Molecular Markers for *Orobanche Crenata* Resistance in Faba Bean (*Vicia Faba* L.) Using Bulked Segregant Analysis (BSA)

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Abstract: The present study aimed to discovery the molecular markers for *Orobanche* tolerant and susceptible in faba bean by using Bulked Segregant Analysis (BSA) using RAPD and ISSR techniques. A cross between x-1722 (tolerant) and Giza-40 (susceptible) was chosen for molecular analysis**.** DNA isolated from the two parents; F_1 and the two extreme groups of F_2 plants using bulked Segregant analysis (BSA) technique. Six RAPD primers (A09, B05, B11, B17, B18 and B20) and four ISSR primers (HB11, HB12, HB13 and 17899B) were used in this study. The results showed that**,** Primer B20 released four positive molecular markers with molecular size of 766, 557,419 and 281 bp**),** these four positive and three negative RAPD markers could be considered as a reliable markers for *Orobanche* tolerance in faba bean. While there were three negative molecular with molecular sizes 949, 355 bp for primer A09 and 441bp for primer B18**.** On the other hand, Primer B05 exhibited unique band for tolerant parent (x-1722) with molecular size 400 bp, as well as primer B11 showed three unique bands with molecular size 1278, 773 and 437 bp. While primer B17 exhibited unique band for sensitive parent (Giza 40) with molecular size 421bp**.** For ISSR primers, Primer HB12 showed one negative molecular marker which was found only in the sensitive parent (Giza 40), F_1 and the sensitive F_2 bulk with molecular size of 880bp. Only one unique band for sensitive parent (Giza 40) released in primer HB12 with molecular size 106.
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1. Introduction

Faba bean (*Vicia faba* L.) is one of the main pulse crops grown for seed in Egypt. It is widely considered as a good source of protein, starch, cellulose and minerals for humans in developing countries and for animals in industrialized countries (Haciseferogullari et al., 2003). In addition, faba bean is one of the most efficient fixers of the atmospheric nitrogen and, hence, can contribute to sustain or enhance total soil nitrogen fertility through biotical N2-fixation (Lindemann and Glover, 2003).

Broomrape (Orobanche sp. Orobanchaceae) is a parasitic plant that attacks and causes yield loss of many important dicotyledonous crops throughout the world (Parker, 1986), by removing carbohydrates and water (Schaffer et al., 1991). Various measures for Orobanche control have been tried including improved cultural practices, use of chemicals (Zahran et al., 1992, and Nassib et al., 1992) and use of high levels of nitrogen fertilization (Nassib and Hussein 1985). However, cultivar resistance remains the most effective and economic control means. Resistant genotypes can grow satisfactorily and yield well in infested fields thus alleviating the hazards of Orobanche attacks. Breeding efforts have been employed for combining genes for adaptability and high yield from elite faba bean genotypes with those for tolerance to Orobanche (Cubero, 1973; Nassib et al., 1979 and El-Deeb et al., 1999).

In faba bean breeding programs, the development of cultivars resistant to O. crenata is a key component and has also become a major research objective. Resistance against most parasitic weeds is, however, difficult to assess, scarce, of complex nature and of low heritability, making breeding for resistance a difficult task (Rubiales 2003). Not much resistance to O. crenata was available in faba bean until the appearance of the Egyptian line F402 (Nassib et al. 1982) that allowed the development of several resistant cultivars (Giza 402, Giza 429, Giza 674, Giza 843, Misr-1 and Misr-3) that are being utilized in crosses to breed for Orobanche resistance (Khalil et al. 2004).

For acceleration of breeding program for development of Orobanche resistant genotypes should be using of molecular approaches, which help to detection of molecular markers related to resistance or susceptible genotypes and subsequent by decrease of the period for development of new Orobanche resistance cultivar in faba bean.

Bulked Segregant Analysis (BSA), can be used to identify markers linked to a gene of interest. This process is highly efficient because it detect only a small percentage of polymorphisms in F2 individual progeny tests. The use of BSA in combination with PCR-based molecular markers has proven to be a very powerful technique for identifying markers tightly linked to or

cosegregating with, genes underlying monogenic traits [Agrama and Moussa (1996) ;Cho et al., (1996) ;Nakamura et al., (2001) ; Rostoks et al. (2002) and Shen et al., (2003)].

On the other hand, randomly amplified polymorphic DNA sequences or RAPD markers are based on the amplification of unknown DNA sequences using single, short, and random oligonucleotide primers. Gutierrez and Kuti (2001) used RAPD markers to differentiate between 10 faba bean genotypes showed resistance and susceptibility to Orobanche crenata. They observed distinct polymorphic DNA bands corresponding to either resistance or susceptibility in 15 tested primers. Genetic characterization and relationships among accessions, lines and cultivars of faba bean based on RAPD molecular markers were reported by Crespo et al., (1995); Potokina et al., (1999); Patto et al., (1999); Wolko et al., (2000) and Weder (2002).

Inter-Simple Sequence Repeats (ISSRs) are a new type of DNA markers, which involve the use of microsatallite sequences directly for DNA amplifications using PCR. ISSR-PCR amplification use a single primer composed of a microsatallite sequence anchored at the 3′ or 5′ end by 2-4 arbitrary nucleotides (Fang and Roose, 1997). This technique enables amplification of genomic DNA and provides information about many loci simultaneously. Due to the technical difficulties in developing SSRs which are species-specific, tedious in their development and are not commonly used for gene tagging in plants, ISSR markers are universal, easy, repeatable useful to develop gene tagging and can be used for finding markers linked to the gene of interest. ISSR has been proposed as a new source of genetic markers which overcomes the technical limitations of RFLP and RAPD (Ratnaparkhe et al., 1998).

The ISSR-directed approach in combination with bulked segregant analysis (BSA) showed a wide application in plant and animal genome mapping. It can be extremely useful in: (1) identifying the markers at clusters of disease resistance gene, (2) filling large gaps in linkage maps, (3) developing the sequence-tagged microsatallite sites and (4) providing marker enrichment at desired regions. Román et al. (2002) used Inter Simple Sequence Repeat markers to assay the variation among and within populations of the parasitic weed Orobanche crenata from Spain and Israel. Fahmy et al. (2006) used ISSRs-PCR technique to get molecular markers for blast resistance. Fifteen primers were used to obtain ISSRs markers for blast disease, only 12 succeeded in the DNA amplification. As a general conclusion, ISSRs are considered good molecular markers for blast disease study, especially when comparing parents, F1 and F2 individual plants.

The aim of this investigation is trail to discovery the molecular markers for Orobanche tolerant and susceptible in faba bean by using Bulked Segregant Analysis (BSA) using RAPD and ISSR techniques.

2. Materials and Methods

The present study was carried out under insect free cages at Gemmeiza Agricultural Research Station, ARC, EGYPT during three successive winter seasons 2008/2009 , 2009/2010 and 2010/2011. For obtaining of F_1 and F_2 seeds and growing of them and their parents [x-1722 (resistance) and Giza-40 (susceptible)] under infested field at season 2010/2011. The pedigree and the important characteristics of these genotypes are given in **Table1 1**.

Table 1: The pedigree and some important characteristics of two faba bean parental genotypes.

Genotype	Pedigree	Characteristics
x-1722	L.667 X (C. 241 X G.461) FCRI	Medium seeded type Tolerant to *Orobanche*
Giza 40	Individual selected plant from Rebaya 40 FCRI	Small seeded type Susceptible to *Orobanche* early maturity Planting zone : Upper Egypt and New vally

Bulk Segregant analysis (BSA):-

A cross between x-1722 (tolerant) and Giza-40 (susceptible) was chosen for molecular analysis, fresh leaves were collected from each plant (parents, F_1 and F_2) at flowering stage. The samples were recorded to determine any molecular marker associated with resistance to *orobanche* in faba bean. DNA isolated from the two parents; F_1 and the two extreme groups of F_2 plants using bulked Segregant analysis (BSA) technique Michelmore *et al.* (1991).

In this study, RAPD-PCR technique, according to the method of Michelmore et al. (1991) was used for the identification of markers associated with the most susceptible and the most resistant faba bean genotypes for Orobanche crenata.

PCR reaction was conducted using six arbitrary 10-mer primers. Their names and sequences are shown in **Table (2)**.

Table (2): List of RAPD primers (Operon Technology USA).

No.	Primer	Sequence (5′ to 3′)
1	OP-A09	5′ GGGTAACGCC 3′
2	OP-B05	5′ TGCGCCCTTC 3′
3	OP-B11	5′ GTAGACCCGT 3′
4	OP-B17	5′ AGGGAACGAG 3′
5	OP-B18	5′ CCACAGCAGT 3′
6	OP-B20	5′ GGACCCTTAC 3′

An alternative method to SSRs, called inter-SSRs (ISSRs)-PCR, according to the method of

Ratnaparkhe *et al.* (1998) has also been used with faba bean genotypes to get molecular markers for *Orobanche crenata* resistance. Four primers were used in this study. **Table (3)** shows names of these primers and their sequences.

Table (3): List of ISSR primers (Operon Technology USA)

No.	Primer	Sequence (5′ to 3′)
1	HB11	5′-GTG TGT GTG TGT CC-3′
2	HB12	5′-CAC CAC CAC GC-3′
3	HB13	5′-GAG GAG GAG GC-3′
4	17899B	5′- CAC ACA CAC ACA GG -3′

3. Results and Discussion

1. Responses of the F_2 plants:-

F_2 plants of (x-1722 "*Orobanche* tolerant" and Giza 40 " *Orobanche* sensitive") presented by 200 individuals were classified into two groups according to their behavior under *Orobanche* infestation. The first group refers to the best growing F_2 plants and the last group refers to the worst ones under *Orobanche* infestation. The F_2 plants were arranged in descending order according to their frequency, so plants with high frequency in group one were chosen as the most tolerant F_2 plants. While the plants in the last group were taken in maturity stage to represent the most sensitive F_2 plants.

According to these classifications, ten F_2 plants were taken to represent the most tolerant to *Orobanche* infestation for each trait as shown in **(Table 4)**, while the most ten sensitive F_2 plants were death in maturity stage as an effect of *Orobanche* infestation and obtaining of leaf at early stage.

These twenty plants were used for Bulked Segregant Analysis to obtain molecular ($RAPD_S$ and $ISSR_S$) markers linked with *Orobanche* tolerance.

2. Molecular genetic markers for *Orobanche* tolerance:-

2.1. RAPD molecular markers:-

DNA isolated from the two contrasting parents, x-1722 as an *Orobanche* tolerant parent and Giza 40 as an *Orobanche* sensitive parent, their subsequent F_1 and DNA bulks of the tolerant and sensitive groups of F_2 segregating population were tested against six preselected primers.

All primers gave polymorphisms with the studied genotypes, while three primers developed molecular markers for *Orobanche* sensitive and tolerance as shown in **(Table 5)**.

Primer B20 showed four positive molecular markers which were found only in the tolerant parent (x-1722), F_1 and the tolerant F_2 bulk with molecular size of 766, 557,419 and 281 bp **(Fig. 6)**, while their were three negative molecular markers which were found only in the sensitive parent (Giza 40), F_1 and the sensitive F_2 bulk with molecular sizes 949 , 355 bp for primer A09 and 441bp for primer B18 **(Fig.1 and 5, respectively)**.These four positive and three negative RAPD markers could be considered as a reliable markers for *Orobanche* tolerance in faba bean. One monomorphic marker released in each primer except primer B18 with molecular size 216,284,516,604 and 92 bp for primers A09, B05, B11, B17 and B20, respectively. **(Figures 1,2,3,4 and 6, respectively)**.

On the other hand, Primer B05 exhibited unique band for tolerant parent (x-1722) with molecular size 400 bp, as well as primer B11 showed three unique bands with molecular size 1278, 773 and 437 bp. While primer B17 exhibited unique band for sensitive parent (Giza 40) with molecular size 421bp **(Table 5).**

Table (4): The most tolerant F_2 (x-1722 X Giza 40) plants according to *Orobanche* tolerance related traits (i.e. Plant height (cm.), First pod height (cm.), No. of branches/ plant, No. of pods / plant, No. of seeds / plant, seed yield (g) and 100-seed weight (g)).

No.	Plant height (cm.)	First pod height (cm.)	No. of branches/ plant	No. of pods / plant	No. of seeds / plant	seed yield (g)	100-seed weight (g)
1	95	10	7	22	66	33.0	50.00
2	100	15	6	10	27	18.7	69.26
3	95	15	5	8	26	20.6	79.23
4	75	15	5	5	12	3.9	32.5
5	75	10	4	13	34	19.5	57.4
6	75	20	5	17	43	19.00	44.2
7	75	15	3	11	35	17.2	49.1
8	75	5	4	14	47	22.3	47.4
9	100	15	7	33	103	51.9	50.4
10	75	15	5	2	4	1.4	35.00

Table 5: RAPD-PCR polymorphic bands of eight primers linked to heat and sensitive tolerance with the two parents, their subsequent F_1 and two bulks of F_2.

Primer name	PBN	M.S (bp)	SP	TP	F_1	Tb	Sb	MT
A09	2	949	1	0	0	0	1	N
	8	355	1	0	0	0	1	N
B05	1	1018	1	1	0	1	1	-
	4	711	0	1	0	1	1	-
	6	530	0	0	0	1	1	-
	7	400	0	1	0	0	0	-
	8	360	1	1	0	1	1	-
B11	1	1396	0	0	1	0	0	-
	1	1371	0	0	0	0	1	-
	1	1278	0	1	0	0	0	-
	1	1111	1	0	0	1	0	-
	4	773	0	1	0	0	0	-
	7	437	0	1	0	0	0	-
	8	359	0	0	0	0	1	-
	9	256	1	1	0	0	1	-
B17	6	535	0	1	0	1	1	-
	7	421	1	0	0	0	0	-
	8	371	1	0	1	1	1	-
	8	321	0	0	0	1	1	-
	9	280	1	0	1	1	1	-
B18	3	808	1	1	1	0	1	-
	4	771	1	0	0	0	0	-
	7	441	1	0	1	0	1	N
	9	265	1	0	1	1	1	-
	10	177	0	0	1	0	0	-
	10	124	0	0	1	0	0	-
B20	1	1295	0	0	0	1	0	-
	1	1019	0	0	0	1	0	-
	4	766	0	1	1	1	0	P
	5	693	0	0	0	1	0	-
	6	557	0	1	1	1	0	P
	7	419	0	1	1	1	0	P
	9	281	0	1	1	1	0	P
	10	171	0	0	1	0	0	-

PBN: polymorphic band number; Tb: tolerant bulk; P: positive; SP: sensitive parent; Sb: sensitive bulk; N: negative; TP: tolerant parent; MT: marker type; F_1: F_1 generation

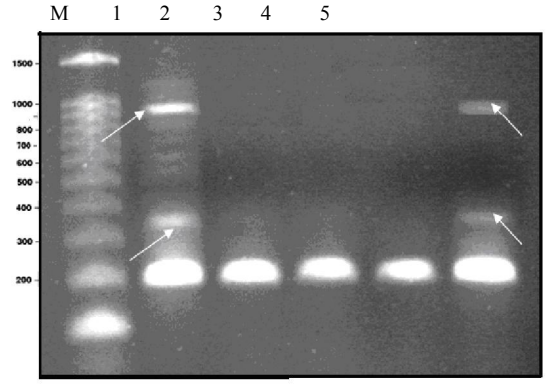

Fig. 1: RAPD-PCR banding patterns of faba bean genotypes under infested field using primer A09. Lane M 1-kb molecular-size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

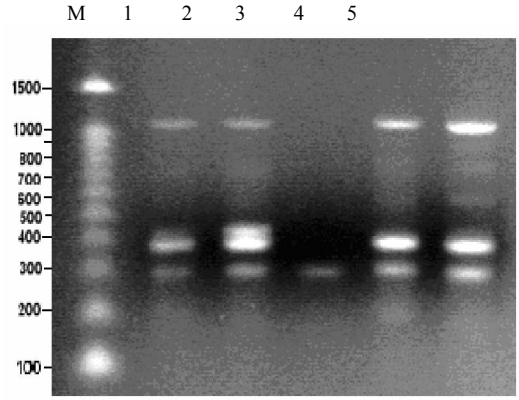

Fig. 2: RAPD-PCR banding patterns of faba bean genotypes under infested field using primer B05. Lane M 1-kb molecular-size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

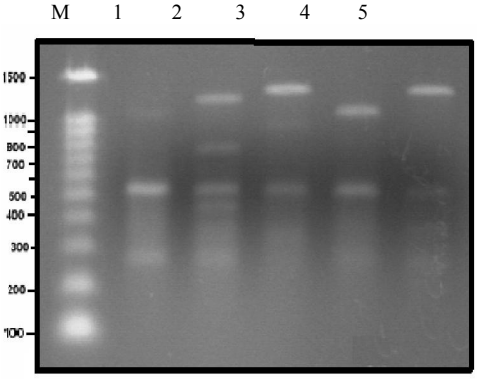

Fig. 3: RAPD-PCR banding patterns of faba bean genotypes under infested field using primer B11. Lane M 1-kb molecular-size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

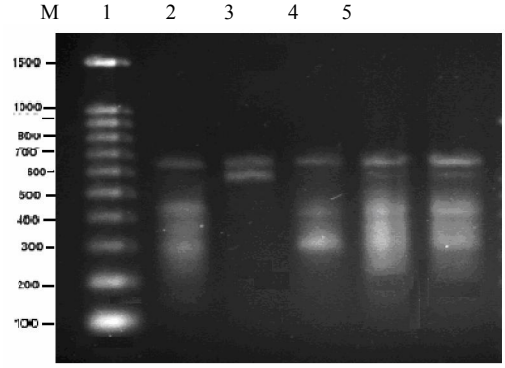

Fig. 4: RAPD-PCR banding patterns of faba bean genotypes under infested field using primer B17. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

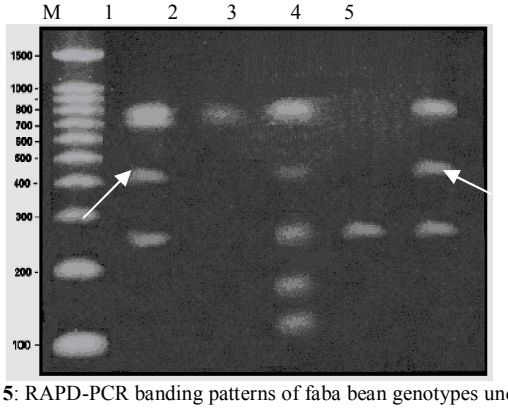

Fig. 5: RAPD-PCR banding patterns of faba bean genotypes under infested field using primer B18. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

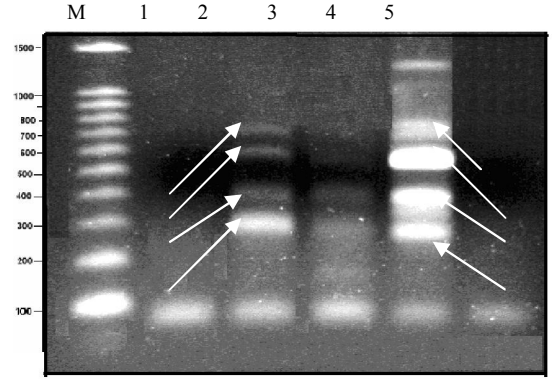

Fig. 6: RAPD-PCR banding patterns of faba bean genotypes under infested field using primer B20. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

2.2. Inter simple sequence repeats (ISSRs) analysis:-

DNA isolated from the two contrasting parents, x-1722 as an *Orobanche* tolerant parent and Giza 40 as an *Orobanche* sensitive parent, their subsequent F_1 and DNA bulks of the tolerant and sensitive groups of F_2 segregating population were tested against four preselected primers.

All primers gave polymorphisms among the studied genotypes except primer 17899B released only one monomorphic marker (Fig. 10). Primer HB12 showed one negative molecular marker which was found only in the sensitive parent (Giza 40), F_1 and the sensitive F_2 bulk with molecular size of 880bp (Fig. 8). Four unique bands for tolerant parent (x-1722) were found, one of them was in primer HB13 with molecular size 1043bp, and another one released in primer HB12 with molecular size 900 bp, while two unique bands were found in primer HB11 with molecular sizes 559 and 288 bp. Only one unique band for sensitive parent (Giza 40) released in primer HB12 with molecular size 106 (Table 6).

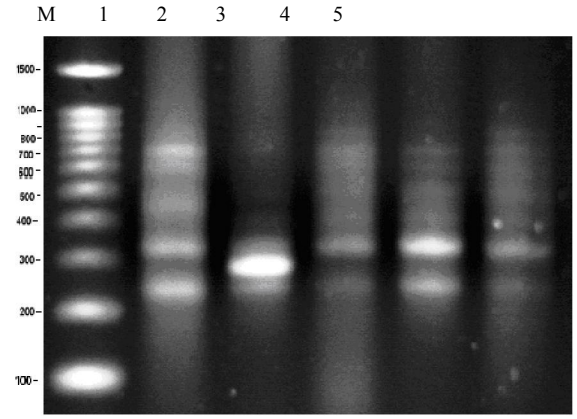

Fig. 7: ISSR-PCR banding patterns of faba bean genotypes under infested field using primer HB11. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

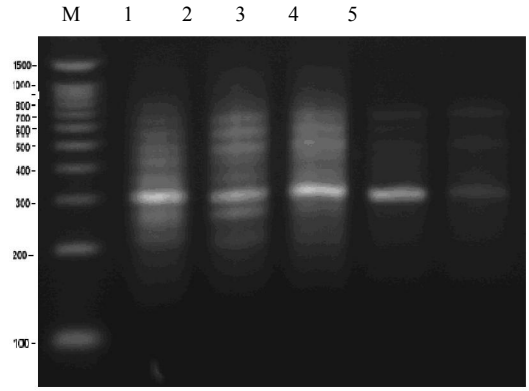

Fig. 8: ISSR-PCR banding patterns of faba bean genotypes under infested field using primer HB13. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bul

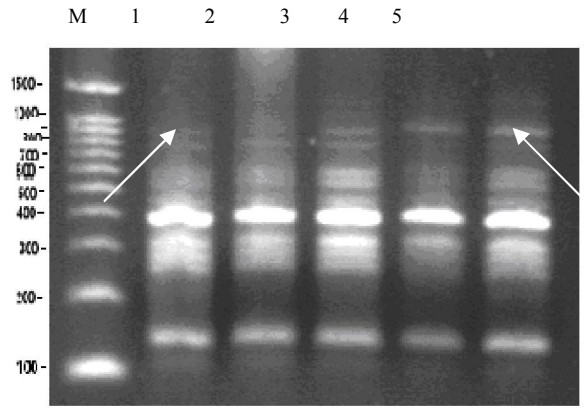

Fig. 9: ISSR-PCR banding patterns of faba bean genotypes under infested field using primer HB12. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

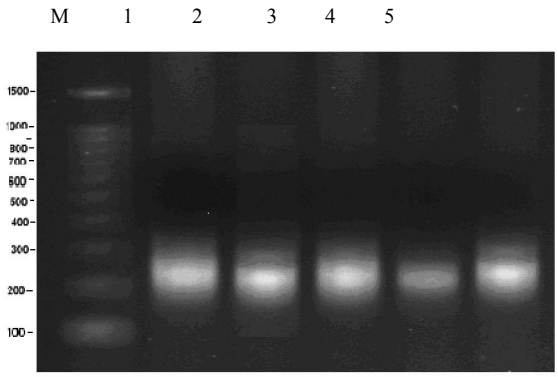

Fig. 10: ISSR-PCR banding patterns of faba bean genotypes under infested field using primer 17899B. Lane M 1-kb molecular- size ladder, 1) sensitive parent (Giza-40), 2) tolerant parent (x-1722), 3) F_1, 4) tolerant F_2 bulk, 5) sensitive F_2 bulk.

These results confirmed the possibility for breeding of new Egyptian lines and hybrids possess high tolerance and yielding in Egyptian cultivars will be acceleration of breeding program for development of new lines and subsequently new hybrids having more tolerance to *Orobanche* and high seed yield.

From this study we can concluded that only four RAPD markers were linked to *Orobanche* tolerance. Thus, BSA allowed us to directly target the gene, as demonstrated by **Michelmore *et al.*,** (1991).

Table 6: ISSR -PCR polymorphic bands of eight primers linked to heat and sensitive tolerance with the two parents, their subsequent F_1 and two bulks of F_2.

Primer name	PBN	M.S (bp)	SP	TP	F_1	Tb	Sb	MT
HB11	2	932	0	0	1	0	1	-
	4	786	0	0	1	0	0	-
	5	639	1	1	0	1	1	-
	6	559	0	1	0	0	0	-
	7	417	0	0	1	1	0	-
	7	411	0	0	0	0	1	-
	9	288	0	1	0	0	0	-
HB12	2	911	0	0	0	1	0	-
	2	900	0	1	0	0	0	-
	3	880	1	0	1	0	1	N
	4	755	1	1	1	0	1	-
	7	430	1	0	1	1	1	-
	9	274	1	1	1	0	1	-
	9	251	0	1	1	0	1	-
	10	106	1	0	0	0	0	-
	10	105	0	1	1	0	0	-
HB13	1	1209	0	1	1	0	0	-
	1	1043	0	1	0	0	0	-
	3	810	1	1	1	0	1	-
	4	700	0	0	1	1	0	-
	5	631	1	1	1	1	0	-
	7	484	1	1	1	0	0	-
	7	433	1	1	1	1	0	-
	8	361	1	1	0	0	1	-
	9	256	1	1	1	0	0	-
	9	218	1	1	1	0	0	-

PBN: polymorphic band number Tb: tolerant bulk P: positive
SP: sensitive parent Sb: sensitive bulk N: negative
TP: tolerant parent MT: marker type F_1: F_1 generation

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