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IDENTIFICATION OF ZYGOTIC AND NUCELLAR SEEDLINGS OF VOLKAMER LEMON, RANGPUR LIME AND CLEOPATRA MANDARIN ROOTSTOCKS USING RAPD TECHNIQUE

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ABSTRACT

The randomly amplified polymorphic DNA (RAPD-PCR) technique was used to identify and distinguish nucellar and zygotic 45-day-old seedlings of three citrus rootstocks namely Volkamer lemon (*Citrus volkameriana* Ten. & Pasq.), Rangpur lime (*C. limonia,* Osbeck), Cleopatra mandarin (*C. reshni* Hort. ex Tan.) with their mother trees. Primers OP-A2, OP-A8, OP-A18, OP-B3, OP-B4 and OP-B6 were used for identifying the nine rootstock genotypes. Seedlings classified as zygotic (sexual) have a different RAPD profile from that of the mother plant or nucellar (vegetative) seedlings. RAPD markers allowed the efficient identification of zygotic and nucellar seedlings. The band pattern in zygotic plants was different from that of the mother plant. All nucellar plants showed 100% similarity. OP-B04 primer was able to identify all zygotic seedlings. The highest similarity value (100%) was scored between nucellar Volkamer lemon and its mother plant, at the same level the nucellar Rangpur and its mother plant and nucellar Cleopatra mandarin and its mother plant. The lowest similarity value was between sexual Cleo and their mother and nucellar plants (77.1%), meanwhile their corresponding values were in Volka and Rangpur (95% and 91% respectively). These results allow us to conclude that nucellar seedlings of Cleopatra mandarin are better in using as a rootstock for grafting other citrus cultivars. Nucellar seedlings of Rangpur were the second then nucellar Volkamer lemon was the last.

INTRODUCTION

Rootstocks can be developed through traditional plant breeding methods; however, the ability to screen and select for economically important traits (such as production of true nucellar seedlings) in an efficient fashion is limited by the difficulties of screening techniques based on whole plant performance. To address these problems, we have used randomly amplified polymorphic DNA (RAPD), thus improving the accuracy of early selection of a citrus rootstock. Recently, Bastianel *et al*., (1998) reported that, molecular markers have been able to analyze DNA directly, without any influence from the environment or tissue age. Among these, random amplified polymorphic DNA markers (RAPD) have been widely used in citrus because of their assumed phenotypic neutrality and their ability to quickly and easily reveal a large number of markers. The technique has been used mainly for genotype typification, phylogenetic studies, mapping and mutant identification. The authors reviewed that, the RAPD technique does not need previous information about the targeted DNA and shows great polymorphism. As, all nucellar plants showed 100% similarity when four random primers were used to distinguish nucellar and zygotic seedlings resulting from crosses between the Montenegrina (*Citrus deliciosa* Tenore) and King (*C. nobilis* Loureiro) tangerines to develop tangerine varieties. Thus, the RAPD technique was efficient in identification and distinguishing nucellar and zygotic seedlings. Also, Cristofani *et al*. (2001) used RAPD markers to identify zygotic and nucellar seedlings in controlled crosses of citrus rootstock varieties. As, RAPD molecular markers technique was used to determine germination frequency of zygotic embryos of sweet orange (*Citrus sinensis*) cv. Caipira and sour orange (*C. aurantium*) hybrid seeds from open-pollination. Hence, RAPD markers allowed the efficient identification of zygotic and nucellar seedlings of *C. reshni* and Robinson (Schafer *et al*., 2004). It is concluded that, techniques based on DNA analyses have been a useful tool for hybrid identification. It is necessary to identify zygotic seedlings at an early stage (as a seedling) (Rodriguez *et al*., 2004) for a more rapid advance in the propagation program; for this purpose, several biochemical methods have been used. Recently, molecular techniques such as RAPD are available. In citrus, they have been used to study the genetic origin of 'Cravo' lemon plants which had been

visually selected as possible hybrids; to identify the mandarin 'Montenegrina' x mandarin 'King' (*Citrus* spp.) hybrids; and to study genetic diversity and to identify interspecific crosses such as 'Volkameriana' lemon x ' Cravo' lemon. Results revealed that, no single primer was able to identify all zygotic seedlings. The authors suggested that when the zygotic embryo is a hybrid, it may be more vigorous, and hence compete better with nucellar embryos, whereas zygotic embryos produced by self-pollination are less vigorous and may not be competitive with nucellar ones. RAPD markers permitted the efficient identification of zygotic and nucellar seedlings of *C. reshni* and Robinson (Rodrigues *et al*., 2005). DNA amplified fingerprinting with single primers was the more successful technique for discriminating between nucellular and zygotic seedlings of progeny of a Volkamer lemon (*Citrus volkameriana* Ten. & Pasq) (Luro *et al*.,1995).

It is necessary to identify hybrids that yield nucellar seeds for rootstock propagation (Rao *et al*., 2007). Where, five random primers were screened to select mandarin (*Citrus reticulata*) and pummelo (*C. grandis*) hybrids that produce primarily nucellar seedlings. The usefulness and efficiency of RAPD-PCR method as a quick screening technique for citrus hybrids are discussed. RAPD-PCR method as a quick screening technique for studies was used for distinguishing nucellars from zygotics rootstock cultivars, to identify mistakes occurring in commercial nurseries. The zygotic and apomictic (non-zygotic) seedlings were confirmed by RAPD analysis.

The objectives of this work were to identify zygotic and nucellar seedlings of *Citrus volkameriana*, C*itrus limonia* and *Citrus reshni* using RAPD.

MATERIALS AND METHODS

The present study was conducted in Molecular Genetics and Genome Mapping Laboratory (MGGM) at the Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Ministry of Agriculture, Giza, Egypt.

1. Collecting plant material

The current experiment was carried out on 45-day-old seedlings of three citrus rootstocks recommended for newly reclaimed lands namely Volkamer lemon (*Citrus volkameriana* Ten. & Pasq.), Rangpur lime (C*itrus limonia,* Osbeck), Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) were used to distinguish their nucellar and zygotic seedlings. All seedlings of each rootstock were obtained from the same mother tree.

2. Genetic identification:

DNA Extraction

Young, fresh and fully expanded leaves were taken at random from the mother plant, placed in plastic bags and kept at -20^{o}C until DNA extraction. Leaves were also taken from analogous seedlings grown from seeds whose all embryos developed. All the selected leaves were free from any physiological or pathogenic symptoms. Leaves were labeled and placed on ice until DNA extraction. Plant tissues were ground under liquid nitrogen to a fine powder, then bulked DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN).

2. 1. Random amplified polymorphic DNA (RAPD)

Polymerase Chain Reaction (PCR) amplification was performed using a set of six random 10-mer- arbitrary primers (synthesized by Operon biotechnologies, Inc. Germany) in the detection of polymorphism among the nine citrus rootstock genotypes with the following sequences (Table, 1):

Table (1): List of the RAPD primers proved for Citrus volkameriana, C. limonia and C. reshni and their sequences.

Primer	Base sequence (5'→ 3')
OP-A2	TGCCGAGCTG
OP-A8	GTGACGTAGG
OP-A18	AGGTGACCGT
OP-B3	CATCCCCCTG
OP-B4	GGACTGGAGT
OP-B6	TGCTCTGCCC

Amplification was conducted in 25 µl reaction volume containing the following reagents: 2.5 µl of dNTPs (2.5 mM), 2.5 µl $MgCl_2$ (25 mM), and 2.5 µl of 10 x buffer, 3.0 µl of primer (10 pmol), 3.0 µl of template DNA (25 ng/µl), 1 µl of *Taq* polymerase (1U/µl)

and 10.5 µl of sterile dd H_2O. The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed as follows: one cycle at 94ºC for 4 min followed by 45 cycles of 1 min at 94ºC, 1 min at 36ºC, and 2 min at 72ºC. The reaction was finally stored at 72ºC for 10 min (Rajapakse *et al*., 1995). Amplified products were size-fractioned (using 1 Kbp ladder marker) by electrophoresis in 1.5 % agarose gels in TBE buffer at 120 V for 1 h. The bands were visualized by ethidium bromide under UV florescence and photographed.

2.2. Molecular data analysis:

The molecular data analysis for the identification of the origin of the seedlings was carried out by comparing band patterns generated by the mother plant, with those generated by each seedling. The bands produced for each DNA sample by six primers were considered polymorphic when they were absent or present in at least one of the seedlings evaluated.

Bands were analyzed using numerical and multivariate analyses (NTSYS-PC) 1.7 Version (Rohlf, 1992). A similarity matrix was generated using SM (simple matching) coefficients and a dendogram constructed using the UPGMA method (unweighted pair group method).

RESULTS AND DISCUSSION

Identification of zygotic and nucellar seedlings using RAPD:

The resulted amplified fragments are shown in Figures (1-6) and their densitometric analyses are illustrated in Tables (2-7). Banding patterns were scored as present (1) or absent (0). All six primers were successfully amplified DNA fragments for all genotypes. Primers produced band numbers ranging from10 (Primers OP-A02, OP-A18, OP-B03 and OP-B04) to 17(Primer OP-B06) across genotypes (Tables 2–7 and Figures 1-6).

Results of amplifying these primers with the nine citrus rootstock genotypes using RAPD-PCR technique are shown as follows:

Primer OP-A02:

The pattern produced by primer OP-A02 showed a maximum number of 10 DNA fragments with molecular sizes (MS) ranging between 242 to 1505 bp (Table, 2 and Figure, 1). Three polymorphic fragments (30%) with numbers of 1,9 and 10 at corresponding

molecular sizes 1505, 265 and 242 bp were detected, whereas the other eight fragments were monomorphic, since they were seen in all genotypes. Mother tree of Volka, nucellar Volka, sexual Volka, mother tree of Rangpur, nucellar Rangpur, sexual Rangpur and sexual Cleo genotypes showed the maximum number of fragments (nine), while the lowest ones (eight) appeared in mother plant of Cleo and nucellar Cleo genotypes.
Two of the detectable polymorphic fragments were genotype-specific markers, one (265bp) as a negative marker and one (242bp) as a positive marker for both them for sexual Cleo.

Primer OP-A08:

Primer OP-A08 exhibited eleven DNA fragments ranging in molecular sizes from 1585 to 215bp (Table,3 and Figure,2). Eight polymorphic fragments (72.73%) with the numbers 1,2,3,4,7,8,9 and 10bp with corresponding molecular sizes of 1585, 1078,836,747,415,378,320 and 271 bp were observed, while the rest of fragments were monomorphic. Mother tree of Volka, nucellar Volka and sexual Volka genotypes gave the maximum number of fragments (ten), while mother tree of Cleo, nucellar Cleo and sexual Cleo genotypes showed the lowest ones (five). No genotype-specific markers were detected.

Primer OP-A18:

Primer OP-A18 resulted in ten DNA fragments with molecular sizes from 232 to 1646 bp (Table,4 and Figure,3). Seven fragments were polymorphic (70.00%). Two of the detectable polymorphic fragments were genotype-specific markers; one with number of 4 at MS 753 bp as a negative marker and the other with the number 10 at MS 232 bp as a positive marker, both of them for sexual Cleo genotype, whereas the remain fragments were monomorphic. Mother tree of Volka, nucellar Volka, sexual Volka and sexual Rangpur showed the maximum fragments number (eight), while the other genotypes had an equal number of fragments (five).

Primer OP-B03:

The results of primer OP-B03 manifested ten DNA fragments with molecular sizes ranging between 222 to 1905 bp (Table,5 and Figure,4) Eight polymorphic fragments (80.00%) with numbers of 1,2,4,6,7,8,9 and 10 at corresponding moleqular sizes of

1905,1371,809,582,373,307, 261 and 222 bp were observed and the other two fragments were monomorphic. Mother tree of Rangpur, nucellar Rangpur and sexual Rangpur genotypes had the maximum number of fragments (nine), while the lowest ones (three) appeared in mother tree of Cleo, nucellar Cleo and sexual Cleo genotypes. No genotype-specific markers were detected.

Primer OP-B04:

The obtained data of primer OP-B04 with the nine genotypes as shown in table (6) and figure (5) revealed the amplification of ten DNA fragments ranging in molecular sizes between 216-1035 bp. Six polymorphic fragments (60.00 %) with numbers of 2,3,6,7,9 and 10 at corresponding molecular sizes of 820,817,420,337,272 and 216 bp respectively were observed, while only four fragments were monomorphic. Mother tree of Volka and nucellar Volka genotypes had the maximum number of fragments (eight), while sexual Cleo genotype gave the lowest one (five). No detectable genotype-specific markers were noticed by OP-B04.

Primer OP-B06:

Primer OP-B06 gave seventeen DNA fragments with molecular sizes ranging from 210 to 1576 bp (Table,7 and Figure,6). From these, fourteen fragments (No. 1,2,3,4,5,6,8,10,12,13,14,15, 16 and 17) with molecular sizes of 1576, 1132, 1122, 947, 767, 686, 533, 417, 337, 282, 258, 231, 211 and 210 bp were polymorphic representing polymorphic percentage of 82.35%, while the other three fragments were monomorphic. Sexual Rangpur obtained the maximum number of thirteen fragments, while mother tree of Cleo and nucellar Cleo gave only seven fragments. Seven of the detectable polymorphic fragments were genotype-specific markers; one of them as a positive marker for sexual Rangpur (211 bp), two as a negative markers (1576 and 767 bp) and four as a positive markers (337, 282, 258 and 231 bp) for sexual Cleo.

Only OP-B04 primer was able to identify all zygotic seedlings.

Table (2): DNA polymorphism using RAPD-PCR for the nine genotypes of citrus rootstocks amplified with primer OP-A02.

		Citrus rootstocks								
Primer No.	M.S. (bp)	*M.P. of Volka	nV	sV	M.P. of Rangpur	nR	sR	M.P. of Cleo	nC	sC
1	1505	1	1	1	1	1	1	0	0	1
2	1032	1	1	1	1	1	1	1	1	1
3	912	1	1	1	1	1	1	1	1	1
4	683	1	1	1	1	1	1	1	1	1
5	604	1	1	1	1	1	1	1	1	1
6	446	1	1	1	1	1	1	1	1	1
7	366	1	1	1	1	1	1	1	1	1
8	306	1	1	1	1	1	1	1	1	1
9	265	1	1	1	1	1	1	1	1	0
10	242	0	0	0	0	0	0	0	0	1
Total		**9**	**9**	**9**	**9**	**9**	**9**	**8**	**8**	**9**

*M.P.= Mother plant
nV= nucellar Volka
nR= nucellar Rangpur
nC= nucellar Cleo
sV= sexual Volka
sR= sexual Rangpur
sC= sexual Cleo

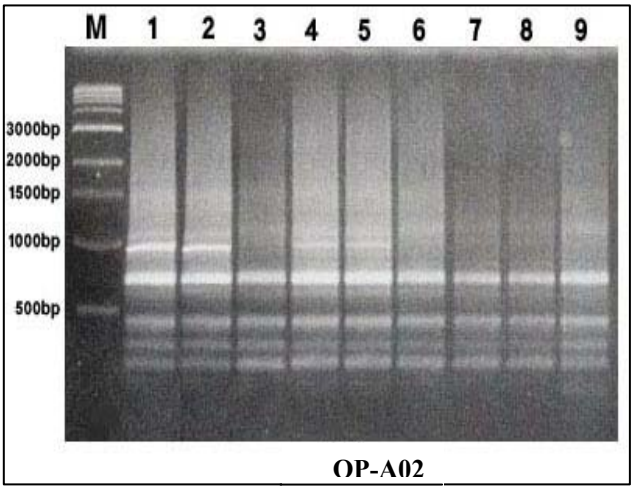

Fig. 1. DNA polymorphism using RAPD-PCR of the nine citrus-rootstock genotypes amplified with primer OP-A02.

(M) DNA ladder marker (bp) (1) Mother tree of Volka (2) nucellar Volka (3) sexual Volka (4) Mother tree of Rangpur (5) nucellar Rangpur (6) sexual Rangpur (7) Mother tree of Cleo (8) nucellar Cleo (9) sexual Cleo

Table (3): DNA polymorphism using RAPD-PCR for the nine genotypes of citrus rootstocks amplified with primer OP-A08.

Primer No.	M.S. (bp)	Citrus rootstocks								
		M.P. of Volka	nV	sV	M.P. of Rangpur	nR	sR	M.P. of Cleo	nC	sC
1	1585	1	1	0	0	0	0	0	0	0
2	1078	1	1	1	0	0	0	0	0	0
3	836	1	1	1	1	1	1	0	0	0
4	747	1	1	1	1	1	1	0	0	0
5	539	1	1	1	1	1	1	1	1	1
6	473	1	1	1	1	1	1	1	1	1
7	415	1	1	1	1	1	1	0	0	0
8	378	1	1	1	0	0	0	1	1	1
9	320	1	1	1	1	1	1	0	0	0
10	271	0	0	1	1	1	1	1	1	1
11	215	1	1	1	1	1	1	1	1	1
Total		**10**	**10**	**10**	**8**	**8**	**8**	**5**	**5**	**5**

*M.P.: Mother plant
nV: nucellar Volka sV: sexual Volka
nR: nucellar Rangpur sR: sexual Rangpur
nC: nucellar Cleo sC: sexual Cleo

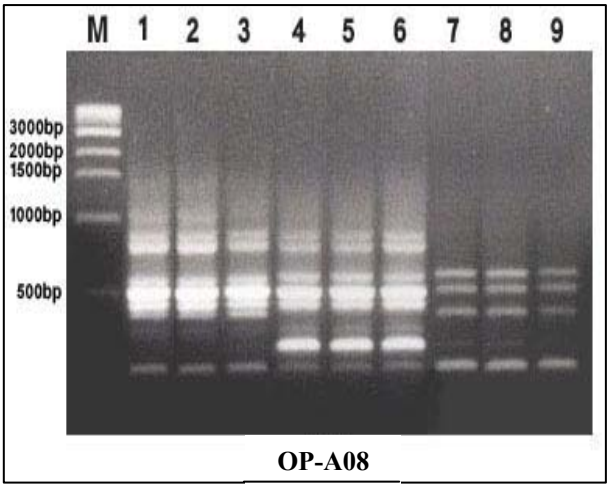

Fig. 2. DNA polymorphism using RAPD-PCR of the nine citrus-rootstock genotypes amplified with primer OP-A08.

(M) DNA ladder marker (bp) (1) Mother tree of Volka (2) nucellar Volka (3) sexual Volka (4) Mother tree of Rangpur (5) nucellar Rangpur (6) sexual Rangpur (7) Mother tree of Cleo (8) nucellar Cleo (9) sexual Cleo

Table (4): DNA polymorphism using RAPD-PCR for the nine genotypes of citrus rootstocks amplified with primer OP-A18.

Primer No.	M.S. (bp)	Citrus rootstocks								
		M.P. of Volka	nV	sV	M.P. of Rangpur	nR	sR	M.P. of Cleo	nC	sC
1	1646	1	1	1	0	0	1	0	0	0
2	1383	1	1	1	0	0	1	0	0	0
3	115	1	1	1	0	0	1	0	0	0
4	753	1	1	1	1	1	1	1	1	0
5	574	1	1	1	1	1	1	1	1	1
6	485	1	1	1	1	1	1	1	1	1
7	369	0	0	0	1	1	1	1	1	1
8	339	1	1	1	0	0	0	0	0	0
9	269	1	1	1	1	1	1	1	1	1
10	232	0	0	0	0	0	0	0	0	1
Total		**8**	**8**	**8**	**5**	**5**	**8**	**5**	**5**	**5**

*M.P.: Mother plant
nV: nucellar Volka
nR: nucellar Rangpur
nC: nucellar Cleo
sV: sexual Volka
sR: sexual Rangpur
sC: sexual Cleo

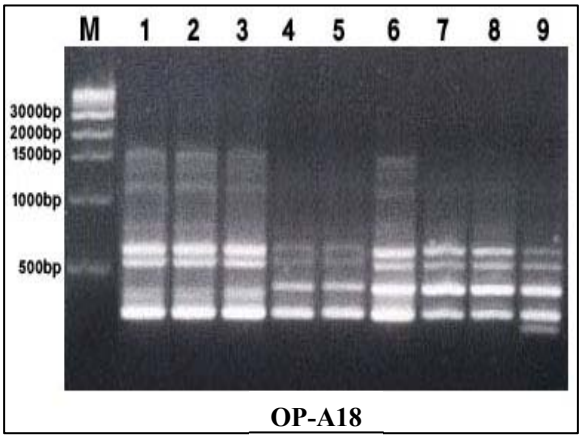

Fig. 3. DNA polymorphism using RAPD-PCR of the nine citrus-rootstock genotypes amplified with primer OP-A18.

(M) DNA ladder marker (bp) (1) Mother tree of Volka (2) nucellar Volka (3) sexual Volka (4) Mother tree of Rangpur (5) nucellar Rangpur (6) sexual Rangpur (7) Mother tree of Cleo (8) nucellar Cleo (9) sexual Cleo

Table (5): DNA polymorphism using RAPD-PCR for the nine genotypes of citrus rootstocks amplified with primer OP-B03.

Primer No.	M.S. (bp)	Citrus rootstocks								
		M.P. of Volka	nV	sV	M.P. of Rangpur	nR	sR	M.P. of Cleo	nC	sC
1	1905	1	1	1	1	1	1	0	0	0
2	1371	0	0	0	1	1	0	0	0	0
3	1061	1	1	1	1	1	1	1	1	1
4	809	1	1	1	1	1	1	0	0	0
5	657	1	1	1	1	1	1	1	1	1
6	582	1	1	1	0	0	1	1	1	1
7	373	1	1	1	1	1	1	0	0	0
8	307	0	0	1	1	1	1	0	0	0
9	261	0	0	0	1	1	1	0	0	0
10	222	0	0	0	1	1	1	0	0	0
Total		**6**	**6**	**7**	**9**	**9**	**9**	**3**	**3**	**3**

*M.P.: Mother plant
nV: nucellar Volka sV: sexual Volka
nR: nucellar Rangpur sR: sexual Rangpur
nC: nucellar Cleo sC: sexual Cleo

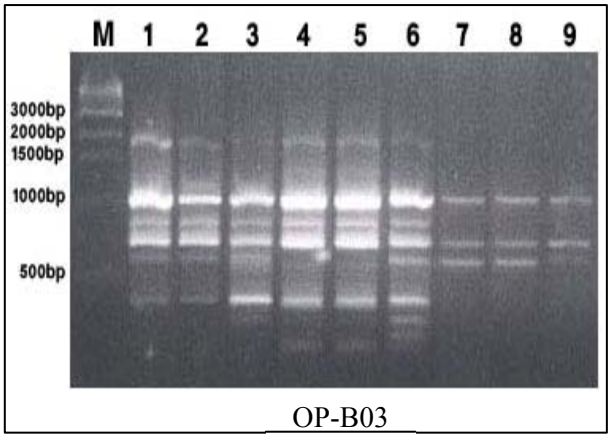

Fig. 4. DNA polymorphism using RAPD-PCR of the nine citrus-rootstock genotypes amplified with primer OP-B03.

(M) DNA ladder marker (bp) (1) Mother tree of Volka (2) nucellar Volka (3) sexual Volka (4) Mother tree of Rangpur (5) nucellar Rangpur (6) sexual Rangpur (7) Mother tree of Cleo (8) nucellar Cleo (9) sexual Cleo

Table (6): DNA polymorphism using RAPD-PCR for the nine genotypes of citrus rootstocks amplified with primer OP-B04.

Primer No.	M.S. (bp)	Citrus rootstocks								
		M.P. of Volka	nV	sV	M.P. of Rangpur	nR	sR	M.P. of Cleo	nC	sC
1	1035	1	1	1	1	1	1	1	1	1
2	820	0	0	0	0	0	1	1	1	0
3	817	1	1	1	1	1	1	0	0	0
4	584	1	1	1	1	1	1	1	1	1
5	525	1	1	1	1	1	1	1	1	1
6	420	1	1	1	1	1	1	0	0	0
7	337	0	0	0	0	0	0	1	1	1
8	296	1	1	1	1	1	1	1	1	1
9	272	1	1	0	0	0	0	0	0	0
10	216	1	1	0	0	0	0	0	0	0
Total		**8**	**8**	**6**	**6**	**6**	**7**	**6**	**6**	**5**

*M.P.: Mother plant
nV: nucellar Volka
nR: nucellar Rangpur
nC: nucellar Cleo
sV: sexual Volka
sR: sexual Rangpur
sC: sexual Cleo

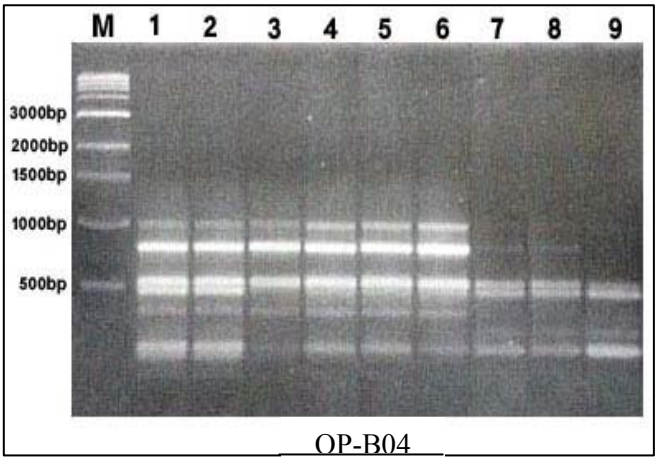

Fig. 5. DNA polymorphism using RAPD-PCR of the nine citrus-rootstock genotypes amplified with primer OP-B04.

(M) DNA ladder marker (bp) (1) Mother tree of Volka (2) nucellar Volka (3) sexual Volka (4) Mother tree of Rangpur (5) nucellar Rangpur (6) sexual Rangpur (7) Mother tree of Cleo (8) nucellar Cleo (9) sexual Cleo

Table (7): DNA polymorphism using RAPD-PCR for the nine genotypes of citrus rootstocks amplified with primer OP-B06.

Primer No.	M.S. (bp)	Citrus rootstocks								
		M.P. of Volka	nV	sV	M.P. of Rangpur	nR	sR	M.P. of Cleo	nC	sC
1	1576	1	1	1	1	1	1	1	1	0
2	1132	0	0	0	0	0	1	1	1	0
3	1122	1	1	1	1	1	1	0	0	0
4	947	0	0	0	0	0	1	1	1	0
5	767	1	1	1	1	1	1	1	1	0
6	686	1	1	1	1	1	1	0	0	1
7	601	1	1	1	1	1	1	1	1	1
8	533	1	1	1	1	1	1	0	0	0
9	471	1	1	1	1	1	1	1	1	1
10	417	1	1	1	1	1	1	0	0	1
11	360	1	1	1	1	1	1	1	1	1
12	337	0	0	0	0	0	0	0	0	1
13	282	0	0	0	0	0	0	0	0	1
14	258	0	0	0	0	0	0	0	0	1
15	231	0	0	0	0	0	0	0	0	1
16	211	0	0	0	0	0	1	0	0	0
17	210	1	1	1	1	1	1	0	0	0
Total		**10**	**10**	**10**	**10**	**10**	**13**	**7**	**7**	**9**

*M.P.: Mother plant
nV: nucellar Volka
nR: nucellar Rangpur
nC: nucellar Cleo
sV: sexual Volka
sR: sexual Rangpur
sC: sexual Cleo

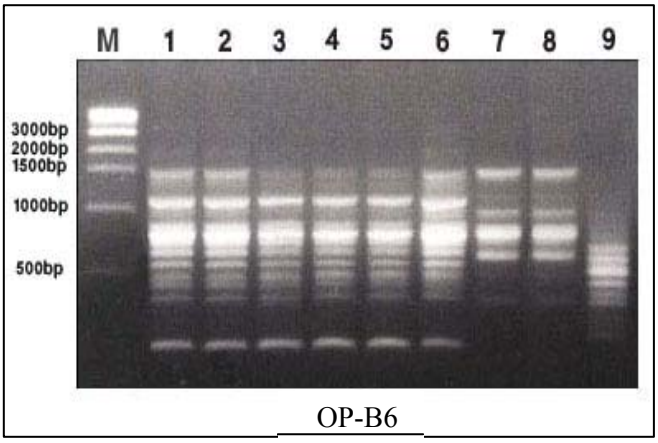

Fig. 6. DNA polymorphism using RAPD-PCR of the nine citrus-rootstock genotypes amplified with primer OP-B06.

(M) DNA ladder marker (bp) (1) Mother tree of Volka (2) nucellar Volka (3) sexual Volka (4) Mother tree of Rangpur (5) nucellar Rangpur (6) sexual Rangpur (7) Mother tree of Cleo (8) nucellar Cleo (9) sexual Cleo

b. Combined identification based on RAPD-PCR analysis:

The number of total amplified fragments (TAF) and polymorphic bands (PB) for each primer, amplified fragments (AF) and specific markers (SM) for each citrus-rootstock genotype using RAPD-PCR markers are presented in table (8).

Data of the amplified fragments based on RAPD-PCR technique using six 10-mer arbitary primers for the nine citrus rootstock genotypes exhibited wide difference in amplifying DNA. All primers showed polymorphism among genotypes with different levels from one primer to another. Primers OP-A02 (30.00%) and OP-B04 (60.00%) exhibited low polymorphism. On the other hand, primers OP-A08 (72.73%), OP-A18 (70.00%), OP-B03 (80.00%) and OP-B06 (82.35%) exhibited high polymorphism levels; therefore they are useful in citrus rootstock genotypes identification.

There were some specific fragments which can be used to discriminate each genotype from the others, since each of these fragments were absent in all genotypes except the assigned one (*i.e.* positive marker) or present in all genotype samples except the assigned one (*i.e.* negative marker). Table (8) showed that, only sexual Rangpur and sexual Cleo had RAPD-PCR specific markers; 10 markers were scored for sexual Cleo, while sexual Rangpur scored one marker. A number of 7 specific markers were scored for the presence of unique band for a given genotype (positive marker), while 4 specific markers were scored for the absence of a common band (negative marker). The largest number of RAPD-PCR cultivar specific marker was generated by primer OP-B06 (7 markers), followed by primers OP-A02 and OP-A08 (2 markers). Seedlings classified as zygotic have a different RAPD profile from that of the mother plant or nucellar seedlings. The band pattern in zygotic plants was different from that of the mother plant, these results agree with those of Rodriguez *et al*., (2004). RAPD markers allowed the efficient identification of zygotic and nucellar seedlings, this result is along with the findings of Schafer *et al*. (2004).

Table (8): Number of amplified fragments and detectable specific markers of the nine citrus rootstock genotypes based on RAPD-PCR analysis.

Primer No.	TAF	PB	M.P. of Volka		nV		sV		M.P. of Rangpur		nR		sR		M.P. of Cleo		nC		sC		TSM
			AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	
OP-A02	10	3	9	--	9	--	9	--	9	--	9	--	9	--	8	--	8	--	9	1(-) 1(+)	2
OP-A08	11	8	10	--	10	--	10	--	8	--	8	--	8	--	5	--	5	--	5	--	--
OP-A18	10	7	8	--	8	--	8	--	5	--	5	--	8	--	5	--	5	--	5	1(-) 1(+)	2
OP-B03	10	8	6	--	6	--	7	--	9	--	9	--	9	--	3	--	3	--	3	--	--
OP-B04	10	6	8	--	8	--	6	--	6	--	6	--	7	--	6	--	6	--	5	--	--
OP-B06	17	14	10	--	10	--	10	--	10	--	10	--	13	1(+)	7	--	7	--	9	2(-) 4(+)	7
Total	**68**	**46**	**51**	--	**51**	--	**50**	--	**47**	--	**47**	--	**54**	**1**	**34**	--	**34**	--	**36**	**10**	**11**

TAF= Total amplified fragments for each primer,
AF= Amplified fragments,
(+)= Positive marker, (-)= Negative marker,
M.P= Mother plant

PB=Polymorphic bands of each primer,
SM= Specific markers,
TSM= Total number of specific markers

c. Cluster analysis of the nine citrus rootstock genotypes as revealed by RAPD data:

Due to the complexity to compare the distribution of polymorphic bands generated by the six primers, the generated data matrix of table (9) were used to create the dendrogram for more simple and precise detection and monitoring the genetic similarities and dissimilarities among different sexual and asexual seedlings of citrus and donor mother trees at the DNA level (Fig.7). It could be concluded that most of the nucellar seedlings are true-to-type of the mother trees. The highest similarity was found (1.000) between mother plants of Volka and nucellar Volka, as same as mother plant of Rangpur and its nucellar seedlings, and at the same level of similarity was the mother plant of Cleo and its nucellar seedlings. It was followed in descending order by the mother plant of Volka and sexual Volka (0.950) then the mother tree of Rangpur and sexual Rangpur, as same as nucellar Rangpur and sexual Rangpur (0.911). On the opposite, the highest diversity was found between mother plant of Volka and sexual Cleo, also, between nucellar Volka and sexual Cleo (0.621).

Similarities between the genotypes nucellar and sexual Volka (lanes 1 and 2) and between the genotypes nucellar and sexual Rangpur (lanes 3 and 4) took place. Genotype nucellar and sexual Cleo (lanes 5 and 6) showed a RAPD profile different from all other tested genotypes. All six field strains showed different RAPD profiles. Two isolates (lanes 1and 2) of nucellar Volka and sexual Volka showed high similarity with the genotype-specific strains of the two genotypes isolates (lanes 3 and 4) of nucellar Rangpur and sexual Rangpur. All nucellar plants showed 100% similarity which go along with Bastianel *et al*. (1998).

Table (9): Similarity index (Pairwise comparison) among the nine citrus rootstock genotypes based on RAPD-PCR.

Genotypes	*M.P of Volka	nucellar Volka	sexual Volka	M.P of Rangpur	nucellar Rangpur	sexual Rangpur	M.P of Cleo	nucellar Cleo
nucellar Volka	1.000							
sexual Volka	0.950	0.950						
M.P of Rangpur	0.837	0.837	0.887					
nucellar Rangpur	0.837	0.837	0.887	1.000				
sexual Rangpur	0.857	0.857	0.904	0.911	0.911			
M.P of Cleo	0.659	0.659	0.690	0.691	0.691	0.727		
nucellar Cleo	0.659	0.659	0.690	0.691	0.691	0.727	1.000	
sexual Cleo	0.621	0.621	0.651	0.651	0.651	0.622	0.771	0.771

* M.P: Mother plant

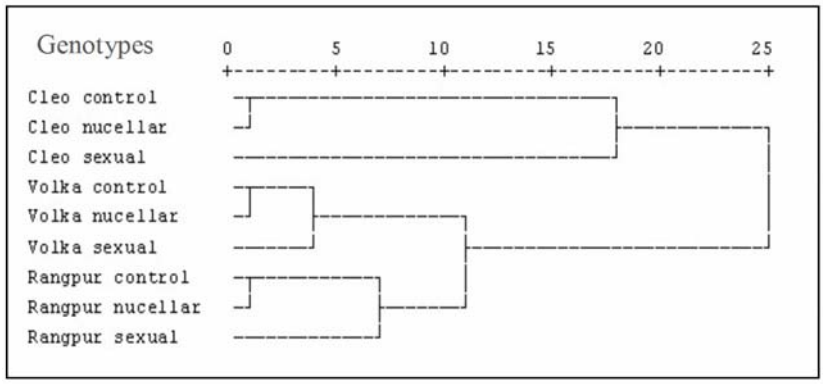

Fig. (7): Dendrogram of the genetic distance among the nine citrus rootstock genotypes based on RAPD-PCR analysis.

The phylogenetic dendrogram for the genetic relationships among the nine citrus rootstock genotypes based on the results of overall markers (RAPD-PCR) (Fig., 7) divided the nine genotypes into two clusters; the first main cluster branched into two subclusters; the first included mother plant of Volka and nucellar Volka, while the second included sexual Volka. The second cluster is divided into two subclusters. The first is branched into two sub-subclusters; the first included mother plant of Rangpur and nucellar Rangpur, while the second sub-subcluster was represented by sexual Rangpur. The second subcluster is divided into two sub-subclusters; the first included mother plant of Cleo and nucellar Cleo, while the second represented in sexual Cleo.

Cluster RAPD-PCR data shown in Table (9) revealed that, the highest similarity value (100.0%) was scored between nucellar Volka and its mother plant, at the same level the nucellar Rangpur and its mother plant and nucellar Cleo and its mother plant, while the lowest similarity value (62.1%) was scored between sexual Cleo and mother plant of Volka, as same as sexual Cleo and nucellar Volka genotypes.

and synonymous with other ones. It is recommended to use vegetative seedlings of Volka as a rootstock for grafting other citrus cultivars. Vegetative seedlings of Rangpur came the second then Cleo was the last.

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تمييز الشتلات الجنسية والنيوسيلية لأصول الفولكامريانا وليمون الرانجبور ويوسفى الكليوباترا باستخدام تكنيك البوادئ العشوائية البلمرة

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تم استخدام تكنيك البوادئ العشوائية البلمرة (RAPD-PCR) لتعريف وتمييز الشتلات النيوسيلية والزيجوتية لشتلات عمر 45 يوم لثلاثة أصول والمسماه ليمون الفولكامريانا، وليمون الرانجبور ويوسفى الكليوباترا ، مقارنة بأشجار الأمهات لتلك الأصول. وتم استخدام البوادئ OP-A2، OP-A8، OP-A18، OP-B3، OP-B4 وOP-B6 لتعريف الأنواع الوراثية التسعة للأصول. الشتلات المصنفة كشتلات زيجوتية (جنسية) كانت ذات سمات جزيئية مختلفة عن تلك الخاصة بالنباتات الأم والشتلات النيوسيلية (الخضرية). ولقد أتاح تكنيك الواسمات الجزيئية العشوائية البلمرة التمييز الفعال للشتلات الزيجوتية والنيوسيلية. اختلفت الحزم الجزيئية بالنباتات الزيجوتية عن تلك المميزة للنباتات الأم. جميع النباتات النيوسيلية أظهرت 100% تشابه. البادئ OP-B04 كان وحده قادرا على تمييز جميع الشتلات الزيجوتية. سجلت أعلى قيمة للتشابه (100%) بين الشتلات النيوسيلية للفولكا وشجرتها الأم، وكانت الشتلات النيوسيلية للرانجبور مع شجرتها الأم وكذلك الشتلات النيوسيلية للكليوباترا مع شجرتها الأم على نفس المستوى من التشابه. أقل قيمة للتشابه كانت بين الكليوباترا الجنسى وكل من النبات الأم والنبات النيوسيلى (77.1%)، فى حين قيما مماثلة كانت بين الفولكامريانا والرانجبور (95% و 91% على التوالى). ولقد أدى بنا ذلك الى استنتاج أن الشتلات النيوسيلية لليوسفى كليوباترا أفضل فى استخدامها كأصل فى تطعيم أصناف الموالح الأخرى. وجاءت الشتلات الخضرية للرانجبور فى المرتبة التالية ثم الشتلات الخضرية لليمون الفولكامريانا فى المرتبة الأخيرة.