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RESEARCH ARTICLE

Genetic variability in Tunisian populations of faba bean (*Vicia faba* L. var. *major*) assessed by morphological and SSR markers

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Abstract The genetic diversity of 21 faba bean populations was examined using morphological and molecular markers. DNA was extracted from 189 individuals and 8 microsatellite markers were genotyped individually in these 21 populations. A total of 53 alleles were obtained in all populations, with an average of 6.62 alleles per locus. The expected and observed heterozygosity was 0.38 and 0.62 respectively. The average polymorphism index content of SSR markers was 0.61, ranging from 0.31 to 0.81. The unweighted pair group method with arithmetic mean dendrogram clustered all the populations into two groups, each for them subdivided into 3 sub-groups according to geographical origin. Morphological variation showed that the populations were not grouped according to their geographical origin. Therefore, patterns of differentiation of morphological traits did not coincide with molecular differentiation, indicating that morphological variation does not reflect genetic subdivision in studied faba bean populations. Analysis of molecular variance revealed high levels of genetic variation (83%) within population and provides a good base for designing genetic improvement programs. The result of Principal Component Analysis (PCA) revealed that three dimensional principal components (PC1, PC2 and PC3) contributed 40.56% of the total variability and accounted with values of 20.64, 11.22 and 8.70%, respectively. Cluster analysis based on PCA indicated three separate groups of populations. The genetic relationships found between the 21 populations samples were the same in both the PCA and STRUCTURE analysis which support the results observed. These data may serve as a foundation for the development of faba bean breeding programs.

Keywords Faba bean (*Vicia faba* L.) · Genetic diversity · SSR markers · Population structure

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Introduction

Grain legumes play an important role in the dietary and nutritional needs of people, particularly in developing countries. Faba bean (*Vicia faba* L.) is one of the major cool-season legume crops in the world as well in the Mediterranean region (Torres et al. 2006). It ranks fourth worldwide in overall production among grain legume crops such as chickpea, pea and lentil and is used as a main source of proteins for both, human nutrition and animal feeding (Mulualem et al. 2012). In Tunisia, faba bean covers more than 68% (70,000 ha) of the total area annually devoted to grain legumes crops (Kharrat and Ouchari

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2011). The average faba bean productivity in Egypt is more than 3 tons/ha, followed by Sudan (2–2.5 tons/ha), China (1.5–2 tons/ha) and Ethiopia (1–1.5 tons/ha) (FAOSTAT 2011). The average productivity in Tunisia is 1.03 tons/ha, nearly 40% below the world average (1.7 tons/ha). Moreover, the national average varies across regions and tremendously from year to year (Kharrat and Ouchari 2011). This fluctuation is due to many factors such as the lack of improved cultivars, the high susceptibility of local material to diseases and pests and the sensitivity of the crop to environmental conditions. Drought is one of the major important abiotic stresses that cause considerable yield loss to faba bean production (Link et al. 1999). Moreover, faba bean shows more sensitivity to drought than some other grain legumes including common bean, pea and chickpea (Amede and Schubert 2003).

Cultivating tolerant genotypes is the most attractive approach to attenuate the negative effects of drought on crop production. Due to the insufficient genetic variability in commercial faba bean cultivars for important traits like drought tolerance, extensive evaluation of local genetic diversity of faba bean is an approach for identifying adapted genotypes, thereby responding to farmers need and providing useful germplasm sources for breeders. Molecular markers are used successfully in the assessment of genetic diversity, germplasm classification and population genetic structure in crop plants (Tehrani et al. 2009; Hajibarat et al. 2015).

Morphological traits (Terzopoulos et al. 2004; Yahia et al. 2012), isozyme markers (Ouji et al. 2011) and molecular markers such as RAPD (Link et al. 1995), SSAP (Ouji et al. 2012), AFLP (Zied et al. 2003), SRAP (Alghamdi et al. 2012), and ISSR (Wang et al. 2012) have been used for detection of genetic diversity in faba bean germplasm. In the other hand, SSRs have been widely applied in plant genetics and breeding, because they are codominant, easy to score, and highly abundant. Despite this, a limited number of previous studies have used SSR markers as a tool for studying the genetic variation in faba bean. Large scale development of faba bean SSR markers was successfully achieved by using next generation sequencing (Yang et al. 2012). In addition, Suresh et al. (2013) developed and characterized 55 polymorphic cDNA-SSR markers for faba bean in order to facilitate the molecular diversity studies in that species.

The objective of this study was to investigate genetic structure, genetic diversity and relationships among 21 populations of faba bean collected from different regions in Tunisia using morphological and SSR markers in order to help and improve the selection process using that germplasm in breeding programs.

Materials and methods

Plant material

Twenty-one populations of cultivated faba bean from different geographical regions of Tunisia, together representing a majority of the cultivated acreage of faba bean in the country, were collected and used in the current study (Table 1). Collecting sites were localized in five provinces of Tunisia, namely, Beja, Jendouba, Sidi Bouzid, Gafsa, and Kasserine (Fig. 1). The experiments were carried out from December 2015 to April 2016 under green house conditions at the Experimental Station of Tunis Biotechnology Center close to the Mediterranean Sea shore, 30 km south-east of Tunis (10°10′E, 36°48′N; 10 m of altitude).

Morphological analysis

All plants were grown in individual pots. The pots were arranged in a simple randomized design with three replications. Each population was represented by a sample consisting of 30 plants (10 plants per replication). Ten morphological characteristics listed in Table 2 according to the faba bean descriptors in the International Board for Plant Genetic Resources (IBPGR 1985) were evaluated. Observations were recorded on five individual plants for each replication (15 plants/population were measured). Days to flowering were recorded as the mean emergence days to the date when 50% plants started flowering. Plant height (cm), number of stems per plant, pods number per node, seeds number per pod, pod length (cm), and the number of leaflets per leaf were measured at different stages of plant growth. After harvest, seed weight was recorded as 100-seed weight (g). Leaflet length (cm), and leaflet width (cm) were measured using calipers.

Analysis of variance was performed for all measured traits in order to test the significance of variation among populations. Mean and standard deviation values of the morphological characters were analysed using the SYSTAT 8.0 software (Wilkinson 1998). To evaluate levels of phenotypic variation among populations, phenological dendrograms were constructed based on Euclidean distance coefficients using MVSP 3.1 (Multi-Variate Statistical Package; Kovach 2007) program.

Molecular analysis

Nine plants per population were randomly selected, and green healthy leaves from each plant were collected, frozen in liquid nitrogen, and then stored at −80 °C prior to DNA extraction. Plant material was ground in liquid nitrogen and total genomic DNA was extracted from individual plants

Table 1 List of the collection sites of Tunisian populations of faba bean (*Vicia faba* L.) and their bioclimatic characteristic

Population code	Location	Climate	Botanical class	Altitude (m)	Latitude	Longitude	Rainfall (mm/year)
P1	Gafsa (Menzel El Gamoudi)	Inferior semi arid	*Major*	380	34.46	9.06	50–150
P2	Gafsa (Zanouch)	Inferior semi arid	*Major*	251	34.35	8.85	50–150
P3	Gafsa (El Aïeïcha)	Inferior semi arid	*Major*	373	34.35	9.11	50–150
P4	Gafsa (Ouled El Hadj)	Inferior semi arid	*Major*	220	34.43	8.71	50–150
P5	Sidi Bouzid (Essaida)	Superior semi arid	*Major*	312	35.07	9.58	100–200
P6	Sidi Bouzid (Boudinar)	Superior semi arid	*Major*	361	35.26	9.42	100–200
P7	Sidi Bouzid (Gouleb)	Superior semi arid	*Major*	319	35.08	9.52	100–200
P8	Sidi Bouzid (Elghiss)	Superior semi arid	*Major*	381	34.98	9.32	100–200
P9	Sidi Bouzid (Elghiss)	Superior semi arid	*Major*	183	34.84	9.79	100–200
P10	Sidi Bouzid (Ksar El ghrib)	Superior semi arid	*Major*	336	35.12	9.37	100–200
P11	Kasserine (Fousana)	Superior semi arid	*Major*	726	35.34	8.65	100–200
P12	Kasserine (Fousana)	Superior semi arid	*Major*	779	35.33	8.71	100–200
P13	Kasserine (Sebha)	Superior semi arid	*Major*	526	35.22	9.11	100–200
P14	Kasserine (Ouled Belnnouma)	Superior semi arid	*Major*	523	35.22	9.12	100–200
P15	Kasserine (Ouled Belnnouma)	Superior semi arid	*Major*	494	35.22	9.17	100–200
P16	Kasserine (Rkhamet)	Superior semi arid	*Major*	643	35.24	8.76	100–200
P17	Kasserine (Sbitla)	Superior semi arid	*Major*	749	34.95	8.58	100–200
P18	Kasserine (Sbitla)	Superior semi arid	*Major*	794	35.00	8.61	100–200
P19	Jendouba (Fernana)	Superior humid	*Major*	288	36.65	8.69	450–550
P20	Jendouba (Fernana)	Superior humid	*Major*	246	36.64	8.71	450–550
P21	Beja	Inferior humid	*Major*	458	36.77	9.08	400–500

according to the cetyltrimethylammonium bromide (CTAB) technique described by Borges et al. (2009). The DNA was quantified using a spectrophotometer (Spectro UV–Vis Double PC 8 Auto Cell, UVS-2007). The quality of the DNA was estimated on a 1% (w/v) agarose gel stained with ethidium bromide. All samples were then brought to a working concentration of 50 ng/µl. Eight simple sequence repeat (SSR) primer pairs were used for molecular analyses. Six primer pairs were obtained from Ma et al. (2011): SSR3, SSR4, SSR5, SSR6, SSR7 and SSR8, and two primer pairs were obtained from Gong et al. (2010): SSR1 and SSR2. The PCR reactions were carried out in a volume of 25 µl containing 50 ng genomic DNA, 0.3 µM forward and reverse primers, 2 mM $MgCl_2$, 0.3 mM dNTPs, 1U Taq DNA polymerase (Thermo Fisher Scientific, Germany), in 1xPCR buffer (10 mM Tris–HCl, pH 8.3 and 50 mM KCl). The reactions were performed in Applied Biosystems 2720 Thermal Cycler using the following temperature profiles: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 55–58 °C according to the primers used (Table 3) and 1 min at 72 °C with a final extension period of 10 min at 72 °C. For each locus, the alleles were separated on a 6% polyacrylamide gel (7 M urea, 1xTBE buffer). Two µl of loading buffer (95% Formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol) were added to 5 µl of each sample; the samples were denatured at 95 °C for 5 min before electrophoresis. The electrophoresis were performed at 80 W constant power for about 3 h (Sequi-Gen GT sequencing cell, Bio-Rad) after a pre-run of the gels for 30 min. Gels were silver-stained according to the protocol of Benbouza et al. (2006). Gels were dried at room temperature and scanned. All PCR reactions and electrophoreses were repeated at least twice and independently scored. DNA fragments were sized using gel_analyser (http://www.sequentix.de/) software.

The SSR profiles were scored based on the size (bp) of fragments amplified across all the 21 populations. Genetic parameters: number of alleles per locus (N), observed heterozygosity (Ho), expected heterozygosity (He) and Shannon's Information Index (I) were performed using GenAlEx software version 6.5 (Peakall and Smouse 2006). The polymorphism information content (PIC) value was calculated following the formula described by Botstein et al. (1980). For genetic distance analysis based on SSR results, the allelic sizes data were transformed to binary data: presence (1) versus absence (0) of each allele, and data were entered as a binary matrix. The resulting matrix was used to estimate genetic similarity among all populations by Dice coefficient of similarity (Nei and Li 1979).

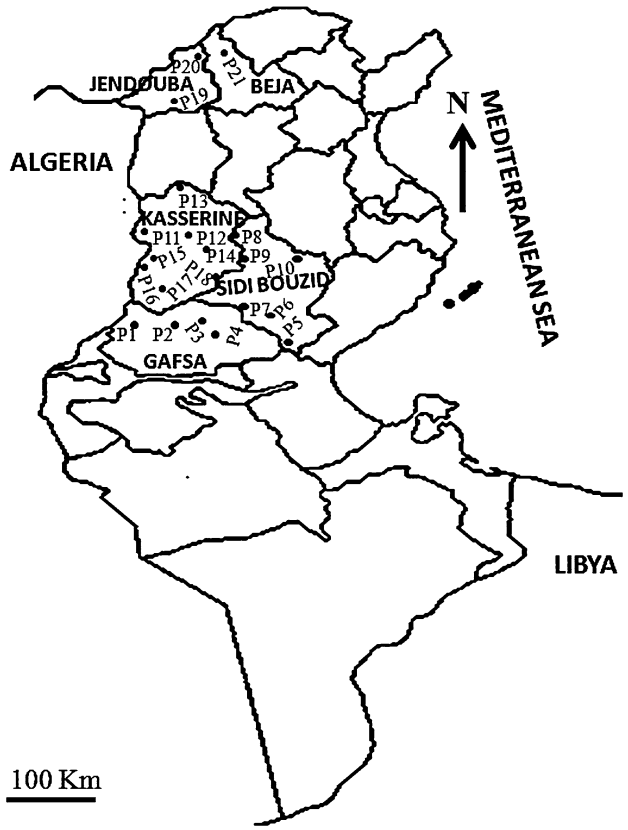

Fig. 1 Geographical distribution of the Tunisian faba bean populations studied

Dendrograms were constructed from these matrices with MVSP 3.1 software using the UPGMA (Unweighted Pair-Group Method with Arithmetic mean) clustering algorithm. A Mantel test was conducted using the program GenAlEx 6.5 for correlation between genetic, geographic and morphological distance. Moreover, three-dimensional principal component analysis (PCA) using microsatellite data was performed using MVSP3.1 program and analysis of the molecular variance (AMOVA) among and within populations was performed using GenAlEx 6.5 program.

Genetic structure

In order to assess the genetic structure of populations, the Bayesian clustering approach implemented in the software STRUCTURE version 2.3.1 (Pritchard et al. 2000) was used. All 189 individuals from the 21 populations were analyzed jointly, without prior population information, under the admixture model with correlated allele frequencies. In order to identify the number of potential genetic clusters (K) capturing the major structure in the data, we used a burn-in period of 500,000 Markov Chain Monte Carlo iterations and a run length of 10^6 iterations, the admixture model with correlated allele frequencies. Five independent runs were performed for each simulated value of K, ranging from 1 to 10 (Falush et al. 2003). The most probable number of Ks was determined using the ΔK statistics method proposed by Evanno et al. (2005). The optimum value of $K = 3$ was then used to determine inferred ancestries.

Results

Morphological characterization

The means of the agro-morphological traits of the twenty-one populations are presented in Table 4. The results revealed that the flowering time extended from 46.33 days (P1) to 85.33 days (P21). With respect to plant height, results showed that the P20 was the tallest with 101.5 cm. The minimum plant height was recorded in P6; P7 and P10 plants were, however, at part in plant height with a mean 58.67 cm. The pod length ranged from 8.5 cm (P3) to 18.58 cm (P14). P6 gave the highest mean value of stems number per plant (2.3) while the lowest one (1.4) was obtained by P1. The highest mean value of number of seeds per pod (4.3) was exhibited by P14 while the lowest mean value was displayed by P5, P6, P7, P3, P11 and P16 (1.6, 1.6, 1.7, 1.9, 2.3 and 2.3 respectively). The 100 seed weight

Table 2 List of the measured agro-morphological traits studied for the 21 faba bean populations

Agro-morphological traits	Abbreviation	Trait
Plant growth	PH	Plant height at flowering (cm)
	PL	Average length of fresh pods (cm)
	LL	Leaflet length (cm)
	LW	Leaflet width (cm)
	NL/L	Number of leaflets per leaf
Plant fertility	NS/PL	Number of stems per plant at flowering
	FD	Number of days to 50% flowering
	NP/N	Number of pods per node
	NS/P	Number of seeds per pods
Yield components	100 SW	100 seed weight (g)

Table 3 Characteristics and diversity information parameters at 8 SSR loci used on 189 faba bean genotypes

SSR locus	Sequence (5′–3′)	Repeat type	Annealing temperature (°C)	N	A	I	Ho	He	uHe	PIC
SSR1	F: ACCAGGCAGAGTTAGATAAGCA R: GTCACTCGGATGTCCAGGTAT	$(GGAACC)_3$	56	6	0.33	0.68	0.96	0.49	0.52	0.68
SSR2	F: GCAAGGGTGTAAGGAAT R: CACAAACCGAGGGAATA	$(TGTTT)_3$	56	3	0.51	0.69	0.98	0.50	0.52	0.49
SSR3	F: AATCACAAGCGACGACGAC R: GCGGAATATGCAGACCAAAT	$(GA)_8$	58	7	0.2	0.65	0.77	0.46	0.49	0.81
SSR4	F: TCGCAATAGCACAGAACCTG R: GATCAAACTCCCCAACCTCA	$(CCACCG)_3$	58	6	0.36	0.48	0.51	0.33	0.35	0.72
SSR5	F: GGATGGATTGATTCTCCAACA R: GCATAACTAACACATTATGCAGGA	$(ATAA)_4$	57	8	0.54	0.55	0.53	0.37	0.39	0.62
SSR6	F: TGCAGAGAAGCTAAGCACCA R: TCGCATGGTACAGTAGCAAAA	$(TC)_8$	56	4	0.78	0.43	0.36	0.28	0.29	0.31
SSR7	F: CGTCGTGAAAATCATGGAGA R: CATTATTATTACACCCGCCTCA	$(ATC)_6$	57	5	0.6	0.47	0.41	0.29	0.31	0.55
SSR8	F: CAACGCGGCAGTTAAAGAAT R: CAGGTATGGCTGACACCTCA	$(GGAACC)_3$	57	14	0.39	0.52	0.49	0.34	0.36	0.76
Mean				6.62	0.46	0.55	0.62	0.38	0.40	0.61
SE				2.63	0.18	0.10	0.24	0.08	0.09	0.16

N number of alleles per locus, *Ho* observed heterozygosity, *He* expected heterozygosity, *I* Shannon's diversity index, *PIC* polymorphism information content

ranged from 102.83 g (P2) to 262.48 g (P16). In general, significant variation among populations was found in all morphological characters, except for number of pods per node (NP/N) and number of leaflets per leaf (NL/L) where the differences were not significant. These results demonstrate that substantial differences exist between studied faba bean populations derived from different environments which may represent genetic differentiation between populations in response to environmental characteristics. Cluster analysis of the 21 populations based on the standardized value of agro-morphological traits was performed by UPGMA method and a dendrogram was constructed as depicted in Fig. 2. Plant populations were divided into four main clusters. The cluster III and IV comprised 4 and 5 population, respectively. The population P12 which had the highest number of stems per plant was separated from all other populations of cluster II, whereas, P16 and P17 which showed the highest pod number per node were separated in a single cluster (Cluster I) from the other populations. The clustering of Tunisian faba bean based on morphological traits was not in agreement with their geographical distribution.

Molecular characterization

Polymorphism among the 189 individual genotypes of the studied 21 faba bean populations was investigated with 8 SSR markers (Table 3). Allelic diversity at SSR loci varied greatly among loci, and was high on average. A total of 53 alleles with an average of 6.62 alleles per locus were detected. The number of alleles ranged from 3 (SSR2) to 14 (SSR8). For all the genotypes the PIC values for the SSR loci ranged from 0.31 (SSR6) to 0.81 (SSR3) and the average PIC value was 0.61. Observed heterozygosity (Ho) ranged from 0.36 (SSR6) to 0.98 (SSR2) with a mean of 0.62, whereas mean expected heterozygosity (He) was revealed to be 0.38. Shannon's Information Index was between 0.43 and 0.69 with an average of 0.55.

Genetic relationships

According to the data from 8 SSR markers, the UPGMA dendrogram of the 21 collected faba bean populations based on Nei & Li's similarity coefficients showed two major clusters each of which is further divided into three sub-groups according to similarity between them (Fig. 3). Cluster I contains all populations of faba bean from Gafsa (subcluster I1) and Sidi Bouzid (subclusters I2 and I3). Cluster II contains all populations from the region of Kasserine, Jendouba and Beja. Subclusters II1 and II2 included 8 populations all of which were from Kasserine, whereas subcluster II3 included populations from Beja and Jendouba.

Table 4 Summary statistics of the agro-morphological traits measured in 21 Tunisian faba bean populations

Population	PH (cm)	PL (cm)	FD	100 SW (g)	LL (cm)	LW (cm)	NP/N	NS/P	NS/PL	NL/L
P1	$68.17^{a} \pm 6.46$	$14.33^{a} \pm 0.41$	$46.33^{a} \pm 3.14$	$156.76^{a} \pm 14.91$	$7.20^{a} \pm 3.29$	$4.23^{a} \pm 2.92$	$1^{a} \pm 0.00$	$3.20^{a} \pm 1.14$	$1.40^{a} \pm 0.52$	$3.50^{a} \pm 0.70$
P2	$66.17^{a} \pm 4.96$	$10.33^{e} \pm 0.52$	$78.67^{b} \pm 3.01$	$102.83^{ab} \pm 22.86$	$5.50^{d} \pm 2.13$	$3.60^{a} \pm 3.61$	$1^{a} \pm 0.00$	$3.00^{a} \pm 0.94$	$1.70^{a} \pm 0.48$	$2.60^{a} \pm 0.97$
P3	$70.00^{a} \pm 2.37$	$8.50^{f} \pm 0.63$	$62.00^{d} \pm 2.28$	$168.08^{a} \pm 1.18$	$5.49^{d} \pm 2.90$	$3.88^{a} \pm 5.52$	$1^{a} \pm 0.00$	$1.90^{ab} \pm 0.88$	$1.90^{ab} \pm 0.88$	$2.00^{a} \pm 0.00$
P4	$77.83^{ac} \pm 6.68$	$12.83^{ac} \pm 2.16$	$58.33^{d} \pm 4.76$	$162.98^{a} \pm 19.26$	$6.58^{a} \pm 2.84$	$3.78^{a} \pm 4.60$	$1^{a} \pm 0.00$	$2.90^{a} \pm 1.10$	$1.90^{a} \pm 0.74$	$2.80^{a} \pm 0.92$
P5	$79.67^{ac} \pm 6.38$	$12.00^{ac} \pm 0.55$	$69.83^{bd} \pm 6.79$	$202.54^{a} \pm 40.82$	$5.76^{d} \pm 3.79$	$3.27^{a} \pm 4.09$	$1^{a} \pm 0.00$	$1.60^{ab} \pm 0.52$	$1.90^{ab} \pm 0.99$	$2.40^{a} \pm 0.70$
P6	$58.67^{ab} \pm 3.72$	$11.75^{ac} \pm 0.61$	$61.17^{d} \pm 7.39$	$215.90^{a} \pm 8.91$	$4.66^{b} \pm 4.71$	$3.19^{a} \pm 3.56$	$1^{ab} \pm 0.00$	$1.60^{ab} \pm 0.52$	$2.30^{ab} \pm 0.82$	$2.40^{a} \pm 0.52$
P7	$58.67^{ab} \pm 4.37$	$11.92^{ac} \pm 0.38$	$46.00^{a} \pm 2.37$	$207.77^{ab} \pm 41.33$	$4.99^{bd} \pm 1.88$	$3.40^{a} \pm 1.50$	$1^{ab} \pm 0.00$	$1.70^{ab} \pm 0.48$	$1.90^{ab} \pm 0.57$	$2.60^{a} \pm 0.84$
P8	$67.17^{a} \pm 6.11$	$15.58^{b} \pm 1.66$	$62.00^{d} \pm 4.56$	$151.10^{a} \pm 22.98$	$7.12^{a} \pm 3.79$	$3.22^{a} \pm 2.00$	$1^{a} \pm 0.00$	$3.80^{a} \pm 0.79$	$1.50^{a} \pm 0.71$	$3.00^{a} \pm 1.15$
P9	$70.17^{a} \pm 4.31$	$17.25^{d} \pm 0.76$	$48.67^{a} \pm 4.08$	$120.13^{ab} \pm 11.13$	$7.16^{a} \pm 2.06$	$2.84^{ab} \pm 1.39$	$1^{a} \pm 0.00$	$3.20^{a} \pm 0.79$	$1.60^{a} \pm 0.52$	$3.00^{a} \pm 1.05$
P10	$58.67^{ab} \pm 5.13$	$16.42^{b} \pm 1.02$	$70.83^{b} \pm 8.89$	$150.72^{a} \pm 0.68$	$4.67^{b} \pm 2.73$	$2.47^{b} \pm 1.94$	$1^{a} \pm 0.00$	$3.10^{a} \pm 0.99$	$1.60^{a} \pm 0.70$	$3.10^{a} \pm 0.88$
P11	$93.17^{b} \pm 4.54$	$14.08^{a} \pm 0.86$	$83.67^{c} \pm 5.43$	$145.94^{a} \pm 8.85$	$3.58^{c} \pm 3.58$	$2.18^{bc} \pm 1.57$	$1^{ab} \pm 0.00$	$2.30^{ab} \pm 0.95$	$2.20^{ab} \pm 0.79$	$2.70^{a} \pm 0.82$
P12	$85.00^{c} \pm 3.69$	$12.75^{ac} \pm 0.88$	$61.83^{d} \pm 13.09$	$123.95^{ab} \pm 11.67$	$3.33^{c} \pm 3.50$	$2.14^{bc} \pm 1.28$	$1^{ab} \pm 0.00$	$3.40^{a} \pm 0.97$	$2.50^{a} \pm 0.85$	$2.20^{a} \pm 0.42$
P13	$76.17^{ac} \pm 2.48$	$17.33^{d} \pm 2.89$	$73.00^{b} \pm 6.48$	$143.07^{a} \pm 11.93$	$3.55^{c} \pm 2.76$	$1.94^{bc} \pm 1.39$	$1^{a} \pm 0.00$	$3.90^{a} \pm 1.10$	$1.90^{a} \pm 0.74$	$3.10^{a} \pm 0.99$
P14	$76.33^{ac} \pm 2.34$	$18.58^{d} \pm 0.66$	$82.67^{c} \pm 6.38$	$170.46^{a} \pm 16.74$	$5.65^{d} \pm 2.85$	$2.33^{b} \pm 1.90$	$1^{a} \pm 0.00$	$4.30^{a} \pm 1.06$	$1.70^{a} \pm 0.82$	$3.40^{a} \pm 0.84$
P15	$88.83^{b} \pm 2.14$	$13.25^{ac} \pm 1.94$	$83.00^{c} \pm 6.66$	$117.92^{ab} \pm 14.26$	$4.71^{b} \pm 2.22$	$2.50^{b} \pm 2.73$	$1^{a} \pm 0.00$	$3.40^{a} \pm 0.52$	$1.80^{a} \pm 0.92$	$2.60^{a} \pm 0.97$
P16	$74.50^{ac} \pm 1.87$	$14.25^{a} \pm 0.94$	$81.33^{e} \pm 0.52$	$262.48^{c} \pm 16.44$	$4.71^{b} \pm 2.22$	$2.50^{b} \pm 2.73$	$1.1^{ab} \pm 0.32$	$2.30^{ab} \pm 1.16$	$2.00^{ab} \pm 0.82$	$2.40^{a} \pm 0.70$
P17	$87.83^{bc} \pm 6.08$	$16.58^{b} \pm 0.58$	$69.50^{f} \pm 0.55$	$147.65^{a} \pm 9.68$	$4.24^{b} \pm 18.48$	$2.18^{b} \pm 8.67$	$1.1^{a} \pm 0.32$	$2.70^{a} \pm 0.95$	$1.90^{ab} \pm 0.99$	$2.90^{a} \pm 0.88$
P18	$85.33^{c} \pm 2.73$	$15.33^{b} \pm 0.61$	$84.17^{e} \pm 2.23$	$154.70^{a} \pm 7.22$	$5.33^{b} \pm 9.66$	$2.49^{b} \pm 1.61$	$1^{a} \pm 0.00$	$2.90^{a} \pm 0.74$	$1.70^{a} \pm 0.82$	$2.60^{a} \pm 0.84$
P19	$82.83^{c} \pm 6.68$	$12.75^{ac} \pm 0.52$	$82.50^{e} \pm 3.94$	$174.35^{a} \pm 18.60$	$5.33^{b} \pm 6.00$	$2.54^{b} \pm 2.62$	$1^{a} \pm 0.00$	$2.80^{a} \pm 0.79$	$2.00^{a} \pm 0.67$	$2.60^{a} \pm 0.70$
P20	$101.50^{d} \pm 5.01$	$14.17^{a} \pm 0.82$	$65.67^{f} \pm 3.20$	$186.82^{a} \pm 21.19$	$3.88^{c} \pm 2.73$	$2.35^{b} \pm 2.17$	$1^{ab} \pm 0.00$	$3.30^{a} \pm 0.95$	$2.20^{a} \pm 1.03$	$2.60^{a} \pm 0.84$
P21	$76.83^{ac} \pm 5.81$	$16.83^{b} \pm 0.61$	$85.33^{e} \pm 1.51$	$139.98^{a} \pm 9.51$	$3.89^{c} \pm 1.13$	$2.13^{bc} \pm 1.17$	$1^{a} \pm 0.00$	$2.90^{a} \pm 0.83$	$1.80^{a} \pm 0.75$	$2.80^{a} \pm 0.98$

Different letters denote significant differences (Tukey's HSD, $P < 0.05$)

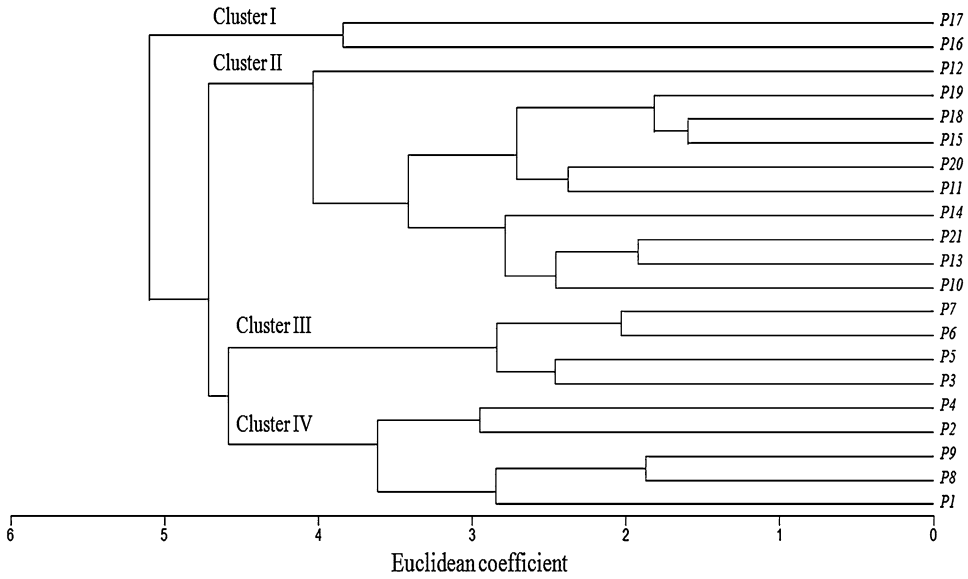

Fig. 2 Morphological variability phenogram of faba bean populations based on Euclidean distance and UPGMA clustering

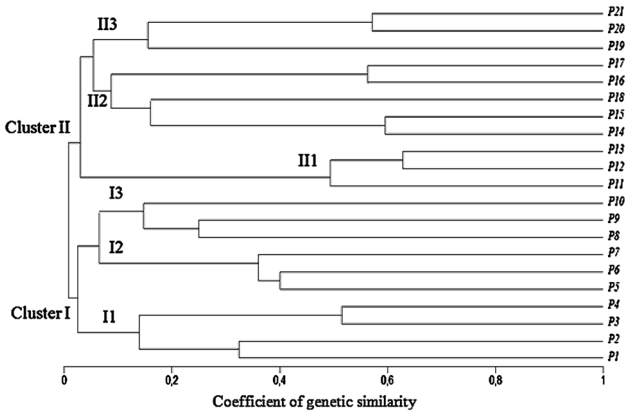

Fig. 3 UPGMA dendrogram of 21 faba bean populations based on SSR data. Genetic distances are based on Nei and Li (1979) similarity coefficients

A Principal Component Analysis (PCA) performed in order to better understand relationships among the populations of different origins showed similar cluster formation to UPGMA method (Fig. 4). The first three components accounted for 40.56% of the total variation, of which principal components 1 (PC1), 2 (PC2) and 3 (PC3) explained 20.64, 11.22 and 8.70% of the variation, respectively. The PCA plots revealed three clusters.

The grouping pattern of cluster I was very similar to the cluster I revealed by UPGMA, with populations from Gafsa and Sidi Bouzid. The cluster II included eight populations from Kasserine, Beja, and Jendouba and is similar to the cluster II of UPGMA data with the exception that, according to PCA analysis, the 3 populations P11, P12 and P13 originating from Kasserine clustered separately from the other populations (Cluster III).

A significant correlation was found between the genetic, morphological and geographic distance matrices (Table 5). Indeed, the Mantel test revealed a significant correlation between the morphological and genetic matrices ($r = 0.41$, $P < 0.01$). Moreover, Mantel test showed a weak but significant correlation among genetic and geographic distances ($r = 0.06$, $P < 0.05$), while, no significant correlation between morphological and geographic matrices ($r = 0.22$, $P = 0.13$) was revealed.

Analysis of molecular variance (AMOVA)

Analysis of Molecular Variance (AMOVA) was used to estimate the partitioning of genetic variance among and within populations (Table 6). AMOVA results based on SSR data revealed that the vast majority of the total genetic variance was due to within population variation (83%) and only 17% of the genetic variation was contained among the populations.

Population structure analysis

The structure of the 21 populations of faba bean was established using STRUCTURE 2.3.4 software to determine the optimum number of genetic clusters reflecting the genetic structure within faba bean. STRUCTURE allocates individuals into a number of clusters (K) independent of population information based on multilocus genotypic data, so as to minimize deviations from Hardy–Weinberg and linkage equilibrium (Aradhya et al. 2013). Structure analysis revealed that the log posterior probabilities (LnP[D]) increased sharply from $K = 1$[LnP(D) = −9324.66] to $K = 3$ [LnP(D) = −4681.18], followed by a gradual decline from $K = 3$ to $K = 10$ (Fig. 5). The ΔK ad hoc

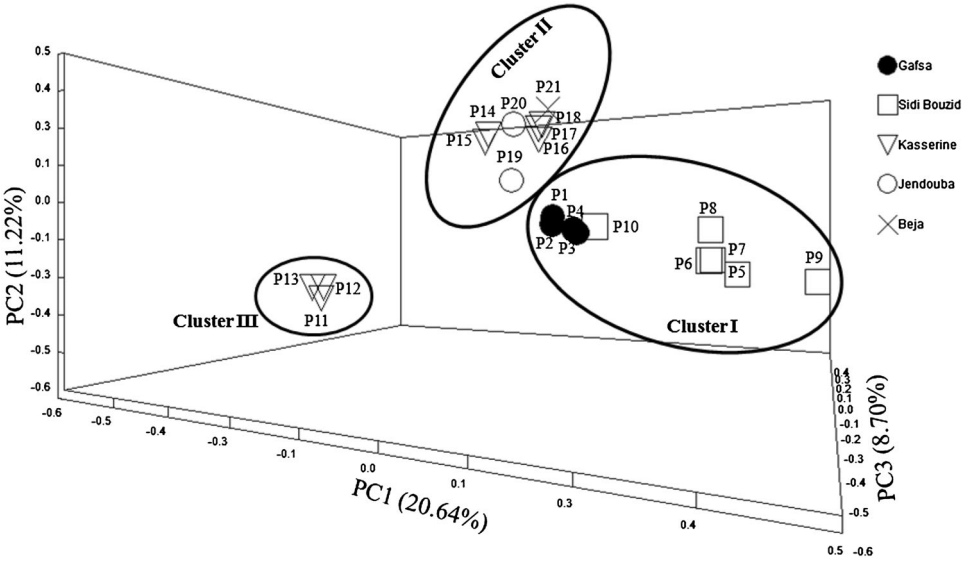

Fig. 4 A three dimensional principal component analysis of 21 faba bean populations using 8 SSR primer pairs. The percentage of variation is presented on each corresponding axis. The circles encompass the three major groups of faba bean populations

Table 5 Correlations between genetic, morphological and geographic differences among 21 populations of faba bean tested with simple Mantel's test

First matrix	Second matrix	*r*	*P* value[a]
Genetic	Geographic	0.06	< 0.05
Morphological	Genetic	0.41	< 0.01
Morphological	Geographic	0.22	0.13

[a] Probability of a significant correlation between genetic, morphological and geographic distances at $P = 0.05$ after 999 permutations

statistic method (Evanno et al. 2005) which is based on the second order rate of change of the likelihood function determined K = 3 to be the most likely number of genetic clusters. This data suggest that faba bean populations are divided into three main clusters (Fig. 6). Genetic structure analysis of the individual samples using STRUCTURE shows that 3 gene pools are represented in the data, and each gene pool is relatively independent (Fig. 7). Faba bean populations (P1-P10) clustered together in a distinct cluster, which was consistent with their clustering into subgroup I1, I2 and I3 revealed by UPGMA method. The second main cluster contained 8 populations (P14-P21), 3 of which (P19-P21) were from northern provinces including Jendouba and Beja, and 5 (P14-P18) were from Kasserine province belonging to the west-central. Interestingly P11, P12 and P13 from Kasserine separated from the other populations and grouped together in a distinct cluster. However, STRUCTURE analysis showed three main clusters within the studied faba bean populations, which were fairly consistent with the PCA analysis.

Discussion

In Tunisia, faba bean is one of the most important grain legumes crops, and it has major impact on agriculture, environment, animal and human nutrition (Kharrat and Ouchari 2011). Both biotic and abiotic stresses can severely limit faba bean production. Drought stress has become the main limiting factor that adversely affects faba bean during the vegetative and reproductive stages and consequently grain yields (Ali et al. 2013). Genetic diversity of plant was used for the development of new improved plant varieties.

Molecular markers have been used in plant breeding programs to estimate genetic variability (Porth and El-Kassaby 2014), detecting quantitative trait loci (Li et al. 2014) and marker-assisted selection (Sakiyama et al. 2014). In this study, we used morphological traits and SSR markers to characterize a set of 21 faba bean populations

Table 6 Analysis of molecular variance (AMOVA) of 21 faba bean populations based on 8 SSRs

SV	d.f.	SSD	MSD	VC	TVP	*P* (rand ≥ data)
Among populations	20	277.556	1.652	1.652	17%	0.001
Within populations	168	1501.032	75.052	8.155	83%	
Total	188	1778.587		9.808	100%	

Source of variation (SV), degrees of freedom (df), sum of squares (SSD), mean squares (MSD), variance component (VC) and total variance percentage (TVP)

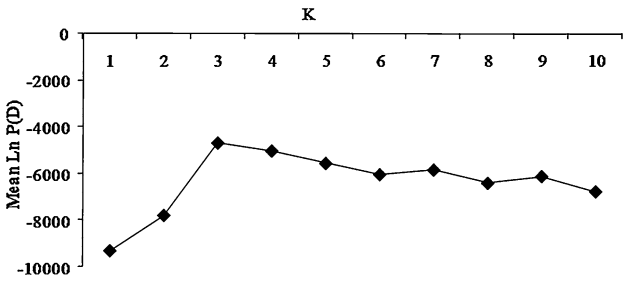

Fig. 5 Estimated log likelihood [LnP(D)] produced by STRUCTURE (Pritchard et al. 2000) at each value of K

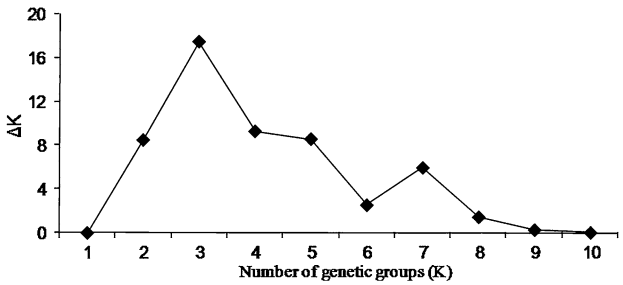

Fig. 6 Estimation of the number of genetic cluster (K) calculated by delta K value (ΔK), according to Evanno et al. (2005)

collected from different regions of Tunisia. Most measured characters showed significant phenotypic differences among faba bean populations. Indeed, in the present study quantitative agromorphological traits such as plant height, leaflet length, leaflet width, number of stems per plant and 100 seed weight revealed a high polymorphism within Tunisian faba bean populations. There were some reports implying genetic variation of faba bean germplasm based on quantitative traits such as nodes per stem, ovules per pod, number of seeds per pod and number of pods per plant (Fikreselassie and Seboka 2012; Yahia et al. 2012). In this study, the presence of significant variability among Tunisian faba bean germplasm indicated the presence of high genetic variability for future faba bean breeding programs. UPGMA cluster analysis of 21 Tunisian faba bean populations based on ten agro-morphological traits was not in agreement with their geographical distribution and divided faba bean germplasm into 4 main clusters. Data showed enough phenotypic variability in the Tunisian faba bean germplasm. Results of present studies are in agreement with those of Yahia et al. (2012). Using 35 morphological traits, these authors found no relationship between the geographical origin and genetic diversity of 42 faba bean landraces from southern Tunisia, belonging to 8 oasis agro-ecosystems.

Assessment of genetic diversity in crop populations using morphological traits is not an easy task because morphological traits can be affected by environmental factors and cultivation conditions. Indeed, molecular markers are increasingly used for the detection of differences at the DNA level among and within crop populations (Hoxha et al. 2004). Although sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Hou et al. 2015) and molecular markers such as ISSR (Salazar-Laureles et al. 2015) and SSR have been widely used to assess genetic diversity in faba bean (Alghamdi et al. 2012; Suresh et al. 2013; Abid et al. 2015), the information on the genetic variability and relationship among Tunisian germplasms is limited.

In the present investigation, SSR markers were employed to assess the genetic diversity among 21 populations of faba bean from Tunisia. Indeed, SSR markers revealed a relatively high observed heterozygosity (Ho) mean value (0.62) which is presumably due to the partially allogamous nature of the species. The expected heterozygosity (He) ranged from 0.28 at locus SSR6 to 0.50 at locus SSR2, with a mean value of 0.38. Similar observations were made for faba bean (Yang et al. 2012); the mean Ho and He values were 0.59 and 0.33 respectively. In fact, we explained the medium level of heterozygoty by the presence of rare alleles in the studied populations.

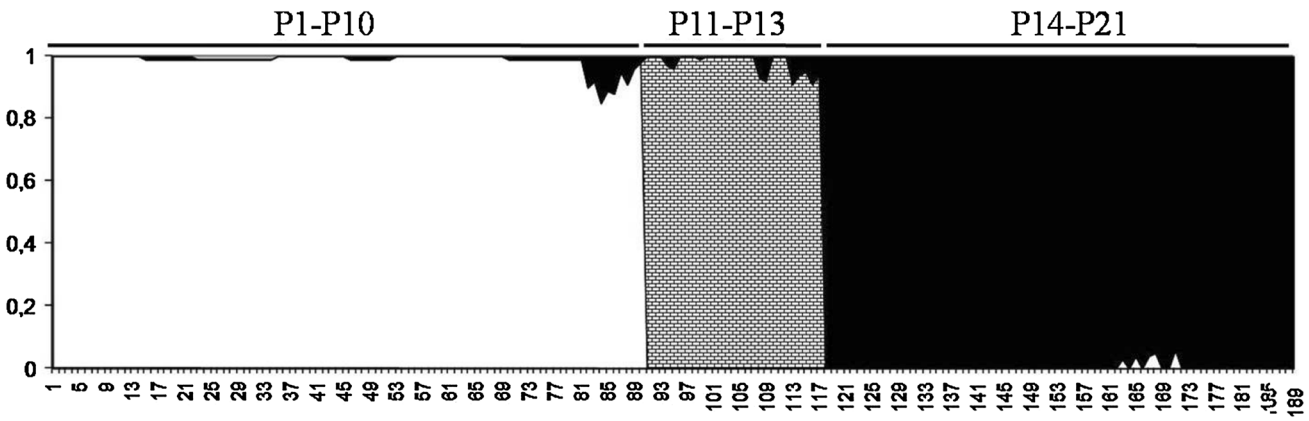

Fig. 7 Diagram of results from STRUCTURE (k = 3)

Polymorphic level among faba bean accessions was evaluated by calculating allelic number and polymorphism information content (PIC) values. The data showed that SSR markers revealed distinct polymorphisms among faba bean germplasm indicating the robustness of microsatellites in revealing polymorphisms. The PIC value is commonly used in genetics as a measure of polymorphism for a marker locus used in linkage analysis (Matin et al. 2012). Table 3 showed that PIC values varied greatly for all the SSR loci tested with an average of 0.61 indicating that the primers could develop high polymorphism which is useful for assessing genetic variation of genotypes studied in this research (Vaiman et al. 1994). The PIC values revealed are comparable to those reported for faba bean cultivars in other countries such as Korea. Indeed, the PIC values for the SSR loci revealed by Suresh et al. (2013) ranged from 0.16 to 0.88 with an average of 0.55. However, the PIC values are also much higher than those reported for 29 faba bean cultivars from China and Europe with an average PIC value of 0.29 (Gong et al. 2011). Moreover, our data revealed that the number of alleles detectable in Tunisian faba bean populations is comparable with the number of alleles detected in traditional Korea population (Suresh et al. 2013). On the other hand, the mean number of alleles was high (6.62) compared with the mean number of alleles per locus for China and some Europen (2.76) faba bean genotypes (Ma et al. 2011). These data suggested that Tunisian faba bean populations are polymorphic, providing a large number of alleles within populations. In general, a relatively high genetic diversity was found in Tunisian faba bean germplasm (Ouji et al. 2012; Backouchi et al. 2015). Many factors act to influence population genetic diversity including mating system, gene flow and seed dispersal, geographic range as well as natural selection (Amos and Harwood 1998). Faba bean is partially allogamous, with rates of out-crossing ranging from 10 to 70% and differing between environments and genotypes, which could explain highly heterogeneous populations and heterozygous plants (Bond 1987; Gnanasambandam et al. 2012). Hence, there is considerable potential to select within Tunisian populations specific traits such as tolerance to drought stress. Moreover, genetic variation between populations of faba bean were reported in this study based on Shannon's information index (I) at each single primer pair which varied from 0.43 to 0.69, with a mean of 0.55. These results indicate that these SSR markers are useful to detect genetic variability of faba bean germplasm.

AMOVA using the SSR data showed that the genetic differentiation was mainly observed within populations. Interestingly, our data are consistent with previous studies in faba bean which revealed a larger genetic variation and differentiation within than between population (Terzopoulos and Bebeli 2008; Ouji et al. 2011; Oliveira et al. 2016). Moreover, this was consistent with the findings from other species such as grapevine (De Andrés et al. 2012), olive (Belaj et al. 2007), apple (Zhang et al. 2012) and lettuce (Rauscher and Simko 2013).

Unlike allogamous species, rice is pre-dominantly autogamous and, hence, gene flow is restricted and a very large differentiation level of genetic variation was detected among populations (Kiambi et al. 2005). This might be a result of historical population processes, such as natural selection, mutation, isolation and genetic drift (Ming-qian et al. 2011; Reed and Frankham 2003). In *Changiostyrax dolichocarpa* which is pollinated mainly by insects such as bees, genetic diversity using SSR markers revealed that high genetic differentiation and low gene flow between populations can result from short-distance gene dispersal either by pollen or by seed (Yao et al. 2007).

Cross-pollination of faba bean is facilitated by insect pollinators like honeybees (*Apis mellifera*), bumblebees (*Bombus* spp.) and diverse solitary bees (*Anthopora* and *Xylocarpa*), with the dominant species varying between regions (Stoddard and Bond 1987; Bond and Kirby 1999). Therefore, we suggest that the high level of genetic variability which was observed within most of the tested populations is presumably due to the partially allogamous reproductive system of faba bean. This is in accordance with a referent study made by Schoen and Brown (1991) mentioning that outcrossing maintains most genetic variations for within population rather than for among populations.

UPGMA cluster analysis separated faba bean population based on geographical origin. These data were in agreement with previous analysis of Yang et al. (2012) which revealed relationship between faba bean germplasm from diverse geographic origin using SSR markers. For some population (P19, P20 and P21), data showed incomplete relationship between the origin of the populations and the molecular clusters. This close genetic relationship observed in P19, P20 and P21 could result from a high genetic overlap among these populations as a result of gene flow through their seed and pollen dispersal.

Principal component analysis (PCA) is a widely-used tool in analyzing genetic variation among plant accessions and determining the most important variables contributing to variation (Price et al. 2006). Indeed, three dimensional plots were performed in order to estimate genetic variance and relationships within 21 Tunisian populations. Faba bean populations clustered into 3 groups. Figure 4 allows assessment of the genetic relationship among populations. We found that there is no obvious relationship between geographical origin and grouping based on genetic similarities. This result suggests that there was considerable gene flow among these studied populations. Therefore, we believe that in addition to outcrossing, human activities

may play a role in distribution of genetic diversity and structure in faba bean germplasm.

Cluster I was the largest group and formed by all individuals of populations originated from two provinces (Gafsa and Sidi Bouzid) which showed very close genetic relationships. The molecular differentiation of the Tunisian populations from these two provinces suggests a great genetic similarity and a common genetic pool as well as an adaptation to local agro-climatic conditions. Frequent gene exchange among faba bean populations from these two provinces could explain a decline in genetic differentiation: indeed individuals from subclusters I1, I2 and I3 (Fig. 3) comprised a principal component. According to the PCA analysis cluster II was formed by eight faba bean populations. Five populations (P14, P15, P16, P17 and P18) among 8 are from Kasserine, P19 and P20 from Jendouba and P21 from Beja. The grouping of populations from Kasserine (Center West), Beja and Jendouba (North West) was probably due to the existence of seed exchanges among farmers of these different locations. The same was reported earlier by other researchers in faba bean (Salazar-Laureles et al. 2015) and other species such as rice (He et al. 2014) and sorghum (Labeyrie et al. 2014). According to Gresta et al. (2010) this could be advantageous in faba bean breeding programs: such materials might be used to maximize the level of variation and to assess these genotypes in contrasting environments that allow broadening their diversification. Cluster III was the smallest cluster and composed of only three populations (P11, P12 and P13) from Kasserine. These 3 populations were clearly discriminated from populations originating from different geographical origins including Kasserine. Indeed, the genetic distances among some faba bean populations were relatively high suggesting the existence of an interesting diversity among these 3 populations and the rest of the studied populations. This probably means that the 3 populations were selected according to the same characters. Genetic variability revealed in P11, P12 and P13 can be related to a differential response to biotic or abiotic stress, to the agronomic management or else to their facility to adapt to other environments. Therefore, these 3 populations could contribute to enrich the genetic foundation for faba bean breeding in Tunisia. Similar results were commented by Terzopoulos and Bebeli (2008) which suggested introducing some Greek faba bean populations as sources of lines appropriate for the development of synthetic varieties.

Liu and Hou (2010) suggested that the classification results of germplasm resources were not only related to the types of markers but also were closely linked to classification method or foundation. STRUCTURE analysis did not show any geographical distribution pattern and suggested the existence of three major groups (Fig. 7). Thus, the result of model based on STRUCTURE analysis is in agreement with the PCA analysis and showed good correspondence with each other. In general the studied populations could not be clustered according to geographical origin which could be explained by a partial sharing of their ancestral genetic polymorphism. This could be associated with gene flow via seed exchanges among different regions which occurred by human transfer trying to mitigate the effects of some diseases like Ascochyta blight of faba bean, caused by *Ascochyta fabae*.

This work shows that SSR markers are a powerful tool for investigating population structure and genetic diversity. Moreover, these markers will be beneficial for both genetic and breeding applications in this crop to facilitate faba bean improvement and accelerate the development of new cultivars.

Conclusion

Genetic variability is important for the development of new and improved cultivars. Indeed, the characterization of the genetic variability between faba bean germplasm could be a good tool of selecting these germplasm in faba bean breeding program. Considerable genetic diversity among the 21 studied populations was observed at both the morphological and molecular levels, which is of importance for germplasm classification, management, and further utilization. The results of the current study show that SSRs markers would be a useful tool for faba bean genetic diversity studies and in breeding programs. Cluster analysis based on genetic distance allowed the 21 populations to be divided into two major groups. Moreover, PCA analysis confirmed clustering results shown by STRUCTURE which divided these faba bean populations into three groups. To increase knowledge about the faba bean genetic structure we also recommend further analysis using a larger number of SSR markers. Overall, the results showed good enough genetic diversity in studied faba bean populations which can be utilized to predict approaches such as parental line selection for faba bean breeding programs in order to obtained new biotic and abiotic-tolerant faba bean varieties.

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