	1.00							
LTOTR	-0.25^{***}	-0.36^{***}	0.12^{*}	0.07^{ns}	0.42^{***}	0.37^{***}	0.11^{*}	0.46^{***}	0.55^{***}	0.53^{***}	0.34^{***}	0.99^{***}	1.00						
PTIG	-0.26^{***}	-0.42^{***}	0.14^{**}	0.09^{*}	0.36^{***}	0.30^{***}	0.03^{ns}	0.52^{***}	0.57^{***}	0.52^{***}	0.19^{***}	0.85^{***}	0.84^{***}	1.00					
NGOU	-0.07^{ns}	-0.09^{*}	0.03^{ns}	-0.08^{ns}	0.37^{***}	0.35^{***}	0.11^{*}	0.02^{ns}	0.12^{**}	0.16^{***}	0.44^{***}	0.43^{***}	0.45^{***}	0.31^{***}	1.00				
PGOU	-0.23^{***}	-0.35^{***}	0.18^{***}	-0.07^{ns}	0.36^{***}	0.35^{***}	0.16^{***}	0.35^{***}	0.42^{***}	0.40^{***}	0.36^{***}	0.70^{***}	0.71^{***}	0.68^{***}	0.56^{***}	1.00			
PTOT	-0.25^{***}	-0.39^{***}	0.18^{***}	-0.03^{ns}	0.38^{***}	0.35^{***}	0.13^{**}	0.42^{***}	0.49^{***}	0.46^{***}	0.34^{***}	0.78^{***}	0.79^{***}	0.81^{***}	0.53^{***}	0.98^{***}	1.00		
P100G	-0.09^{*}	-0.18^{***}	0.10^{*}	0.05^{ns}	-0.19^{***}	-0.22^{***}	-0.11^{*}	0.23^{***}	0.15^{***}	0.09^{*}	-0.31^{***}	0.01^{ns}	-0.01^{ns}	0.16^{***}	-0.71^{***}	0.08^{ns}	0.10^{*}	1.00	
REP	0.09^{ns}	0.18^{***}	0.02^{ns}	-0.21^{***}	-0.09^{ns}	-0.01^{ns}	0.12^{*}	-0.29^{***}	-0.27^{***}	-0.23^{***}	0.13^{**}	-0.34^{***}	-0.32^{***}	-0.55^{***}	0.20^{***}	0.20^{***}	0.01^{ns}	-0.12^{**}	1.00

Significance levels; ^{ns} = not significant ($P > 0.05$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

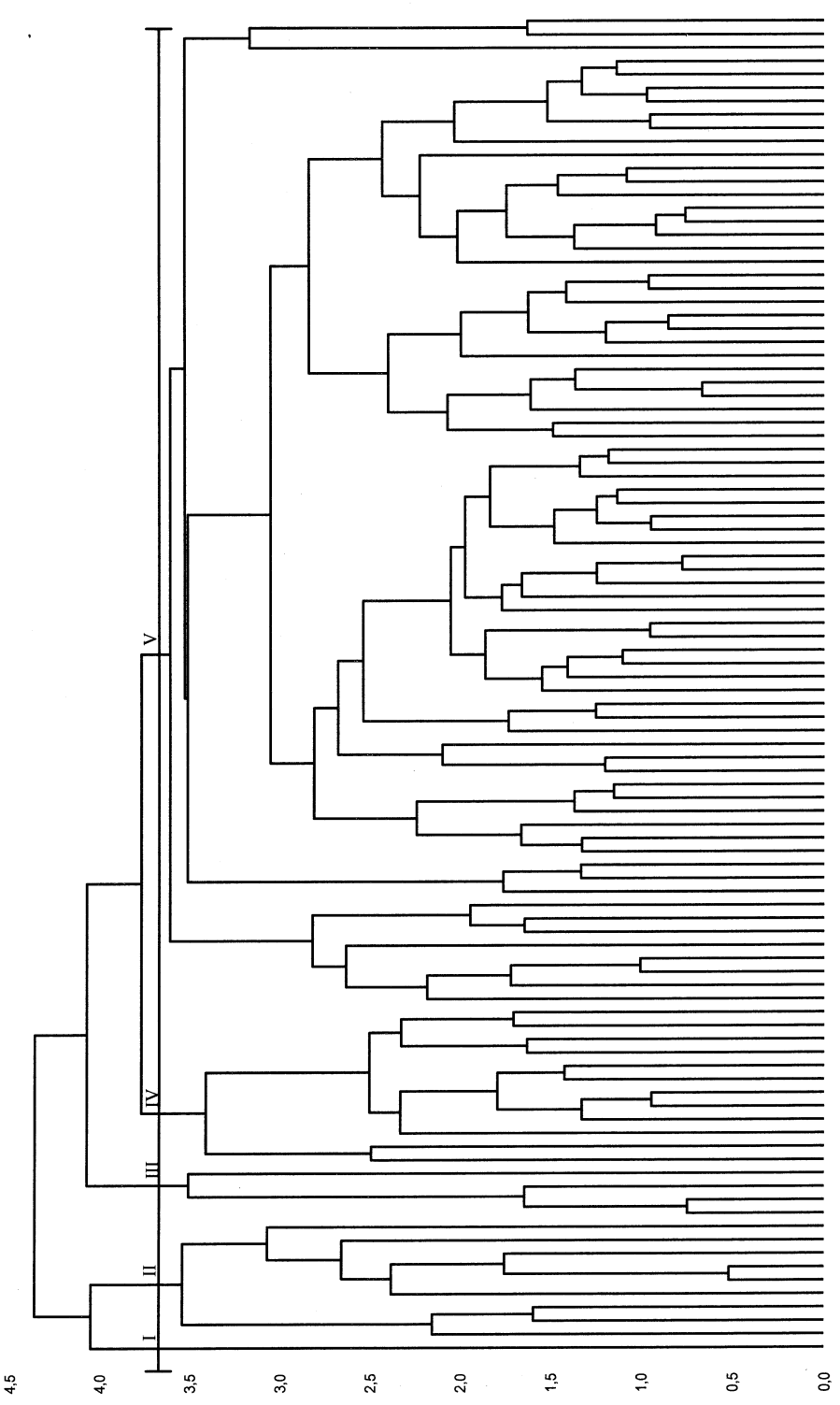

Figure 2. Unweighted pair group method with arithmetic averages (UPGMA) dendrogram between studied lines of *M. laciniata* based on Euclidean distances of dissimilarity estimated using all principal component analysis (PCA) scores. 1, El Ghouilet; 2, Jelma; 3, Amra; 4, Deguache; 5, Majel Bel Abbes; 6, Khmouda; 7, Kairouan; 8, Djerba; 9, Medenine; 10, Tataouine; b, lines belonging to *brachyacantha* variety.

Table 7. Microsatellite diversity averaged over SSR loci, in natural populations of *M. laciniata.*

	TNL1	TNL2	TNL3	TNL4	TNL5	TNL6	TNL7	TNL8	TNL9	TNL10	TNL
N	10	10	10	10	10	10	10	10	10	10	
Geno	5	9	9	10	7	6	7	7	7	5	72
H_o	0	0	0	0	0	0	0	0	0	0	0.00
H_e	0.27	0.28	0.27	0.46	0.38	0.21	0.24	0.42	0.37	0.31	0.32
U_a	1	1	1	4	0	1	1	3	0	4	1.60
LD%	3.21	66.65	53.07	52.94	21.04	64.07	42.35	28.66	29.64	17.34	37.90

TNL1–TNL10, natural populations (refer footnote of table 1); TNL, *M. laciniata* species. *N*, Line numbers; *Geno*, number of multilocus genotypes; H_o, the mean observed heterozygosity, the gene diversity (H_e, expected heterozygosity; Nei 1978), the number of unique alleles, i.e. alleles found in only one population (U_a) (Turpeinen *et al.* 2001), and linkage disequilibria (*LD%*).

Table 8. Pair-wise population differentiation at SSR markers (F_{ST}).

	TNL1	TNL2	TNL3	TNL4	TNL5	TNL6	TNL7	TNL8	TNL9	TNL10
TNL1	0.00									
TNL2	0.16	0.00								
TNL3	0.40	0.37	0.00							
TNL4	0.56	0.52	0.49	0.00						
TNL5	0.40	0.28	0.34	0.39	0.00					
TNL6	0.44	0.43	0.46	0.56	0.49	0.00				
TNL7	0.48	0.47	0.46	0.57	0.50	0.47	0.00			
TNL8	0.47	0.42	0.44	0.35	0.42	0.42	0.43	0.00		
TNL9	0.51	0.46	0.51	0.41	0.47	0.49	0.52	0.11	0.00	
TNL10	0.64	0.60	0.64	0.40	0.53	0.65	0.66	0.39	0.37	0.00

TNL1–TNL10, natural populations (refer footnote of table 1).

Quantitative traits versus SSR markers

We found three different patterns of population differentiation across the measured traits for *M. laciniata* (table 5). First, we found that 13 of these traits displayed no significant difference in the level of Q_{ST} and F_{ST}, while four of these traits exhibited significantly lower Q_{ST} than F_{ST}. Second, we found that 2 of these 19 traits displayed significantly higher Q_{ST} than F_{ST}. There were no consistent patterns of associations between CV_g at multi-trait level and H_e over all loci ($r = 0.47; P = 0.18$), and between CV_g at specific-trait level and H_e of each locus ($P > 0.00019$) (data not shown).

Associations of quantitative traits and SSR markers with eco-geographical factors

The site-of-origin environmental factors explain about 18.13% and 23.40% of total genetic variation among populations at quantitative traits and SSR markers respectively. For 114 possible correlations between measured traits and eco-geographical variables, 59 were significant and 24 of them were positive (table 9). The environmental factors with the greatest influence on quantitative genetic variation among natural populations of *M. laciniata* are assimilated phosphorus (P_20_5) and mean annual rainfall (20.34% of significant correlations), followed by climate and soil texture (18.64%), altitude (13.56%) and organic matter (8.47%).

On the other hand, significant ($P < 0.05$) associations of gene diversity H_e at five loci, as well as mean gene diversity over all loci (H_e all) with eco-geographical factors were found (table 10). Positive correlations were established for soil texture (MTIC35) and the climate (B21E13). Negative correlations were observed for soil texture (MTSA5, MTIC169), organic matter (MTSA5), altitude (MTSA5) and mean annual rainfall (B21E13, MTIC451, H_e all). In all, nine (11.54%) of 78 possible correlations between gene diversity (H_e) and eco-geographical factors were significant. This proportion (11.54%) of significant associations exceeds the 5% level expected by chance (binomial test, $P < 0.01$; Siegel and Castellan 1988).

Discussion

Levels of quantitative and molecular genetic diversity in M. laciniata

A high level of polymorphism was detected both within and among natural populations at quantitative traits and SSR markers. The distribution of genetic variation within-populations and among-populations is of importance to the adaptation and adaptability of a species (Slatkin 1987) and for gene conservation efforts (El-Kassaby and Ritland 1996). As a self-fertilizing species, the variances between populations of *M. laciniata* are slightly higher than that found

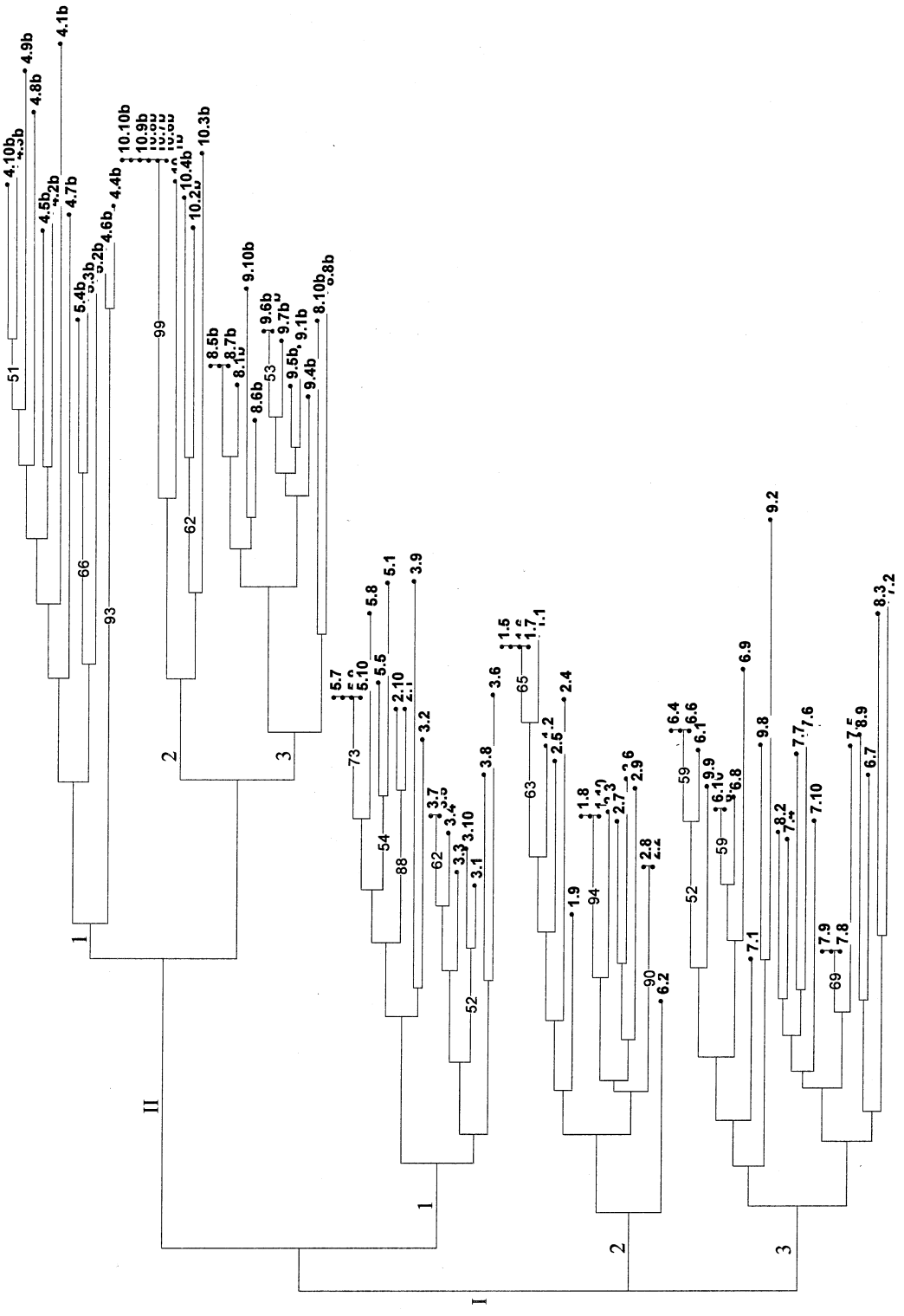

Figure 3. Neighbour-joining analysis based on microsatellite loci data among studied lines of *M. laciniata.* The numbers on the branches indicate bootstrap values (expressed in percentages) and are shown for all clusters with >50% bootstrap support. 1, El Ghouilet; 2, Jelma; 3, Amra; 4, Deguache; 5, Majel Bel Abbes; 6, Khmouda; 7, Kairouan; 8, Djerba; 9, Medenine; 10, Tataouine; b, lines belonging to *brachyacantha* variety.

Table 9. Estimated correlations between 19 measured traits for *M. laciniata* and six eco-geographical factors.

	Texture	O.M	$^{a}P_2O_5$	Climate	Ann. rain	Altitude
D1F	0.23*	−0.07	0.01	0.07	−0.01	0.04
D6F	0.19*	−0.32*	−0.31*	0.20*	−0.09	−0.11
SFT6	−0.21*	0.06	−0.06	0.10	−0.18*	−0.19*
FLOR	−0.02	0.01	0.26*	−0.23*	0.21*	0.25*
LP1BF	−0.13	0.10	−0.11	0.11	−0.20*	−0.16*
LP20	−0.05	−0.08	−0.27*	0.15	−0.17*	−0.18*
CP/J	0.09	−0.29*	−0.33*	0.12	−0.04	−0.10
LS1BF	−0.24*	0.20*	0.30*	−0.31*	0.22*	0.20*
LS20	−0.25*	0.10	0.28*	−0.31*	0.24*	0.10
CS/J	−0.23*	0.04	0.24*	−0.27*	0.21*	0.04
LOR	0.02	−0.25*	−0.48*	0.33*	−0.27*	−0.28*
LPLAR	−0.22*	−0.06	0.07	−0.17*	0.14	−0.02
LTOT	−0.21*	−0.07	0.03	−0.14	0.12	−0.04
PTIG	−0.27*	0.07	0.22*	−0.33*	0.28*	0.06
NGOU	0.12	−0.10	−0.32*	0.35*	−0.36*	−0.21
PGOU	−0.08	0.06	−0.04	−0.08	0.07	−0.02
PTOT	−0.14	0.07	0.03	−0.15	0.13	0.00
P100G	−0.21*	0.23*	0.44*	−0.49*	0.49*	0.28*
REP	0.21*	−0.06	−0.35*	0.34*	−0.31*	−0.17*

*Significant after using Bonferroni correction at $\alpha = (0.05/114 = 0.00044)$. $^{a}P_2O_5$, assimilated P_2O_5 (ppm); O.M, organic matter; ann. rain, mean annual rainfall.

Table 10. Spearman rank correlations between gene diversity (H_e) in 12 SSR loci and six eco-geographical factors in 10 populations of *M. laciniata.*

	Texture	O.M	P2O5	Climate	Ann. rain	Altitude
ATPase456	0.48^{ns}	0.08^{ns}	-0.51^{ns}	0.36^{ns}	-0.30^{ns}	-0.21^{ns}
B21E13	0.34^{ns}	-0.12^{ns}	-0.48^{ns}	0.71*	−0.75*	-0.46^{ns}
MTIC80	0.37^{ns}	0.16^{ns}	-0.49^{ns}	0.44^{ns}	-0.35^{ns}	-0.10^{ns}
MTIC451	-0.24^{ns}	0.09^{ns}	-0.05^{ns}	-0.09^{ns}	-0.05^{ns}	-0.12^{ns}
MtN25	0.03^{ns}	-0.42^{ns}	-0.36^{ns}	0.47^{ns}	-0.58^{ns}	-0.57^{ns}
MTSA5	−0.66*	−0.76*	-0.13^{ns}	0.25^{ns}	-0.41^{ns}	−0.75*
TP36B	-0.34^{ns}	0.23^{ns}	-0.01^{ns}	-0.32^{ns}	0.21^{ns}	-0.33^{ns}
TPG20C	-0.08^{ns}	0.22^{ns}	0.07^{ns}	-0.07^{ns}	0.07^{ns}	0.15^{ns}
MTIC452	0.46^{ns}	0.14^{ns}	-0.02^{ns}	0.57^{ns}	−0.68*	-0.08^{ns}
MTIC35	0.78**	0.14^{ns}	-0.11^{ns}	0.44^{ns}	-0.44^{ns}	0.14^{ns}
MTIC153	-0.47^{ns}	-0.33^{ns}	0.24^{ns}	0.19^{ns}	-0.44^{ns}	-0.46^{ns}
MTIC169	−0.80**	-0.03^{ns}	0.43^{ns}	-0.43^{ns}	0.34^{ns}	-0.16^{ns}
He all	0.13^{ns}	-0.08^{ns}	-0.37^{ns}	0.61^{ns}	−0.76*	-0.59^{ns}

For abbreviations, refer to tables 3 and 9, significance levels; ^{ns}= not significant ($P > 0.05$), $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

within populations for both quantitative traits and SSR markers. This finding is most likely a direct consequence of the mating system; inbreeding reduces the effective population size, and consequently enhances the effect of genetic drift (Charlesworth 2003). Djerba, which is the only studied population coming from an island, exhibited the highest within-population genetic variation for quantitative traits, while Deguache was the most diverse in SSR markers. One potential explanation of this finding is the location of these two populations within metapopulations. Indeed, the molecular phylogenetic relationships between studied lines of *M. laciniata* (figure 3) revealed that while the constitutive lines of Deguache population cluster together with the three lines of Majel Bel Abbes of *brachyacantha* var. those of Djerba form one group with Medenine lines. This is expected, given that Deguache and Majel Bel Abbes, and Djerba and Medenine populations are geographically proximate. Correlations established between the 19 measured traits revealed that earlier emerging seedlings (D6F) were the earliest flowering (FLOR) and they allocated less REP. These results are consistent with those found in French populations of *M. truncatula* (Bonnin *et al*. 1997). Further, natural populations of *M. laciniata* seemed to allocate more REP than French and Tunisian populations of *M. truncatula* (Bonnin *et al*. 1997; Badri *et al*. 2007). In this collection of *M. laciniata*, no heterozygous genotypes were identified among the SSR loci. The predominantly selfpollinating mating system of this species is an obvious reason for this finding. Further, for the four studied populations (Jelma, Amra, Deguache and Majel Bel Abbes) any out-crossing events might be lost after the two selfing generations performed in the greenhouse. Accordingly, a low level of heterozygosity, less than 0.6%, was found for natural populations of *M. truncatula* (Bonnin *et al*. 1996; F. Lazrek, J. Ronfort, G. Cardinet, F. Chardon, T. Huguet and M. E. Aouani, unpublished data). As expected for a predominantly selfing organism, we found a high level of molecular differentiation (F_{ST}) among natural populations of *M. laciniata* (Stenoien *et al*. 2005). Phylogenetic relationships between studied lines of *M. laciniata* based on quantitative traits and SSR markers showed that, by contrast to traits, lines belonging to *laciniata* and *brachyacantha* varieties form two distinguishable groups for SSR loci. The relationship between F_{ST} and geographic distances indicates that an isolation by distance effect may occur in natural populations of this species. On the other hand, in contrast to Badri *et al*. (2007), we found high Q_{ST} values in *M. laciniata*. One possible reason behind this finding is the number of studied populations. While, only four populations of *M. laciniata* were analysed in Badri *et al*. (2007), 10 populations were used in this study.

Quantitative traits versus SSR markers

We found three different patterns of population differentiation across the measured traits for *M. laciniata*. First, we found that the majority of measured traits (13/19) displayed no significant difference in the level of Q_{ST} and F_{ST}, indicating that the effects of drift and selection are indistinguishable, probably being of similar magnitude. Further, the absence of significant correlation between geographical distance and environmental characteristics ($r = 0.22; P = 0.15$), as well as the isolation by distance among studied populations at SSR markers suggest that natural selection should be invoked to explain the phenotypic divergence among populations. Second, we found that 4 of these 19 traits exhibited significantly smaller Q_{ST} than F_{ST}, consistent with the action of uniform

selection. Third, the remaining of these 19 quantitative traits (2/19) exhibited significantly larger Q_{ST} than F_{ST}, suggesting that directional selection for local adaptation played a significant role in the evolution of these traits. The presence of significant correlations found between these traits and eco-geographical factors may suggest that these particular characters are adapting in response to the regional differences detected in these environmental factors. Further, estimated Q_{ST} and F_{ST} values indicated a high level of differentiation among analysed populations of *M. laciniata*. Theoretically, the high level of population differentiation may be explained by several factors, including breeding system, genetic drift or genetic isolation of populations (Hogbin and Peakall 1999). The relative importance of genetic drift and natural selection as determinants of populations' differentiation in quantitative traits has been one of the central themes in evolutionary biology (Morgan *et al*. 2005). A review by Merilä and Crnokrak (2001) reported that more than 20 studies have compared F_{ST} and Q_{ST}. Estimates of Q_{ST} have been based on the average of quantitative traits, where those of F_{ST} in most of these studies have been based on allozymes, RAPD, RFLP, but few on SSR markers. In agreement with our results, the majority of studies which used SSR makers for the estimation of F_{ST} generally showed that F_{ST} is on average equal or greater than Q_{ST} (Merilä and Crnokrak 2001; Porcher *et al*. 2006; Badri *et al*. 2007). For the rest of molecular markers, the overall trend revealed by those studies is that quantitative traits often show a higher level of population differentiation than molecular markers ($Q_{ST} > F_{ST}$), suggesting that diversifying selection is commonly operating on the former, at least, on a broad-scale (i.e. among-populations) (Podolsky and Holtsford 1995; Bonnin *et al*. 1996; Kremer *et al*. 1997). Differences in F_{ST} values were dependent on marker type, as well as on plant species. Indeed, Latta and Mitton (1997) observed such differences between allozymes and random amplified polymorphic DNA (RAPD) in limber pine (*Pinus flexilis* James). However, Isabel *et al*. (1999) and Szmidt *et al*. (1996) found essentially no differences in population differentiation estimates between allozymes and RAPD markers in three conifers.

We found no significant correlation between genetic variation at quantitative traits and SSR markers. Similarly, numerous studies have now been published that detail the discordant patterns of molecular and morphological variation (Spitze 1993; Podolsky and Holtsford 1995; Reed and Frankham 2001; Palo *et al*. 2003; Volis *et al*. 2005). Several genetic and environmental factors might be behind the absence of consistent association between genetic variation at quantitative traits and molecular markers. These factors include a nonadditive genetic component such as epistasis or dominance (Crnokrak and Roff 1995), the conversion of nonadditive genetic variance components to additive through genetic drift (Lopez-Fanjul *et al*. 2003), variation in mutation rates across loci, different genomic patterns of variation in regions rich in SSRs and other small repeat markers and the regions underlying QTLs in quantitative traits of interest (Latta 1998), and variation in selection pressures and eco-geographical effects (Reed and Frankham 2001). Nevertheless, in some cases quantitative and neutral molecular distances have been reported to show a significant positive correlation (Merilä and Crnokrak 2001; Stenoien *et al*. 2005; Zhan *et al*. 2005), suggesting that variation at molecular markers can sometimes be used as a predictor of the degree of quantitative differentiation. A review covering more than 70 studies carried out on various species indicated that correlations between genetic variation at quantitative traits and molecular markers are not significant, with an overall correlation coefficient of −0.08 (Reed and Frankham 2001).

Associations of quantitative traits and SSR markers with eco-geographical factors

An additional factor that may cause genetic divergence of populations is the presence of local adaptation, often via genotype-by-environment interactions. Established correlations, in the current study, between quantitative traits and environmental factors suggest that these particular characters are adapting in response to the regional differences detected in eco-geographical factors. In accordance with our results, significant correlations between quantitative traits and eco-geographical factors were observed in populations of *Triticum dicoccoides* (wild emmer wheat) (Li *et al*. 2001) and in *Hordeum spontaneum* (wild barley) (Huang *et al*. 2002) showing a significant micro-geographical genetic differentiation in response to climate. This micro-geographical adaptive differentiation with respect to climate was also found in some tree species such as *Pinus edulis* (pinon pine) (Mitton and Duran 2004). The high number of significant correlations between quantitative traits and eco-geographical factors and the large Q_{ST} values found in *M. laciniata* suggest that a large amount of the genetic variation could be adaptive.

Further, our results showed significant associations between SSR markers and eco-geographical factors in natural populations of *M. laciniata* suggesting that these markers are not necessarily neutral. These findings are consistent with those observed in wild emmer wheat (Li *et al*. 2000), in wild barely (Turpeinen *et al*. 2001; Ivandic *et al*. 2002) and in the selfing plant, *Arabidopsis thaliana*, (Innan *et al*. 1997). Therefore, studied environmental factors are likely to impose strong selection on *M. laciniata* populations, leading to local adaptation in each regional population and creating the high differentiation among populations. On the other hand, Badri *et al*. (2007) reported no significant correlation between H_e and environmental factors in *M. laciniata* although a great number of SSR markers are common between these two studies. This finding may be due to the smaller number of populations examined by Badri *et al*. (2007).

Strong genetic differentiation among analysed populations of *M. laciniata* at Q_{ST} and SSR markers (F_{ST}) were found. Morphological and SSR diversity were correlated with ecological factors. At any rate, this correlation suggests

that SSRs may not be neutral. Overall, our results suggest a prominent role for natural selection in accounting for patterns of genetic differentiation at quantitative traits among natural populations of *M. laciniata*. We found no significant correlation between quantitative and molecular genetic variation within populations. Further study is needed to examine the mechanisms underlying adaptive genetic diversity within and among natural populations of *M. laciniata* in relation to ecological factors.

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