

# MOLECULAR GENETIC MARKERS FOR ROOT-KNOT NEMATODE RESISTANCE IN SOME PEACH ROOTSTOCKS

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**P**each (*Prunus persica* (L.) Batsch) is a member of the Rosaceae, which contain many important fruit, nut and ornamental species. Peach is a diploid plant with chromosome haploid number ( $n=8$ ), and has a comparatively small genome;  $5.9 \times 10^8$  bp or 0.61 pg/diploid nucleus, with a haploid size of 300 Mb (Baird *et al.*, 1994). Rootstocks (The below-ground portions of fruit tree) play a major role in modern orchards. The most important agricultural traits of the tree as a biotic unit, such as vigor, blossom initiation, fruit set, fruit size and fruit flavor, etc., may be, substantially, influenced by the rootstock (Dozier *et al.*, 1984). The root-knot nematodes *Meloidogyne* spp. especially (*Meloidogyne incognita* and *M. javanica*) cause severe damage to several species of *Prunus*, damage can seriously affect early stages of plant development in the nursery or when rootstocks are transplanted into the field, also the extent of growth reduction caused by these pathogens has been documented for many *Prunus* rootstocks used for peach and nectarine varieties (Pinochet *et al.*, 1997). Some cultivars or selections, such as "Nemaguard", "Nemared", and "Okinawa" etc., showed various levels of resistance (Lu *et al.*, 1996; Scorza and Sherman,

1996). Genetic studies of resistance to root-knot nematodes show that inheritance patterns vary from simple to complex. Lu *et al.* (1996) proposed a two-gene model for resistance to root-knot nematodes. Genetic analysis indicates that resistance to *M. incognita* and *M. javanica* are controlled by two dominant genes (*Mi* or *Mij*; and *Mj* or *Mij* respectively), where the shared gene (*Mij*) may be required for resistance to both species. Because it is very difficult to observe morphological traits of rootstocks after grafting, so that DNA markers greatly facilitate rootstock identification. Molecular markers are interesting to plant geneticists and breeders as a source of new genetic information on plant genomes and for use in trait selection. Randomly Amplified Polymorphic DNA analysis (RAPD) has tremendous potential for use in cultivar identification; it has been used to study genetic relationships in peach varieties (Chaparro *et al.*, 1994; Warburton and Bliss, 1996) and in peach rootstocks (Lu *et al.*, 1996; Casas *et al.*, 1999). RAPD markers have been used in peach genetics and breeding programs (Dirlewanger and Bodo, 1994; Rajapakse *et al.*, 1995). Microsatellite or Simple Sequence Repeats (SSRs) are the best available choice of markers for peach genetics

and breeding, they are generally codominant, highly polymorphic, and can be found in large numbers covering the whole genome of any species (Sosinski *et al.*, 2000). Many SSRs have been developed in peach (Testolin *et al.*, 2000; Aranzana *et al.*, 2002; Dirlewanger *et al.*, 2002). The objectives of this study were to:

1. Characterize these used rootstocks through RAPD and SSR-PCR techniques.
2. Find some molecular genetic markers (RAPD and SSR) of these used rootstocks to nematode resistance.

## MATERIALS AND METHODS

### 1. Plant material

This study included six peach rootstocks (*P. persica* (L.) Batsch)  $2n = 16$ , which were supplied by Deciduous Fruits Trees Department, Horticulture Research Institute (HRI), ARC, Giza, Egypt. Code numbers, rootstocks names, chromosome number and their relative resistance to nematodes are shown in (Table 1). These rootstocks were propagated by seeds, and their seedlings were planted at the orchard of HRI in Ali-Moubarak village, South Eltahir, El-Behera Governorate.

### 2. Genomic DNA extraction

Young and fresh leaf samples were collected separately from 10 seedlings for each peach rootstock genotype then bulked DNA extraction was performed using DNeasy Plant Mini Kit (QIAGEN).

DNA concentration was quantified spectrophotometrically (Gene Quant, Amersham Pharmacia Biotech) and DNA quality was examined by electrophoresis in 0.8% agarose.

## 3. Randomly amplified polymorphic DNA (RAPD-PCR)

### 3.1. DNA amplification

RAPD-PCR reactions were conducted using 31 arbitrary 10-mer primers. Their codes and sequences are shown in (Table 2). Amplification reactions were performed in 30  $\mu$ l total volumes according to Williams *et al.* (1990). The amplification procedures were carried out in a DNA thermocycler (MWG-BIO TECH Primuse) programmed as follows: initial pre-denaturation step at 94°C for 5 min, followed by 45 cycles of 1 min at 94°C, 90 sec 36°C and 2 min at 72°C followed by 7 min incubation period at 72°C. The amplification products were stored at 4°C before analysis.

### 3.2. DNA electrophoresis

The amplified products were separated in 1.2% agarose gel electrophoresis using 1 x Tris-Boric acid-EDTA buffer, (Ethidium bromide, (5  $\mu$ l) was added to the melted gel after the temperature became 55°C).

## 4. Simple Sequence Repeats (SSRs)

### 4.1. DNA amplification

RAPD-PCR reactions were conducted using two primers pairs according to Wang *et al.* (2002). Their codes and

sequences are shown in (Table 3). The amplification procedures were carried out in a DNA thermocycler (MWG-BIO TECH Primuse) programmed as follows: initial pre-denaturation step at 94°C for 5 min, followed by 45 cycles of 30 sec at 94°C, 45 sec 62°C and 2 min at 72°C followed by 7 min incubation period at 72°C. The amplification products were stored at 4°C before analysis.

#### **4.2. DNA electrophoresis**

The amplified products were separated in 2.5% agarose gel electrophoresis using 1x Tris-Boric acid-EDTA buffer, (Ethidium bromide (5 µl) was added to the melted gel after the temperature became 55°C.)

## **RESULTS AND DISCUSSION**

### ***Molecular genetic markers related to root-knot nematode***

#### **1. RAPD markers**

Thirty one arbitrary 10-mer random primers were used to obtain some molecular genetic markers for root-knot nematode resistance, out of these used thirty one random primers only twelve gave twenty molecular markers related to root-knot nematode as grouped in Table (4) and (Figs. 1 & 2).

Primer OP-A01 (Fig. 1A) indicated the appearance of three negative molecular markers for root-knot nematode resistance, at molecular sizes 750, 330 and 250 bp in the three susceptible rootstocks Hegazy, Shami and Sultani. At the same

time, primer OP-A11 (Fig. 1C) showed three negative markers at 745, 516 and 255 bp, which were present only in the susceptible rootstocks, while they were absent in the resistant ones. Another three negative RAPD markers at 970, 865 and 755 bp were obtained using primer OP-C12 (Fig. 1E), which were absent in the resistant rootstocks, while were present in the three susceptible rootstocks. Moreover, primers OP-B08 (Fig. 1D), OP-C19 (Fig. 2A), OP-L13 (Fig. 2C) and OP-O05 (Fig. 2E) showed one negative molecular marker for each primer at 1100, 670, 650 and 1270 bp, respectively, which disappeared only in resistant rootstocks; Okinawa, Nemaguard and Nemared. However, some primers exhibited one positive RAPD marker for each of primers OP-A09 (Fig. 1B), OP-C13 (Fig. 1F), OP-L12 (Fig. 2B), OP-L16 (Fig. 2D) and OP-O10 (Fig. 2F) at 1765, 750, 760, 1290 and 1050 bp, respectively. These fragments could be considered as positive molecular markers for the resistant rootstocks; Okinawa, Nemaguard and Nemared. At the same time, primer OP-O05 (Fig. 2E) exhibited two fragments at 1750 and 650 bp in the resistant rootstocks Okinawa, Nemaguard and Nemared which could be considered as positive RAPD markers for nematode resistance.

Previous review confirmed that Okinawa, Nemaguard and Nemared are nematode resistant rootstocks (Malo, 1967; Ramming and Tanner, 1983). While Hegazy, Shami and Sultani are considered nematode susceptible (Abdel-Aziz *et al.*, 1985). Di Vito *et al.* (2002) indicated that

resistance of *Prunus* to root-knot nematodes (*Meloidogyne* spp.) is controlled by several different genes confirming previous findings (Lecouls *et al.*, 1997; Pinochet, 1997). On the other hand, these positive and negative molecular markers using RAPD-PCR analysis must be confirmed when F<sub>2</sub> and F<sub>3</sub> segregants obtained through bulked segregant analysis technique which is beyond, the scope of this study.

## 2. SSRs molecular markers

The results of the two primer pairs Pchgms 26-1 and Pchgms 26-2 for SSRs technique were used to get molecular markers for root-knot nematode resistance. Primer Pchgms 26-1 (Fig. 3A) exhibited three fragments at molecular sizes of 310, 165, 130 bp. The fragment at 310 bp was present in all rootstocks except Nemaguard rootstock (resistant), while the fragment at 165 bp was present in the two resistant rootstocks Okinawa and Nemaguard, therefore this fragment may be considered as a SSR molecular marker for nematode resistance (appeared in two resistant rootstocks out of three). However, primer pairs Pchgms 26-2 exhibited two fragments only at 360 and 336 bp (Fig. 3B) which did not show any relation with root-knot nematode resistance. These results agreed with the findings of Wang *et al.* (2002) who found that most SSRs are not specifically linked to gene loci of immediate interest and developing an SSR map is very time consuming and expensive. Either these results were in agreement with Testolin *et al.* (2000) who

gave some examples to the utility of SSRs for pedigree determination of several peach cultivars. Aranzana *et al.*, (2003) reported that the available SSRs on the *Prunus* saturated map allowing them to develop a resource useful for map comparison or MAS in fruit crops. Georgi *et al.* (2002) and Ahmed *et al.* (2004) reported that SSR molecular markers could be used to identify and characterize peach cultivars.

## Genetic similarity based on RAPD markers

Results of similarity index among the six peach rootstock cultivars based on RAPD-PCR with the twelve primers using UPGMA computer analysis is shown in (Table 5). The highest similarity value recorded was (0.930), which was observed between Shami and Sultani rootstocks, while the lowest similarity value recorded was (0.818) between Okinawa and Sultani rootstocks. A dendrogram for the genetic relationships among the six peach rootstocks across the twelve primers results was carried out and is shown in (Fig. 4). The six peach rootstocks were separated into two clusters; cluster 1 included Hegazy, Shami, and Sultani, while cluster 2 comprised Okinawa, Nemared and Nemaguard. Within cluster 1, two subclusters appeared; one comprised Hegazy and Shami rootstocks, while the second subcluster contained Sultani only. Cluster 2 was also divided into two subclusters; the first one contained the two rootstocks Okinawa and Nemared, while

the remaining subcluster contained Nemaguard.

***Similarity and relationships based on combined data of RAPD-PCR and SSR-PCR analyses***

Cluster analysis based on RAPD-PCR and SSR-PCR analysis as shown in (Table 6) was carried out using UPGMA computer program. The highest similarity index recorded was (94.1%) between the two rootstocks Shami and Hegazy, while the lowest similarity index (81.8%) was observed between the two rootstocks Okinawa and Sultani. Dendrogram for the genetic relationships among the six peach rootstocks across the two techniques results were carried out and are shown in (Fig. 5), it was similar to the previous dendrogram based on 12 RAPD primers.

In this study of genetic diversity the use of RAPD-PCR seemed to be a satisfactory tool and could discriminate among the six peach rootstocks. Our results were in partial agreement with of Casas *et al.* (1999) who confirmed that RAPD-PCR results appear to play an important role in the differentiation among different cultivars.

Because of the differences in the rootstocks traits and the molecular genetic diversity of these rootstocks, it would be useful to introgress root-knot nematode resistance genes into elite rootstock germplasm. However, the extended juvenile stage of peach impedes such progress by traditional methods. Therefore, it is

recommended to use marker-assisted selection (MAS) in peach rootstocks breeding programs, under biotic stresses such as root-knot nematode (*Meloidogyne incognita* and *M. javanica*), as an efficient short cut and cost-effective tool for evaluating peach rootstocks.

**SUMMARY**

Six peach rootstocks were collected according to their susceptibility to infestation of root-knot nematodes; *Meloidogyne incognita* and *M. javanica*. These rootstocks were Okinawa, Nemaguard and Nemared (nematode resistant) and Hegazy, Shami and Sultani (nematode susceptible), which screened by randomly amplified polymorphic DNA (RAPD) markers using 31 arbitrary 10-mer primers and two SSR primer pairs. Twenty molecular markers related to root-knot nematode have been detected (using 12 primers), 13 of which were negative since they appeared in susceptible rootstocks and were absent in the resistant ones, and seven were positive markers which were present in the resistant rootstocks. Dendrogram tree generated across RAPD and SSR analysis demonstrated that the highest similarity was scored between Shami and Sultani (94.1%) while the lowest similarity was scored between Okinawa and Sultani (82.3%). Because of the differences in the rootstocks traits and the molecular genetic diversity of these rootstocks, marker-assisted selection (MAS) in peach rootstocks breeding programs, under biotic stresses (*Meloidogyne*

*incognita* and *M. javanica*), is recommended as an efficient tool for improving peach rootstocks resistance.

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Table (1): List of the six peach rootstocks and chromosome numbers.

Code No.	Rootstocks names	Chromosome number	Nematode resistance
1	Okinawa	16	R
2	Nemaguard	16	R
3	Nemared	16	R
4	Hegazy	16	S
5	Shami	16	S
6	Sulltani	16	S

R = Resistant

S = Susceptible



Table (2): List of the primers codes and nucleotide sequences.

No.	Code	Sequence	No.	Code	Sequence
1	OP-A01	5' CAG GCC CTT C 3'	17	OP-D16	5' AGG GCG TAA G 3'
2	OP-A09	5' GGG TAA CGC C 3'	18	OP-E01	5' CCC AAG GTC C 3'
3	OP-A11	5' CAA TCG CCG T 3'	19	OP-L12	5' GGG CGG TAC T 3'
4	OP-A18	5' AGG TGA CCG T 3'	20	OP-L13	5' ACC GCC TGC T 3'
5	OP-B01	5' GTT TCG CTC C 3'	21	OP-L16	5' AGG TTG CAG G 3'
6	OP-B02	5' TGA TCC CTG G 3'	22	OP-L20	5' TGG TGG ACC A 3'
7	OP-B03	5' CAT CCC CCT G 3'	23	OP-O01	5' GGC ACG TAA G 3'
8	OP-B06	5' TGC TCT GCC C 3'	24	OP-O02	5' ACG TAG CGT C 3'
9	OP-B08	5' GTC CAC ACG G 3'	25	OP-O03	5' CTG TTG CTA C 3'
10	OP-B20	5' GGA CCC TTA C 3'	26	OP-O05	5' CCC AGT CAC T 3'
11	OP-C01	5' TTC GAG CCA C 3'	27	OP-O07	5' CAG CAC TGA C 3'
12	OP-C12	5' TGT CAT CCC C 3'	28	OP-O10	5' TCA GAG CGC C 3'
13	OP-C13	5' AAG CCT CGT C 3'	29	OP-O14	5' AGC ATG GCT C 3'
14	OP-C15	5' GAC GGA TCA G 3'	30	OP-O19	5' GGT GCA CGT T 3'
15	OP-C18	5' TGA GTG GGT G 3'	31	OP-O20	5' ACA CAC GCT G 3'
16	OP-C19	5' GTT GCC AGC C 3'			

Table (3): List of primer pairs names, their nucleotide sequences and melting temperature.

Locus	Primer sequence	T <sub>M</sub> (°C)
Pchgms 26-1	F: 5' TGAACGGGTTCTATCCGTGT 3'	60.0
	R: 5' AAACGGTTGCGTCCAATAAG 3'	60.0
Pchgms 26-2	F: 5' TTTGATAGGATCCCAAGGGTA 3'	58.0
	R: 5' TTGGCTGGCAGTTATCATCA 3'	60.0

Table (4): Molecular genetic markers for root-knot nematode resistance based on RAPD-PCR.

Primer	Rootstock cultivar	Molecular marker
Primer OP-A01	Hegazy, Shami and Sultani (S)	<sup>-</sup> 750 bp, <sup>-</sup> 330 bp and <sup>-</sup> 250 bp
Primer OP-A09	Okinawa, Nemaguard and Nemared (R)	<sup>+</sup> 1765 bp
Primer OP-A11	Hegazy, Shami and Sultani (S)	<sup>-</sup> 745 bp, <sup>-</sup> 516 bp and <sup>-</sup> 255 bp
Primer OP-B08	Hegazy, Shami and Sultani (S)	<sup>-</sup> 1100 bp
Primer OP-C12	Hegazy, Shami and Sultani (S)	<sup>-</sup> 970 bp, <sup>-</sup> 865 bp and <sup>-</sup> 755 bp
Primer OP-C13	Okinawa, Nemaguard and Nemared (R)	<sup>+</sup> 750 bp
Primer OP-C19	Hegazy, Shami and Sultani (S)	<sup>-</sup> 670 bp
Primer OP-L12	Okinawa, Nemaguard and Nemared (R)	<sup>+</sup> 760 bp
Primer OP-L13	Hegazy, Shami and Sultani (S)	<sup>-</sup> 650 bp
Primer OP-L16	Okinawa, Nemaguard and Nemared (R)	<sup>+</sup> 1290 bp
Primer OP-O 05	Okinawa, Nemaguard and Nemared (R)	<sup>+</sup> 1750 bp, <sup>+</sup> 650 bp and <sup>-</sup> 1270 bp
Primer OP-O10	Okinawa, Nemaguard and Nemared (R)	<sup>+</sup> 1050 bp

R = Resistance, S = Susceptible, + = Band is present, - = band is absent.

Table (5): Similarity indices among the six peach rootstocks based on RAPD-PCR using 12 primers.

Rootstocks	Okinawa	Nemaguard	Nemared	Hegazy	Shami
Nemaguard	0.890				
Nemared	0.911	0.898			
Hegazy	0.888	0.851	0.887		
Shami	0.865	0.869	0.885	0.941	
Sultani	0.823	0.840	0.861	0.906	0.933

Table (6): Similarity indices among the six peach rootstocks based on both RAPD-PCR and SSR-PCR combined analyses.

Rootstocks	Okinawa	Nemaguard	Nemared	Hegazy	Shami
Nemaguard	0.889				
Nemared	0.907	0.897			
Hegazy	0.885	0.845	0.885		
Shami	0.863	0.862	0.884	0.940	
Sultani	0.818	0.831	0.858	0.904	0.930

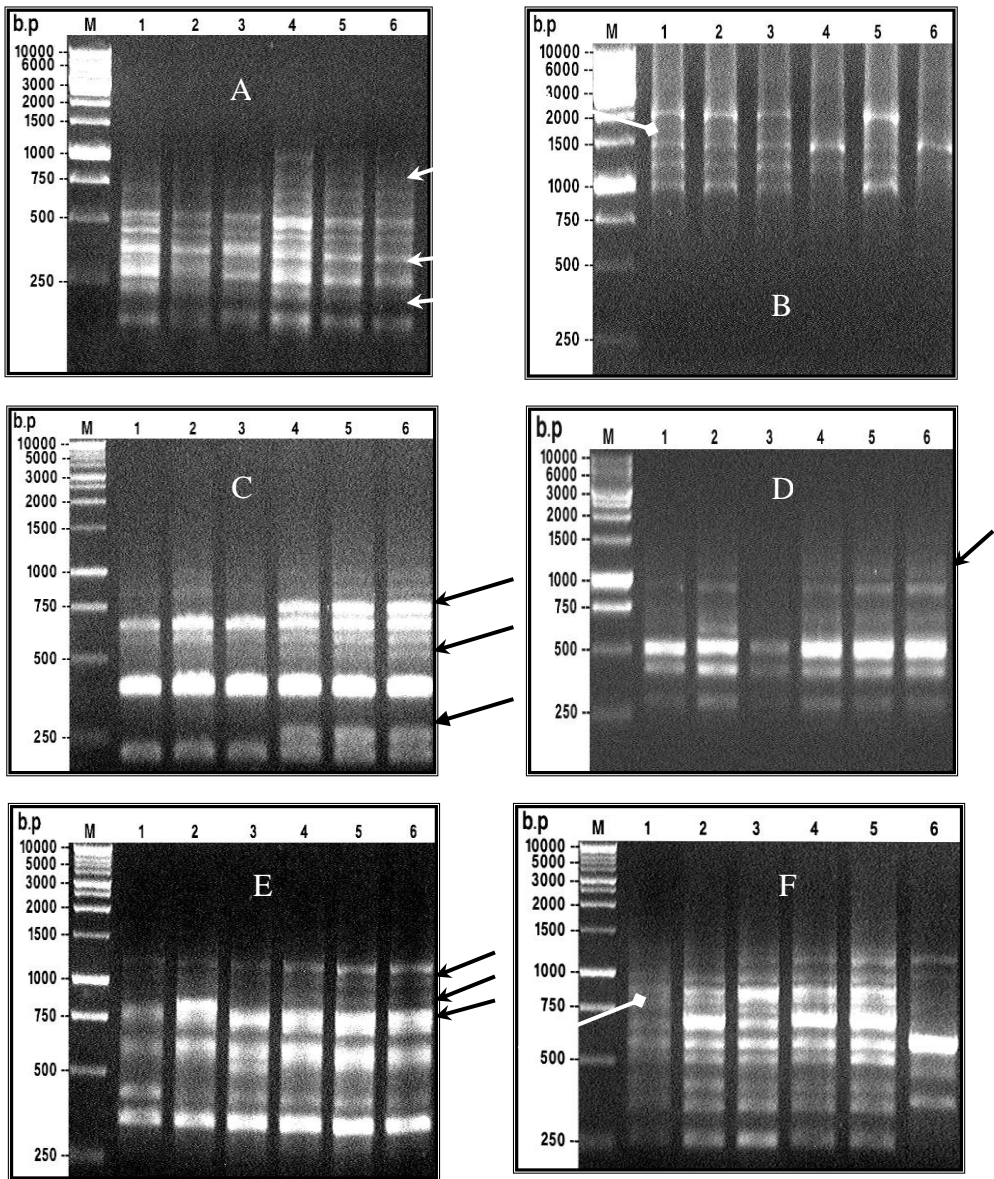


Fig. (1): DNA polymorphism of the six peach rootstock cultivars amplified with primer:  
 A: OP-A01 B: OP-A09 C: OP-A11 D: OP-B08 E: OP-C12 F: OP-C13  
 (1) Okinawa (2) Nemaguard (3) Nemared (4) Hegazy  
 (5) Shami (6) Sultani (M) DNA ladder marker (bp)

◆ Positive marker (appeared in the 3 resistant rootstocks)  
 → Negative marker (appeared in the 3 susceptible rootstocks)

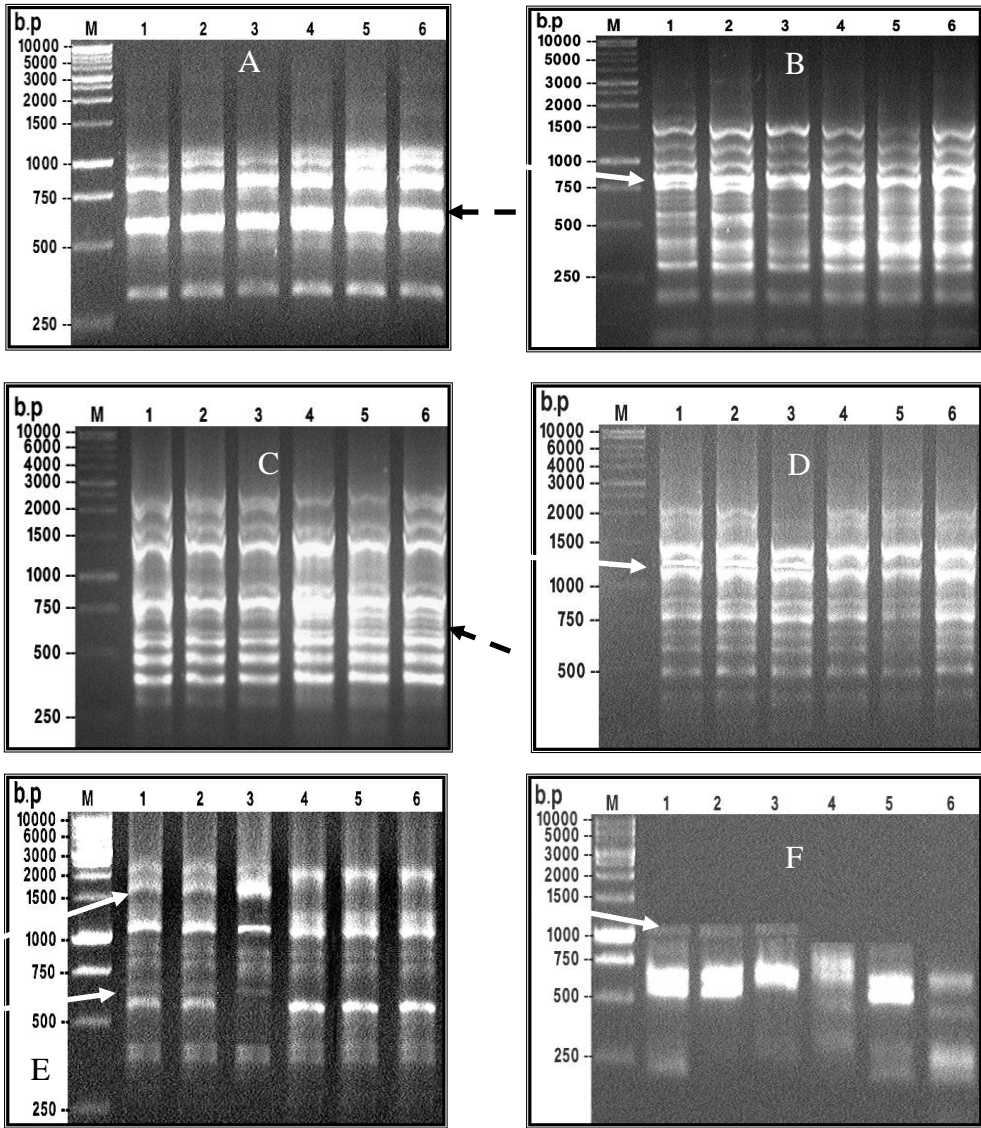


Fig. (2): DNA polymorphism of the six peach rootstock cultivars amplified with primer:  
 A: OP-C19 B: OP-L12 C: OP-L13 D: OP-L16 E: OP-O05 F: OP-O10  
 (1) Okinawa (2) Nemaguard (3) Nemared (4) Hegazy  
 (5) Shami (6) Sultani (M) DNA ladder marker (bp)

—————> Positive marker (appeared in the 3 resistant rootstocks)

- - - - -> Negative marker (appeared in the 3 susceptible rootstocks)

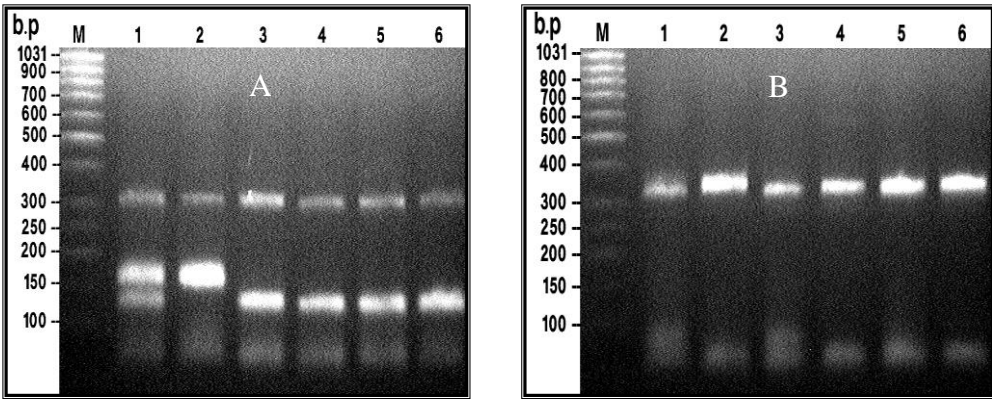


Fig. (3): Amplified SSR marker A: pchgms 26-1 B: pchgms 26-2  
 (1) Okinawa (2) Nemaguard (3) Nemared (4) Hegazy  
 (5) Shami (6) Sultani (M) DNA standard

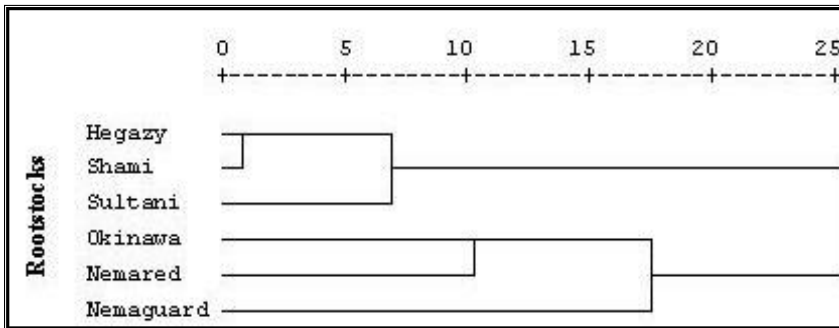


Fig. (4): Dendrogram for the genetic distances relationships among the six peach rootstocks based on similarity indices data of RAPD analysis.

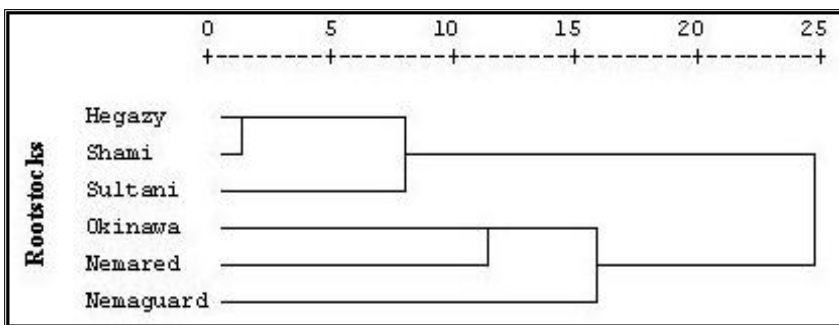


Fig. (5): Dendrogram for the genetic distances between the six peach rootstocks based on RAPD-PCR and SSR-PCR analyses.