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Genetic diversity in *Vicia faba* L. populations cultivated in Tunisia revealed by simple sequence repeat analysis

Yassine Yahia[1]*, Hédia Hannachi[1], Antonio Jose Monforte[2], James Cockram[3], Mohamed Loumerem[1], Belkacem Zarouri[4] and Ali Ferchichi[1]

[1]*Arid and Oases Cropping Laboratory, Arid Lands Institute (IRA), Médenine 4119, Tunisia,* [2]*Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universidad Politécnica de Valencia (UPV)-Consejo Superior de Investigaciones Científicas (CSIC), Ciudad Politécnica de la Innovación (CPI), Ed. 8E, Ingeniero Fausto Elio s/n, 46022 Valencia, Spain,* [3]*National Institute of Agricultural Botany (NIAB), Huntington Road, Cambridge CB3 0LE, UK and* [4]*Departamento de Investigación Agroalimentaria, Instituto Madrileño de Investigación y Desarrollo, IMIDRA, Madrid, Spain*

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Abstract

Faba bean (*Vicia faba* L.) is one of the most important legumes in the world. Little is known about the genetic resources of faba bean in Southern Tunisia. In the present study, genetic diversity within Tunisian faba bean germplasms was investigated using 16 simple sequence repeat markers. In total, 50 alleles were detected. The number of alleles per marker ranged from 2 to 6, with an average of 3. Genetic diversity and polymorphism information content values averaged, respectively, 0.43 (range 0.34–0.51) and 0.36 (range 0.28–0.43). The mean heterozygosity value was 0.27. A model-based structure analysis based on neighbour-joining tree and factorial correspondence analysis revealed the presence of two subpopulations, consistent with the clustering based on genetic distance (GD). The overall F_{is} value was 0.36, indicating the importance of selfing in these populations. Analysis of molecular variance revealed that the within-population genetic variance component was much higher than the between-population or between-subpopulation variance component. The genetic relationships based on Nei's GD revealed that AGD (Aguadulce) and SAG (Super Aguadulce) and TF1 and TF2 (Tafartassa-Gafsa) were the most closely related populations. Assessment of genetic variation within faba bean populations will be informative for the conservation of germplasms and the implementation of effective breeding programmes in Tunisia.

Keywords: genetic diversity; plant genetic resource conservation; population structure; simple sequence repeats (SSRs); *Vicia faba* L.

Introduction

Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes in the world, first cultivated in the Neolithic period (Metayer, 2004). In Tunisia, faba bean cultivation is common among traditional farmers, especially in Southern regions (Khaldi and Zekri, 2002). In recent years, the area under faba bean production has reduced due to low yield and yield instability (Gresta *et al.*, 2009), mostly as a result of environmental conditions and inefficient agronomical management.

* Corresponding author. E-mail: yahia.yassine@gmail.com

Various studies have described genetic variations in local faba bean collections in the Mediterranean region using morphological traits (Zeid *et al*., 2003; Terzopoulos *et al*., 2003, 2008; Terzopoulos and Bebeli, 2008). Indeed, the morphological and agronomical evaluation of faba bean populations is imperative for effective exploitation in plant breeding schemes, as well as for their efficient conservation and management. The advent of molecular marker systems has provided new tools for evaluating faba bean germplasms and assessing genetic diversity (Zeid *et al*., 2003; Terzopoulos and Bebeli, 2008; Zong *et al*., 2010; Gong *et al*., 2011). Molecular markers are not affected by environmental factors or by developmental stages (Bebeli and Kaltsikes, 1993). Furthermore, molecular data can contribute to a more holistic picture of genetic diversity within a collection of populations (Curley and Jung, 2004).

Genetic variability in *V. faba* populations is relatively high in Tunisia, and information on such variability is essential for their conservation, breeding and commercial production. Screening methods based on isozymes (Ouji *et al*., 2011a) and agromorphological (Ouji *et al*., 2011b) and biochemical (Chaieb *et al*., 2011) traits have all been used previously to assess faba bean genetic diversity. However, molecular marker systems have proven particularly useful for evaluating germplasms and assessing genetic diversity (Zeid *et al*., 2003; Terzopoulos and Bebeli, 2008). Link *et al.* (1995) employed random amplified polymorphic DNA markers to study genetic diversity within European and Mediterranean faba bean germplasms. Zeid *et al.* (2003) used amplified fragment length polymorphism (AFLP) markers to assess genetic diversity among elite faba bean inbred lines, while Terzopoulos and Bebeli (2008) described the genetic diversity of 20 Greek faba bean local populations with inter-simple sequence repeat (ISSR) markers. However, simple sequence repeat (SSR) markers have been postulated to be of more relevance to faba bean germplasm characterization (Kaur *et al*., 2012; Yang *et al*., 2013), DNA fingerprinting and genetic mapping (Ma *et al*., 2013), due to their high information content, co-dominant and relatively simple-to-interpret properties (Powell *et al*., 1996) and potential for analysis of intra-cultivar variability (Cipriani *et al*., 2002; Lopes *et al*., 2004).

A previous study investigating faba bean populations from Southern Tunisia based on agromorphological traits (Yahia *et al*., 2012) did not reveal any population–geographical area interaction. However, some traits proved useful for the classification of populations. The aim of the present study was to describe genetic diversity and population structure of local faba bean germplasms from Tunisia using SSR markers. Our results provide a framework for future studies aimed at the conservation, improvement and management of cultivated faba bean in Tunisia.

Materials and methods

Plant material

The germplasm studied consisted of 16 southern Tunisian faba bean local populations from the collection described by Yahia *et al.* (2012). Populations have been traditionally divided into *Vicia faba minor*, *Vicia faba equina* and *Vicia faba major* on the basis of seed weight. Accordingly, based on the criteria proposed by Henelt (cited by Lawes *et al.* (1983)) and Duc (1997), ten of the populations were classified as *major* (MR1, MR3, MD1, MD2, MD3, MD4, BK1, TF1, FRT and TZ2), five as *equina* (MR2, TF2, BK2, HMG and TZ1) and one as *minor* (MD5). The commercial varieties 'Aguadulce' and 'Super Aguadulce' (*equina* Var.) were used as references, coded, respectively, as AGD and SAG. The population codes, localities of origin and 1000-seed weight of each entry are listed in Table 1. Each population was represented by randomly selected pools of individual plants.

DNA extraction and polymerase chain reaction (PCR) procedure

Nucleic acid extraction

Samples (2–3 g) of very young, healthy leaf tissue harvested from each bulk of three plants were lyophilized and kept at −20°C. Due to the high anthocyanin content of faba bean leaf tissue, genomic DNA extraction was carried out using the protocol described by Sreelakshmi *et al.* (2010). DNA quality was tested using 1% agarose gel electrophoresis and DNA concentration was determined spectrophotometrically. DNAs of all the samples were diluted to 5 ng/μl using sterile PCR-grade water.

SSR genotyping

Twenty-four SSR markers previously developed by Zeid *et al.* (2009) were employed in the present study, of which 16 were polymorphic (Table 2). The SSR primers were synthesized by Invitrogen (Bacelona, Spain). A 20-nucleotide sequence from the M13 cloning vector (5′-CACGACGTTAAAACGACC-3′) was attached to the 5′ end of each forward primer to incorporate fluorescent dyes during the PCRs by adding oligonucleotides complementary to the M13 cloning vector sequence labelled with 6-FAM (6-carboxyfluorescein), NED, PET or VIC (Applied Biosystems, Foster City, USA) (Schuelke, 2000). PCRs were carried out in a total volume of 25 μl, containing 2.5 μl of 10 × PCR buffer, 0.6 mM of dNTPs, 3 mM of $MgCl_2$, 0.5 μM of each primer (forward and reverse), 0.3 μM of labelled primer, 1 unit of Taq polymerase

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Table 1. Geographical data and 1000-seed weight of *Vicia faba* L. populations collected from the oases of Southern Tunisia

Population code	Yahia *et al.*'s (2012) population code	Oasis	Province	Latitude (N)	Longitude (E)	Altitude (m)	1000-Seed weight (g)
MR1	P1	Mareth	Gabes	33°37′	10°16′	46	1888.50
MR2	P7	Mareth	Gabes	33°37′	10°16′	46	1486.48
MR3	P8	Mareth	Gabes	33°37′	10°16′	46	1996.77
MD1	P10	Medenine	Medenine	33°20′	10°29′	48	1673.25
MD2	P12	Medenine	Medenine	33°20′	10°29′	48	2140.73
MD3	P14	Medenine	Medenine	33°20′	10°29′	48	1769.00
MD4	P16	Medenine	Medenine	33°20′	10°29′	48	1759.77
MD5	P24	Medenine	Medenine	33°20′	10°29′	48	0885.08
BK1	P26	Beni Khedache	Medenine	33°20′	10°29′	48	1744.39
TF1	P30	Tafartassa	Gafsa	34°24′	8°46′	294	1678.68
TF2	P31	Tafartassa	Gafsa	34°24′	8°46′	294	1175.14
FRT	P32	Ferch	Tataouine	32°55′	10°27′	235	1827.02
BK2	P33	Beni Khedache	Medenine	33°15′	10°11′	506	1425.28
HMG	P37	El Hamma	Gabes	33°52′	9°47′	64	1263.86
TZ1	P38	Tozeur	Tozeur	33°55′	8°08′	43	1230.03
TZ2	P41	Tozeur	Tozeur	33°55′	8°08′	43	1641.14
AGD[a]	AGD	–	–	33°29′	10°38′	20	1438.20
SAG[a]	SAG	–	–	33°29′	10°38′	20	1332.30

[a]Commercial varieties (Aguadulce 'AGD' and Super Aguadulce 'SAG').

(ECOTAQ; Ecogen srl, Barcelona, Spain) and 50 ng of genomic DNA.

PCRs were carried out in a Robocycler Gradient 96 thermocycler (Stratagene, La Jolla, CA, USA) using the following cycling profile: a denaturation step at 94°C for 5 min, followed by 35 cycles of a denaturation step at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 45 s. After the last cycle, a final elongation step of 72°C for 5 min was included. PCR products were visualized using the 3130xl Genetic Analyzer (Applied Biosystems). The loading mixture contained 0.5 µl of PCR product, 0.5 µl of 500 LIZ® Size Standard (Applied Biosystems) and 12 µl of Hi-Di™ formamide (Applied Biosystems). Band molecular sizes were determined using Genemapper 3.0 (Applied Biosystems).

Statistical analysis

Summary statistics including the allele number, major allele frequency and polymorphism information content (PIC) were calculated for each primer (Table 2) using PowerMarker version 3.25 (Liu and Muse, 2005). The same program was employed to calculate genetic diversity (H_e), number of effective alleles (N_e), heterozygosity (H_o), percentage of polymorphic loci (P), PIC and fixation index (F_{is}) for each population. Analysis of molecular variance (AMOVA) was conducted for 56 individuals sampled from 18 faba bean populations using 16 SSR markers.

Genetic distance (GD) between the genotypes (Nei, 1983) was estimated using PowerMarker version 3.25, and neighbour-joining (NJ) trees were constructed using MEGA 4 (Kumar *et al.*, 2008). Factorial correspondence analysis (FCA) based on allele frequencies was carried out using NTSYS-PC version 2.1 (Rohlf, 1997).

Results

Microsatellite genotyping

Of the 24 tested SSR markers, 16 were polymorphic in the germplasm collection studied. A total of 50 alleles were observed. The number of alleles per marker ranged from 2 to 6, with an average of 3 (Table 2). SSR marker M34 exhibited the highest major allele frequency (0.69), whereas SSR marker M4 exhibited the lowest (0.34). The PIC value of the 16 SSRs ranged from 0.33 to 0.68, with an average of 0.51.

Genetic diversity within and among the faba bean populations

The percentage of polymorphism within the faba bean populations varied from 80.6 (TZ1) to 95.0% (MR1 and BK2) with an average of 91.1% (Table 3), excluding the eight SSRs found to be monomorphic in all accessions. The genetic diversity value ranged from 0.34 (TZ1) to 0.50 (MR1 and BK2). The heterozygosity value ranged from 0.16 (TZ1) to 0.38 (MD4).

The highest PIC values were observed for the MR1 population (0.43), originating from Mareth (Gabes)

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Table 2. Information on polymorphic simple sequence repeat markers detected among the studied faba bean genotypes

Locus (codes)	Primer sequences		Repeat type and length	Expected size (bp)	Major allele frequency	Allele number	PIC
	Forward (5′–3′)	Reverse (5′–3′)					
VfG 3 (M3)	TTCTTTCGTCCTCTCTCTATC	GCACTGTTGTTGCTGATACAA	(AG)7 + 14	159	0.50	2	0.37
VfG 4 (M4)	AAGGCGAGGGCATAACAGAA	AATCCGCAAGGGTCTTCTTT	(AG)5 + 10	218	0.34	3	0.59
VfG 10 (M10)	ACCAAAACGCGCACTTATCA	AAGAGAGAGAAGAGAGCTTC	(AG)5	219	0.40	4	0.66
VfG 11 (M11)	GCAAAAGGAGAGGCAAGGGAA	CGAAACAGGGGGGACATTTTGT	(AG)8	301	0.35	4	0.67
VfG 13 (M13)	GGTTGGGATCTTTTAGGTTGAA	TGGCCTTATATCCGTCCAAT	(AG)10	191	0.61	2	0.36
VfG 15 (M15)	TCGATAGGGTTTCAGATTGA	GATGTTGACGGTGGTGTTT	(AG)6 + 15	205	0.36	6	0.65
VfG 19 (M19)	AGCGATGGTGCTCATGCTTA	TCTCTCACGGAATCACATCTTT	(AG)9	174	0.63	2	0.35
VfG 22 (M22)	GGCTATTGTCACGAACAAAT	GATTCAGACCCGGATACATT	(AG)15	147	0.35	4	0.68
VfG 28 (M28)	AGAGTCCCAAAGAGTGGGTT	CCAAAGGCAAAAATGAGGGCTT	(AG)19	220	0.41	3	0.57
VfG 34 (M34)	GCACTCGAAGGAATTAATTTT	GAACAGTTGTTTCGTGTCGTA	(AG)12	205	0.69	2	0.33
VfG 41 (M41)	AGCCCATGGTTCAAATGCAA	GCAGTCATGCCACTGCTTA	(AG)7 + 10	217	0.53	3	0.52
VfG 47 (M47)	CGATTGTTTGCAGAGGAGATA	ACAGAGAGGGACAGAGAGAA	(AG)8	282	0.68	2	0.34
VfG 53 (M53)	GGTTCATGAAAAGAGGTTAG	CATTTTCCGTTCTCTCTCTA	(AG)9	230	0.45	3	0.55
VfG 55 (M55)	ATCATCCAGGAGGGAGAAAA	ATGGGCAGAGAGGATAAAAA	(AG)12	148	0.44	4	0.57
VfG 81 (M81)	GTCCTGGAAAAAAGAAAGAGA	AAAGAAACCTCTCTCTCCAT	(AG)7	175	0.51	2	0.37
VfG 87 (M87)	AGGGCCAGCGTGATCCAATA	TGGGTTGGGATCTTTTGGTTG	(AG)10	242	0.36	4	0.61
Average					0.47	3	0.51

PIC, polymorphism information content.

province, followed by the BK2 (0.42) and BK1 (0.41) populations, both from Beni Khedache (Medenine) province. The lowest PIC value was detected within the TZ1 population from Tozeur province (0.28). Mean effective allele values were found to be highest for the MR1 and BK2 populations ($N_e = 2.50$), followed by the BK1, MD2, MD5 and MR2 populations (2.43). For all the other populations, N_e values ranged from 1.93 to 2.37.

Fixation index (F_{is}), or the differentiation of individuals within populations, was quite high (mean $F_{is} = 0.36$) for all populations, indicating that the degree of selfing may be high within these populations (Table 3).

The AMOVA revealed that the majority of the observed genetic variability was among individuals with subpopulation variation (53.8%). There was a significant amount of variation (8.7%) among the faba bean populations based on geographical origin. Variation among the subpopulations within populations accounted for 7.6% of the total variation (Table 4).

SSR data were used to establish genetic relationships among the faba bean populations, analysed using Nei's GD (Nei, 1983). On the whole, GDs among the faba bean populations analysed in this study varied from 0.06 to 0.42. For the most closely related populations, GDs were 0.06 (TF1 and TF2) and 0.07 (AGD and SAG and MD4 and MD5). For the most divergent populations, GDs were 0.38 (MD1 and FRT) and 0.42 (MD1 and TZ1) (Table S1, available online). The GD between members from the same geographical site was lower than that between individuals from different geographical sites.

Cluster analysis

SSR data were used to construct a NJ tree based on pairwise GDs (Fig. 1). The populations resolved into two major clusters, reflecting their regional or geographical origin: populations from Medenine (MD4, MD5, MD3, MD1 and MD2), Mareth (Gabes) (MR2, MR1 and MR3) and Beni Khedache (Medenine) (BK1 and BK2) clustered together, whereas the eight remaining populations from four different localities [HMG (Hamma-Gabes), TF2 and TF1 (Tafartassa-Gafsa), TZ1 and TZ2 (Tozeur), FRT (Tataouine) and both commercial populations SAG and AGD] formed a separate cluster.

Factorial correspondence analysis

Three FCA axes explained 32.7% of the total genetic variability (Fig. 2). The first and second axes, which accounted for 24.5% of the variance, clearly separated the Beni Khedache (BK1 and BK2), Medenine (MD1, MD2, MD3, MD4 and MD5) and Mareth (MR1, MR2

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Table 3. Parameters of genetic variability in faba bean populations based on 16 simple sequence repeat markers

Population	Yahia *et al.*'s (2012) population code	Genotype no.	Genetic diversity (H_e)	N_e	Heterozygosity (H_o)	P (%)	PIC	F_{is}
AGD	AGD	1.87	0.40	2.06	0.35	90.90	0.33	0.12
BK1	P26	2.31	0.48	2.43	0.32	94.87	0.41	0.33
BK2	P33	2.56	0.50	2.50	0.23	95.00	0.42	0.53
FRT	P32	2.31	0.38	2.12	0.23	85.29	0.32	0.39
HMG	P37	1.81	0.42	2.06	0.29	90.90	0.33	0.30
MD1	P10	2.25	0.43	2.37	0.31	92.10	0.37	0.27
MD2	P12	2.31	0.48	2.43	0.37	94.87	0.40	0.22
MD3	P14	2.31	0.41	2.31	0.22	89.18	0.36	0.46
MD4	P16	2.37	0.48	2.37	0.38	94.73	0.40	0.21
MD5	P24	2.37	0.41	2.18	0.25	88.57	0.34	0.39
MR1	P1	2.43	0.50	2.50	0.24	95.00	0.43	0.51
MR2	P7	2.50	0.46	2.43	0.29	92.30	0.39	0.37
MR3	P8	2.18	0.43	2.18	0.28	91.42	0.36	0.33
SAG	SAG	1.75	0.39	2.06	0.35	87.87	0.32	0.11
TF1	P30	2.50	0.46	2.31	0.24	94.59	0.39	0.47
TF2	P31	2.37	0.45	2.31	0.28	94.59	0.38	0.37
TZ1	P38	1.93	0.34	1.93	0.16	80.64	0.28	0.52
TZ2	P41	2.06	0.40	2.06	0.19	87.87	0.32	0.52
Mean	–	2.23	0.43	2.25	0.27	91.14	0.36	0.36

H_e, genetic diversity; N_e, mean number of effective alleles; H_o, heterozygosity; P, percentage of polymorphic loci; PIC, polymorphism information content; F_{is}, fixation index.

and MR3) germplasms with a south-eastern origin from the other group with mostly south-western origin together with the AGD and SAG varieties. FCA, similar to the NJ tree, revealed a higher variability within the south-western germplasm than within the south-eastern germplasm.

Discussion

In the present study, we used SSRs to examine genetic diversity in a collection of faba bean germplasms from Southern Tunisia and identified a relatively high degree of genetic variability in the collection. Genetic diversity scores were found to be higher at the intragroup level than at the inter-group level, with a positive fixation index. These results indicate partial cross-pollination, as expected, for local faba bean populations due to their partially allogamous nature. Indeed, previous studies have shown that Mediterranean-type populations are mixtures of *V. faba minor*, *V. faba equina* and *V. faba major* (Link *et al.*, 1996; Yahia *et al.*, 2012) and that, more generally, faba bean populations represent heterogeneous mixtures of inbreds and hybrids (Terzopoulos *et al.*, 2008). Collectively, these results are in accordance with those reported by Hamrick and Godt (1990), who stated that reproductive biology is the most important factor determining the genetic structure of plant populations and that out-crossing maintains most of the genetic variation within populations rather than among populations. Similarly, Gabriella *et al.* (1996) confirmed that intrapopulation diversity was higher than interpopulation diversity in out-crossing plants. High levels of intrapopulation variance have been revealed previously in faba bean populations using ISSR (Terzopoulos and Bebeli, 2008; Wang *et al.*, 2012) and isozyme markers (Sonnante *et al.*, 1997; Ouji *et al.*, 2011a), as well as in other out-crossed species such as soybean (*Glycine max*) (Jin *et al.*, 2006), *Lathyrus* populations (Belaid *et al.*, 2006) and *Haloxylon ammodendron* (Sheng *et al.*, 2005). In crops, such results could be attributed to the lack

Table 4. Data from analysis of molecular variance for 56 individuals sampled from 18 populations of faba bean using 16 simple sequence repeat markers

Sources of variation	DF	SS	MS	Variance components	Percentage of variation	P value
Among populations (regions)	6	44.96	7.49	0.56	8.70	0.001
Among subpopulations within populations	17	39.31	2.31	0.49	7.67	0.001
Among individuals within subpopulations	56	265.80	4.75	3.43	53.80	0.001
Total	79	350.07	14.55	4.48		

DF, degree of freedom; SS, sum of squares; MS, mean squares.

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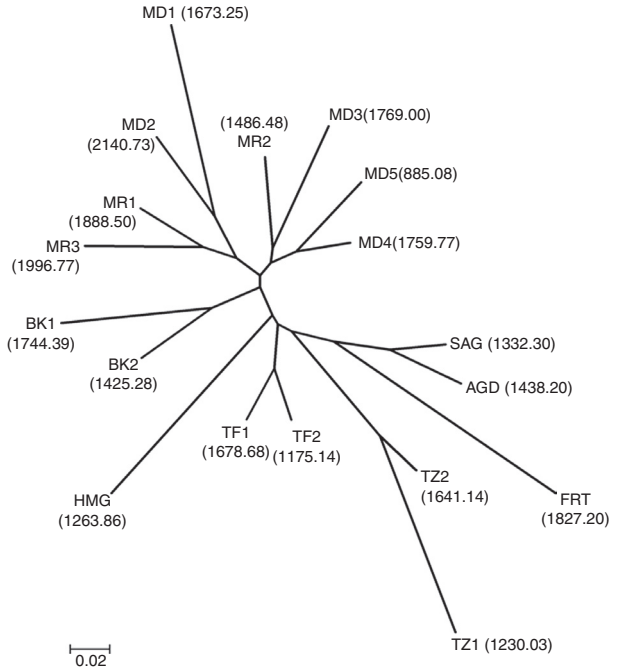

Fig. 1. Unrooted neighbour-joining tree depicting relationships among faba bean germplasms and commercial varieties (Aguadulce and Super Aguadulce) based on Nei *et al.*'s (1983) genetic distance computed from simple sequence repeat marker frequencies (value of seed weight given in brackets in g).

of genetic material exchange between farmers, use of traditional cultivation methods (e.g. no mechanical harvesting) and consecutive cycles of inbreeding and selection carried out over a long period by farmers. It has been suggested that traditional cultivation practices may have led to increased genetic homogeneity within populations, which was higher than that reported for Spanish bean landraces (Alvarez *et al.*, 1998) and Greek wheat landraces (Mantzavinou *et al.*, 2005).

The NJ tree clustered genotypes into two main groups, correlating well with geographical origin and seed weight. The first cluster included ten faba bean populations from three provinces in geographical proximity: Mareth (Gabes), Beni Khedache (Medenine) and Medenine. This group represented faba bean populations from Southeastern Tunisia, with the exception of MD5, and represented populations with the heaviest seeds. The second cluster included eight faba bean populations originating from Southwestern Tunisia and represented populations with the lowest seed weights. The Aguadulce and Super Aguadulce varieties clustered within group 2, had medium-weight seeds, and were classified as *V. faba* L. Var. *equina* (Ouji *et al.*, 2011b). The grouping structure identified in this study using SSR data was in good agreement with previous results based on morphological characters (Yahia *et al.*, 2012), in which 1000-seed weight was found to be the most discriminate descriptor, subdividing faba bean populations into three groups (*minor*, *equina* and *major* types). High correlations between molecular markers and morphological traits have been reported in other plant species, such as maize (Karanja *et al.*, 2009) and *Bromus tectorum* (Ramakrishnan *et al.*, 2004). In the present study, the observation that seed weight correlates well with genetic differentiation confirms that seed weight could represent a key morphological trait for the classification of faba bean germplasms. Indeed, previous morphological classifications of faba bean populations have depended mainly on 1000-seed weight, in combination with additional

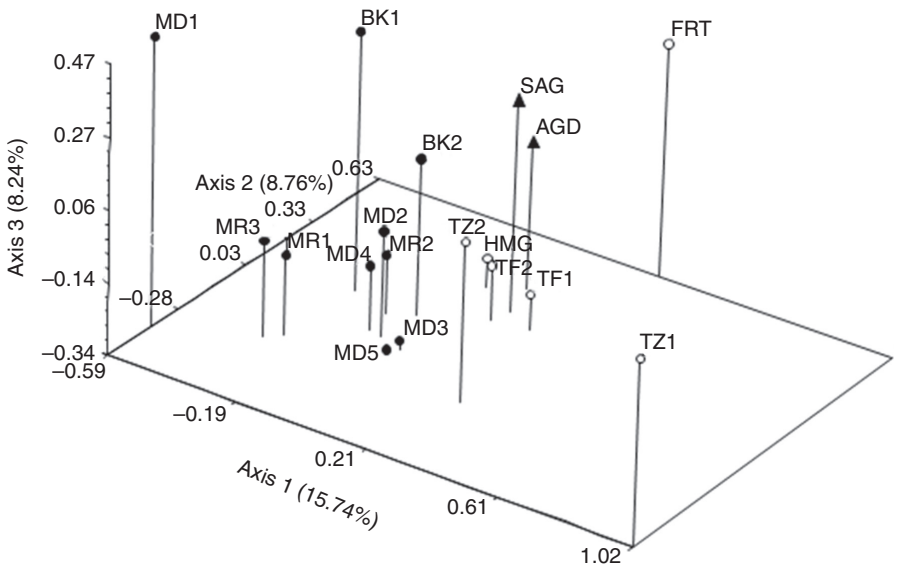

Fig. 2. Multivariate analysis (factorial correspondence analysis) of simple sequence repeat data for 16 faba bean germplasms from Southeastern ● and Southwestern ○ Tunisia and commercial varieties Aguadulce and Super Aguadulce ▲.

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traits such as number of flowers per raceme, number of pods per truss, pod width, number of ovules per pod (including seeds) and stems per plant (Yahia *et al.*, 2012). Similar results correlating genetic variation with seed traits have been reported in other crops. For example, Pradhan *et al.* (2004) showed that genotype was clearly correlated with phenotypic variability of seed traits in radish cultivars. Similarly, Johnson *et al.* (1996) reported that the *Phs* locus in *Phaseolus vulgaris* accounted for 18–33% of total phenotypic variation in seed weight. Using the AFLP markers, Fatokun *et al.* (1992) showed that two unlinked genomic regions in cowpea contained Quantitative Trait Loci (QTLs) accounting for 52.7% of the variation in seed weight and that four unlinked genomic regions in mung bean accounted for 49.7% of the variation in seed weight.

Understanding genetic relationships among varieties can be particularly useful for planning crosses, defining heterotic pools and assigning lines to specific heterotic groups (Hallauer and Miranda, 1988). Furthermore, knowledge of genetic diversity is of critical importance for plant genetic resource management in gene banks, facilitating reliable classification of accessions, detection of duplicates and identification of useful accessions for specific breeding purposes (Engels *et al.*, 2002; Mohammadi and Prasanna, 2003). In general, very few studies have investigated genetic diversity in faba bean populations from Tunisian oasis agroecosystems. For the effective conservation of faba bean germplasms and breeding, as well as for utilization in oasis agroecosystems, analysis and documentation of genetic diversity are essential. This study represents a critical component towards safeguarding Tunisian faba bean germplasms and will help inform the development of new varieties adapted to the environmental conditions of Southern Tunisia.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262114000021

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