Genetic analysis in some citrus accessions using microsatellites - and AFLP-based markers

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ABSTRACT

Twelve ISSR primers, five SSR primer pairs and five AFLP primer combinations were used to estimate the level of polymorphism among 14 Citrus genotypes. The two types of ISSR primers (unanchored and anchored) showed different levels of polymorphism. All of the unanchored primers successfully amplified bands with a total number of 54 of which 44 were polymorphic, representing a percentage of polymorphism of 79.14% and 8.8 polymorphic bands per primer. While, three out of the four 5'-anchored primers amplified scorable bands revealing a total number of 34 bands of which 28 were polymorphic, corresponding to 82.4% polymorphism and an average of 9.3 polymorphic bands per primer. On the other hand, the 3'-anchored primers failed to amplify reproducible patterns even with the modification of the PCR-reaction and temperature profile. Four sets out of five SSR primer pairs amplified a total number of 23 alleles revealing 100% polymorphism, with an average of 5.8 polymorphic alleles per loci. The SSR markers revealed allele frequency ranging from 0.04 to 0.52 . The expected heterozygosity ranged from 0.65 to 0.83 and the effective alleles per locus ranged from 2.9 to 5.9. The five AFLP primer combinations amplified a total number of 311 amplicons, where 304 were polymorphic representing 97.7% polymorphism and 60.8 polymorphic amplicons per primer combination. The microsatellite based-markers revealed genetic similarity ranging from 55.4 [between Ruby red grapefruit (RRG) and Troyer citrange (TC)] to 94.9 [between Washington navel orange (WNO) and Valencia orange (VO)]. While, the highest similarity percentage (73.9%) detected by AFLP markers was between Volkamer lemon (VOL) and Rangpure lime (RL), and the lowest percentage of similarity (8.3%) was observed between Troyer citrange (TC) and Balady sour orange (BSO).Generally, the dendrograms based on the two types of markers (microsatellites and AFLPs) revealed nearly similar topology separating Troyer citrange from the other Citrus genotypes. Moreover, accessions belonging to the same species always clustered together except for the sweet orange group (Valencia orange, Washington navel orange and Robertson navel orange) only in the AFLP dendrogram. All the tested ISSR primer (8) identified ten accessions, and both of Mic4 and Amic1 detected the highest number of identified accessions (4). Moreover, four SSR primer pairs identified six Citrus accessions (Rangpure lime, Willowleaf mandarin, Cleopatra mandarin, Troyer citrange, Robertson navel orange and Abdel Razek mandarin). In addition, SSR markers exhibited species-specific markers. Three species out of eight (C. aurantium, C. deliciosa and C. paradisi) were characterized by specific allele sizes. The AFLP had the potentiality to characterize all the

tested accessions by unique markers. Combination $M_{cag}XE_{aag}$ characterized the highest number of Citrus genotype (10). While, combination $M_{ctc}XE_{agc}$ characterized the lowest number of accession (5). In addition, the genomic DNA of Valencia orange scions grafted onto three different rootstocks (Volkamer lemon, Troyer citrange and Cleopatra mandarin) was analyzed with five different AFLP primer combinations. All the tested combinations revealed new bands (1-6) in the graft-induced variants except for combination M_{ctt} X E_{act} with Troyer citrange and M_{cag} X E_{act} with Cleopatra mandarin. On the other hand, some bands which were present in the scion and (or) stock disappeared in the graft induced variant. The number of these bands ranged from 1 to 7. Moreover, it seems that the examined rootstocks transmitted bands to the graft induced variants, ranging in number from 1 to 6. While, the conserved bands from the scion ranged from 2 (with combination $M_{cac}XE_{acc}$ on both of Troyer citrange and Cleopatra mandarin) to 9 (with combination $M_{ctc}XE_{agc}$ on Volkamer lemon). These results indicated the presence of a genetic relationship between the scion and the stock, however, further studies are needed to elucidate this relationship at the molecular level.

Key words: *Citrus, ISSR, SSR, AFLP, Genotype-specific markers, Scion-stock relationships.*

INTRODUCTION

Genetic analysis including assessment of genetic diversity, relatedness between or within species, population and individuals as well as genotype characterization, are central tasks for many disciplines of biological sciences. Conventionally, genetic analysis was dependent on morphological and/or biochemical markers. During the past few decades, classical strategies of genetic analysis have been increasingly complemented by molecular techniques. The most fundamental of these molecular techniques are DNA markers which portray genome sequence composition, thus, enabling to detect differences in the genetic information carried by the different individuals. Therefore, these markers are powerful tools in genotype identification or fingerprinting and the estimation of relatedness between genotypes. Consequently, they provide the means to utilize our existing germplasm resources to understand fundamental plant processes and mechanisms. Furthermore, marker-mediated genetic analysis elucidates the genetic basis of agronomic characters and leads to their direct manipulation by plant breeders.

Several molecular marker systems have been applied in the last few decades and the development of the polymerase chain reaction (PCR) technology has introduced a considerable number of useful molecular markers. RAPD (Williams *et al.*, 1990) was the first PCR-based marker system used in genetic analysis. Subsequently, a large number of PCR-based marker systems have been developed including microsatellite-based markers and AFLPs.

Microsatellites consist of highly variable tandem repeats of very short motifs (1-6 bp) (Litt and Lutty, 1989). Based on microsatellites two types of DNA markers could be generated, i.e., simple sequence repeats (SSRs) and inter simple sequence repeats (ISSRs). In SSRs, the polymorphism is detected by PCR amplification using primers complementary to unique flanking sequences. While generation of ISSR markers involve PCR amplification of DNA using a

single primer composed of a microsatellite repeated sequence.

SSRs are becoming the markers of choice in genetic studies because they are transferable, multiallelic codominant markers, easily reproducible, randomly and widely distributed along the genome (Rafalski *et al.*, 1995). However, ISSRs have the advantage that they do not require a prior knowledge of sequence.

Amplified fragment length polymorphism (AFLP) is a relatively new DNA fingerprinting technique (Vos *et al.*, 1995), which uses selective amplification of restriction fragments. This PCR-based method is able to generate complex banding patterns, DNA fingerprints of up to at least 100 DNA fragments in each reaction. AFLP is, therefore, potentially very useful in genetic analysis (Hill *et al.*, 1996; Tohme *et al.*, 1996; Paul *et al.*, 1997; Schut *et al.*, 1997; Kardolus *et al.*, 1998; Hussein *et al.*, 2002).

Citrus is one of the most important fruit crops in Egypt and many parts of the world. Genetic studies and improvement of Citrus are hindered by a long juvenility period, high heterozygosity, sterility, sexual incompatibility and high degree of polyembryony. Moreover, in Citrus, grafting scion on different rootstocks produce graft-induced variants. This phenomenon has been also reported in different plant species (Yagishita and Hirata, 1987; Protopapadalis, 1988). However, little is currently known about the molecular basis of scion-stock relationship. The application of molecular markers technology offers great potentiality for Citrus genetic analysis, characterization and improvement.

This paper is aiming at (1) exploring the level of polymorphism among 14 Citrus genotypes using SSRs, ISSRs and AFLPs (2) assessing the genetic relationship among these genotypes (3) characterizing the different accessions by unique markers (4) investigating the relationship between the scion and stock at the molecular level.

MATERIALS AND METHODS

Plant materials

The study included fourteen Citrus assessions provided by the Horticulture Research Institute, ARC, Giza, Egypt. These accessions comprise 7 rootstocks and 7 scions and belong to eight different species as described by Hussein *et al.* (2003). In addition, Valencia orange derived either from nucellar trees or grafting on three different rootstocks (Troyer citrange, Volkamer lemon and Cleopatra mandarin) were used to investigate the genetic relationship between the scion and the rootstock using the AFLP technique.

DNA isolation

Genomic DNA was extracted from leaf samples as described by Porebski *et al.* (1977). For each accession, equal amounts of DNA from 10 individual trees were pooled together to form the sample representing the accession.

ISSR-PCR reaction and thermocycling profile

PCR was performed in 25 μl reaction mix containing 1 X PCR buffer, 2 mM MgC12, 0.2 mM of each dNTPs, 1 μM oligonucleotide primer, 25 ng genomic DNA and 1 unit of hotstart taq DNA polymerase. A high stringency touchdown and hot start thermocycling profile was performed as follows: an initial hot start and denaturation step at 94°C for 15 min followed by one cycle at 94°C for 1 min; (55°C for some primers) for 1 min and 72°C for 2 min.The annealing temperature was lowered each cycle 1° C during nine cycles. This was followed by thirty cycles at 94°C for 1 min; 55°C (or 45°C for some primers) for 1 min and 72°C for 2 min

and an extension cycle at 72°C for 7 min. The PCR products were separated on 1.8 % agarose gel.

SSR assay

Five published sets of primers flanking five (TAA and CAC) SSR regions in the Citrus genome (Kijas *et al.*, 1997) were analyzed to directly amplify these regions. The PCR reaction was conducted in 25 μl reaction volume containing 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 μM of forward and reverse primers, 1U hotstart Taq polymerase (GIBCO, BRL) and 25 ng genomic DNA. Hot start and touchdown profile was used as follows: An initial denaturing step at 94°C for 15 min followed by 35 cycles at 94°C for 30 sec, annealing temperature (Ta) (Table 1) for 30 sec, and primer elongation at 72°C for 1 min. A final extension step at 72°C for 5 min was performed. The PCR products were detected by electrophoresis on polyacrylamide non-denaturing gels stained with ethidium bromide.

AFLP analysis

The AFLP technique was performed as described by Vos *et al.* (1995). Genomic DNA (250 ng) was restricted with Eco RI/Mse I (2.5 U each) in a restriction buffer (50 mM Tris HCl, pH 7.5, 50 mM Mg-acetate, 250 mM K-acetate) in a final volume of 25 ul. Eco RI and Mse I adapters were subsequently ligated to the digested DNA fragments. The adapter-ligated DNA (diluted 1:10) was pre-amplified with AFLP primers each having one selective nucleotide using the following cycling parameters: 20 cycles of 30 sec at 94°C, 60 sec at 56°C and 60 sec at 72°C. The pre-amplified DNA was diluted (1:50), and an aliquot was used for selective amplification with Eco RI and Mse I primers having three selective nucleotides at the 3` ends. The cycling parameters for selective amplification were as follows: 1 cycle of 30 sec at 94°C, 30 sec at 65°C and 60 sec at 72°C. The annealing temperature was then lowered by 0.7°C per cycle during the first 12 cycles and then 23 cycles were performed at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec. The reaction products were resolved on 6 % polyacrylamide sequencing gels followed by silver staining. Five selective primer combinations were used in this study.

Data analysis

The banding patterns generated by microsatellite-based markers or AFLP markers analyses were compared to determine the genetic relatedness of the 14 Citrus genotypes. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical. The genetic similarity and similarity matrix among genotypes were estimated according to Dice coefficient (Sneath and Sokal, 1973). Dice formula: GS(ij)=2a / (2a + b +c), where GS (ij) is the measure of genetic similarity between individuals I and j, (a) is the number of bands shared by I and j, (b) is the number of bands present in I and absent in j , and (c) is the number of bands absent in I and present in j. Cluster analysis was based on similarity matrix obtained with the unweighted pair group method using arithmetic averages (UPGMA) and relationships between genotypes were illustrated as a dendrogram. The observed heterozygosity was calculated as the ratio between heterozygous genotypes and the total analyzed genotypes for each locus. However, the expected heterozygosity for the polymorphic loci (Hav (p)) was calculated as follows: B= np/ (np+nnp) , where B is the fraction of polymorphic loci, np is the number of polymorphic loci and nnp is the number of non- polymorphic loci. Then, Hav(p)= B(SUM Hp/np) (Powell *et al.*, 1996). The effective

alleles per locus were calculated according to Weir (1989) using the formula : 1/(1-Hep), where Hep is the genetic diversity per locus.

RESULTS AND DISCUSSION

Polymporphism detected by inter simple sequence repeats (ISSRs)

In the present study, 12 ISSR primers (6 unanchored, 3 5` anchored and 3 3` anchored) were tested. Among the six unanchored primers only five produced good, reproducible and scorable patterns. The total number of amplified amplicons was 54 fragments, and the number of amplified DNA fragments by each primer ranged from 7-15 fragments. Mic4 amplified the highest number of fragments (15 bands) while, Mic9 produced the lowest number of amplicons (7 bands). The average number of fragments/ primer was 10.8, and the size of these fragments ranged from 200-3000 bp. All the used primers produced polymorphic bands (Fig. 1 and Table 1), ranging in number from 4 to 14 with an average polymorphism/ primer of 8.8. The percent of polymorphism revealed by the different primers ranged from 50% to 100%. In this context, Luro *et al.* (1995) reported that the use of two short repetitive nucleotide sequences (GTG)5 and (GACA)4, which correspond to Mic9 and Mic3, respectively in the present study, produced good amplification patterns in Citrus. Moreover, Gupta *et al.* (1994) obtained good amplification patterns with other genomes derived from different plant species using the (GACA)4 primers. Fang and Roose (1997) stated that, the number of amplified products, generated by ISSR-PCR, depends on the frequency and the occurrence of the particular repeat used as primer in the target genome.

All 3' anchored primers used in this study produced smears or fuzzy patterns that could not be scored. Many factors may affect amplification such as the concentration of Mg++, template concentration, temperature profile, thermalcycler used and even the source of DNA polymerase. However, the modification of PCR amplification conditions did not improve the patterns much. The poor results with these three 3' anchored primers could be due to the characteristics of the primers or to the relative abundance of priming sites in Citrus DNA. It may be necessary to use more 3' anchored primers to ensure the defect of such primers with Citrus. This has been also suggested by Fang *et al.* (1997b). On the other hand, 3 primers out of the four 5' anchored primers amplified scorable fragments (Fig. 1). The total number of amplified fragments was 34 (Table 1) with an average number of 11.3 fragments per primer. The molecular weight of these amplicons ranged from 200 to 3000 bp. The primer Amic1 revealed the highest percentage of polymorphic bands (90.9%), while Amic4 showed the lowest percentage (72.7%). Thus, the average number of polymorphic bands/primer was 9.3. In this respect, Zietkiewicz *et al.* (1994) noted that primers anchored at the 5' end displayed broader specificity than those anchored at the 3' end. On the other hand, Fang *et al.* (1997b) found that in Citrus, the 5'-anchored primers generally produce clearer patterns but fewer and larger fragments than 3'-anchored primers.

Table(1): Primer names and sequences, annealing temperatures, total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentages of polymorphism as revealed by ISSR markers among the 14 Citrus genotypes.

Primer name and sequence	Annealing temp.	Total # ampl.	Monom. ampl.	Polymor-phic ampl.	Percent of polymorphism
Unanchored repeats					
Mic3 =$(GACA)_4$	45	8	4	4	50
Mic4 =$(GGAT)_4$	45	15	3	12	80
Mic7 =$(CCTA)_4$	45	10	2	8	80
Mic8 =$(CGA)_5$	65-50	14	0	14	100
Mic9 =$(GTG)_5$	50-45	7	1	6	85.7
Total		54	10	44	
Average		10.8	2	8.8	79.14
5'Anchored repeats					
Amic1=CGC$(GATA)_4$	65-50	11	1	10	90.9
Amic4=CGA$(GATA)_4$	60-50	11	3	8	72.7
Amic5=GAC$(GATA)_4$	60-50	12	2	10	83.8
Total		34	6	28	
Average		11.3	2	9.3	82.4
Grand total		88	16	72	81.8

Polymorphism as detected by SSRs

In the present study five published primer pairs flanking simple sequence repeats were employed to investigate the level of polymorphism among the 14 Citrus accessions. (Kijas *et al.*, 1997). One pair of primers (TAA52) failed to amplify any product, even when re-synthesized and modification of conditions was performed. The four SSR primer sets revealed 23 alleles across the 14 accessions, all these alleles were polymorphic, thus revealing a level of 100% polymorphism (Table 2).

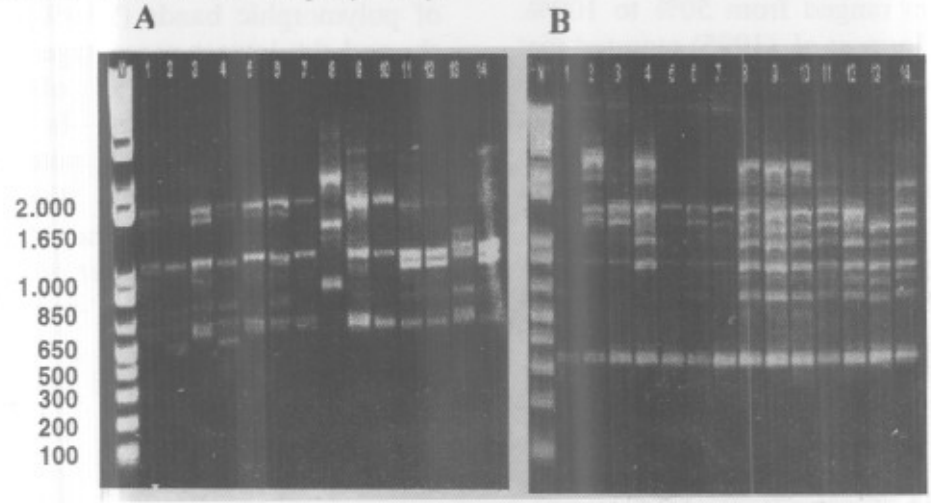

Fig. (1): Electrophoretic separation pattern of the ISSR- PCR products (as revealed on 1.8% agarose gel) using the primers Mic8 (A) and Mic4 (B). M: 1Kb ladder plus DNA marker. Lanes 1 to 14 represent the Citrus genotypes: WNO, WM, VO, AWM, MSG, RNO, RRG, BSO, SSO, RSO, RL, VOL, TC and CM, respectively.

The number of alleles per locus ranged from 4 to 8 with an average of 5.8 alleles per locus. Hokanson *et al.* (2001) detected a comparable number of alleles per locus (5 to 8) among 142 Malus species. While, Yamamoto *et al.* (2001) obtained 79 alleles from 7 SSR primers, with an average of 11 alleles per locus and a range from 9 to 14 alleles per locus in pear. As shown in Table (2), the size of the detected alleles ranged from 148 to 205 bp. This reflects a large difference in the number of repeats between the different alleles.

In agreement with the present results, Kijas *et al.* (1995) used the SSR primers specific for the TAA1 locus in citrus and obtained alleles with sizes ranging from 161 to 188 bp. As shown in Fig. (2), TAA15 revealed a multi-band pattern. This pattern did not change even, when modification of the reaction components and raising annealing temperature during the touchdown profile was performed. These multiple products may result from multiple priming sites along the genome. This phenomenon is quite common since microsatellite sequence may be associated with highly repetitive DNA (Smith and Davey, 1994).

Table(2): Total number of allelic forms, their description and the level of polymorphism among the 14 Citrus genotypes.

Locus and primer sequence		Annealing temp.	Observ. size (bp)	Total # of Alleles	Polym. Alleles	% of Polym.
TAA1	F=GACAACATCAACAACGCAAGAGC R=AAGAAGAAGAGCCCCCATTAGC	65-55	161-181	5	5	100
TAA15	F=GAAAGGGTTACTTGACCAGGC R=CTTCCCAGCTGCACAAGC	65-55	148-200	8	8	100
TAA27	F=GGATGAAAAATGCTCAAAATG R=TAGTACCCACAGGGAAGAGAGC	65-55	150-173	4	4	100
CAC23	F=ATCACAATTACTAGCAGCGCC R=TTGCCATTGTAGCATGTTGG	65-55	176-205	6	6	100
Total				23	23	100
Average				5.75	5.75	100

Moreover, Kijas *et al.* (1997) found up to six bands per diploid genome of Citrus at the CAC33 locus. They suggested that this may be due to the amplification of microsatellite array duplicated within the genome.

In the present investigation, allele frequencies ranged from 0.04 to 0.52 in the different genotypes (Table 3). The lowest allele frequency was obtained for one allele (179 bp) of the TAA1 locus, three different alleles of the locus TAA15 (191 bp, 172 bp and 148 bp), and one allele (196 bp) of the locus CAC23. In this respect, Sanchez-Escribano *et al.* (1999) found allele frequencies ranging from 0.01 to 0.78 in Vitis.

While, Perera *et al.* (2000) detected allele frequencies ranging from 0.035 to 0.768 in coconut. On the other hand, Hokanson *et al.* (2001) reported that frequencies for individual alleles within 142 Malus accessions at all the tested loci were generally low, with only four

alleles having values greater than 20% at a locus.

The amplification of more than one band per genotype by some SSR primers may be due to residual heterozygosity. In this investigation, the observed heterozygosity was calculated as the ratio between heterozygous genotypes and the total analyzed genotypes for each locus (Table 3). The observed heterozygosity ranged from 14% to 93%. On the other hand, expected heterozygosity is the possibility that two individuals taken at random from a given sample will have different alleles at a locus. As shown in Table (3), the expected heterozygosity ranged from 0.65 to 0.83. Sanchez-Escribano *et al.* (1999) found that the observed and expected heterozygosity within a collection of 43 table grape cultivars ranged from 38%-80% and from 39% to 95%, respectively. While, Hokanson *et al.* (2001) showed in Malus species an expected heterozygosity ranging from 42.3% to 94.8% and an observed heterozygosity ranging from 11.3% to 90.9%. The expected heterozygosity value did not always correspond with the level of heterozygosity at a given locus. For example, eventhough the direct count heterozygosity at TAA27 was the lowest in this study (2), the expected heterozygosity value at the locus was 0.69. This is due to the fact that expected heterozygosity is a statistical estimate based on Hardy- Weinberg expectation. In contrast, the direct count heterozygosity statistical is an actual count of heterozygous genotypes. Another type of genetic parameters for evaluating marker loci is the effective alleles per locus (Aep). Effective alleles per locus were calculated according to Weir (1989) using the formula: 1/(1-Hep) where Hep is the genetic diversity per locus. The effective alleles per locus ranged from 2.9 for the TAA1 locus to 5.9 for the TAA15 locus (Table 3).

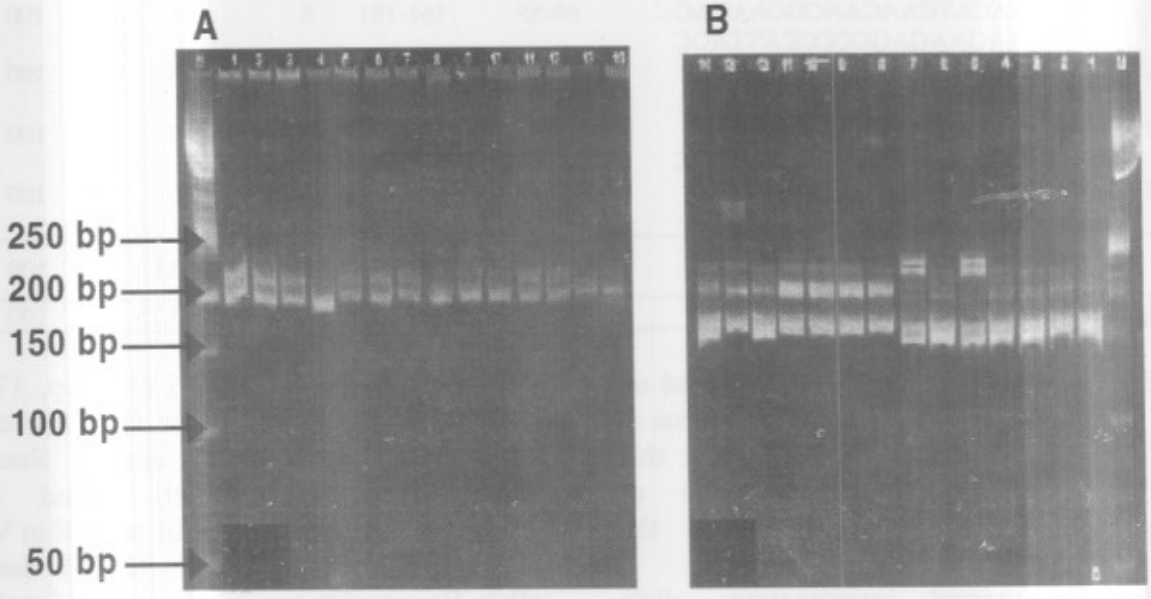

Fig. (2): The separation pattern of SSR-PCR products on 6% non-denaturing polyacrylamide gel. Using the flanking primer sets Mico5 (A) and Mico3 (B). Lanes 1 to 14 represent Citrus genotypes: WNO, WM, VO, AWM, MSG, RNO, RRG, BSO, SSO, RSO, RL, VOL, TC and CM. M: DNA molecular weight marker (50 bp ladder).

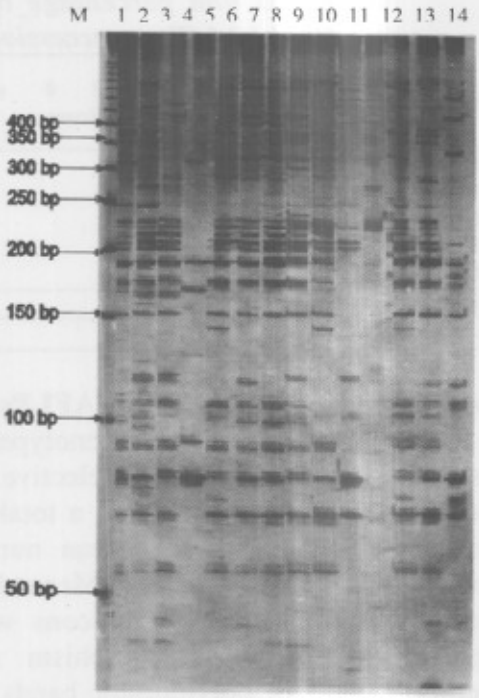

Fig. (3): AFLP profiles of the 14 citrus accession as reveled as by the primer combination M_{cac} X E_{acc}. Lanes 1-14 represent: WNO, WM, VO, AMW, MSG, RNO, RRG, BSO, SSO, RSO, RL, VOL, TC and CM. M: DNA molecular weight marker (50 bp DNA ladder).

Table (3): The allele sizes, allele frequencies, expected heterozygosity ($H_{av(p)}$), number of heterozygotes (% of observed heterozygosity) and effective alleles per locus.

Locus	Allele size (bp)	Allele frequency	Expected $H_{av(P)}$	Number of $H_{av(P)}$ (Observed $H_{av(P)}$)	A_{ep}
TAA1	181	0.22	0.65	9 (64%)	2.9
	179	0.04			
	170	0.13			
	167	0.09			
	161	0.52			
TAA15	200	0.07	0.83	13 (93%)	5.9
	197	0.11			
	191	0.04			
	183	0.07			
	175	0.26			
	172	0.04			
	162	0.37			
	148	0.04			
TAA27	173	0.43	0.69	2 (14%)	3.2
	167	0.31			
	152	0.13			
	150	0.13			
CAC23	205	0.39	0.72	9 (64%)	3.6
	202	0.09			
	200	0.31			
	196	0.04			
	188	0.13			
	176	0.40			

Table (4): Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by AFLP markers among the 14 Citrus accessions.

Primer combination	Total # of amplicons	Monomorphic amplicons	Polymorphic amplicons	% of polymorphism
Mcct X Eact	79	5	74	93.7
Mcag X Eaag	75	0	75	100
Mcac X Eacc	73	1	72	98.6
Mcag X Eact	49	1	48	98
Mctc X Eagc	35	0	35	100
Total	311	7	304	97.7
Average	62.2	1.4	60.8	

Polymorphism as detected by AFLPs

The fourteen Citrus genotypes were assayed for AFLP using 5 selective primer combinations, which generated a total of 311 amplicons (Table 4). The mean number of amplicons per assay was 62.2. Meanwhile, the number of polymorphic amplicons was 304 representing 97.7% polymorphism and an average number of polymorphic bands of 60.8 per AFLP primer combination. The high percent of polymorphism reflects the genetic differences among the tested accessions which belong to 8 different species. This finding is in agreement with Negi *et al.* (2000), who found an average of 82% polymorphic fragments per assay within Withania species. They attributed the high level of polymorphism to the use of different species. As shown in Fig. (3), the size of AFLP fragments generated by the different primer combinations ranged from 50-650 bp and the number of amplicons produced by the different primer combinations ranged from 35 (M_{ctc} X E_{agc}) to 79 ($M_{cct}XE_{a}$. In this concern, Han *et al.* (2000) stated that the AFLP amplified fragments ranged from 65 to 450 bp in tea species. Goulao *et al.* (2001) reported a range of 34 to 66 fragments per primer pair with an average of 47.6 fragments per primer in apple cultivars and a range of fragment sizes from 100 to 1000 bp. Combination M_{cag} X E_{aag} and M_{ctc} X E_{agc} exhibited the highest percent of polymorphism (100%). Therefore, these combinations were the most informative. However, combination M_{cct} X E_{act} showed the lowest percentage of polymorphism (93.7%). These results confirmed the high multiplex ratio expected with AFLP markers, as reported by Paran *et al.* (1998) and Degani *et al.* (2001).

Genetic Relationships and cluster analysis as revealed by micosatellite based-markers. The scoring data resulting from ISSR and SSR were compiled and analyzed using the Dice similarity coefficient. The genetic similarity matrices based on the Dice coefficients were used in the cluster analysis to generate the dendrogram using the UPGMA. The estimated similarities among the 14 Citrus genotypes ranged from 55.4 [between Ruby red grapefruit (RRG) and Troyer citrange (TC)] to 94.9 [between Washington navel orange (WNO) and Valencia orange (VO)].

The microsatellite-based dendrogram obtained from UPGMA cluster analysis of genetic distances (GDs) is presented in Fig (4).

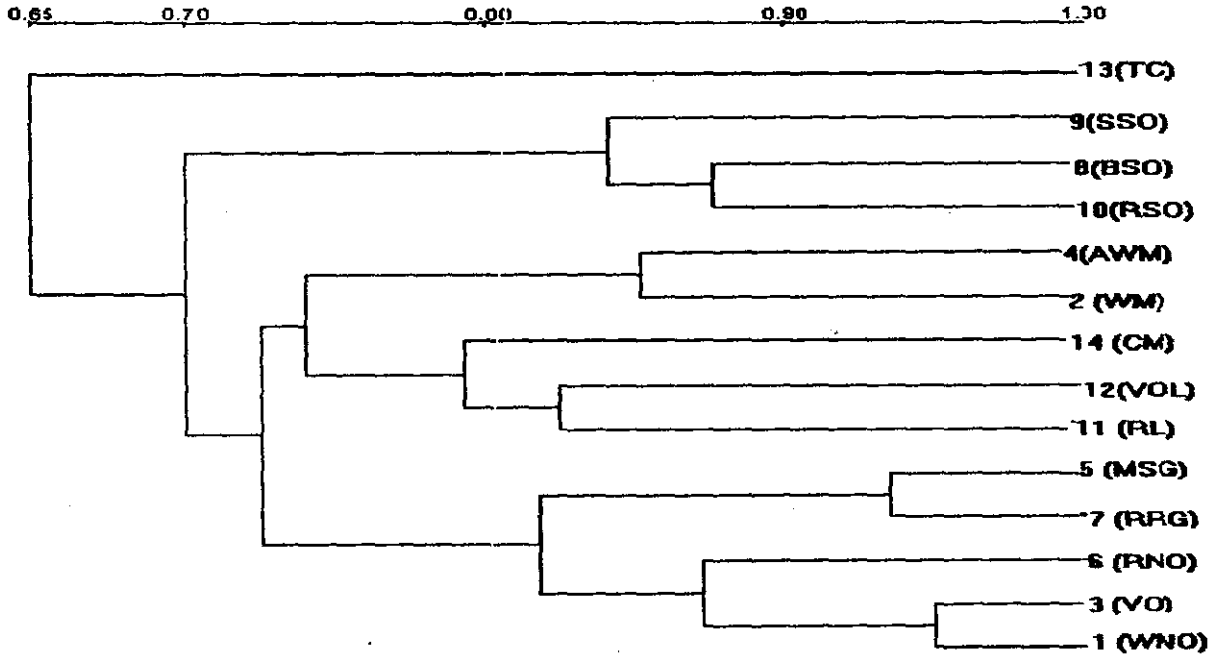

Fig. (4): Dendrogram for the 14 Citrus genotypes constructed from microsatellites data using unweighted pair-group arithmetic average (UPGMA) and similarity matrices computed according to Dice coefficients.

The dendrogram revealed that Troyer citrange (TC) was separated from the remaining 13 genotypes. The cluster containing the 13 genotypes was divided into two main subclusters, one collecting the Sour orange accessions (Spanish, Balady and Brazilian sour orange) belonging to the species C. aurantium. The second subcluster was divided into two groups, the first group divided into two subgroups, one containing the Washington navel orange (WNO), Valencia orange (VO) and Robertson navel orange (RNO) belonging to C. sinensis, and the second subgroup containing Marsh seedless grapefruit (MSG) and Ruby red grapefruit (RRG) both belonging to C. paradisi. The second group was also divided into two subgroups, the first subgroup collecting the Rangpure lime (RL) (C. limonia), Volkamer lemon (VOL) (C. volkameriana) and Cleopatra mandarin (CM) (C. reticulata). However, the second subgroup contained the Willowleaf mandarin (WM) and Abdel Razek willowleaf mandarin (AWM), both belonging to C. deliciosa.

Genetic relationships and cluster analysis as revealed by AFLPs

The highest similarity percentage was observed between Volkamer lemon (VOL) and Rangpur lime (RL) (73.9%). On the other hand, the lowest percentage of similarity was exhibited by Troyer citrange (TC) with all the remaining Citrus accessions ranging from 8.3 to 11.8, reflecting the different genetic background of Troyer citrange.

The AFLP-based dendrogram (Fig. 5) was divided into three main clusters, two of these clusters included only one genotype, i.e., one cluster included Troyer citange (TC). While the second cluster included Cleopatra mandarin (CM). On the other hand, the third cluster was divided into two subclusters, one of them was divided into two groups, the first

group contained only Spanish sour orange (SSO). However, the second group was divided into two subgroups, one containing Brazilian and Balady sour orange, and the other containing Rangpure lime (RL) and Volkamer lemon (VOL). The second subcluster was divided into three groups, the first group was divded into two subgroups, one collected Abdel Razek willowleaf mandarin (AWM) and Willowleaf mandarin (WM). While, the second subgroup contained Ruby red grapefruit (RRG), Marsh seedless grapefruit (MSG) and Robertson navel orange (RNO). On the other hand, the second and third groups contained Washington navel orange (WNO) and Valencia orange (VO), respectively. The lower genetic distances among the studied sweet orange accessions (Washington navel orange, Robertson navel orange and Valencia orange) and the scattering of these accessions in different groups may be due to the simultaneous mutations arisen within C. sinensis (Fang and Roose, 1997).

The AFLP assay was efficient enough to distinguish CM as a separate taxonomic entity. This is in accordance with its taxonomic status as a distinct species (C. reticulata).

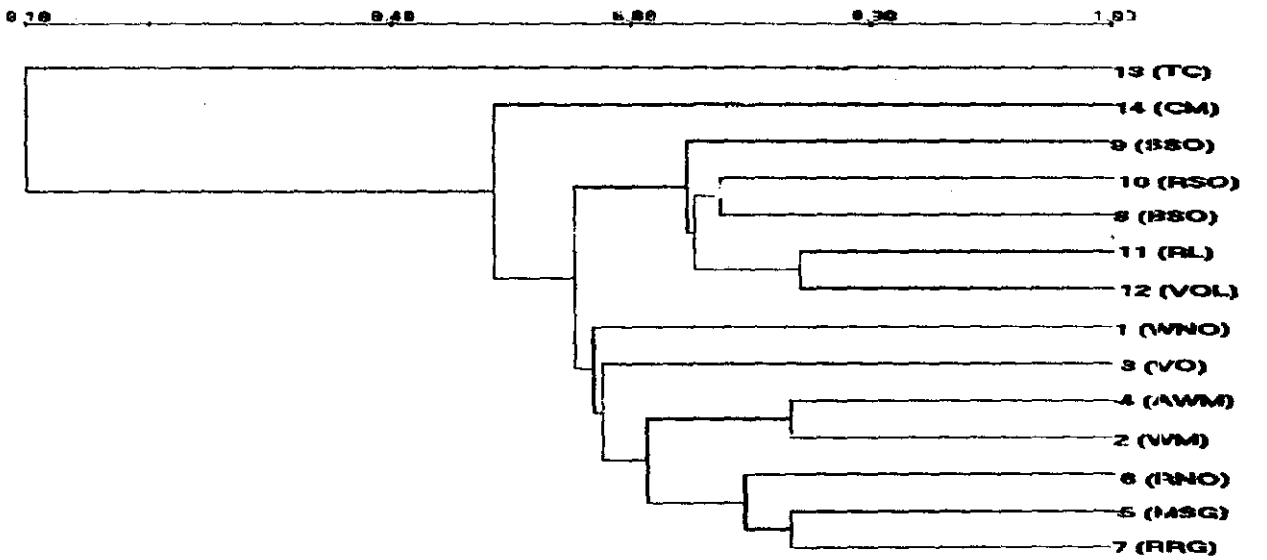

Fig. (5): Dendrogram for the 14 Citrus genotypes from AFLPs data using unweighted pair group arithmetic average (UPGMA) and similarity matrices computed according to Dice coefficients.

Genotype identification by unique ISSR markers

Genotype-specific ISSR unique markers could distinguish 10 out of the 14 Citrus accessions. The ISSR-primers generating the markers and the markers approximate size as shown in Table (5). Among these 10 genotypes, Troyer citrange and Balady sour orange were characterized by both positive and negative unique ISSR markers. However, the remaining accessions were characterized by only positive or negative unique markers. Twenty-four unique markers were generated from 8 primers (with an average of 3.0 markers / primer). Troyer citrange was characterized by 33.3% of these unique markers. This reflects its unique genetic background as a hybrid between C. sinensis X

P. trifoliata. Primers Mic4 and Amic1 together represent putative informative primers that could identify 7 different Citrus genotypes. While, the remaining primers were able to identify one or two genotypes. Fang and Roose (1997) used ISSR to distinguish between cultivars within the same variety of Citrus and they distinguished 14 of 33 sweet orange, one of seven grapefruit and five of six lemon cultivars. Moreover, Fang *et al.* (1998) concluded that accessions with many unique ISSR fragments could be useful for expanding the germplasm base of Citrus breeding programs.

Genotype identification by unique SSR markers

As shown in Table (6), all the tested SSR primer pairs characterized 6 different genotypes. Rangpure lime was characterized by the 179 bp allele at the TAA1 locus. However, the TAA15 characterized 3 different genotypes (Willowleaf mandarin, Cleopatra mandarin and Troyer citrange). The CAC23 locus characterized the Robertson navel orange and Abdel Razek mandarin by the presence of the 196 bp and 176 bp alleles, respectively. While, the TAA27 did not reveal any unique genotype-specific marker. Furthermore, three SSR primer pairs were informative in revealing unique species-specific markers, as shown in Table (6). Perera *et al.* (2000) found that eight microsatellite primer pairs uniquely discriminated 116 out of 130 coconut genotypes. Moreover, Teulat *et al.* (2000) stated that the SSRs were informative in revealing genetic differences among fourteen populations of coconut.

Genotype identification by unique AFLP markers

In the present study, the AFLP technique was successful in characterizing all the tested Citrus accessions as shown in Table (7). A total of seventy unique AFLP markers were identified in the fourteen Citrus accessions of which 65 were unique positive markers and five were unique negative markers. The total number of unique markers per genotype ranged from 1 to 10. The highest number (10) of unique markers was exhibited by Cleopatra mandarin (CM). While the lowest number (1) of unique markers was revealed by both of Brazilian sour orange (RSO) and Robertson navel orange (RNO). All the 14 Citrus accessions were characterized by unique positive markers except Brazilian sour orange which was characterized only by one unique negative marker (170 bp) with the primer combination M_{cag} X E_{aag}. Three other Citrus genotypes were characterized by unique negative markers, i.e., Troyer citrange which was characterized by two unique negative markers (130 bp and 112 bp) with primer combination M_{ctt} X E_{act}, and Rangpur lime which was characterized by a 165 bp unique negative marker with the same primer combination. Paran *et al.* (1998) stated that, two AFLP primer pairs (M_{cag} X E_{acg} and M_{cta} X E_{aca}) were sufficient to distinguish four cultivars of pepper from each other.The results of the present study assessed the usefulness of the molecular markers in identifying the Citrus accessions. The AFLP technology was able to distinguish all the tested accessions.

While, the microsatellite-based markers identified a lower number of accessions. However, taking into consideration the number of primers or primer combinations used in each marker type, the cost and simplicity of the technology, it could be concluded that the different types of markers are equally efficient in characterizing the Citrus genotypes and provide important advantages for genotype profiling and should play a major role in breeding programs.

Table (5): Citrus genotypes characterized by unique positive and / or negative ISSR markers, markers size and total number of markers identifying each genotype.

Genotype	Unique positive markers			Unique negative markers			Grand total
	Size (bp) of the marker band	Primer	Total # of markers/ accession	Size (bp)of the marker band	Primer	Total # of markers/ accession	
Troyer citrange	500 400 250 900 1000	Mic9 Mic4 Mic4 Mic8 Amic1	5	600 1000 1000	Mic9 Amic1 Amic5	3	8
Balady sour orange	1000 3000 1400 1200	Mic3 Mic8 Mic8 Mic8	4	1000 500	Mic8 Mic8	2	6
Brazilian sour orange				650	Mic7	1	1
Cleopatra mandarin	250 500 1200	Mic9 Mic4 Amic4	3				3
Rangpure lime	450	Mic4	1				1
Volkamer lemon	550	Amic1	1				1
Robertson navel orange				850	Mic4	1	1
Willowleaf mandarin	200	Amic4	1				1
Abdel Razek mandarin	2950	Amic1	1				1
Marsh seedless grapefruit	3000	Amic1	1				1
10 genotypes		6 primers	17		6 primers	7	24

Table (6): Unique genotype - and species - specific SSR markers and their corresponding allele sizes at four different loci.

Locus	Genotype	Species	Allele size (bp)	Marker type
TAA1	Rangpure lime		179	Positive
		C. aurantium	170	Positive
		C. deliciosa	167	Positive
		C. deliciosa	161	Negative
TAA15	Willowleaf mandarin		191	Positive
	Cleopatra mandarin		172	Positive
	Troyer citrange		148	Positive
		C. deliciosa	200	Positive
		C. aurantium	197	Positive
TAA27		*C. paradisi*	152	Positive
		C. paradisi	150	Positive
CAC23	Robertson navel orange		196	Positive
	Abdel Razek mandarin		176	Positive

Analysis of scion-stock genetic relationships

An attempt was performed to examine the genetic changes produced by various scion / stock combinations of Valencia orange (VO) grafted on three rootstocks: Volkamer lemon (VOL), Troyer citrange (TC) and Cleopatra mandarin (CM) at the molecular level using five different AFLP primer combinations. As shown in Fig. (6) and Table (9), Valencia orange (VO) grafted on the three rootstocks revealed new amplification products not observed in either the scion or the rootstock. The new bands ranged from 1 to 5 and from 0 to 6 in Valencia orange grafted on Volkamer lemon (VO/VOL) and in Troyer citrange (VO/TC) and Cleopatra mandarin (VO/CM), respectively. In addition, comparing the AFLP profiles of the graft-induced variants with the scion profiles it was noticed that some bands were conserved, while others were absent. The number of absent bands ranged from 1 to 6 in VO/VOL, from 1 to 7 in VO/TC and from 4 to 8 in VO/CM. Moreover, comparing the graft-induced variant AFLP profiles with the rootstock profiles, it was noticed that some bands seem to be transmitted from the rootstock to the graft-induced variants. In accordance with the present results, Taller *et al.* (1998) detected RAPD markers in pepper which were found only in the stock cultivar [Spanish paprika (Sp)] and graft induced variants (G_5S_{25}) but were absent in the scion cultivar [Yatsubusa (Y)]. They found new graft induced variant-specific markers, while the loss of Y markers were recognized.

The scoring data (1 for the presence and 0 for the absence) resulting from each of the five AFLP primer combinations were used to compute the similarity matrices among the scion, stock and graft induced variants according to Dice coefficients. Table (8) reveals that the similarity between the scion and the graft induced variants was always higher than the similarity percentage between the graft induced variants and all the tested rootstocks. This confirms that most of the genetic background of the graft induced variants was introduced from the scion.

In conclusion, the results revealed that the scion/ stock relationship is a complicated aspect and differs from one combination to another, depending on the used rootstocks (Table 9). The average number of transmitted bands from the stock varied from one stock to another, i.e., Volkamer lemon stock showed the highest average number of polymorphic transmitted bands (3.2) followed by Troyer citrange (2) and then Cleopatra mandarin (1.8). The number of conserved bands in the scion and the graft induced variants was also influenced by the stock, the scion on Troyer citrange revealed the highest average number of conserved bands (6.4). While, Volkamer lemon and Cleopatra mandarin detected an average of 5 and 4.2 conserved bands, respectively. These results confirm the findings of Protopapadadis (1988), who reported that the enzymatic activities of peroxidase for one variety of Citrus evolves according to the nature of the rootstock.

Although, all the AFLP primer combinations revealed the influence of the grafting on the graft induced variants, however, this effect varied according to the rootstock. This variation concerning the influence of the rootstock in the different scion-stock combinations may be due to several reasons: Hartmann and Kester (1978) suggested a possible interpretation on the basis of fusion of nuclei of vegetative cells coming from scion and stock. On the other hand, Ohta and Choung (1975) suggested that hereditary changes of certain Mendelian traits in red pepper after grafting are induced by a mechanism similar to the transformation in higher organisms.

Moreover, Hirata and Yagishita (1986) attributed the variation obtained by different grafting combinations to the unstable transmission of changed gene(s). Moreover, the possibility of foreign gene(s) transported from stock to scion through vascular system, integration into the genome, and sexual transmission to the scion plant progeny in graft systems was proposed by Yagishita *et al.* (1990) and Taller *et al.* (1998) in pepper. Therefore, further studies might be required to elucidate the scion-stock relationships at the molecular level.

Table (7): AFLP markers of the fourteen citrus accessions.

Genotype	Unique positive markers			Unique negative markers			Grand total
	Primer combination	Molecular weight	Total	Primer combination	Molecular weight	Total	
Troyer citarange	Mcag X Eaag Mcac X Eacc Mcag X Eact Mctc X Eagc	200 350,340,225,126 300 650	1 4 1 1	Mctt X Eact	130 112	2	9
Rangpur lime	Mcag X Eaag Mcag X Eact Mctc X Eagc	67 340,185 165	1 2 1	Mctt X Eact	165	1	5
Volkamer lemon	Mcag X Eact Mctc X Eagc	251,171 500	2 1				3
Cleopatra mandarin	Mctt X Eact Mcag X Eaag Mcac X Eacc Mcag X Eact	260 155 420,410,225,220 234,215,169	1 1 4 3	Mcag X Eaag	188	1	10
Spanish sour orange	Mctt X Eact Mcac X Eacc Mcag X Eact	56 188,173 267	1 2 1				4
Balady sour orange	Mcag X Eaag	1500,50	2				2
Brazilian sour orange				Mcag X Eaag	170	1	1
Washington navel orange	Mctt X Eact Mcag X Eaag Mcag X Eact Mctc X Eagc	102,101,75,74 183,179,89 800 390	4 3 1 1				9
Robertson navel orange	Mcag X Eaag	140	1				1
Willowleaf mandarin	Mctt X Eact Mcag X Eaag Mcac X Eacc	83 165,124,100,74,65 260	1 5 1				7
Abdel Razek mandarin	Mcag X Eaag Mcac X Eacc	645,150,140,103 275	4 1				5
Valencia orange	Mcac X Eacc Mcag X Eact	155,138,131,124 317	4 1				5
Ruby red grapefruit	Mctt X Eact Mcag X Eaag Mcac X Eacc Mcag X Eact	146 557,230 252 320,90	1 2 1 2				6
Marsh seedless grapefruit	Mcac X Eacc Mctc X Eagc	254,219 267	2 1				3
Total			65			5	70

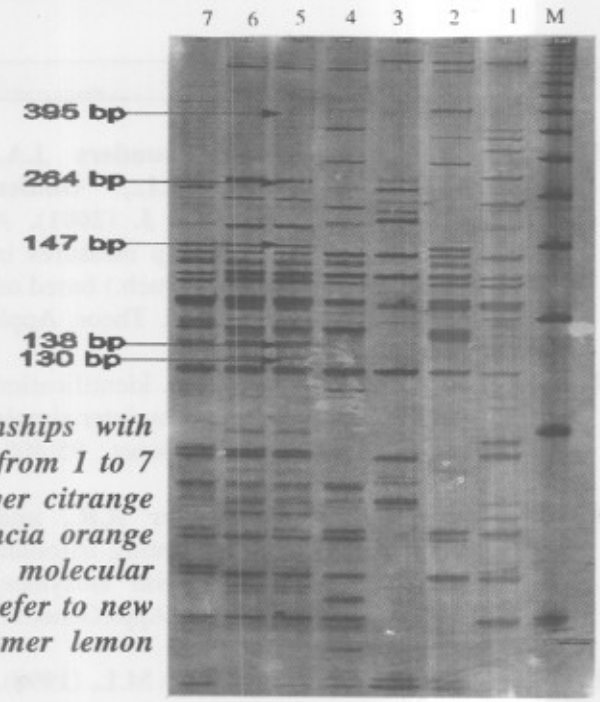

Fig. (6): AFLP profiles of scion-stock relationships with primer combinations $M_{cag}XE_{aag}$. Lanes from 1 to 7 represent Volkamer lemon (VOL), Troyer citrange (TC), Cleopatra mandarin (CM), Valencia orange (VO), VO/VOL, VO/TC, VO/CM. M: molecular weight marker (50 bp ladder). Arrows refer to new bands appeared in the GIV on Volkamer lemon stock.

Table (8): Genetic similarity matrices computed according to Dice coefficients from the scion-stock relationships.

Vol	100					
CM	63.7					
T.C	61.7	71.1				
VO	53.5	56.2	60.0			
VO/CM	51.0	50.9	46.4	52.8		
VO/TC	48.3	43.6	46.8	58.7	62.0	
VO/Vol	46.2	48.3	43.6	53.3	47.4	52.5

Table (9): Summary of the number of new amplicons (NA) in the graft induced variants, absent amplicons in the graft (AA), amplicons conserved in the graft (CG) and amplicons transmitted to the graft from the stocks (AST).

Primer combination	Volkamer lemon				Troyer citrange				Cleopatra mandarin			
	NA	AA	CG	AST	NA	AA	CG	AST	NA	AA	CG	AST
Mctt X Eact	1	4	8	2	0	3	8	2	4	4	5	1
Mcag X Eaag	5	5	3	5	3	3	7	2	6	7	3	2
Mcac X Eacc	4	1	4	6	6	1	2	3	6	6	2	1
Mcag X Eact	3	6	6	1	3	6	7	2	0	5	7	1
Mctc X Eagc	3	6	9	2	2	7	8	1	1	8	4	4
Total	16	22	20	16	14	20	32	10	17	31	21	9
Average	3.2	4.4	5	3.2	2.8	4	6.4	2	3.4	6.2	4.2	1.8

REFERENCES

Degani C., Rowland L.J., Saunders J.A., Hokanson S.C., Ogden E.L., Golden Goldhirsh A. and Galletta G. J. (2001). A comparison of genetic relationship measures in strawberry (Fragaria X ananassa Duch.) based on AFLPs, RAPDs and pedigree data. Theor. Appl. Genet. 117:1-12.

Fang D.Q. and Roose M.L. (1997). Identification of closely related Citrus cultivars by inter simple sequence repeats. Theor. Appl.Genet. 95:408-417.

Fang D.Q., Roose M.L., Kruger R.R., and Federici C.T. (1997b). Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs and ISSR markers. Theor. Appl.Genet.95 :211-219.

Fang D., Krueger R. R. and Roose M.L. (1998). Phylogenetic relationships among selected Citrus germplasm accessions revealed by inter simple sequence repeat (ISSR) markers. J. Amer. Soc.Hort. Sci.123 (4): 612-617.

Goulao L., Cabrita L., Oliverira C.M. and Leitao J.M. (2001). Comparing RAPD and AFLP analysis in discrimination and estimation of genetic similarity among apple (Malus domestica Borkh.) cultivars. Euphytica 119:259-270.

Gupta M., Chyi Y.S., Remero-Severson J. and Owen J.L. (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple sequence repeats. Theor.Appl. Genet.89: 998-1006.

Han T., Jeu M., Eck H.V. and Jacobsen E. (2000). Genetic diversity of Chilean and Brazilian Alstroemeria species assessed by AFLP analysis. Heredity 84:564-569.

Hartmann H. T. and Kester D.E. (1978). Plant Propagation, Principles and Practices. Third edition. pp: 193-194.

Hill M., Witsenboer, H., Zabeau, M., Vos, P., Kesseli, R. and Michelmore, R. (1996). PCR-based fingerprinting using AFLP as a tool for studying genetic relationship in Lettuce spp. Theor. Appl. Genet. 93:1202-1210.

Hirata Y. and Yagishita N. (1986). Graft-induced changes in soybean storage proteins.I. Appearance of the changes. Euphytica 35:395-410.

Hokanson S.C., Lamboy W.F., Szewc-Mcfadden A.K.and McFerson J.R. (2001). Microsatellite (SSR) variation in a collection of Malus (apple) species and hybrids. Euphytica 118: 281-294.

Hussein Ebtissam.H.A., Adawy S.S., El-Kishin D., Moharam H. and El-Itriby H.(2002). Genetic variablity studies and fingerprinting of some Egyptian date palm (Phoenix dactylifera L.) cultivars. 1- A preliminary study using RAPD markers. Arab J.Biotec.5 (2): 217-224.

Hussein Ebtissam.H.A., Abd-alla, S.M., Awad Nahla, A., and Hussein, M.S. (2003). Assessment of genetic variability and genotyping of some Citrus accessions using molecular markers. Arab J.Biotec.6 (2):165-178.

Kardolus G.P., Vaneck, H.J. and Vanberg, R.G. (1998). The potential of AFLPs in biosystematics : a first application in Solanum taxonomy. Pl. Syst. Evol. 210: 87-103.

Kijas J.M.H., Fowler J.C.S. and Thomas M.R. (1995). An evaluation of sequence tagged microsatellite site markers for genetic analysis within Citrus and related species. Genome 38:349-355.

Kijas J.M.H., Thomas M.R., Fowler J.C.S., and Roose M.L. (1997). Integration of trinucleotide microsatellites into a linkage map of Citrus. Theor. Appl. Genet. 94: 701-706.

Litt M. and Lutty J.A. (1989). A hypervariable microsatellite revealed by in vitro amplification of nucleotide repeat within the cardiac muscle actin gene. Amer. J. Hum.Genet.44:397-401.

Luro F., Laigret F.,Bove J.M. and Ollitrault P.(1995). DNA amplified fingerprinting: A useful tool for determination of genetic origin and diversity analysis in Citrus. Hort. Sci. 30(5):1063-1067.

Negi M.S., Singh A. and Lakshmikumaran M. (2000). Genetic variation and relationship among and within Withania species as revelead by AFLP markers. Genome 43: 975-980.

Ohta Y. and Choung V.P. (1975). Hereditary changes in Capsicum annuum L. I. Induced by ordinary grafting. Euphytica 24:355-368.

Paul, S., Wechire, S.N., Powell, W. and **Waugh, R. (1997).** Diversity andgenetic differentiation among populations of Indian and Kenyan tea (Camellia sinensis L. O. kuntze) revealed by AFLP markers. Theor. Appl. Genet. 94:255-263.

Paran I., Aftergoot E. and Shifriss C.(1998). Variation in Capsicum annum revealed by RAPD and AFLP markers. Euphytica 99:167-173.

Perera L., Russell J.R., Provan J. and Powell W.(2000). Use of microsatellite DNA markers to investigate the level of genetic diversity and population genetic structure of coconut (Cocos nucifera L.). Genome 43:15-21.

Porebski S., Bailey L.G. and Baum B.R.(1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharides and polyphenol components. Plant Mol.Biol. Rep.15:8-15.

Powell W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey S. and Rafalski A. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 3:225-238.

Protopapadadis E.E. (1988). Effect of rootstocks on isoenzymic composition of Citrus. Proceeding of the Sixth International Citrus Congress. pp.609-614.

Rafalski J.A., Morgante, M., Vogel, J.M., Powell, W. and Tingey, S.V.(1995). Generating and using DNA markers in plants . In: Birren B. and Lai, E (eds). Non-mammalian Genome Analysis: apractical guide. Academic Press, London New York, pp. 75-134.

Sanchez-Escribano E.M., Martin J.P.,Carreno J.and Cenis J.L.(1999). Use of sequence- tagged microsatellite site markers for characterizing table grape cultivars. Genome 42: 87-93.

Smith D.N. and Davey M.E. (1994). Occurrence and inheritance of microsatellites in Pinus radiata. Genome 37: 977-983.

Sneath P.H.A. and Sokal R.R. (1973). Numerical Taxonomy. Freeman, San Francisco, California.

Taller J., Hirata Y., Yagishita N., Kita M. and Ogata S. (1998). Graft induced changes and the inheritance of several characteristics in pepper (Capsicum annuum L.). Theor. Appl. Genet. 97: 705-713.

Teulat B.,Aldam C., Trehin R., Lebrun R., Barker J.H.A.,Arnold G.M., Krap C.M., Bauduin L.and Rognon F.(2000). An analysis of genetic diversity in coconut (Cocos nucifera) populations from across the geographic range using sequence-tagged microsatellites (SSRs) and AFLPs. Theor.Appl.Genet.100:764-771.

Tohme J., Gonzalez, D.O., Beebe, S. and Duque, M.C. (1996). AFLP analysis of gene pool of a wild bean core collection. Crop Science 36: 37-53.

Vos P., Hogers R., Bleeker M., Reijans M., Vande Lee T., Hornes M., Freijters A., Pot J., Peleman J., Kuiper M. and Zabeau M. (1995). AFLP :A new technique for DNA fingerprinting. Nucleic. Acids Res. 23:4407-4417.

Weir B.S. (1989). Sampling properties of gene diversity. In : Brown A.H.D., Clegg M.T., Kahler H.L. and Weir B.S. (Eds.), Plant Population Genetics, Breeding and Genetic Resources. Sinauer Associates, Sunderland, Mass, pp. 23-246.

Williams J.G.K., Kublik A.R., Livak K.J., Rafalsky J.A. and Tingey S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acid.Res.(18):6531-6535.

Yagishita N. and Hirata Y. (1987). Genetic nature of bushy plant type in the variant strain induced by grafting in Capsicum annuum L. Euphytica 35:17-23.

Yagishita N., Hirata Y., Mizukami H., Ohashi H. and Yamashita K.(1990). Genetic nature of low capsaicin content in the variant strains induced by grafting in Capsicum annuum L. Euphytica 46:249-252.

Yamamoto T., Kimura T., Sawamura Y., Kotobuki K., Ban Y., Hayashi T. and Matsuta N. (2001). SSRs isolated from apple can identify polymorphism and genetic diversity in pear.Theor.Appl.Genet.102:865-870.

Zietkiewicz E., Rafalski A.and Lab Unda D. (1994). Genome fingerprinting by simple sequence repeat (SSR). Anchored polymerase chain reaction amplification. Genomics 20: 176-183.

الملخص العربي

التحليل الوراثى لبعض أصناف الموالح باستخدام الواسمات الجزيئية (الميكروستالايت و الAFLP)

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تم دراسة التباين الوراثى بين ١٤ صنف من الموالح بواسطة ثلاثة من تقنيات الواسمات المعتمدة على الـ PCR وهى الـ ISSR ، SSR ، والـ AFLP حيث تم إستخدام ١٢بادئ ISSR ، ٥ أزواج من بادئات الـ SSR و ٥ توليفات من بادئات الـ AFLP. وقد أوضح نوعى البادئات التابعة للـ ISSR (anchored & unanchored) مستويات مختلفة من التباين بين الأصناف حيث نجحت بادئات الـ unanchored فى إظهار ٥٤ شظية من ال د ن أ منها ٤٤ شظية تظهر تباين بين الأصناف وبذلك تكون نسبة التباين ٧٩,١٤% ومتوسط عدد الشظايا المتباينة التى يظهرها البادىء الواحد هو ٨,٨ . بينما أعطت ثلاثة من بادئات الـ anchored -'5 الاربعة المستخدمة فى الدراسة ٣٤ شظية د ن أ منها ٢٨ شظية تظهر تباين حيث كانت نسبة التباين فى هذه الحالة ٨٢,٤% ومتوسط عدد الشظايا المتباينة لكل بادىء ٩,٣. بينما فشلت بادئات الـ anchored-'3 فى إعطاء نتيجة واضحة حتى مع تغيير ظروف التفاعل أو درجات الحرارة .كما أوضحت الدراسة باستخدام ٤ أزواج من بادئات الـ SSR ٢٣ أليل جميعها أظهرت تباينات بين الأصناف المدروسة و بذلك كانت نسبة التباين ١٠٠% و كان متوسط عدد الاليلات على مستوى الموقع الواحد ٥,٨. تراوح معدل تكرار واسمات الـ SSR من ٠,٠٤ حتى ٠,٥٢ كما تراوحت الهيتروزيجوزتى (Heterozygosity) من ٠,٦٥ حتى ٠,٨٣ تراوح عدد الأليلات الفعالة (effective alleles)لكل موقع SSR بين ٢,٩ حتى ٥,٩ أما الخمس توليفات المستخدمة فى تقنية الـ AFLP فقد أظهرت ٣١١ شظية د ن أ كعدد كلى منها ٣٠٤ توضح تباينات على مستوى الأصناف المدروسة مما اعطى نسبة من التباين تعادل ٩٧,٧ % ومتوسط ٦٠,٨ شظية متباينة لكل توليفة من البادئات المستخدمة. اما الميكروستالايت فأعطت نسبة تشابه تتراوح بين ٥٥,٤ بين جريب فروت الروبى رد والترويز سيترانج و ٩٤,٩% بين البرتقال بسره الواشنجطن وبرتقال الفالنشيا ٠ بينما أظهرت تقنية الـAFLP أعلى نسبة تشابه (٧٣,٩%) بين ليمون الفولكا ماريانا و ليمون الرانجبور و أقل نسبة تشابه (٨,٣%) بين الترويز سيترانج والنارنج البلدى .استخدمت درجات التشابه الناتجة من تقنية الميكروستالايت و الـ AFLP لرسم الدندروجرام الذى يوضح درجات القرابة بين الاصناف تحت الدراسة واشتركت التقنيات المستخدمة فى فصل الترويز سيترانج فى مجموعة منفردة ٠ بالإضافة إلى أن الأصناف التى تنتمى لنفس النوع كانت دائما تقع فى مجموعة واحدة فيما عدا مجموعة البرتقال الحلو فى دندروجرام الـ AFLP. ميزت بادئات الـ ISSR ١٠ أصناف حيث امكن باستخدام كل من البادئين Mic4, Amic1 تمييز أكبر عدد من الأصناف المدروسة (٤) .بالإضافة إلى ذلك فإن ٤ أزواج من بادئات الـ SSR ميزت ٦ أصناف مختلفة من الموالح (ليمون رانجبور ، اليوسفى البلدى ، يوسفى كليو باترا ، الترويز سيترانج ، يوسفى عبد الرازق ، البرتقال بسره روبرتسون). و كذلك فان بادئات الـSSR أظهرت واسمات خاصة بتعريف النوع حيث عرفت ٣ من ٨ أنواع من الموالح المستخدمة بالدراسة . اما الـAFLP فكان اكثر التقنيات كفاءة فى التميز بين الاصناف حيث امكن باستخدام ٥ توليفات من البادئات تحديد واسمات فريدة خاصة بكل صنف من أصناف الموالح الـ١٤. إستطاع زوج البادئ $M_{cte}XE_{agg}$ ان يميز اكبر عدد من الاصناف (١٠) بينما ميز زوج البادئ $M_{cte}XE_{age}$ اقل عدد من السلالات (٥).تم تحليل ال د ن أ الناتج من جيل طعوم الفالنشيا على أصول الفولكا ماريانا، الترويز سيترانج، يوسفى كليوباترا بإستحدام ٥ توليفات من بادئات الـ AFLP.كل التوليفات المستخدمة فى الدراسة حيث أظهرت عدد من شظايا الـ د ن أ الجديدة (١-٦) فى الطعم فيما عدا $M_{ctt}XE_{act}$ مع الترويز سيترانج و $M_{cag}XE_{act}$ مع يوسفى كيلوباترا. من ناحية أخرى بعض الشظايا التى كانت موجودة فى الاصل و (أو) الصنف البذرى اختفت من الطعم بعد التطعيم وتراوح عدد هذة الشظايا من ١-٧. كما يبدو ان الاصول المستخدمة فى الدراسة نقلت شظايا د ن أ الى الطعم و كان عددها يتراوح من ١-٦.بالاضافة الى ذلك كان هناك عدد من الشظايا التى استمرت فى التواجد فى الطعم و كانت موجودة فى الصنف البذرى حيث تراوح عددها بين ٢ و ٩.هذة النتائج تدل على وجود علاقة وراثية بين الطعم و الاصل لكن هناك حاجة لمزيد من الدراسات لتفسير هذة العلاقة على المستوى الجزيئ.