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Full Length Research Paper

Genetic diversity of faba bean (*Vicia faba* L.) populations revealed by sequence specific amplified polymorphism (SSAP) markers

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This study used sequence specifc amplifcation polymorphism (SSAP) markers to investigate the genetic diversity and population genetic structure of nine Tunisian *Vicia faba* populations belonging to the *minor* and *major* faba bean's sub-species. Three primers were used (*PDR1, Tps19 and Tvf4*) in this study. These primers gave good SSAP marker profiles, high number of bands obtained per gel and a high percentage of polymorphic bands as confirmed in a previous study. Indeed, these primers provided a total of 173 amplified bands, with 123 of them being polymorphic. Shannon indexes ranges from 0.166 to 0.248 with an average of 0.207. The genetic diversity within population of 0.743 was clearly higher than that of among population genetic diversity (Dst = 0.138), indicating an out-crossing predominance in the studied populations. The Dst value showed that 15.6% of the total genetic variation resided among populations, a little lower than that of out-crossing species. The dendrogram grouping the populations by unweighted pair-group method with arithmetic averages (UPGMA) method revealed three main clusters. The local *major* faba bean 'Batata' was the most divergent population and was separated from other population.

Key words: *Vicia faba*, SSAP marker, Genetic diversity, Tunisia.

INTRODUCTION

It is generally accepted that the wide variation in genome size observed among eukaryotic species is more closely correlated with the amount of repetitive DNA than with the number of coding genes.

Transposons are mobile genetic elements capable of changing their location in the genome with the potential to produce a wide array of changes in the genomes of their hosts (Kidwell and Lisch, 2000). They constitute a significant fraction of plant genomes (Grzebelus, 2006).

Long terminal repeat (LTR) retrotransposons [which have been classified into two main groups, the *Ty1-copia* group and the *Ty3-gypsy* group, on the basis of conserved sequence features and gene order (Xiong and Eickbush, 1990)], tend to be the dominant retro-transposon class in plants. They are found in the genomes of many plant species and can constitute more than half of the entire genome in some cases (Kumar and Bennetzen, 1999). In addition, LTR retrotransposons provide particularly useful markers for the analysis of genetic diversity, because they typically reveal high levels of polymorphism between individuals (Waugh et al., 1997; Ellis et al., 1998; Tam et al., 2005) and their insertions are irreversible and therefore stable over millions of years (Jing et al., 2005).

Several systems for identifying transposon insertion sites have been developed over the last 10 years, including sequence-specifc amplifcation polymorphism (SSAP). Among the analysis methods developed for

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revealing the polymorphic retrotransposon insertions, SSAP has a high multiplex ratio and flexibility with regard to copy number of retrotransposon families analyzed (Ellis et al., 1998; Jing et al., 2005; Waugh et al., 1997). This approach has been successfully used for genetic mapping, diversity and evolutionary studies in many plant species (Ellis et al., 1998; Lou and Chen, 2007; Pearce et al., 2000; Queen et al., 2004; Sanz et al., 2007; Syed et al., 2005, 2006; Tam et al., 2005; Vukich et al., 2009; Waugh et al., 1997).

The SSAP is an anchored polymerase chain reaction (PCR) approach derived from amplified fragment length polymorphism (AFLP) (Vos et al., 1995), which amplifies the region between a transposon insertion and an adjacent restriction site approach (Waugh et al., 1997); it seems to be one of the most popular transposon-based molecular marker methods. Many applications such as phylogeny, genetic diversity and the functional analyses of genes using retrotransposons were developed (Kalendar and Schulman, 1999; Flavell et al., 1998; Waugh et al., 1997).

These methods have been in use extensively ever since, in many plant species; for instance, in barley (Leigh et al., 2003), wheat (Queen et al., 2004) and Aegilops species (Nagy et al., 2006), oat (Yu and Wise, 2000), apple (Venturi et al., 2006), artichoke (Lanteri et al., 2006), lettuce (Syed et al., 2006), pea (Ellis et al., 1998), pepper and tomato (Tam et al., 2005) and sweet potato (Tahara et al., 2004). SSAP analysis is also used as cladistic molecular markers to show evolutionary history in a given species. In this regard it was used in tobacco (Petit et al., 2007), *Vicia* species (Sanz et al., 2007), Rice (Gao et al., 2004), wheat (Qween et al., 2004) and Zea (García and Martínez, 2003).

Faba bean (*Vicia faba* L.) is a diploid species with 2n=12 chromosomes. It is considered as a valuable protein-rich food for human and animals (Fernandez et al., 1996). Based on differences in seed weight, shape, and size, Muratova (1931) and Cubero (1974) referred to four botanical varieties: *V. faba paucijuga* (particular shape), *V. faba major* (more than 2.0 g per seed), V*. faba equina* (0.45-1.1 g per seed) and *V. faba minor* (0.2 to 0.5 g per seed). *V. faba* is a partially insect cross-pollinated crop. The pollinators can carry out both self-pollinations by the tripping process when they trip the flower and out crossing when they visit other flowers of other plants (Hanelt and Mettin, 1989). Bond and Poulsen (1983) revealed the rate of cross-fertilization could range from 4 to 84%, with averages around 35%.

The aim of the present work was to assess the genetic variation present in nine Tunisian faba bean populations using SSAP markers.

MATERIALS AND METHODS

Plant material

A total of nine faba bean populations (*V. faba* L.) were used in this study. These populations were kindly received from the laboratory of field crops of the National Institute of Agricultural Research of Tunisia. They belong to the two subspecies (*minor* and *major*) of *V. faba* (Table 1).

DNA extraction

Plant DNAs were extracted from the leaves of *V. faba* by the method described by Torres et al. (1993) and using Qiagen Plant DNeasy 96 kits while adhering to the manufacturer's instructions.

LTR sequences and SSAP molecular marker analysis

LTRs used in this study were *PDR1, Tps19 and Tvf4*. These LTR based primers were chosen because they give good SSAP marker profiles, high number of bands obtained per gel and a high percentage of polymorphic bands (Sanz et al., 2007).

For all primers, the SSAP procedure was performed as described by Syed et al. (2005). Selective amplifications were performed with primer pairs containing two or three selective nucleotides on *Mse*I or *Pst*I adapter primers and one selective nucleotide on the γ^{33}P-labeled retrotransposon- based primers. Adaptor primers with selective bases were used in the following combinations with retrotransposon primers: *PDRI* with Mse ATA and Mse ATG; *Tps19* with Pst AA and Pst AT and *Tvf4* with Pst AA. The amplified fragments were separated on 6% polyacrylamide sequencing gels and visualized by autoradiography. SSAP electrophoretic profiles were evaluated by visual inspection of the autoradiograms and amplified DNA bands were scored for their presence (1) or absence (0).

Statistical analyses

The number of total and polymorphic bands, the observed number of alleles (na), the effective number of alleles (Ne), and gene diversity value (h) were calculated for SSAP data. The effective number of alleles identified per locus was calculated as:

$Ne= 1/1-\sum p_i^2$

Where, p_i is the frequency of the *i*th marker allele (Kimura and Crow, 1964).
Gene diversity was evaluated according to:

$H=n/n-1\ (1- \sum p_i^2),$

Where, p_i is the frequency, in each group, of the i^{th} allele at each SSAP locus; and n is the number of individuals in each group of samples (Nei, 1978). Analysis of genetic diversity was conducted according to Nei (1978) method using FSTAT ver.2.9.3.2 program (Goudet, 2001). The total gene diversity (H_T) can be divided into gene diversity within populations (H_S) and between populations (D_{ST}) where:

$H_T = H_S + D_{ST}$.

H_T was calculated on the weighted average allele frequencies over all populations:

$$H_T = 1 - \sum_1^i p_i^2$$

Where p_i is mean frequency of i^{th} allele.
Shannon's H' index was also estimated using POPGENE ver.1.32 program (Yeh and Boyle 1996):

Table 1. Populations common name, origins/pedigree and botanical class.

Populations common name	Origin/pedigree	Botanical class
Malti	Local landrace	Major
Batata	Local large seeded landrace collected from Boussalem (Tunisia)	Major
Chahbi	Selection from cross S83182-22 / (New Mamoth x Local Tunisian faba bean) – Commercial variety (INRAT)	Major
Super aguadulce	Commercial variety	Major
Aguadulce	Commercial variety	Major
Chemlali	Local landrace	Major
Badï	Selection from Tunisian population – Commercial variety (INRAT)	Minor
Bachaar	Pure line developed from FLIP84-59FB (S82166) – Commercial variety (INRAT)	Minor
Masri	Local small seeded landrace Collected from Boussalem (Tunisia)	Minor

Table 2. Diversity information for SSAP markers.

Rétrotransposon	Sample size	na (observed number of alleles/ locus)	ne (effective number of alleles/ locus)	Number of band		Percentage of polymorphic bands	h (Nei's gene diversity)
				poly	mono		
Tvf4/Pst AA	90	1.53	1.30	*25*	*13*	65.8	0.20
Tps 19/PstAA	90	1.62	1.15	18	11	62	0.11
Tps 19/ PstAT	90	1.76	1.34	26	8	76.5	0.21
PDR1/MseATG	90	1.69	1.16	24	11	68.6	0.12
PDR1/MseATA	90	1.81	1.19	30	7	78.4	0.14
Mean	90	1.68	1.22	123	51	71.10	0.15
S.D		0.455	0.256				0.151

$H' = (-\Sigma \pi i \log 2 \pi i)/L$

Where πi is the frequency of the ith SSAP band in that population and L is the number of loci.
The dendrogram separating populations was constructed using UPGMA method based on matrix distance (Nei, 1978).

RESULTS

Genetic diversity analysis

All primers corresponding to *PDRI*, *Tps19* and *Tvf4* produced high quality and reliability of banding patterns. SSAP analysis of nine faba bean populations provided a total of 173 amplified bands (Table 2). The average amplified bands per primer combination over all populations varied from 29 (*Tps19/* PST AA) to 38 (*Tvf4*/ pst AA). Furthermore, the average number of SSAP products detected per polymorphic primers combinations was 34.6. Primer *PDR1*/MseATA generated the highest number of polymorphic fragments among the primers used. The mean polymorphism for all combined bands was 71% (123/173). The heterozygosity value detected as the observed number of alleles (na), the effective number of alleles (ne) and the gene diversity (*h)* showed values of 1.68, 1.22 and 0.15, respectively.

Genetic structure and distance

Nei's genetic diversity index was estimated for all populations (Table 3). The total genetic diversity (H_T) and

Table 3. Estimated Nei's diversity indexes and gene flow in the 9 populations of *Vicia faba* L.

Rétrotransposon	Sample size	H_S	H_T	D_{ST}	Nm
Tvf4/Pst AA	90	0.699	0.862	0.163	2.14
Tps 19/PstAA	90	0.766	0.894	0.128	3.00
Tps 19/ PstAT	90	0.734	0.890	0.156	2.33
PDR1/MseATG	90	0.773	0.893	0.119	3.24
PDR1/MseATA	90	0.744	0.870	0.125	2.96
Mean	90	0.743	0.882	0.138	2.73

H_S, Within population genetic diversity; H_T, total genetic diversity; D_{ST}, genetic diversity between populations; *Nm*, estimated gene flow = 0.5 (1-GST)/Gst.

Table 4. Shannon's diversity index among populations (H'p) and for all populations (H'pop).

Amorce	H'p									
	Chemlali	Bachar	Massri	Aguad	S,Agua	Malti	batata	Chahbi	Badi	H'pop
Tvf4/Pst AA	0,338	0,273	0,131	0,189	0,294	0,222	0,301	0,212	0,315	0,253
Tps 19/PstAA	0,199	0,134	0,229	0,171	0,104	0,149	0,178	0,148	0,133	0,161
Tps 19/ PstAT	0,256	0,309	0,274	0,282	0,228	0,279	0,356	0,229	0,213	0,270
PDR1/MseATG	0,153	0,167	0,128	0,167	0,143	0,193	0,240	0,168	0,115	0,164
PDR1/MseATA	0,207	0,284	0,272	0,231	0,198	0,170	0,166	0,078	0,058	0,185
All primers	0,231	0,233	0,207	0,208	0,193	0,203	0,248	0,167	0,166	0,207

the intra-population genetic diversity (H_S) were 0.882 and 0.743, respectively. In addition, the within genetic diversity (H_S) was higher than genetic diversity between *V. faba* population (D_{ST}) which was a little increased. Furthermore, the contribution of inter-population genetic diversity (D_{ST}) in the total genetic diversity was 15.6%.

Shannon's index (H') was used to classify the constituents between landraces per primer combinations (Table 4). In fact, the *Tvf4/Pst AA* Shannon's diversity index per populations showed an average of H'pop=0.253 and ranged from 0.131 ('Massri') to 0.338 ('Chemlali'). Also, *Tps 19/PstAA* primer combination showed the lowest Shannon's diversity index for all populations (H'pop= 0.161), ranging from 0.104 ('S.Aguadulce') to 0.229 ('Massri').

Population 'Batata' showed the highest Shannon's diversity index (H'pop= 0.356) with *Tps 19/ PstAT* combination. However, population 'Badi' showed the lowest value (H'p= 0.213). According to PDR1/MseATG combination, Shannon's diversity index per population (H') ranges from 0.115 (population 'Badi') to 0.240 (population 'Batata') with an average of H'pop=0.164. Furthermore, with PDR1*/MseATA* population 'Badi' showed the lowest Shannon's diversity index per population (H'pop= 0.058) and population 'Bachar' showed the highest value (H'p= 0.284).The mean value for all populations was H'pop= 0.185.

Shannon's diversity index (H'pop) for all populations, when primers were taken together, ranged from 0.166 (population 'Badi') to 0.248 (population 'Batata') with an average of 0.207. Nei's genetic distances ranged from 0.009 (between 'Massri' and 'Chemlali') to 0.040 (between 'Batata' and 'Aguadulce'), with an average of 0.024 (Table 5). The resulting dendrogram assigned the different populations into three groups (Figure 1). The local major faba bean landrace 'Batata' was the most distant and clustered separately from the remaining other populations, which were grouped into two main clusters: one cluster included 'Chemlali' and 'Aguadulce' (*V. faba major*), 'Massri' and 'Bachar' (*V. faba minor*). The other cluster included 'Malti', 'Chahbi' and 'Super Aguadulce ' (*major*), and 'Badi' (*minor*).

DISCUSSION

The current study was conducted to assess the pattern of genetic relations among nine faba bean populations based on SSAP markers. In previous studies, DNA based molecular markers have provided new tools for evaluating germplasm and assessing the genetic diversity in faba bean populations (Van de Ven et al., 1993; Link et al., 1995; Zeid et al., 2003; Roma´n et al., 2004; Gresta et al., 2010). Recently, retrotransposon-based molecular markers have been widely used; they were developed based on the features of retro-transposons such as ubiquitous distribution in the higher plant genome, presence in high copy numbers as highly heterogeneous populations, wide dispersion on chro-mosome, and insertional polymorphism both within and between species in plants (Schulman, 2007) in particular in faba bean (Sanz et al., 2007).

Table 5. Nei's genetic distance among 9 faba bean populations.

Parameter	Aguadulce	Malti	Badi	Batata	Chahbi	Chemlali	Massri	Bachar	S -aguad
Aguadulce	0								
Malti	0.034	0							
Badi	0.034	0.025	0						
Batata	0.040	0.025	0.034	0					
Chahbi	0.037	0.020	0.025	0.026	0				
Chemlali	0.019	0.025	0.027	0.032	0.019	0			
Massri	0.019	0.030	0.030	0.038	0.018	0.009	0		
Bachar	0.020	0.026	0.033	0.027	0.021	0.011	0.011	0	
S aguad	0.028	0.016	0.019	0.023	0.020	0.013	0.019	0.021	0

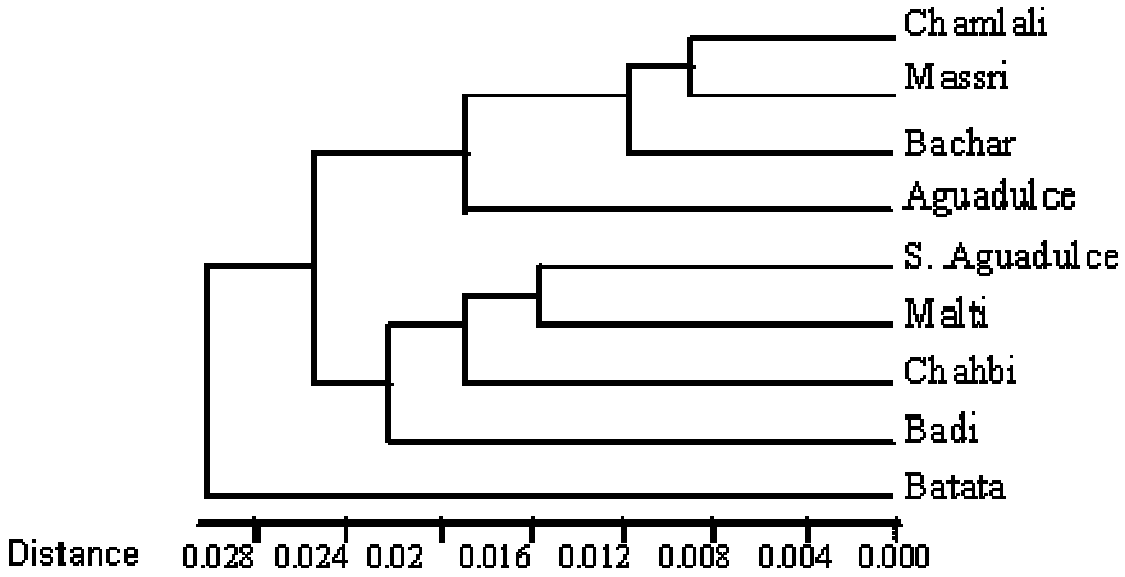

Figure 1. Dendrogram based on Nei's (1978) genetic distance: UPGMA modified method from neighbor procedure of PHYLIP version 3.5.

It has been generally shown that SSAP markers provides more diversity and higher levels of polymorphism than AFLP when a direct comparison was made, (Waugh et al., 1997; Syed et al., 2005). Moreover, with a relatively small number of SSAP primers, it should be possible to generate abundant genetic information with sufficient precision and at reasonable cost analysis. Additionally, in SSAP analysis, because one of the primers is based on specific retrotransposon sequences, utilization of SSAP molecular marker allows for tracking of a retrotransposon insertion event and its subsequent vertical radiation through a pedigree of phylogeny (Kumar and Bennetzen, 1999). We have not tested whether this is also true in *V. faba* or not and the only other published report for *V. faba* (Zeid et al., 2003) cannot be compared to our results, as it reports percentage polymorphic bands across larger numbers of lines (79 vs. 20), that have no overlap with our accessions.

In the present study, it is expected that there is a higher variation with SSAP markers than that revealed by Ouji et al. (2011) with enzyme markers. This is because SSAP often detects much higher genetic diversity than allozyme data. Thus, biochemical (enzyme) markers tend to have the disadvantages of a low degree of polymorphism; relatively few loci, limiting the density of maps which can be produced; and environmentally variable expression, complicating scoring as well as the determination of genotype (Schulman, 2007).

Since faba bean is a largely out-crossing species (Suso et al., 2006), the variation within populations is expected to be higher (Smýkal, 2006). Our result reveals a higher distribution of genetic variation within populations. Indeed, analysis of variance indicated that the source of variation among and within populations accounted for 15.6 and 84.4%, respectively, of the total variation, values in the range of out-crossing species. A similar

result was obtained in the analysis of genetic variation of allogamous forage species. In *Dactylis glomerata*, Kolliker et al. (1999) found 85.1% of variation within cultivars. On the other hand Vieira et al. (2004) reported that 98% of total diversity in annual ryegrasses was intra-populational. Furthermore, results obtained by Terzopoulos and Bebeli (2008) on 20 local Greek faba bean populations using four ISSR primers, suggested that the majority of the observed genetic variability was due to within population variation (75.4%). Later, Gresta et al. (2010), based on AFLP analysis, achieved a considerable level of genetic variation within faba bean accessions of 'Larga di Leonforte' landrace. In this study, V*. faba* populations has a large genetic variability, which should be taken into account when planning conservation strategies or when faba bean variability is used in breeding programs. For these reasons, the molecular diversity analysis should not be used to replace traditional morphological characterization but rather as a complement to it. This information is particularly important in faba bean with the floral biology intermediate between allo- and autogamous (Hanelt and Mettin, 1989). Decreased heterozygosity and heterogeneity of populations will decrease vigor and productivity. Furthermore, the low value of the effective number of alleles per locus for SSAP was reflected in low values of expected heterozygosity as confirmed by Demey et al. (2004).

Most of the genetic variation of faba bean populations was found within populations as it is typical for out crossing species (Nybom and Bartish, 2000). Furthermore, Hamrick and Godt (1989) confirmed that in out-crossing type diversity, within population is larger than among population. Whereas, self-crossing type exhibits intra-population diversity that is lower than that of among population. This is in accordance with our results, suggesting that our studied faba bean populations were predominantly out crossing.

The landrace 'Batata' was the most distant from the other faba bean populations analyzed. The results indicate that this population (showing high protein content in glutelin and prolamin fractions in a previous study (Ouji et al. 2010)) was genetically divergent from the investigated faba bean populations, and therefore a potential source of novel, favourable alleles could be used to improve this species.

Retrotransposon primers in our work were successfully applied to both within and between populations, implying that the corresponding retrotransposons are ubiquitous across faba bean populations and that these primers could be shared between intra- and inter- populations.

The fact that the retrotransposon primers produced many bands and abundant polymorphisms suggested that these elements are extensively heterogeneous with high copy number or that they are clustered in the genome, as have been shown elsewhere (Antonius-Kemola et al., 2006). Furthermore, ubiquitous distribution, abundant copy number, high heterogeneity and insertional polymorphism both within and between plant species make retrotransposons very informative as molecular markers (Kumar and Bennetzen, 1999).

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