Nature of Gene Action and Efficiency of Molecular Markers for Evaluation of Genetic Polymorphism for *Orobanche* Tolerance in Faba Bean (*Vicia faba* L.).

Dora, S. A.[1]; M. M. Rady[2]; A. A. Abou-Shosha[1]; Aziza A. Aboulila[1]* and Shymaa F. A. Kalboush[2].
[1] Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Egypt.
[2] Food Legumes Res. Section, FCRI, ARC, Giza, Egypt.
*Corresponding author; E-mail: aa_aboulila@yahoo.com & aziza.aboulila@agr.kfs.edu.eg

ABSTRACT

The present study was carried out to identify the best cross(es) for *Orobanche* tolerance using genetic variance components and molecular analyses among four parents of faba bean, their six F_1 crosses and six F_2 populations which were produced via half-diallel mating design under *Orobanche* infected soil condition. Significant mean squares were recorded for parents and crosses for the studied traits. The GCA/SCA ratio was higher than unity for all studied traits, except for number of *Orobanche* spikes/plant and 100-seed weight. Highly significant positive and negative correlation coefficients were obtained among almost studied traits. Broad sense heritability ranged from intermediate to high and from low to intermediate for narrow sense. Five RAPD and five SSR primers were used for marker-assisted selection for *Orobanche* tolerance in parents and their F_2 populations. In the case of RAPD primers, three of them showed higher resolving power (RP) rates and were found to be more suitable for genetic diversity analysis in the genotypes under study. In the case of SSR markers, the average value of resolving power was 5.24 per primer. Moreover, primer GA4 was found to be the most suitable for genetic diversity analysis among genotypes under study while, it recorded the highest RP value (7.64). The average values of polymorphic information content were 0.27 and 0.34 per primer based on RAPD and SSR data, respectively. The molecular distance among four faba bean parents and their F_2 bulks ranged from 0.099 to 0.556 by using RAPD and SSR combined data. Positive genotype-specific markers were recorded in some genotypes with molecular sizes ranged from 180 bp to 1584 bp by using both DNA markers. These positive specific markers may play important roles in *Orobanche* tolerance in faba bean.

Keywords: *Orobanche* tolerance, half-diallel, RAPD, SSR, principal coordinate analysis and DNA barcoding.

INTRODUCTION

Faba bean (*Vicia faba* L.) is a diploid species with 2n =12 chromosomes. It is one of the major pulse crops grown in Egypt and many countries. It is used as a protein source in human and animal nutrition (Larralde, 1982). The cultivated area of faba bean was reduced in the last years due to competition with other winter crops. Increase of the cultivated area may not be feasible and hence increasing productivity through developing new high yielding varieties; improving cultural practices and adopting intercropping are very essential.

Broomrape (*Orobanche crenata* Forsek.) is a holoparasitic weed that attacks legume crops such as faba bean and a large number of wild legumes (Cubero and Moreno 1983). Broomrape causes considerable losses in faba bean crop and has become a limiting factor for faba bean production in many regions. These parasitic weeds are difficult to be controlled while they are closely associated with the host root and remain underground for most of their life cycle.

The use of Random amplified polymorphic DNA (RAPD) technique offers a simple, fast, efficient and inexpensive method (Basheer-Salimia *et al.*, 2012). Furthermore, it does not need knowledge of marker sequences and can produce abundant polymorphic DNA fragments (Kocsis *et al.*, 2005 and Achtak *et al.*, 2009). Therefore, RAPD is a powerful and accurate tool for analyzing the genetic relatedness and diversity in many species. Simple sequence repeats (SSRs) are abundant, codominant, markers with great genome coverage (Kalia *et al.*, 2011). SSR analysis has not been used extensively for molecular studies that lack information on DNA sequence because of the high costs of development. Thus, markers from well-studied species can be used in species with no or low amount of available molecular data. So far, DNA markers like

RAPD, ISSRs, AFLPs and SSRs were extensively used in assessing genetic diversity in faba bean (Wang *et al.*, 2012).

This study aimed to estimate the gene action and to assessment the genetic diversity and to identify the molecular markers associated with *Orobanche crenata* tolerance and/or susceptibility in different genotypes of faba bean.

MATERIALS AND METHODS

This study was carried out at the laboratories of Genetics Dept., Fac. of Agric., Kafrelsheikh University and the Experimental Farm of Sakha Agricultural Research Station, Agricultural Research Center (ARC), Egypt during faba bean growing seasons 2013/2014, 2014/2015 and 2015/2016.

Plant material

Four faba bean cultivars were used in this study. Pedigree and reaction to broomrape (*Orobanche sp.*) of the studied cultivars are presented in Table 1. A half diallel mating design was applied for the four faba bean cultivars under free from insects cage during 2013/2014 faba bean growing season.

Table 1. Pedigree and reaction to broomrape (*Orobanche sp.*) of faba bean parental cultivars under study.

Cultivar	Pedigree	Reaction to *Orobanche*
Misr1 (P_1)	Giza 3/123A/45/76	Tolerant
Giza843 (P_2)	461/845/ 83 x 561/2076/85	Tolerant
Sakha2 (P_3)	Rena Balanka x 461/845/83	Susceptible
Nubaria1 (P_4)	Individual plant selection from Rena Blancka.	Susceptible

In 2014/2015 season, the parental cultivars were planted once again under same conditions and crossed to obtain more F_1 hybrid seeds and the F_2 seeds were obtained from the F_1 plants raised under cages. In 2015/2016, four faba bean parents and six F_1 crosses

along with their six F_2 populations were grown in Randomized Complete Block Design (RCBD) with three replicates under heavy natural infected soil with *Orobanche crenata* seeds.

Data were recorded on number of days to 50% flowering (DF), plant height (PH) cm, number of branches per plant (BP), number of pods/plant (PP), number of seeds/plant (SP), seed yield/plant (YP) g, number of *Orobanche* spikes per plant (OS) and 100-seed weight (100-SW) g.

Statistical analysis

Analysis of variance and genetic variance components for all traits were estimated using Griffing (1956) diallel cross analysis designated as method 2 model 1.

Molecular analysis

Genomic DNA isolation

Total genomic DNA was isolated from young leaves (flowering stage) using Cetyl trimethyl ammonium bromide (CTAB)-based procedure for plants (Murray and Thompson, 1980).

RAPD and SSR primers

Five randomly amplified polymorphic DNA (RAPD) primers (OPH-01, OPH-02, OPH-03, OPH-04 and OPH-05) and five simple sequence repeat (SSR) primers (GA4, GAII-8, GAII-30, GAII-59 and JFI-GA3) were used in this investigation to study genetic diversity and to identify markers related to *Orobanche* tolerance in four faba bean parental genotypes, six F_1 crosses and their seven F_2 bulked crosses, while the analyses of molecular data was applied for the parental and F_2 genotypes only. In the case of cross $P_1 \times P_2$, there were two different bulks, the first was resistant $P_1 \times P_2$ (R) and the second was susceptible $P_1 \times P_2$ (S).

Amplification condition

Amplification reactions were performed using 20 μl reaction mixture containing the following; 1 μl of template DNA (40 ng/μl), 1.0 μl of RAPD primer (10 pmol/ μl) and 1.0 μl of each SSR primer (10 pmol/ μl), 10 μl 2X PCR Master mix solution ((i-Taq^{TM}) iNtRON Biotechnology) and 7-8 μl of sterile ddH2O. The reaction mixtures were overlaid with drops of mineral oil per sample. PCR amplification condition was carried out in thermal cycle (Perkin Emer Cetus) programed. The reaction in RAPD analysis was subjected to one cycle at 94 °C for 2 min. (initial denaturation), then 40 cycles of 20 sec. at 94 °C, 30 sec. at 30 °C and 30 sec. at 72 °C, final extention for 5 min at 72 °C (one cycle). PCR amplification condition was carried out in SSR analysis with the following specification. Initial denaturation for 5 min. at 94 °C (one cycle), 40 cycles of 1 min. at 94 °C, 1 min. at 55 °C and 1.5 min. at 72 °C, final extention for 7 min. at 72 °C (one cycle), followed by a final hold at 4 °C.

Separation of amplification products was achieved by horizontal gel electrophoresis unit using 1.5% agarose gel. Electrophoresis was carried out under 70 volts for 15 min., then 90 volts for 90 min. Bands were detected on Benchtop UV-transilliminator and photographed using photo Doc-It TM imaging system. The molecular sizes of the amplified products were determined against 1 Kb DNA ladder with stain (SibEnzyme).

Data analysis

DNA banding patterns generated from RAPD and SSR procedures were analyzed by GelAnalyzer 3 program. Amplification with each RAPD primer was repeated for three times, and consistent bands were selected for data generation. Only consistent and reproducible bands were considered to run the corresponding statistical analysis. DNA polymorphic bands were registered as discrete variables considering "1" presence and "0" absence to construct a binary data matrix. The molecular distances (MD) were estimated using Nei & Li coefficients (Nei and Li, 1979) by computational package MVSP 3.1. Also, depending on this matrix, Cluster analysis and Principal Coordinate (PCo) analysis were performed using the same program. The resulting matrix was analyzed according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

The informational certainly of primers to differences among genotypes was analyzed by means of the estimation of their Resolving Power (RP) and Polymorphic Information Content (PIC). Resolving Power (RP) was calculated according to Prevost and Wilkinson (1999). PIC was calculated using the formula described by Roldan-Ruiz *et al*., (2000). The PIC for each primer was recorded using the average PIC value from all loci of each primer.

RESULTS AND DISCUSSION

Analysis of variance in Tables 2 and 3 showed highly significant differences among genotypes for all traits in both F_1 and F_2 generations. Mean squares of parents and crosses were significant and highly significant for all traits in both F_1 and F_2 generations. While, parents *vs* crosses mean squares were highly significant for days to 50% flowering, pods/plant, seeds/plant, seed yield/plant, *Orobanche* spikes/plant and 100-seed weight in F_1 crosses. Mean squares of parents *vs* crosses showed highly significance for days to flowering, plant height, seed yield/plant, *Orobanche* spikes per plant and 100-seed weight in F_2 generations. This indicates the superiority of crosses over parents. The results indicated wide variability for all materials under study.

Data in Tables 2 and 3 showed significant and highly significant mean squares due to GCA and SCA for all studied characteristics in the two generations, except that due to SCA for pods/plant in F_1 crosses and 100-seed weight in F_2 generations which were insignificant. SCA mean squares for plant height scored a significant value in F_1.

The ratio of GCA/SCA variance as an indication of the relative importance of the two types of gene action was more than unity for number of days to 50% flowering, branches/plant, pods/plant, seeds/plant and seed yield/plant in both generations. This indicates that the additive genetic effect was more important and played the major role in the inheritance of the mentioned traits. While, the lower GCA/SCA ratio than unity for plant height and *Orobanche* spikes/plant in F_2 could be

encouraging the heterozygosity in producing hybrids. Similar results were obtained by Darwish *et al.*, (2005) who found that GCA/SCA ratio exceeded unity for flowering date, maturity date and 100-seed weight. GCA/SCA ratio revealed the preponderance of additive gene action for number of pods, number of seeds, seed yield and 100-seed weight. Abdel Sattar and El-

Mouhamady (2012) found that significant mean squares were detected for genotypes, general and specific combining ability effects for the studied traits, except number of branches/plant indicating that additive gene action was more important than non-additive gene action in the inheritance of these traits.

Table 2. Mean square estimates of combining ability analysis in F_1 crosses for all studied traits.

S.O.V	df	DF	PH	BP	PP	SP	YP (g)	OS	100-SW (g)
Replications	2	112.9	2926.8	5.4	17.8	194.04	106.4	8.8	3.3
Genotypes	9	342.8**	2079.6**	6.44**	86.9**	728.5**	470.6**	5.0**	346.1**
Parents (p)	3	677.41**	4268.33**	11.40**	195.6**	1642.20**	1035.75**	7.71**	231.36*
Crosses (c)	5	200.22**	1182.25*	4.61**	33.48**	305.41**	221.42**	4.34**	574.78*
P vs C. (h)	1	52.27**	0.02	0.71	27.43**	102.73**	20.73**	0.43	1046.80**
Error	18	1.72	193.3	0.45	0.53	10.18	15.68	0.18	52.0
GCA	3	302.0**	1796.4**	6.1**	70.6**	627.7**	398.4**	4.0**	97.1**
SCA	6	20.4**	141.6*	0.17	8.1**	50.4**	36.1**	0.5**	124.5**
GCA/SCA		2.53	3.74	49.58	1.48	2.21	2.12	1.49	0.12
Error	18	0.57	64.45	0.15	0.17	3.39	5.23	0.06	17.33

* and ** indicated significant and highly significant at 0.05 and 0.01 level of probability, respectively. DF= Days to 50% flowering, PH= plant height, BP= Branches/plant, PP= Pods/plant, SP= Seeds/plant, YP= Seed yield/plant, OS= *Orobanche* spikes/plant and 100-SW= 100-seed weight (seed index).

Table 3. Mean square estimates of combining ability analysis in F_2 generations for all studied traits.

S.O.V	df	DF	PH	BP	PP	SP	YP (g)	OS	100-SW (g)
Replications	2	36.2	268.0	0.97	8.44	2.17	4.49	5.76	27.16
Genotypes	9	421.8**	1628.7**	5.24**	77.59**	707.13**	451.26**	5.28**	153.9*
Parents (p)	3	677.41**	4268.33**	11.40**	195.6**	1642.20**	1035.75**	7.71**	231.36*
Crosses (c)	5	242.5**	198.81**	2.54**	21.63**	287.49**	187.20**	2.30**	122.47*
P vs C. (h)	1	551.25**	859.463**	0.26	3.293	0.11	18.085**	12.887**	78.811**
Error	18	12.9	41.45	0.09	0.53	4.89	2.07	0.39	48.74
GCA	3	322.7**	1007.1**	4.69**	71.28**	686.37**	441.2**	2.24**	17.66**
SCA	6	49.5**	310.8**	0.28**	3.16**	10.38**	5.03**	1.52**	18.13
GCA/SCA		1.17	0.56	3.11	3.98	13.04	16.92	0.25	0.13
Error	18	4.3	13.82	0.03	0.18	1.63	0.69	0.13	16.25

* and ** indicated significant and highly significant at 0.05 and 0.01 level of probability, respectively. DF= Days to 50% flowering, PH= plant height, BP= Branches/plant, PP= Pods/plant, SP= Seeds/plant, YP= Seed yield/plant, OS= *Orobanche* spikes/plant and 100-SW= 100-seed weight (seed index).

Genetic variance components and heritability

Genetic variance components were calculated for the studied traits and results are found in Tables 4 and 5. It was clear that the non-additive genetic variance ($\sigma^2 D$) was lower than the additive genetic variance ($\sigma^2 A$) for the studied characters in both generations, except for plant height (F_2), *Orobanche* spikes/plant (F_2) and 100-seed weight in both generations, indicating that non-additive gene action played a major role in the inheritance of these traits, F_1 hybrids could be produced to utilize obtained heterosis ($\sigma^2 D$ was higher than $\sigma^2 A$). While, selection would be effective in improving days to flowering, branches/plant, pods/plant, seeds/plant and yield/plant because ($\sigma^2 A$ was higher than $\sigma^2 D$). Obiadalla-Ali *et al.*, (2013) showed that the magnitude of additive genetic variance ($\sigma^2 A$) which was positive and lower than those of non-additive ($\sigma^2 D$) for the studied traits.

Heritability in both broad and narrow senses was estimated for all studied traits. The results in Tables 4 and 5 indicated also that heritability estimates in broad sense (H^2 %) were larger than their corresponding values of narrow sense heritability (h^2 %) for the studied traits in both generations. Heritability in broad sense ranged from 89.57% for 100-seed weight to 99.41% for days to flowering in F_1 crosses and from 58.11% for 100-seed weight to 99.41% for seed yield/plant in F_2 generations. Whereas, narrow sense heritability values ranged from 14.61% for 100-seed weight to 82.66% for branches per plant in F_1 crosses and from 11.38% for 100-seed weight to 95.07% for seed yield/plant in F_2 generations. The findings of this investigation agree with those reported by Sharifi (2015) and Soliman (2016).

Table 4. Genetic variance components and heritability of studied traits for F_1 crosses.

Parameter	DF	PH	BP	PP	SP	YP (g)	OS	100-SW (g)
$\sigma^2 e$	0.57	64.45	0.15	0.17	3.39	5.23	0.06	17.33
$\sigma^2 A$	75.50	449.10	1.53	17.65	156.93	99.60	1.00	24.28
$\sigma^2 D$	20.40	141.60	0.17	8.10	50.40	36.10	0.50	124.50
$\sigma^2 G$	95.90	590.70	1.70	25.75	207.33	135.70	1.50	148.78
$\sigma^2 PH$	96.47	655.15	1.85	25.92	210.72	140.93	1.56	166.11
h^2	78.26	68.55	82.66	68.09	74.47	70.67	64.10	14.61
H^2	99.41	90.16	91.87	99.34	98.39	96.29	96.15	89.57

DF= Days to 50% flowering, PH= plant height, BP= Branches/plant, PP= Pods/plant, SP= Seeds/plant, YP= Seed yield/plant, OS= *Orobanche* spikes/plant and 100-SW= 100-seed weight (seed index).

Table 5. Genetic variance components and heritability of studied traits for F_2 generations.

Parameter	DF	PH	BP	PP	SP	YP (g)	OS	100-SW (g)
σ^2 e	4.30	13.82	0.03	0.18	1.63	0.69	0.13	16.25
σ^2 A	80.68	251.78	1.17	17.82	171.59	110.30	0.56	4.42
σ^2 D	49.50	310.80	0.28	3.16	10.38	5.03	1.52	18.13
σ^2 G	130.18	562.58	1.45	20.98	181.97	115.33	2.08	22.55
σ^2 PH	134.48	576.40	1.48	21.16	183.60	116.02	2.21	38.80
h^2	59.99	43.68	79.09	84.22	93.46	95.07	25.34	11.38
H^2	96.80	97.60	97.98	99.15	99.11	99.41	94.12	58.11

DF= Days to 50% flowering, PH= plant height, BP= Branches/plant, PP= Pods/plant, SP= Seeds/plant, YP= Seed yield/plant, OS= *Orobanche* spikes/plant and 100-SW= 100-seed weight (seed index).

Correlation coefficients among the studied traits

Correlation among plant growth and yield traits was showed in Table 6 and the results showed that days to 50% flowering was highly significant negative correlated with plant height, branches/plant, pods/plant, seeds/plant, seed yield/plant and 100-seed weight. Plant height was highly significant positive correlated with branches/plant, pods/plant, seeds/plant, seed yield per plant and 100-seed weight. While, it was significant positive correlated with *Orobanche* spikes/plant. Number of branches/plant was highly significant positive correlated with pods/plant, seeds/plant, seed yield/plant,

Orobanche spikes/plant and 100-seed weight. Pods/plant was highly significantly positively correlated with both seeds/plant and seed yield/plant and significantly positively correlated with 100-seed weight. Seeds/plant was highly significant positive correlated with seed yield/plant and 100-seed weight. Seed yield/plant was highly significant positive correlated with 100-seed weight (r=0.487). Number of *Orobanche* spikes/plant was significant positive correlated with 100-seed weight (r=0.266). These results are in agreement with those obtained by Kalboush (2013) and Abdalla *et al.*, (2015).

Table 6. Correlation coefficients among studied traits of faba bean genotypes from the combined data.

	DF	PH	BP	PP	SP	YP (g)	OS	100-SW (g)
DF	1							
PH	-0.4**	1						
BP	-0.604**	0.841**	1					
PP	-0.677**	0.810**	0.814**	1				
SP	-0.697**	0.827**	0.858**	0.974**	1			
YP (g)	-0.721**	0.818**	0.884**	0.953**	0.983**	1		
OS	-0.157	0.292*	0.467**	0.097	0.160	0.187	1	
100-SW (g)	-0.382**	0.337**	0.538**	0.292*	0.345**	0.487**	0.266*	1

* and ** indicated significant and highly significant at 0.05 and 0.01 level of probability, respectively. DF= Days to 50% flowering, PH= plant height, BP= Branches/plant, PP= Pods/plant, SP= Seeds/plant, YP= Seed yield/plant, OS= *Orobanche* spikes/plant and 100-SW= 100-seed weight (seed index).

Molecular diversity assessment
RAPD markers analysis

Five RAPD primers were used to study the genetic differences and relationships among the four faba bean cultivars and their seven bulks of F_2 generations Table 7 and Figure 1. A total of 54 amplified fragments (loci) with sizes ranged from 113 bp to 1647 bp were obtained with an average of 10.8 loci per primer. Out of them 44 (81.48%) were polymorphic. Total number of polymorphic DNA fragments ranged from four (OPH-05) to 13 (OPH-01). While, the polymorphism percentage ranged from 50% (OPH-05) to 100% (OPH-02).

The primers OPH-01, OPH-03 and OPH-05 showed higher resolving power (RP) rates (18.18, 13.27 and 11.09, respectively). Therefore, they were found to be more suitable RAPD primers to assess the genetic diversity in the faba bean genotypes under study. The PIC values ranged from 0.19 to 0.31 with an average of 0.27, which indicated the presence of high genetic variability among the studied genotypes. Basheer-Salimia *et al.*, (2013) exhibited that 94 DNA fragments (loci) were detected by using 11 RAPD markers with an average of 8.54 loci for each primer and sizes ranged from 160 to 1370 bp and the estimated resolving power (RP) value was 26.316.

Table 7. Number and types of the amplified DNA fragments as well as the polymorphism percentage generated by the five RAPD primers.

Primer	Sequence 5'→3'	Molecular size range (bp)	Monomorphic	Polymorphic bands without genotype-specific markers	(+ or –) specific markers	Total	P (%)	RP	PIC
OPH-01	GGTCGGAGAA	215-1647	2	9	4	15	73.30	18.18	0.29
OPH-02	TCGGACGTGA	194-995	0	6	5	11	100.00	10.91	0.31
OPH-03	AGACGTCCAC	333-1584	3	6	2	11	72.73	13.27	0.26
OPH-04	GGAAGTCGCC	280-1343	1	5	3	9	88.89	8.91	0.30
OPH-05	AGTCGTCCCC	113-758	4	4	0	8	50.0	11.09	0.19
Total			10	44	54		81.48	62.36	1.35
Average			2	8.8	10.8			12.47	0.27

P (%): polymorphism percentage, RP: resolving power and PIC: polymorphic information content.
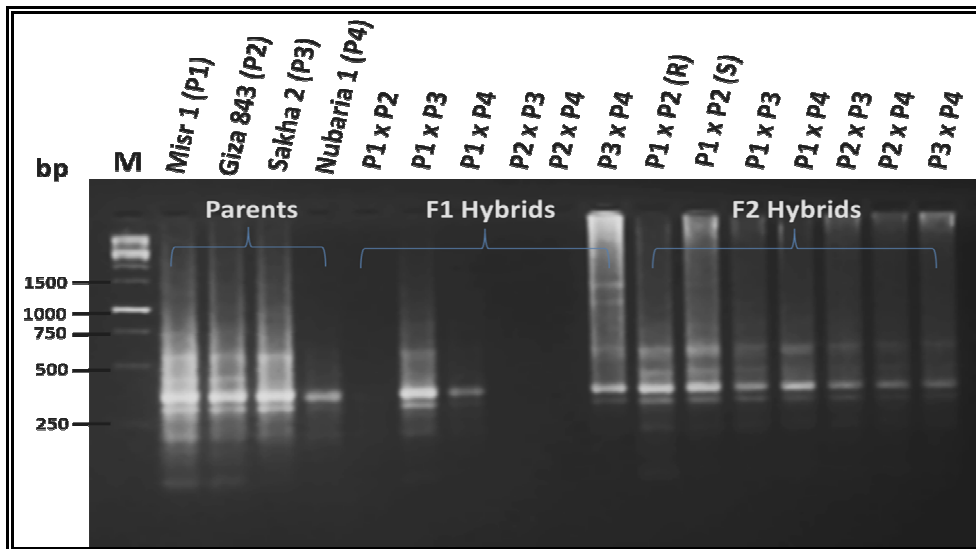
Figure 1. RAPD fingerprint for the four parents, six F$_1$ crosses and the seven bulks of F$_2$ generations using OPH-05 RAPD marker.

SSR markers analysis

Genetic differences and relationships among the four faba bean parents and their seven bulks of F$_2$ based on five SSR primers are shown in Table 8 and Figure 2. A total of 26 DNA fragments (loci) were obtained with an average of 5.21 loci for each primer. Out of them 22 (84.62 %) were polymorphic. Number of polymorphic bands ranged from two (GAII-30 and GAII-59) to seven (JFI-AG3). While, the polymorphism percentage ranged from 66.67 % (GAII-59) to 100 % (GAII-30 and JF1-AG3). Also, the expected DNA fragments with different sizes for the five SSR primers were detected in some of the used parental and hybrid genotypes.

The values of RP ranged from 2.73 to 7.64 with an average of 5.24 per primer, so that primer GA4 was proved to be the most useful SSR primer to evaluate genetic diversity among genotypes under study which presented the highest RP value. The PIC values ranged from 0.28 for primer GA4 to 0.43 for primer GAII-30 with an average of 0.34 which indicated the presence of genetic variability among genotypes under study. Yahia *et al.*, (2014) used SSR and RAPD markers to evaluate the genetic diversity of 13 Tunisian faba bean genotypes and showed that the polymorphic fragments percentages were 100 and 60.63% for SSR and RAPD markers, respectively. Also, PIC test values were 0.370 and 0.319 for SSR and RAPD primers, respectively.
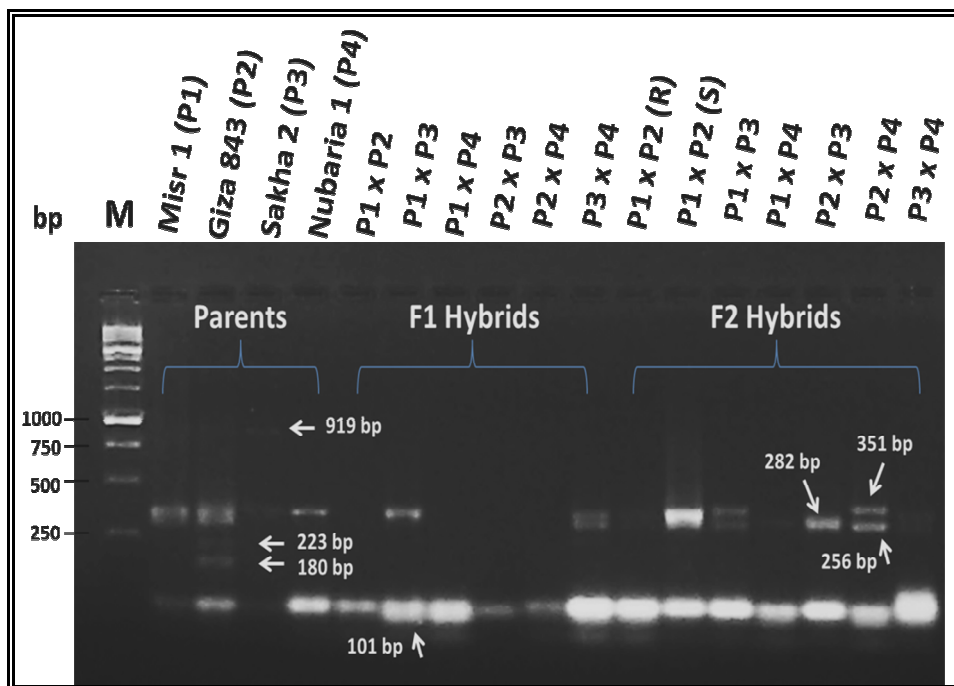

Figure 2. SSR fingerprint for the four parents, six F$_1$ crosses and the seven bulks of F$_2$ generations using GAII-8 SSR marker.

Table 8. Numbers and types of the amplified DNA bands as well as the polymorphism percentage generated by the five SSR primers.

Primer name	Primer sequence (5'→ 3')	Expected size (bp)	Amplified Bands		P (%)	RP	PIC
			TB	PB			
GA4	F: GAACTAAGGTGTACACGCGGG R: GGGGGGTAGTCTTGTTTTTTCC	232	7	5	71.43	7.64	0.28
GAII-8	F: GTTATTATTATGTACGCGCGTGC R: GAATAAGCAGAAACGCGACGT	351	7	6	85.71	5.64	0.29
GAII-30	F: GGAAAATATGATGAAAAAGCCGC R: GAGTCGATATCACGTCGGAGG	281	2	2	100	2.73	0.43
GAII-59	F: GTAATGTGGCCCAATCCAATT R: GTGAATTGTTGAAGATGGATGAA	250	3	2	66.67	4.0	0.33
JFI-AG3	F: ATGCTGAGGATGCAGGATCGA R: TAATTTGTTGGTCTCAGTGC	350	7	7	100	6.18	0.35
Total			26	22	84.62	26.19	1.68
Average			5.2	4.40		5.24	0.34

TB: Total amplified bands, PB: polymorphic bands, P (%): polymorphism percentage, RP: resolving power and PIC: polymorphic information content.

Molecular distance (MD), cluster analysis and principal coordinate analysis (PCoA) based on RAPD and SSR combined data

Molecular distance (MD) on the basis of RAPD and SSR combined data among the four faba bean genotypes and their seven F_2 bulks ranged from 0.099 to 0.556. The highest MD value (0.556) was scored between Nubaria1 and the bulk of cross P_3xP_4 as shown in Table 9, which indicates that the two genotypes are distantly related. While, the lowest MD value (0.099) was found between the two tolerant parents Misr1 (P_1) and Giza843 (P_2), indicating that there is high similarity between both genotypes which might be introduced and produced from the same region and parents. Tahir (2015) found that genetic distance coefficients between the genotypes ranged from 0.400 to 0.725. Basheer-Salimia *et al*., (2013) revealed that genetic distance based on Jaccard coefficient ranged from 0.358 to 0.069, with a mean of 0.213.

Table 9. Nei molecular distance between Pairwise faba bean parents and F_2 generations based on combined data.

Genotype	Misr1 (P_1)	Giza843 (P_2)	Sakha2 (P_3)	Nubaria1 (P_4)	P_1xP_2 (R)	P_1xP_2 (S)	P_1xP_3	P_1xP_4	$P_2x P_3$	$P_2x P_4$	P_3xP_4
Misr1 (P_1)	0										
Giza843 (P_2)	0.099	0									
Sakha2 (P_3)	0.186	0.195	0								
Nubaria1(P_4)	0.406	0.415	0.467	0							
P_1 x P_2 (R)	0.341	0.371	0.405	0.452	0						
P_1 x P_2 (S)	0.226	0.271	0.294	0.525	0.308	0					
P_1 x P_3	0.269	0.255	0.281	0.463	0.231	0.174	0				
P_1 x P_4	0.325	0.309	0.342	0.407	0.308	0.354	0.229	0			
P_2 x P_3	0.265	0.293	0.319	0.528	0.250	0.263	0.208	0.250	0		
P_2 x P_4	0.318	0.303	0.286	0.419	0.302	0.231	0.143	0.154	0.229	0	
P_3 x P_4	0.265	0.293	0.319	0.556	0.229	0.193	0.188	0.273	0.170	0.208	0

The dendrogram showed two main clusters based on RAPD and SSR combined data as shown in Figure 3. Nubaria1 was separated alone in the first cluster at MD=0.464. The second cluster separated into two sub-clusters at MD=0.302. The first sub-cluster contained three parental genotypes (Misr1, Giza843 and Sakha2), also Misr1 and Giza843 were located at the same molecular distance as shown in the dendrogram. P_1 x P_2 (R) was separated from sub-cluster two at MD=0.271. These results are confirmed with those obtained by Salazar-Laureles *et al*., (2015) who observed six defined groups according to UPGMA analysis.
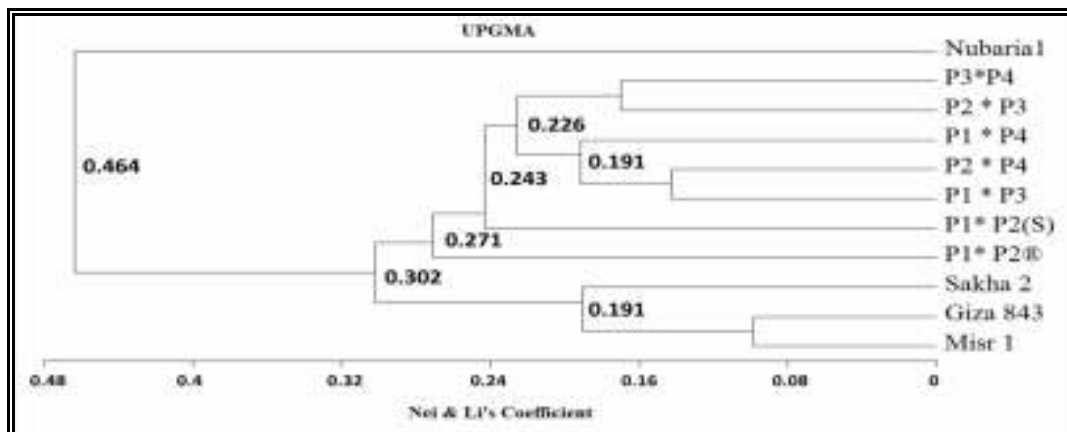

Figure 3. Dendrogram from the UPGMA grouping analysis, using Nei & Lei coefficient based on RAPD and SSR combined data in 11 genotypes of faba bean.

The combined RAPD and SSR data were used to perform the principal coordinate analysis (PCoA) based on molecular distance for all the eleven genotypes as shown in Figure 4. The PCoA explained (45.62 %) and (14.49 %) of total variation for axis 1 and axis 2, respectively. In this analysis, three genotypes; Misr1, Giza 843 and Sakha2 were observed in one group of PCoA. It was similar to that obtained by UPGMA clustering. Nubaria1 and the sensitive bulk cross of $P_1xP_2(S)$ were observed in a group of PCoA. While, Nubaria1 was found in a separate cluster of UPGMA

dendrogram at MD=0.464. Also, Sakha2 was separated from the sub-cluster of UPGMA dendrogram at MD=0.191. The rest genotypes were found in one group of PCoA. This result was also similar to that obtained by UPGMA clustering. All genotypes were grouped in a cluster in UPGMA dendrogram at MD=0.302. Therefore, it can be suggested that both UPGMA and PCoA should be performed for genetic diversity analysis (Yadav *et al.*, 2012 and Aboulila, 2016). These results are confirmed with those concluded by Salazar-Laureles *et al.*, (2015) and Sallam and Martsch (2016).
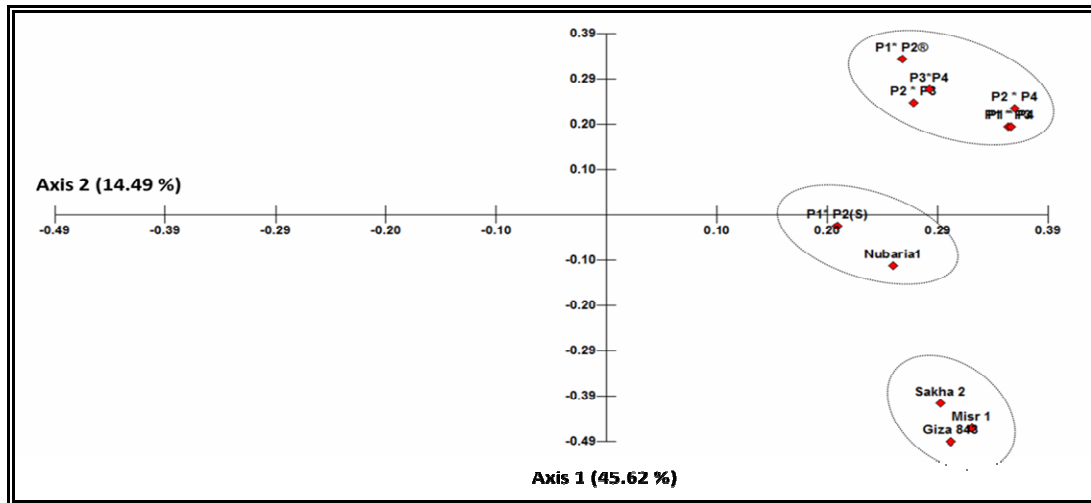

Figure 4. Principal coordinate analysis (PCoA) of the 11 genotypes of faba bean produced by RAPD and SSR combined data.

DNA barcoding and genotype-specific marker

DNA barcoding for data that was obtained from Table 7 and Figure 1 using five RAPD primers as shown in Figure 5. For parental genotypes, Nubaria1 revealed the lowest number of fragments (14). While, the genotype Misr1 showed the highest number of fragments (31). In the case of F_2 generations, cross P_1 x P_4 showed the lowest number of fragments (29) among

the F_2 generations. While, the susceptible bulk of cross Misr1 x Giza843 [(P_1 xP_2 (S)] revealed the highest number of fragments in all genotypes (41). All genotypes gave a total of 343 DNA fragments with an average of 31.18 fragments for each genotype. Data in Table 10 showed that the total number of unique fragments was 14.
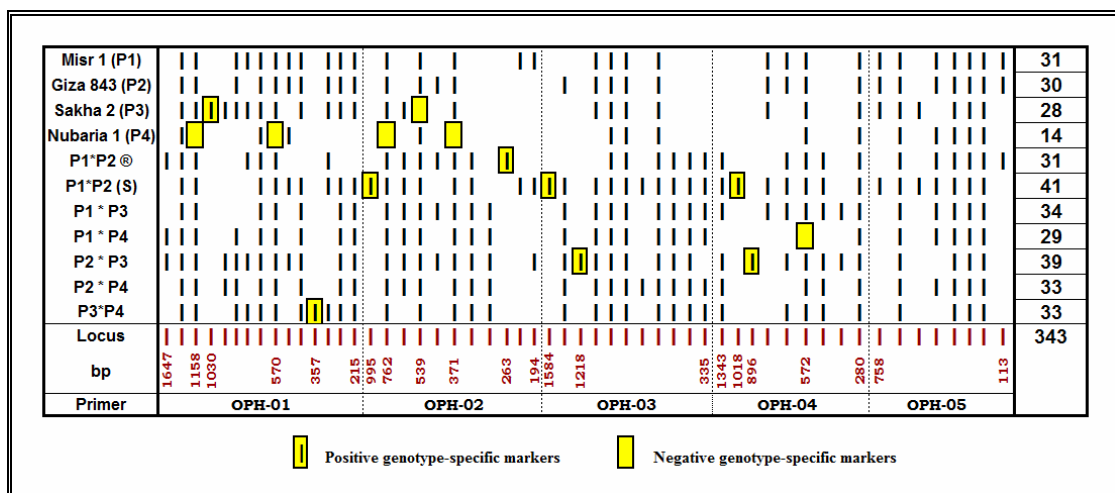

Figure 5. DNA barcoding for 11 genotypes of faba bean with the amplified fragments by using five RAPD primers.

Presence of a new unique band for a genotype is considered as positive marker, while the absence of a normal band is referred as negative marker. These bands

could be used as genotype specific markers. In respect to the positive genotype-specific markers, the highest number (3) was recorded by the genotype P_1 x P_2(S) with molecular sizes of 995 bp, 1018 bp and 1584 bp.

Genotype P_2 x P_3 exhibited two positive genotype-specific markers with sizes of 896 bp and 1218 bp. There were three different genotypes which showed only one positive genotype-specific marker, Sakha2 (1030 bp), P_1xP_2 (R) (263 bp) and P_3 x P_4 (357 bp). For the negative genotype-specific markers as shown in Figure 5 and Table 10. The genotype Nubaria1 exhibited the highest number of bands (4) with molecular sizes of 371 bp, 570 bp, 762 bp and 1158 bp. On the other hand, only one negative genotype-specific marker was appeared with each of genotypes Sakha2 (539 bp) and P_1 x P_4 (572 bp).

No unique bands were found in genotypes Misr1, Giza 843, P_1xP_3 and P_2 x P_4. The highest RAPD specific markers number was generated by the primer OPH-02 (2 positive and 3 negative specific markers) as in Table 10. These results are agreed with those obtained by Tahir (2015) who found that out of 75 polymorphic bands, a total of 7 unique bands (4 positive and 3 negative) were registered, and could be deemed for marker assisted selection.

Table 10. Faba bean genotypes characterized by positive and negative genotype-specific markers and their molecular sizes (bp) using RAPD and SSR analysis.

Genotypes	Genotype-specific markers using RAPD marker			Genotype-specific markers using SSR marker		
	Positive	Negative	Total	Positive	Negative	Total
Giza843 (P_2)	---	---	-	180 & 223 bp (GAII-8)	---	2
Sakha2 (P_3)	1030 bp (OPH-01)	539 bp (OPH-02)	2	---	---	-
Nubaria1 (P_4)	--- --- --- ---	570 bp (OPH-01) 1158 bp (OPH-01) 371 bp (OPH-02) 762 bp (OPH-02)	4	---	100 bp (JF1-AG3)	1
P_1 x P_2 (R)	263 bp (OPH-02)	---	1	---	---	-
P_1 x P_2 (S)	995 bp (OPH-02) 1584 bp (OPH-03) 1018 bp (OPH-04)	--- --- ---	3	512 bp (GA4)	---	1
P_1 x P_4	---	572 bp (OPH-04)	1	---	---	-
P_2 x P_3	1218 bp (OPH-03) 896 bp (OPH-04)	--- ---	2	---	---	-
P_3 x P_4	357 bp (OPH-01)	---	1	---	---	-
Total	8	6	14	3	1	4

DNA barcoding based on data of SSR is shown in Figure 6 and Table 10. For the parental genotypes, Nubaria1 revealed the lowest number of fragments (5), while, the genotype Giza843 showed the highest number of fragments (16). For F_2 bulk crosses, the cross P_1x P_4 exhibited the lowest number of fragments (6). On the other side, the susceptible bulk of cross P_1xP_2 (S) and P_3xP_4 revealed the highest number of fragments on all genotypes (parents and genotypes) with 20 fragments. All genotypes gave a total of 144 fragments with an average of 13.09 fragments per genotype.

From data summarized in Table 10, the genotypes Giza843 and P_1xP_2(S) showed three unique fragments (180 bp and 223 bp for Giza843 and 512 bp for the susceptible bulk of P_1xP_2 as positive markers, while the parental genotype Nubaria1 recorded only one unique fragment (100 bp) as a negative marker. These results are similar to that concluded by Tahir (2015).
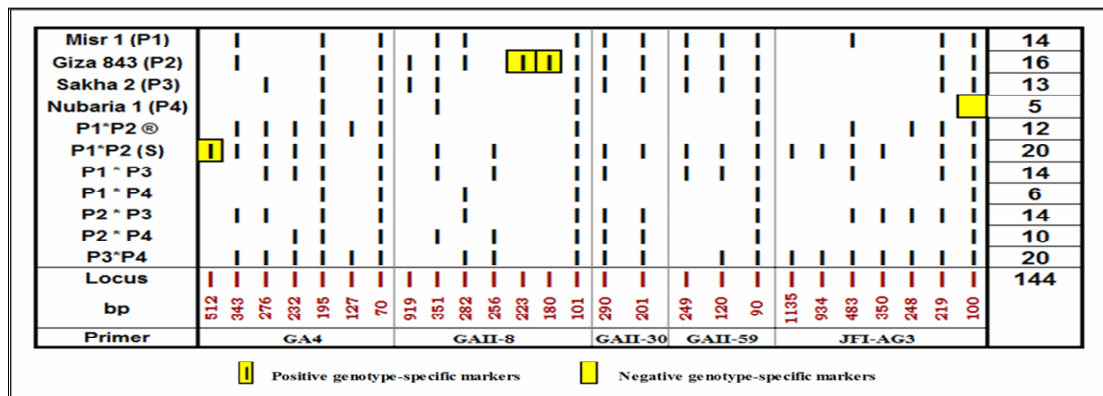

Figure 6. DNA barcoding for 11 genotypes of faba bean with the amplified fragments by using five SSR markers.

CONCLUSION

Based on results obtained from the present study it was clear that the non-additive genetic variance (σ^2D) was lower than the additive genetic variance (σ^2A) for the studied traits in both generations, except for plant height (F_2), *Orobanche* spikes/plant (F_2) and 100-seed weight in both generations, this indicates that non-additive gene action played a major role in the inheritance of these traits, F_1 hybrids could be produced to utilize obtained heterosis (σ^2D higher than σ^2A). Also, number of *Orobanche* spikes/plant was positively significant correlated with 100-seed weight (r=0.266). All RAPD and SSR primers used in this study are recommended to examine the genetic variability among the studied faba bean genotypes.

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طبيعة الفعل الجيني وكفاءه المعلمات الجزيئيه في تقييم التنوع الوراثي لتحمل الهالوك في الفول البلدي
سعيد عبدالسلام دره [1]، مصطفي محمود راضي [2]، علي أحمد أبوشوشه [1]، عزيزه أحمد أبوليله [1] و شيماء فرج كلبوش [2]
[1] قسم الوراثه ــ كليه الزراعه ــ جامعه كفرالشيخ ــ مصر.
[2] قسم بحوث البقوليات ــ معهد المحاصيل الحقليهــ مركز البحوث الزراعيه ــ الجيزه ــ مصر.

أجريت هذه الدراسة بهدف معرفة أفضل الهجن المتحملة للإصابة بالهالوك باستخدام مكونات التباين الوراثي والتحليل الجزيئي بواسطة تكنيكات الوراثه الجزيئيه بين أربعة أصناف من الفول البلدي استخدمت كآباء في التهجين اثنان متحملين للإصابة بالهالوك (مصر1، جيزة 843) واثنان حساسين للإصابة بالهالوك (سخا2 و نوباريه1) وستة هجن في الجيل الأول وستة هجن في الجيل الثاني بنظام تزاوج النصف داياليل (الغير شامل على الهجن العكسية) لثمان صفات وهي (عدد الأيام حتى 50% تزهير، ارتفاع النبات، عدد الأفرع للنبات، عدد القرون للنبات، عدد البذور للنبات، محصول البذور للنبات بالجرام، عدد شماريخ الهالوك للنبات ووزن المائة بذرة بالجرام). أظهر كل من متوسط مجموع مربعات التراكيب الوراثية (الآباء والهجن) تأثيرات معنوية لجميع الصفات المدروسة. في حين كانت النسبة بين تباين القدرة العامة والقدرة الخاصة على الائتلاف أعلى من الوحدة لكل الصفات فيما عدا صفتي عدد شماريخ الهالوك للنبات ووزن المائة بذرة بالجرام. كان معامل الارتباط عالي المعنوية (موجب أو سالب) بين معظم الصفات المدروسة. وتراوحت قيم المكافئ الوراثي في المدى الواسع بين المتوسط الى المرتفع في حين تراوحت قيم المكافئ الوراثي في المدى الضيق من منخفض لمتوسط لجميع الصفات المدروسة في كل من الجيلين الأول والثاني. تم استخدام خمسه بوادئ عشوائيه وخمسه بوادئ متخصصه في تقييم التنوع الجزيئي بين التراكيب المدروسه من خلال تكنيكات الـ RAPD والـ SSR والتي أظهرت تباين في التنوع الوراثي بمعدل 81٫48 ، 84٫62 % علي التوالي. تراوحت المسافة الوراثية بين أربع آباء من الفول البلدي و مجموعات الجيل الثاني من 0٫099 إلى 0٫556 باستخدام بيانات RAPD و SSR معاً. أوضحت البوادئ المستخدمه فعاليه في التفرقه بين التراكيب الوراثيه المدروسه حيث تم تحديد معلمات جزيئية متخصصه موجبة تراوحت بين 180 - 1584 زوج من القواعد لبعض التراكيب الوراثيه والتي قد تستخدم كدلائل لصفة تحمل الهالوك في الفول البلدي. وبالتالي يمكن استخدام المعلمات الجزيئية في تربية الفول البلدي لتحمل الهالوك عن طريق الإنتخاب.