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Genetic diversity in Tunisian populations of faba bean (*Vicia faba* L.) based on morphological traits and molecular markers

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ABSTRACT. Genetic diversity within *Vicia faba* L. is key to the genetic improvement of this important species. In this study, morphological traits and RAPD molecular markers were used to assess the levels of polymorphism across 12 Tunisian populations, three major and nine minor from different locations. Analysis of morphological traits indicated that the three major populations showed significant differences and the nine minor populations exhibited considerable variation for most traits. The grain yield of the Alia population could be increased by inoculation. Of the seven primers tested, it was clear that the Cs12 primer would be recommend for genetic diversity analysis of *V. faba*. Within population genetic diversity exhibited 94% of total diversity. Intra-population genetic diversity (H_S) was 0.16, which was clearly higher than between population genetic diversity (D_{ST} = 0.06) UPGMA showed a high level of genetic variation between major and minor populations of *V. faba L.* Particularly the minor populations showed a high level of diversity and was divided into two subclusters. Ltaifia was separated from the other populations. In addition to a high grain yield,

these populations showed the lowest Nei and Shannon indices (H = 0.08 and I = 0.13) justifying their homogeneity. For these reasons, these cultivars can be considered a selected population. However, the Takelsa population showed the highest Nei and Shannon indices (H = 0.13 and I = 0.21), indicating that this population was the most heterogeneous, which is interesting for breeding programs.

Key words: Genetic diversity; morphological traits; RAPD; Tunisian populations; UPGMA analysis; *Vicia faba* L.

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most important legumes, and it is extensively cultivated in the world. It not only provides an important source of human dietary protein, but is also a good source for the feed market (Gong et al., 2011).

Due to its partial cross-pollination, there are various faba bean varieties and they are highly heterogeneous, which makes the conservation of genotype resources more expensive and difficult (Duc et al., 2010).

However, despite its long history of cultivation and economic importance, there have been few studies about the diversity of faba beans (Gong et al., 2011).

Morphological studies are used to study multiple characters at the same time, to help discriminate heterogeneous populations, and they are vital tools to predict mechanisms of spread and dispersal of species (Benor et al., 2012).

Morphological characterization of plant species is mainly used to conduct a thorough investigation of genetic diversity in germplasm collections, contributing valuable information for breeding programs and conservation strategies for taxa of concern (Benor et al., 2012).

One of the most extensively used molecular markers is RAPD, which has been applied to address genetic diversity issues in plants (Vilanova et al., 2001; Gichuki et al., 2003). RAPD analysis may be used for many plant samples, using small quantities to study molecular mapping, taxonomy and phylogeny and genetic diversity. It is also used successfully for both the identification and classification of plant cultivars (Li et al., 2006). Furthermore, RAPD is effective, simple and more rapid compared to other molecular methods that use small oligonucleotides that differ in their DNA sequence (Shehata, 2004).

In the present study, the extent of genetic diversity between 12 minor and major populations of *Vicia faba* L. collected from some area of Tunisia was assessed by using morphological and molecular markers.

MATERIAL AND METHODS

Plant materials

We used twelve populations of *V. faba*, three major (Mateur, Alia, and Eljem) and seven minor (Mateur, Takelsa, Korba, Fahes, Eljem, Mahdia, and Ltaifia), collected from different regions in Tunisia during 2010, and two reference varieties Locale and Saber 02 (Table1).

Table 1. Bioclimatic characteristic of the collection sites of Tunisian populations of *Vicia faba L.*

Population code	Location	Altitude (m)	Latitude	Longitude	Rainfall (mm/year)
1	Mateur	51	37.04	9.66	550-600
2	Alia	42	37.16	9.98	450-550
3	Takelsa	168	36.81	10.66	500-600
4	Korba	52	36.61	10.81	450-500
5	Fahes	170	36.36	9.90	350-550
6	Eljem	107	35.30	10.71	200-300
7	Mahdia	11	35.50	10.95	200-300
8	Ltaifia	13	34.74	10.76	200-250
9	Locale	ND	ND	ND	ND
10	Saber 02	ND	ND	ND	ND

ND: Not Determined.

Morphological traits

Twelve genotypes of *V. faba* were analyzed. To account for environmental variance, fifteen seeds per genotype were used. A total of 180 plants were studied. Seeds were surface sterilized with $HgCl_2$ (0.2%) for 3 min and rinsed 10 times with sterile-distilled water. Soaked seeds were sown in Petri dishes on 0.9% agar. Besides, seedlings were transplanted in plastic pots, which were placed in a greenhouse at the Biotechnology Centre of Borj Cedria (CBBC).

Eleven morphological traits related to the different developmental phases of plants were measured (Table 2). For dry weight determination, plant organs were dried at 70°C for 48 h.

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

Trait	Abbreviation
Plant height (cm)	PlH
Number of flowers	NFl
Aerial fresh weight (g)	AFW
Aerial dry weight (g)	ADW
Length of roots (cm)	LR
Number of nodule	NNod
Root fresh weight (g)	RFW
Root dry weight (g)	RDW
Number of pods	NP
Number of seeds	NS
Weight of 100 seeds (g)	W100S

Molecular traits

DNA extraction

Total genomic DNA from the young leaves was isolated following the modified method of Geuna (2004). Ten plants from each population were used in this experiment. DNA was quantified spectrophotometrically by taking the absorbance at 260 nm using a Biophotometer. The quality of the DNA was estimated on an agarose gel (0.8%) stained with ethidium bromide.

Amplification reactions

Polymerase chain reaction (PCR) amplification of DNA was performed with each of seven decamer primers obtained from Operon Technologies: P4 (GTTAGGTCGT); P6 (TCG CCCCATT); P8 (GTCCCGTTAC); P11 (CTGTGCTGTG); P105 (CAGTCGCGTG); P137 (ATCTGCGACA), and Cs12 (GCGACGCCTA).

PCR was performed in a 20-µL reaction mixture containing 20 to 40 ng DNA template, 2 µL 10X reaction buffer, 2 µL 10 µM primer, 2.5 Mm dNTPs, 25 mM $MgCl_2$ and 1.5 U Taq polymerase. To reach the expected goals, amplifications were carried out in a Biometra thermal cycler for 3 min at 94°C, followed by 40 cycles of 30 s at 36°C, 1 min at 72°C, and 18 s at 94°C, and a final extension for 8 min at 72°C.

These amplification products were separated by electrophoresis in 1.5% agarose gels made with 1X TAE buffer (pH 8), which were stained with ethidium bromide and visualized under UV light. As a size marker, a 100-bp DNA Ladder (Promega) was run in each gel.

Data analysis

Data were subjected to analysis of variance (ANOVA) using the STATISTICA software system and the means of morphological traits measured were compared between populations with the HSD Tukey test.

RAPD markers were scored as present (1) or absent (0). A data matrix based on all the observed bands was constructed.

On the basis of the binary matrix obtained, genetic diversity indices [the percentage of polymorphic bands, the observed number of alleles (N_A), the expected number of alleles (N_E), and Shannon's information index of diversity (I)] were calculated by POPGENE 1.32 (1997). Nei's genetic differentiation index [$G_{ST} = [(H_T - H_S)/H_T]$) and gene flow [$N_m = 0.5\ (1-G_{ST})/G_{ST}$] were calculated using the population mean diversity and the total diversity indices (H_S was the population mean of Nei's diversity index, and H_T was the total of Nei's diversity index).

RESULTS

Morphological diversity

There was a remarkable difference between the three major populations of *V. faba* for the morphological traits (Table 3).

The Eljem and Mateur populations showed remarkable similarity for ten of eleven morphological traits. The Alia population differed from other populations by plant height, number of flowers, aerial fresh weight, aerial dry weight, length of roots, number of nodules, root fresh weight and root dry weight. Alia showed similarity for seed yield which could be increased by inoculation (Table 3).

The nine minor populations of *V. faba* exhibited considerable variation for most of the morphological characters (Table 4). It can be seen from the results that there was a remarkable similarity between the Korba, Mateur, and Takelsa populations for most of the morphological traits. The Eljem population had the highest seed yield (Table 4).

Table 3. Means of morphological traits measured of three major populations of *Vicia faba* used in this study.

Populations	PlH	N Fl	AFW	ADW	LR	NNod	RFW	RDW	NP	NS	W100S
Mateur	59.5^{b}	56.5^{a}	118.25^{a}	15.75^{a}	58^{a}	180.5^{a}	44.75^{a}	2.80^{a}	2.33^{a}	2.55^{a}	198.97^{a}
Alia	65.25^{b}	19.75^{b}	54.5^{b}	6.25^{b}	24.75^{c}	92.5^{b}	16.25^{b}	1.33^{b}	2.11^{a}	2.88^{a}	191.07^{a}
Eljem	79.25^{a}	54.75^{a}	120.75^{a}	15.69^{a}	39.75^{b}	189^{a}	47.5^{a}	3.28^{a}	2.33^{a}	3.11^{a}	179.91^{a}

Means in each column having similar letters are not significantly different using HSD Tukey test at 5% level.

Table 4. Means of morphological traits measured of nine minor populations of *Vicia faba* used in this study.

Populations	PlH	N Fl	AFW	ADW	LR	NNod	RFW	RDW	NP	NS	W100S
Eljem	79.25^{c}	25.50^{c}	89.00^{bc}	11.25^{b}	59.50^{ab}	112.75^{a}	33.50^{a}	1.64^{d}	2.83^{b}	5.50^{b}	144.25^{a}
Saber02	101.75^{a}	42.25^{b}	66.75^{d}	7.625^{cd}	33.75^{c}	56.25^{c}	19.31^{c}	1.54^{de}	5.16^{ab}	11.66^{ab}	47.28^{b}
Fahes	81.75^{c}	68.50^{a}	81.25^{cd}	11.25^{b}	66.50^{a}	123.25^{a}	30.50^{ab}	2.46^{bc}	4.00^{b}	10.00^{b}	42.87^{b}
Mahdia	83.25^{c}	23.25^{c}	111.50^{a}	12.50^{ab}	52.75^{b}	115.25^{a}	22.75^{bc}	2.02^{cd}	2.00^{b}	3.33^{b}	91.22^{a}
Ltaifia	63.25^{d}	15.75^{c}	42.25^{e}	3.69^{e}	24.75^{c}	81.50^{b}	4.57^{d}	0.57^{f}	2.66^{b}	3.50^{b}	78.23^{b}
Korba	77.50^{c}	77.50^{a}	101.50^{ab}	15.25^{a}	54.25^{b}	89.25^{b}	36.40^{a}	3.66^{a}	7.83^{ab}	14.16^{ab}	50.96^{b}
Mateur	86.25^{bc}	68.00^{a}	113.00^{a}	12.50^{ab}	50.25^{b}	59.50^{c}	33.68^{a}	2.40^{bc}	12.66^{a}	30.83^{a}	41.02^{b}
Takelsa	96.5^{ab}	50.00^{b}	103.00^{ab}	9.75^{bc}	57.50^{ab}	107.75^{a}	32.09^{a}	2.97^{b}	8.83^{ab}	21.33^{ab}	49.05^{b}
Locale	78.00^{c}	12.66^{c}	73.66^{cd}	5.41^{de}	31.33^{c}	48.66^{c}	6.18^{d}	0.84^{ef}	5.33^{ab}	13.00^{ab}	45.18^{b}

Means in each column having similar letters are not significantly different using HSD Tukey test at 5% level.

RAPD genetic diversity

For all the populations, a total of 218 RAPD fragments were amplified with 7 selected primers (Table 5), in a size range of 100-2700 bp, with 218 polymorphic bands (100%). The number of bands produced by each primer varied from 23 (CS12) to 36 (P11), with an average of 31. With a 95% threshold, the percentage of polymorphism ranged from 62.86 (P4) to 92.59% (P6), with an average of 75.96%. The Cs12 primer also showed a substantial percentage of polymorphism (86.96%).

The level of polymorphism within a population ranged from 32.11 (Mahdia and Ltaifia populations) to 51.38% (Takelsa and Locale populations), showing that the Takelsa and Locale populations were the most polymorphic. Several genetic diversity indices were measured: the observed number of alleles (N_A), the effective number of alleles (N_E), gene diversity (H) and Shannon Information index (I) (Table 6).

Table 5. RAPD primers and bands produced for Tunisian populations of *Vicia faba L.*

Primers	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism (%)
P4	35	22	13	62.86
P6	27	25	2	92.59
P8	27	20	7	74.07
P11	36	24	12	66.67
P105	35	25	10	71.43
P137	35	27	8	77.14
CS12	23	20	3	86.96
Total	218	163	55	-
Means	31	23	8	75.96

Table 6. Genetic diversity for RAPD primers.

Primer	Simple size	N_A*	N_E*	*H**	*I**
P4	120	2	1.17	0.13	0.23
P6	120	2	1.26	0.18	0.31
P8	120	2	1.18	0.14	0.25
P11	120	2	1.26	0.17	0.28
P105	120	2	1.2	0.14	0.25
P137	120	2	1.31	0.21	0.34
Cs12	120	2	1.45	0.27	0.42
Mean	120	2	1.26	0.17	0.29
SD		0	0.27	0.15	0.2

*N_A = Observed number of alleles. *N_E = Effective number of alleles [Kimura and Crow (1964)]. **H** = Nei's (1978) gene diversity. ***I*** = Shannon's Information index [Lewontin (1972)].

N_A was 2. N_E ranged from 1.17 (P4) to 1.45 (Cs12), with a mean of 1.26. *H* varied from 0.13 (P4) to 0.27 (Cs12) with a mean of 0.17. I ranged from 0.23 (P4) to 0.42 (Cs12), with a mean of 0.29. Hence, the results showed that Cs12 revealed a high level of genetic variation.

The Takelsa, Fahes, and Locale populations showed the highest Nei's genetic diversity and Shannon's information index (0.13 and 0.21, respectively), while the Ltaifia population showed the lowest values (0.08 and 0.13, respectively).

The average gene diversity within population was 0.16 (H_S) and within total population 0.17 (H_T). Investigations proved that genetic differentiation between populations (G_{ST}) had an mean of 0.06, indicating a moderate level of genetic differentiation between the populations of *V. faba* L analyzed. Gene flow (N_m) was found to be 8.21, indicating notable gene flow between populations (Table 7). Nei's genetic distances ranged from 0.028 (between F Alia and F Mateur) to 0.131 (between F Eljem and Ltaifia), with a mean of 0.079 (Table 8).

From the UPGMA dendrogram (Figure 1), it was discernible that the Ltaifia (LT) and Mahdia (MH) populations were the most distant and clustered separately from other populations of *V. faba,* which were grouped into two main clusters, cluster I (CI) grouped the major populations and cluster II (CII) contained minor populations. Interestingly, cluster (CI) appeared separately from second cluster (CII), showing a high level of genetic variation between major and minor populations of *V. faba*. The first one was made up of three populations of *V. faba major*: Eljem (FEJ), Alia (FA) and Mateur (FM).

Cluster II showed a high level of diversity and it was further divided into two subclusters. Subcluster I (ScI) had five samples [Saber02 (SB), Locale (LC), Fahes (FH), Eljem (EJ) and Takelsa (TK)]. Subcluster II was found to contain two samples [Mateur (MT) and Korba(KB)].

Table 7. Genetic diversity index.

Locus	Sample Size	H_T	H_S	G_{ST}	$N_m(G_{ST})$*
Mean	120	0.1730	0.1631	0.0574	8.2127
St. Dev		0.0214	0.0187		

H_T = total variability. H_S = variability within population. G_{ST} = diversity among populations. N_m = gene flow (0.5 (1 - G_{ST}) / G_{ST}).

Table 8. Nei's genetic distances between pairwise *Vicia faba L.*populations.

Parameter	Ltaifia	Takelsa	Eljem	F Alia	F Mateur	Korba	Fahes	Locale	Mahdia	Mateur	F Eljem	Saber 02
Ltaifia	0											
Takelsa	0.073	0										
Eljem	0.068	0.047	0									
F Alia	0.084	0.040	0.055	0								
F Mateur	0.116	0.062	0.068	0.028	0							
Korba	0.085	0.045	0.062	0.056	0.079	0						
Fahes	0.082	0.049	0.047	0.059	0.077	0.043	0					
Locale	0.070	0.037	0.036	0.041	0.063	0.047	0.031	0				
Mahdia	0.124	0.086	0.092	0.085	0.105	0.072	0.060	0.068	0			
Mateur	0.103	0.049	0.059	0.036	0.059	0.046	0.048	0.048	0.065	0		
F Eljem	0.131	0.070	0.080	0.052	0.046	0.075	0.074	0.073	0.093	0.064	0	
Saber 02	0.096	0.056	0.051	0.050	0.062	0.063	0.045	0.041	0.061	0.047	0.066	0

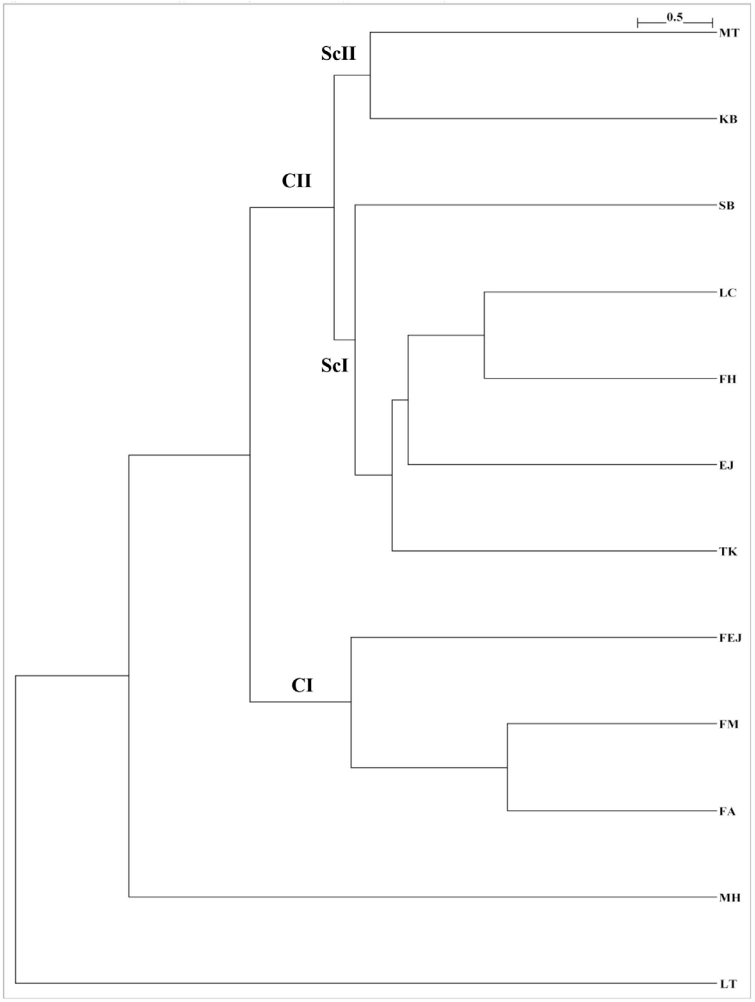

Figure 1. Dendrogram based on Nei's (1978) genetic distance: method UPGMA modified from neighbor procedure of PHYLIP version 3.5.

DISCUSSION

Morphological traits indicated that major and minor populations of *V. faba* exhibited considerable variation. Particularly the nine *V. faba* minor populations showed considerable variation for most of the morphological characters in agreement with a previous observation describing a large diversity for morpho-agronomic traits in Greek and Ethiopian landraces (Keneni et al., 2005; Terzopoulos et al., 2008). Recently in Tunisia, Yahia et al. (2012) described a substantial phenotypic variability in faba bean germplasm using morphological descriptors. Moreover, the study showed interesting populations, such as Alia with significant gain yield despite poor nodulation. Its grain yield could be improved by efficient soil bacteria inoculation. In this context, Trabelsi et al. (2011) reported that inoculation of *Phaseolus vulgaris* with two indigenous rhizobia strains induced a significant increase in nodulation and grain yield.

The results obtained from RAPD analysis used to assess the genetic diversity of Tunisian *V. faba* populations showed that the primer Cs12 was recommended to examine the genetic diversity of *V. faba* populations. Our results reflected similar findings as reported earlier in studies of the genetic variation in other species at the cultivar level by using RAPD markers (Singh et al., 2012; Kalpanaa et al., 2012; Badfar-Chaleshtori et al., 2012; Basheer-Salimia et al., 2012; Rajesha et al., 2013) and among Palestinian faba bean landraces (Basheer-Salimia et al., 2013).

Furthermore, investigations reported that the species maintained a high genetic diversity within populations. The minor populations of *V. faba* exhibited a high level of diversity and were further divided into two subclusters, which agrees with a previous study of genetic diversity in European and Mediterranean faba bean germplasm using RAPD markers (Link et al., 1995). In addition, Terzopoulos and Bebeli (2008) studied 20 local Greek faba bean populations using four ISSR primers and proposed that the majority of the observed genetic variability was due to within population variation (75.4%).

Our results showed that genetic diversity within population was 94% of total diversity and intra-population genetic diversity (H_S) was 0.16, which was clearly higher than between population genetic diversity (D_{ST} = 0.06). Similarly, on the basis of AFLP analysis, Gresta et al. (2010) observed a considerable level of genetic variation within faba bean accessions. Recently, Ouji et al. (2012) demonstrated that the majority of the genetic variation of faba bean populations was found within populations.

Overall, the Ltaifia population was the most homogeneous, which can be considered a selected cultivar with very interesting agronomic characters, and this cultivar can be proposed as obtained for direct use by farmers.

In addition, the Takelsa population was the most heterogeneous which represents a interesting reservoir of genes for breeding programs.

There was agreement on a variety relationship between the morphology and molecular methods. Similar results were obtained by many authors when comparing the findings of morphological and molecular analyses in different species (Wang et al., 2002; Li et al., 2006; Talbi et al., 2008; Benor et al., 2012).

The significant positive correlations between the matrices obtained from the RAPD markers and the morphological trait matrices also indicate that morphological characters can provide a useful measure of genetic diversity between inbred lines (Ye et al., 2008). This correlation can be used to study populations efficiently in plant breeding programs.

CONCLUSIONS

In the present study, data on morphological traits and RAPD markers used for evaluating genetic diversity in *V. faba* revealed different levels of polymorphism between populations. The primer Cs12 was recommended to examine the genetic diversity in the collections of *V. faba* populations. Our results showed that the Ltaifia population was the most homogeneous, which can be considered a selected population, and that the Takelsa population was the most heterogeneous, which is interesting for breeding program.

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