MOLECULAR FINGERPRINTING AND MARKER-ASSISTED SELECTION IN *CITRUS* SPECIES

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Citrus is one of the major fruit crops all over the world. The genus *Citrus* belongs to the subtribe Citrinae, tribe Citreae, subfamily Aurantioideae of the family Rutaceae. Using morphological traits, it is difficult to distinguish between many *Citrus* taxa because some taxa are distinguishable only by fruit traits and *Citrus* trees usually do not bear fruits until 3-4 years after planting. Moreover, conventional plant breeding methods have a very little impact on the development of new improved varieties of *Citrus*. A long juvenility period, high heterozygosity, sterility, sexual incompatibility and high degree of polyembryony in several species are the main roadblocks for *Citrus* improvement. DNA marker techniques offer a great potential as a tool for a wide range of areas in *Citrus* improvement (Cabrita *et al*., 2001).

A wide variety of DNA-based markers have been developed in the past few years. RFLPs (Restriction fragment length polymorphism) were the first molecular markers (Botstein *et al*., 1980) generated for genome analysis and mapping. However, the development of the polymerase chain reaction (PCR) technology has introduced a considerable number of useful molecular markers, e.g. Randomly Amplified Polymorphic DNA (RAPDs), microsatellites (SSRs), Inter Simple Sequence Repeats (ISSRs) and Amplified Fragment Length Polymorphism (AFLPs). PCR-based markers share a number of general advantages over RFLP technology, the major advantage is the speed with which results are generated.

RAPD analysis can be used to identify many useful polymorphisms quickly and efficiently and as such it has tremendous potential for use in cultivar identification, (Koller *et al*., 1993). RAPD markers usually show dominant expression and are scored for the presence or absence of each amplified band and they have been developed for a number of species including several fruit crops. (Chaparro *et al*., 1994).Microsatellite – based markers consist of highly variable tandem repeats of very short motifs (1- 6 bp), which are dispersed throughout the genomes (Litt and Lutty, 1989). ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite repeated sequence. The

Amplified Fragment Length Polymorphism (AFLP) technology has been developed (Vos *et al*., 1995) as a powerful fingerprinting technique. AFLP markers detect the polymorphism on the level of restriction enzyme sites. It is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonuclutide adaptors of few nucleotide bases. This method generates a large number of restriction fragments facilitating the detection of polymorphic. Therefore, the present investigation has been carried out to address the following objectives; to collect some species of *Citrus* and develop fingerprints and molecular markers to assist selection for these species.

MATERIALS AND METHODS

This study included nineteen accessions of *Citrus* collected from the research farm of the Horticulture Department, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima Egypt. These accessions belong to different species (Table 1).

Methods

1. Randomly amplified polymorphic DNA (RAPD)

Isolation of DNA leaves was done using DNeasy plant Mini Kit (QIAGEN). PCR reaction was conducted using 18 arbitrary 10-mer primers (Operon Technologies, Inc.). Their names and sequences are shown in Table (2).

The reaction conditions were optimized and the reaction mixtures (30-µl total volume) according to Williams *et al.* (1990) that contained the following:

dNTPs	2.4 µl
$MgCl_2$	3.0 µl
10X buffer	3.0 µl
Primer (10 µM)	2.0 µl
Template DNA (50 ng/µl)	2.0 µl
Taq (5 U/µl)	0.3 µl
H_2O (dd)	17.3 µl

Amplification was carried out in a Perkin Elmer Cetus thermocycler programmed for 47 cycles as follows; Denaturation (one cycle) at 94°C for 5 min, annealing (45 cycles) at 94°C for 1 min, 36°C for 90 sec, 72°C for 2 min, extension (one cycle) 72°C for 7 min, then 4°C infinit. Agarose (1.2%) electrophoresis was used for resolving the PCR products.

2. Inter simple sequence repeats (ISSRs)

PCR reaction was conducted using five ISSR primers. Their names and sequences are shown in Table (3). PCR was performed in 25µl volume tubes according to (Wang *et al*., 2002) The amplification was carried out in a DNA thermocycler (TECHNE-512) Programmed as follows: Denaturation (one cycle) at 94°C for 5 min., annealing (45 cycles) at 94°C for 30 sec., 62°C for 45 sec., 72°C for 2 min., extension (one cycle) 72°C for 7 min, then 4°C infinit.

3. Amplified fragment length polymorphism (AFLP)

AFLP analysis was performed according to Vos *et al*. (1995). Two types of pairs of restriction enzymes, *Mse*l/*Pst*I (1.2 units/µl), and *Mse*I/ *Eco*RI (1.2 units/µl each in 10mM tris-HCL (pH 7.5) 50 mM EDTA, 1mM DTT 0.1 mg/ml BSA, and 50% glycerol) in 25µl total volume were used to digest the genomic DNA. *Mse*l is a frequent cutter with a T/TAA cutting site, whereas *Pst*I and *Eco*RI are 6-base rare cutters (*Pst*I is methylation sensitive).

To the restricted DNA, 24 µl of the adapter ligation solution [*Eco*R1/ *Mse*1adapters, 0.4 mM ATP, 10 mM Tris HCL (pH 7.5), 10 mM Mg acetate, 50 mM K acetate] and 1 µl T4 ligase (1unit/µl) were added.

Pre-amplification of DNA was performed in a total volume of 51 µl which consisted of 5µl of the 10 fold diluted ligation mix, 40 µl pre-amp primer mix, 5µl of 10x PCR buffer plus Mg and 1µl Taq polymerase. PCR conditions were 11 cycles at 94°C for 30 sec, 65°C for 60 sec, and 22 cycles at 94°C for 30 sec, 56°C for 30 sec, and at 72°C for 60 sec, then soaking at 4°C. For selective amplification of DNA, pre-amplified products were used as templets using two AFLP primers, each containing three selective nucleotides. Two mixes were prepeared; Mix1 with a total volume of 501 (5 µl *Eco*R1 primer and 45 µl *Mse*1 primer with dNTPs), Mix2 with a total volume of 100 µl (79 µl dd H_2O, 2 µl PCR buffer and 1µl Taq DNA polymerase). The reaction was performed in a 20 µl total volume of 5µl diluted pre-amplification product, 5 µl Mix1 and 10 µl Mix 2. The reactions were carried out using the following cycling parameters: 11 cycles of 30 sec at 94°C, 30 sec at 65°C (-0.7ºC/cycle) 30, 1 min at 72ºC, followed by 22 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1min at 72°C. All PCR reactions were performed using a Perkin Elmer 9600 thermocycler. Products from the selective amplification were separated on a 6% denaturing polyacrylamide sequencing gel. The DNA silver staining system was used for band detection.

RESULTS AND DISCUSSION

1. Fingerprinting by RAPD

Data of the amplified fragments using eighteen 10-mer arbitrary primers for the nineteen *Citrus* cultivars indicated successful amplification of PCR products. Polymorphism levels differed from one primer to the other (Table 4a, b).Six primers; OP-C09, OP-A19, OP-D15, OP-G17, OP-L13, OP-L16 showed no polymorphic differences among the species, while some primers exhibited low polymorphism such as OP-D07 (37%), OP-L12 (11.1%), OP-L20 (12.5%), OP-Z03 (9%) and OP-B11 (16.6%).On the other hand, some primers exhibited high levels of polymorphism such as OP-B07 (80%), OP-B12 (90.9%), OP-C10 (63.3%), OP-C13 (100%), OP-C15

(50%), OP-D01 (66.6%) and OP-F06 (58.3%) (Fig. 1).

These results are in agreement with those of Cabrita *et al.* (2001) who analyzed a group of 22 *Citrus* cultivars (sweet oranges, lemons, grape fruits, clementines, and several other mandarin biotypes) by isoenzyme and RAPD –PCR analysis in order to assess their genetic relationships. They stated that RAPD technique, discriminating among all the species and distinguishing among the mandarin cultivars, including Carvalhais and Fremont. However, it was unable to discriminate among the different cultivars within the remaining *Citrus* species (biotypes).

Genetic similarity and cluster analysis based on RAPD markers

The RAPD data were used to estimate the genetic similarity among 19 *Citrus* taxa by using UPGMA computer analysis (Table 5). The highest similarity index recorded was (0.985), between the Navel orange and Sour orange, while the lowest similarity index recorded was (0.852), between Fortunella marigarata and Navel orange.

A dendrogram for the genetic relationships among the 19 *Citrus* taxa was carried out as in Fig. (2). The 19 *Citruses* taxa were separated into two clusters; cluster 1 included Tanarief orange, Jaffa orange, Balady orange, Succari orange, Balady Mandarine, Valancia orange, Egyptian Jaffa orange, Lemon, Grapefruit, Fortunella Japonica and Fortunella marigarata, while cluster 2 included Blood orange, White Khallili orange, Sour orange, Navel orange, Trifoliata orange, Selection Malawy Clementine, Lime and Santra Clementine.

Within cluster 1, two sub clusters were observed, the first one contained Fortunella marigarata and Fortunella Japonica, while the second was divided into two sub-sub clusters as Lemon and Grapefruit (in the first sub-sub clusters) and the second sub-sub clusters was divided into 3 groups, the first contained Tanarief orange, Jaffa orange and Balady orange in one division and Succari orange in the other division. The second group contained Balady Mandarine and Valancia orange (in the first division), while Egyptian Jaffa orange was in the second division. The second cluster was divided into two sub clusters, the first one contained Lime and Santra Clementine, while the second sub cluster divided into two sub-sub clusters as Blood orange and White Khallili in the first one, the second sub-sub cluster contained Sour orange and Navel orange in one-division and Trifoliata orange in the second division, while the third division contained Selection Malawy Clementine.

2. Fingerprinting and inter simple sequence repeats (ISSRs)

Data of the amplified fragments using the aforementioned five ISSR primers (Table 3) for the nineteen *Citrus* cultivars indicated successful amplification of PCR products. Polymorphism

levels differed from one primer to the other .The main results are presented in Table (6)

Only two primers HB-14 and HA-98 showed 100% polymorphic differences among the cultivars, while three primers exhibited high polymorphism such as HB12 (94%) and HA99 (93%) (Fig. 3).

The previous results agreed with those of Fang and Roose (1997) who used 22 ISSR primers with 94 trees of 68 *Citrus* taxa. Within *C. sinensis* and *C. paradise*, ISSR markers distinguished 14 of 33 sweet orange and 1 of 7 grapefruit taxa. Five of six lemon taxa were discriminated by ISSR markers. Many differences were found among mandarin taxa; however, all five Satsuma taxa analyzed had identical ISSR fingerprints Fang *et al.* (1997) also used eleven ISSR among 48 vegetatively propagated trifoliate orange accessions. ISSR amplifications generated multiple banding profiles with an average of 58 fragments/ primer/ accession.

Genetic similarity and cluster analysis based on ISSRs markers

The ISSR data were used to estimate the genetic similarity among 19 *Citrus* taxa by using UPGMA computer analysis as shown in Table (7). The highest similarity index (0.957) recorded between the two taxa Jaffa orange and Egyptian Jaffa orange, while the lowest similarity index (0.286) was observed between Lemon and Succari orange.

They classified the 48 trifoliate orange accessions into four major groups based on polymorphic ISSR markers. Fang *et al*. (1998) studied the phylogenetic relationships among 46 *Citrus* accessions repesenting 35 species using 10 ISSR primers. They classified these 46 accessions into five major groups: 1- *C. indica* 2- *C. maxima* 3- *C. limon* (lemon) or *C. aurantifolia* (lime) 4- *C. halimii* 5- sour orange (*C. aurantium*), mandarins and their hybrids. Group 5 was further divided into three subgroups.

A dendrogram for the genetic relationships among the 19 *Citurs* taxa was carried out Fig. (4). The 19 *Citurs* taxa were separated into two major clusters; the first cluster included Jaffa orange, Egyptian Jaffa orange, Balady orange, Succari orange, Tanarief orange, Navel orange, Sour orange, Blood orange, White Khallili orange and Trifoliata orange, while the second cluster included Lemon, Grapefruit, Lime, Fortunella marigarata, Valancia orange, Fortunella Japonica, Santra Clementine, Selection Malawy Clementine and Balady Mandarine.

Within the first cluster, two sub clusters were observed, the first one contained Blood orange and White Khallili (in one division) and Trifoliata orange (in other division), while the second sub cluster was divided into two sub-sub clusters; the first one contained Sour orange and the second sub-sub cluster was divided into two groups; the first one contained Jaffa orange and

Egyptian Jaffa orange, while the second group was divided into two sub-groups; the first one contained Navel orange, while the second one had Balady orange and Succari orange (in one division) and Tanarief orange (in the second division). The second clusters was divided into two sub clusters, the first one contained Lemon and Grapefruit, while the second sub cluster was divided into two sub-sub clusters as Balady Mandarine in the first one,while the second was divided into two groups; as Lime and Fortunella marigarata (in the first one), while the second contained Valancia orange and Fortunella Japonica (in the first division) and Santra Clementine and Selection Malawy Clementine (in the second division).

3. Combined identification based on RAPD-PCR and ISSR-PCR analyses

Cluster analysis based on RAPD and ISSR-PCR analyses (Table 8 and Fig. 13) was carried out using UPGMA computer program. The highest similarity index recorded (0.967) was between the two Jaffa orange and Egyptian jaffa taxa, while the lowest similarity index (0.797) was observed between the two taxa of Naval orange and Fortunella marigarata. Dendrogram for the genetic relationships among these taxa (Fig. 5) showed that the 19 *Citrus* taxa were separated into two clusters; the first cluster included Jaffa orange, Egyptian Jaffa orange, Balady orange, Succari orange, Tanarief orange, Blood orange, White Khallili, Sour orange, Navel orange and Trifoliata orange, while the second cluster included Fortunella marigarata, Fortunella Japonica, Lemon, Grapefruit, Balady Mandarine, Valancia orange, Santra Clementine, Selection Malawy Clementine and Lime.

Within the first cluster there were two subclusters; the first one was divided into two sub-sub clusters as Jaffa orange, Egyptian Jaffa orange in the first one, and Balady orange, Succari orange and Tanarief orange in the second, while the second sub-cluster was divided into two sub-sub clusters as Blood orange and White Khallili in the first division and Sour orange, Navel orange and Trifoliata orange in the second division.

The second cluster, showed three subclusters; the first one included Fortunella marigarata and Fortunella Japonica, the second sub cluster included Lemon and Grapefruit, while the third sub cluster was divided into two sub-sub clusters as Balady Mandarine and Valancia orange in the first division and Santra Clementine, Selection Malawy Clementine and Lime in the second division.

Our studeis indicated that RAPD and ISSR techniques are useful in the establishment of the genetic fingerprinting and estimation of relationships among *Citrus* genotypes.

Also, these techniques could detect enough polymorphism in the studied *Citrus* genotypes to distinguish each

genotype from all others by at least one unique band or a group of combined banding pattern (Tables 5 and 6). Furthermore, the use of these data in the future is important for *Citrus* germplasm management, improvement as well as for the selection strategies of parental lines that facilitate the prediction of crosses in order to produce hybrids with higher performance (Hassan *et al.*, 2002)

In general, the overall results indicated the possible use of the above-mentioned analysis to detcet species-specific and characteristic-specific markers for 19 *Citrus* cultivars that can be used to discriminate among the species and the genotypes. Also, detection of genetic relationships among these cultivars can be used in breeding programs. The molecular genetic studies of 19 *Citrus* cultivars and their genetic diversity are efficient tools for the characterization of these cultivars for fruit traits, which could be used in assisted-marker selection (MAS) in *Citrus* cultivars breeding program and for providing data for gene bank.

The study of genetic diversity using RAPD and ISSR-PCR analyses seemed to be powerful tools in characterizing these *Citrus* taxa which agreed with Pasquale *et al.,* (2006) who reported significant morphological differences between five clones of sour orange (*Citrus aurantium* L.). The genetic studies were undertaken by the use of molecular markers developed by PCR-based techniques (ISSR and RAPD). ISSR markers appear to be suitable for mapping, as evidenced by the successful incorporation of 88% of the putative marker loci into the *Citrus* genetic linkage map (Sanker and Moore, 2001).

4. Fingerprinting by AFLP

AFLP markers are a reliable method for genetic fingerprinting and have been successfully used for characterization and evaluation of genetic relationships in several cultivars. AFLP analysis was linked to the three citrus cultivars: Balady orange, Balady mandarine and Lime.

Combination I: AFLP analysis of the three *Citrus* cultivars (three species) using two pairs of primers; *Eco* RI –AT (5'- GAC TGC GTA CCA ATT AT- 3') and *Mse* I- CAA (5'- GAT GAG TCC TGA GTA CAA- 3') provided a total of 23 bands ranging from 1774 to 203 bp (Fig. 6 and Table 9). 17 polymorphic fragments (74%) with numbers 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19, 20, 21 and 22 with corresponding molecular sizes of 1383, 1279, 1230, 1183, 1138, 1095, 974, 937, 867, 713, 635, 65, 382, 340, 280, 249 and 213 bp, respectively, were observed, while the other bands were monomorphic.

The fragments with MS of 1138, 974 and 280 bp appeared exclusively in Balady orange. So these fragments could be used as molecular markers for Balady orange.

The fragment with 213 bp appeared only in Balady mandarine. There-

fore, this fragment could be considered as molecular marker for Balady mandarine. The fragments with MS of 1383, 1279, 937, 867, 713, 635, 465 and 340 bp appeared only in Lime. So, these fragments could be regarded as molecular markers for Lime.

Combination II: Cultivars using two pairs of primers *Eco* RI –AG (5'- GAC TGC GTA CCA ATT AG- 3') and *Mse* I-CAG (5'- GAT GAG TCC TGA GTA CAG- 3') provided a total of 20 bands ranging from 1600 to 167 bp (Fig. 6 and Table 9). Eight polymorphic fragments (40%) with numbers 2, 3, 9, 16, 17, 18, 19 and 20 with corresponding molecular sizes of 1480, 1434, 517, 249, 232, 186, 180 and 167 bp, respectively, were observed, while the other bands were monomorphic. The fragments with 517 and 180 bp appeared uniquely in Balady orange. So, these fragments could be used as molecular markers for Balady orange. The fragment with 186 bp appeared in Balady mandarine only, so this fragment could be used as molecular marker for Balady mandarine. The fragments with 1480 and 1434 bp appeared in Lime only, and these two fragments could be used as molecular markers for Lime.

Combination III: AFLP analysis of the three *Citrus* cultivars using two pairs of primers *Eco* RI –TG (5'- GAC TGC GTA CCA ATT TG- 3') and *Mse* I- CAC (5'- GAT GAG TCC TGA GTA CAC- 3') provided a total of 22 bands ranging from 2396 to 238 bp (Fig. 6 and Table 9).Thirteen polymorphic fragments (63%) with numbers 1, 2, 3, 4, 7, 11, 12, 14, 17, 18, 19, 21 and 22 with corresponding molecular sizes of 2396, 1744, 1616, 1494, 1102, 910, 843, 723, 533, 493, 475, 267 and 238 bp, respectively, were observed, while the other bands were monomorphic. The fragments with 493, 267 and 238 bp appeared in Balady orange only, so, these fragments could be used as molecular markers for Balady orange.The fragments with 910, 843 and 723 bp appeared in Balady mandarine only, so, these fragments could be used as molecular markers for Balady mandarine.The fragments with 2396, 1102 and 533 bp appeared uniquely in Lime, therefore, these fragments could be used as molecular markers for Lime.

Combination IV: AFLP analysis of the three *Citrus* cultivars using these two pairs of primers *Eco* RI –TC (5'- GAC TGC GTA CCA ATT TC- 3') and *Mse* I-CAA (5'- GAT GAG TCC TGA GTA CAA- 3') provided a total of 22 bands ranged from 1341 to 352 bp (Fig. 6 and Table 9). Fifteen polymorphic fragments (68%) with numbers 1, 2, 3, 5, 6, 8, 9, 12, 13, 14, 16, 18, 19, 20 and 21 with corresponding molecular sizes of 1341, 1302, 1246, 1089, 1058, 997, 885, 658, 620, 584, 519, 447, 434, 409 and 385 bp, respectively, were observed, while the other bands were monomorphic.

The fragments with 1341, 1058, 885 and 584 bp appeared in Balady orange only, so, these fragments could be used as molecular markers for Balady orange. The fragments with 1302, 1089

and 434 bp appeared exclusively in Balady mandarine, so, these fragments could be used as molecular markers for Balady mandarine. The fragments with 1246 and 997 bp appeared in Lime only. So, these fragments could be used as molecular markers for Lime.

Data of the amplified fragments using the four combinations; where each combination included two pairs of primers with three *Citrus* cultivars indicated different levels of polymorphism from one combination to the other. The main results could be presented as following (Table 10).

Combination I had 17 polymorphic fragments (74%), it exhibited 10 amplified fragments and 3 specific markers in Balady orange. While in Balady mandarine, it exhibited 12 amplified fragments and one specific marker, but in lime it showed 18 amplified fragments and eight specific markers. On the other hand, the combination II exhibited eight polymorphic fragments (40%), 15 amplified fragments and two specific markers in Balady orange, while in Balady mandarine, it exhibited 16 amplified fragments and one specific marker, but in lime, it showed 16 amplified fragments and two specific markers. Combination III had 13 polymorphic fragments (63%), 12 amplified fragments and three specific markers in Balady orange, while in Balady mandarine, it exhibited 16 amplified fragments and two specific markers, but in lime it showed 16 amplified fragments and three specific markers. Combination IV exhibited 15 polymorphic fragments (68 %), 11 amplified fragments and four specific markers in Balady orange, while in Balady mandarine, it exhibited 16 amplified fragments and three specific markers, but in lime, it showed 15 amplified fragments and two specific markers.

Our results agreed with those of Besnard *et al.* (2001) who analyzed a group of 22 *Citrus* cultivars (sweet oranges, lemons, grape fruits, clementines, and several other mandarin biotypes) by isoenzymes and RAPD markers in order to assess their genetic relationships. They stated that RAPD technique, though discriminating among all the species and distinguishing among the mandarin cultivars, including Carvalhais and Fremont, was unable to discriminate among the different cultivars within the remaining *Citrus* species (biotypes). Testolin *et al.* (2001) constructed a genetic map of Kiwi fruit using AFLP markers. The AFLP markers were produced using *Mse* l and *Eco*RI restriction enzymes and 15 primer combinations. Two linkage maps were produced, one for each parent (*Actinidia chinensis* and *A. callosa*).

Genetic similarity and cluster analysis based on AFLP markers

The AFLP data were used to estimate the genetic relationships among three *Citrus* taxa by using UPGMA computer analysis as shown in Table (11). In combination I, the highest similarity index recorded was 0.667 which was

observed between the two taxa Balady mandarine and Lime, while the lowest similarity index recorded was 0.429, which was observed between Balady orange and Lime. In combination II, the highest similarity index recorded was 0.875, which was observed between the two taxa Balady mandarine and Lime, while the lowest similarity index recorded was 0.774, which was observed between Balady orange and Lime. In combination III, the highest similarity index recorded was 0.813, which was observed between the two taxa Balady mandarine and Lime, while the lowest similarity index recorded was 0.643, which was observed between Balady orange and Balady mandarine and between Balady orange and Lime. In combination IV, the highest similarity index recorded was 0.839, which was observed between Balady mandarine and Lime, while the lowest similarity index (0.519) which was observed between Balady mandarine and Balady orange.

A dendrogram for the genetic relationships among the 3 *Citures* species was carried out (Fig. 7). They were separated into two clusters; the first cluster included Balady Mandarine and Lime, while the second cluster included Balady orange in all four AFLP combinations.

Generally speaking, each of the three PCR–based systems gave sufficient discrimination between the citrus taxa used in the present study. However, combined analysis gave higher resolution for distinction between these taxa. In addition, specific markers were obtained for some species. The Citrus tree breeding could evidently benefit from the use of such DNA molecular markers associated with genes for yield-related traits through marker-assisted selection (MAS). MAS would also allow early screening for economically important traits in seedlings, which is especially useful for traits expressed only in fully mature trees (Warburton *et al.*, 1996).

SUMMARY

Nineteen citrus cultivars were collected; their fingerprint and molecular markers were developed to assist selection for these cultivars.

In this study, RAPD was used for the identification of markers associated with Citrus taxa genotypes using 18 arbitrary 10-mer primers. Primers OP-A19, OP-C09, OP-D15, OP-G17, OP-L13 and OP-L16 showed monomorphic fragments with no detected polymorphism. The results of primers OP-B07, OP-B11, OP-12, OP-C10, OP-C13 and OP-L20 gave polymorphic fragments with different molecular sizes. No taxa- specific markers were observed for these primers. Primers OP-C15, OP-D01, OP-D07, OP-F06, OP-L12 and OP-Z03 showed taxa-specific markers.

PCR reaction was conducted using five ISSR primers. Only two primers HB14 and HA98 showed 100% polymorphic differences among the cultivars, while three primers exhibited high polymorphism such as HB12 (94%) and HA99 (93%).

Two types of pairs of restriction enzymes, *Mse*1 and *Pst*I, *Mse*I and *Eco*RI, were used to digest the genomic DNA which was used for AFLP analysis. The three *Citurs* taxa were separated into two clusters; the first cluster included Balady mandarine and Lime, while the second cluster included Balady orange in all four AFLP combinations.

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Table (1): List of the nineteen species of *Citrus* used in this study

Accession number	Common name	Species	Vernacular name
1	- Blood orange	*Citrus sinensis*	Abo dammo
2	- White khalili	*Citrus sinensis*	----
3	-Trifoliata orange	*Poncirus trifoliate*	----
4	-Sour orange	*Citrus aurantium*	Nareng
5	- Naval orange	*Citrus sinensis*	Abo sorra
6	- Balady orange	*Citrus sinensis*	Balady
7	- Succari orange	*Citrus sinensis*	Succari
8	- Tanarief orange	*Citrus sinensis*	----
9	- Jaffa orange	*Citrus sinensis*	Yaffawi
10	- Egyptian-Jaffa orange	*Citrus sinensis*	Yaffawi masry
11	- Balady mandarine	*Citrus reticulata*	Usifi balady
12	- Valencia orange	*Citrus sinensis*	Saify
13	- Lemon	*Citrus lemon*	Adalia
14	- Grapefruit	*Citrus paradisii*	Grapefruit
15	- Lime	*Citrus aurantifolia*	Lemon balady
16	- Santra clement	*Citrus reticulata*	----
17	- Selc. Malawy clem	*Citrus reticulate*	----
18	- Fortunella marigarata	*Fortunella marigara*	Kemquat
19	- Fortunella japonica	*Fortunella japonica*	Kemquat

Table (2): List of RAPD primers and their nucleotide sequences used in this study

No.	Name	Sequence	No.	Name	Sequence
1	**OP-A19**	**5' CAATCGCCGT 3'**	**10**	**OP-D07**	**5' CAGCACCCCA 3'**
2	**OP-B07**	**5' AGGTGACCGT 3'**	**11**	**OP-D15**	**5' CAATCGCCGT 3'**
3	**OP-B11**	**5' GACGGATCAG 3'**	**12**	**OP-G17**	**5' CTCACCGTCC 3'**
4	**OP-B12**	**5' CCTTGACGCA 3'**	**13**	**OP-F06**	**5' CCTTGACGCA 3'**
5	**OP-C09**	**5' CTCACCGTCC 3'**	**14**	**OP-L12**	**5` GGGCGGTACT 3`**
6	**OP-C10**	**5` TGTCTGGGTG 3`**	**15**	**OP-L13**	**5` ACCGCCTGCT 3`**
7	**OP-C13**	**5` AAGCCTCGTC 3`**	**16**	**OP-L16**	**5` AGGTTGCAGG 3`**
8	**OP-C15**	**5` GACGGATCAG 3`**	**17**	**OP-L20**	**5` TGGTGGACCA 3`**
9	**OP-D01**	**5' ACCGCGAAGC 3'**	**18**	**OP-Z03**	**5' CAGCACCCCA 3'**

Table (3): List of the primer names and their nucleotide sequences used in this study (ISSR)

No.	Primer	Sequences
1.	HA-98	5' CACACACACACAGT 3'
2.	HA-99	5' CACACACACACAAG 3'
3.	HB-12	5' CACCACCACGC 3'
4.	HB-13	5' GAGGAGGAGGC 3'
5.	HB-14	5' CTCCTCCTCGC 3'

Table (4a): Cultivar-specific RAPD markers of the 10 *Citrus* species with 18 RAPD primers.

Primers Op-			Blood orange		White khallili		Trifoliata orange		Sour orange		Naval orange		Balady orange		Succari orange		Tanarief orange		Jaffa orange		Egyptian jaffa org.	
	TAF	PB	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM
A19	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0
B07	10	8	7	0	6	0	2	0	2	0	2	0	7	0	10	0	10	0	10	0	10	0
B11	6	1	6	0	6	0	6	0	6	0	6	0	6	0	5	0	6	0	6	0	6	0
B12	11	10	5	0	6	0	5	0	3	0	2	0	8	0	11	0	9	0	9	0	9	0
C09	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0
C10	11	7	5	0	5	0	7	0	7	0	7	0	11	0	11	0	11	0	11	0	11	0
C13	14	12	5	0	3	0	8	0	5	0	6	0	11	0	11	0	10	0	10	0	7	0
C15	12	6	6	1	7	1	7	1	7	1	7	1	7	1	11	2	7	0	6	0	6	0
D01	9	6	6	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0	7	0
D07	8	3	6	0	6	0	6	0	6	0	6	0	6	0	6	0	6	0	6	0	6	0
D15	9	0	9	0	9	0	9	0	9	0	9	0	9	0	9	0	9	0	9	0	9	0
F06	12	7	7	1	11	0	11	0	10	1	8	0	11	0	11	0	11	0	8	0	6	0
G17	18	0	18	0	18	0	18	0	18	0	18	0	18	0	18	0	18	0	18	0	18	0
L12	9	1	8	0	8	0	8	0	8	0	8	0	8	0	8	0	8	0	8	0	8	0
L13	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0
L16	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0
L20	8	1	8	0	8	0	8	0	8	0	8	0	8	0	8	0	7	0	7	0	8	0
Z03	11	1	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0
Total	197	63	155	2	153	1	151	1	155	2	145	1	176	1	183	2	178	0	174	0	170	0

TAF: total amplified fragment PB: polymorphic bands AF: amplified fragment SM: specific marker

Table (4b): Cultivar-specific RAPD markers of the 9 *Citrus* species with 18 RAPD primers.

Primers Op-	Balady mandarine		Valencia orange		Lemon		Grapefruit		Lime		Santra clement		Sel. Malawy clent.		Fortunella japonica		Fort. marigarata	
	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM
A19	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0	15	0
B07	10	0	9	0	8	0	4	0	8	0	2	0	4	0	10	0	10	0
B11	6	0	6	0	6	0	6	0	6	0	5	0	6	0	6	0	6	0
B12	8	0	9	0	10	0	9	0	5	0	5	0	7	0	11	0	11	0
C09	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0
C10	11	0	10	0	5	0	5	0	5	0	10	0	7	0	10	0	8	0
C13	5	0	5	0	10	0	10	0	8	0	8	0	4	0	12	0	12	0
C15	6	0	6	0	7	1	6	0	6	0	6	0	6	0	8	0	9	0
D01	7	0	7	0	7	0	7	0	7	0	6	0	6	0	6	1	6	2
D07	6	0	6	0	6	0	6	0	6	0	6	0	6	0	6	0	7	2
D15	9	0	9	0	9	0	9	0	9	0	9	0	9	0	9	0	9	0
F06	11	0	11	0	10	0	8	0	11	0	11	0	11	0	8	0	6	0
G17	18	0	18	0	18	0	18	0	18	0	18	0	18	0	18	0	18	0
L12	8	0	8	0	8	0	8	0	9	1	8	0	8	0	8	0	8	0
L13	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0
L16	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0
L20	7	0	8	0	8	0	7	0	8	0	7	0	7	0	7	0	7	0
Z03	10	0	10	0	10	0	10	0	11	1	10	0	10	0	10	0	10	0
Total	171	0	172	0	171	1	162	0	164	2	160	0	158	0	178	1	176	4

TAF: total amplified fragment PB: polymorphic bands AF: amplified fragment SM: specific marker

Table (5): Similarity indices among the 19 *Citrus* Taxa based on RAPD-PCR using 18 primes.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Blood Orange																		
2. White khallili	.956																	
3. Trifoliata org.	.934	.957																
4. Sour org.	.940	.956	.978															
5. Naval org.	.932	.940	.963	.985														
6. Balady org.	.913	.935	.942	.920	.919													
7. Succari org.	.885	.907	.914	.892	.884	.965												
8. Tanarief org.	.900	.922	.929	.907	.892	.974	.972											
9. Jaffa org.	.909	.917	.925	.909	.901	.971	.962	.990										
10.Egyption Jaffa	.932	.919	.913	.911	.903	.947	.939	.961	.970									
11.Balady mandarine	.905	.927	.913	.919	.904	.947	.939	.967	.964	.966								
12.Valencia org.	.908	.937	.924	.929	.914	.937	.942	.951	.947	.956	.977							
13.Lemon	.911	.933	.927	918.	.910	.947	.939	.947	.950	.932	.940	.949						
14.Grapefruit	.908	.924	.932	.923	.930	.932	.911	.926	.935	.910	.917	.927	.972					
15.Lime	.906	.929	.915	.920	.912	.909	.895	.910	.905	.914	.928	.945	.942	.919				
16.Santra Clement	.886	.916	.931	.985	.929	.918	.897	.912	.907	.909	.931	.941	.902	.914	.940			
17.Sel. Malawy Clem.	.925	.941	.949	.955	.940	.920	.899	.921	.917	.912	.940	.951	.933	.945	.942	.945		
18.Fortunella Japonica	.882	.884	.891	.875	.867	.932	.950	.952	.961	.937	.931	.934	.931	.915	.899	.901	.903	
19.Fort. marigarata	.881	.869	.877	.860	.852	.906	.924	.926	.935	.930	.904	.907	.917	.901	.885	.873	882	.968

Table (6): ISSR banding patterns of the 19 *Citrus* species with five ISSR primers.

Primers			Blood orang		White khallili		Trifoliata orange		Sour orange		Naval orang		Balady orange		Succari orange		Tanarief orange		Jaffa orange		Egyptian jaffa org.	
	TAF	PB	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM
HB12	17	16	14	1	12	1	8	0	10	0	12	1	12	1	11	1	11	1	9	0	9	0
HB13	14	13	12	0	5	0	4	0	5	0	6	0	6	0	6	0	4	0	11	0	10	0
HB14	20	20	11	6	11	6	10	6	11	5	13	7	13	7	14	7	14	6	13	6	13	6
HA98	17	17	10	4	11	4	13	6	8	3	13	6	14	6	15	7	15	7	15	7	15	7
HA99	15	14	7	2	6	2	1	0	7	2	2	1	12	5	12	5	7	3	7	1	6	1
Total	83	80	54	13	45	13	36	12	41	10	46	15	57	19	58	20	51	17	55	14	53	14

	Balady mandarin		Valencia orange		Lemon		Grapefrui		Lime		Santra clement		Sel. Malawy clent		Fortunella japonica		Fort. marigarat	
Primers	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM
HB12	11	0	10	0	9	0	12	0	9	0	13	1	6	1	8	0	12	0
HB13	5	0	10	0	4	0	10	0	12	0	11	0	12	0	12	0	12	0
HB14	6	0	9	0	0	0	2	0	10	0	3	0	5	0	7	0	10	0
HA98	6	0	7	0	2	0	2	0	4	0	9	0	7	0	8	0	5	0
HA99	4	0	4	0	6	0	5	0	5	0	5	0	4	0	4	0	6	0
Total	32	0	40	0	21	0	31	0	40	0	41	1	34	1	39	0	45	0

TAF: total amplified fragment PB: polymorphic bands AF: amplified fragment SM: specific marker

Table (7): Similarity indices among the 19 *Citrus* Taxa based on ISSRs-PCR using five primers.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Blood Orange																		
2. White khallili	.887																	
3. Trifoliata org.	.765	.854																
4. Sour org.	.780	.809	.711															
5. Naval org.	.761	.785	.718	.851														
6. Balady org.	.783	.807	.762	.816	.897													
7. Succari org.	.759	.782	.736	.788	.906	.958												
8. Tanarief org.	.737	.769	.750	.745	.870	.932	.949											
9. Jaffa org.	.769	.721	.710	.724	.831	.867	.876	.891										
10. Egyption Jaffa	.759	.745	.736	.731	.838	.874	.883	.898	.975									
11. Balady mandarine	.622	.643	.600	.513	.549	.538	.511	.522	.547	.532								
12. Valencia org.	.646	.624	.584	.552	.580	.608	.602	.547	.615	.621	.779							
13. Lemon	.438	.418	.381	.393	.351	.316	.286	.347	.333	.338	.471	.500						
14. Grapefruit	.595	.462	.432	.472	.424	.414	.386	.395	.494	.455	.516	.563	.756					
15. Lime	.619	.505	.460	.565	.531	.540	.515	.525	.588	.554	.560	.714	.586	.696				
16. Santra Clement	.687	.624	.584	.575	.600	.647	.621	.594	.673	.641	.727	.767	.567	.704	.714			
17. Sel. Malawy Clem.	.609	.535	.512	.550	.538	.547	.542	.511	.598	.563	.629	.684	.453	.594	.727	.810		
18. Fortunella Japonica	.673	.609	.614	.535	.566	.614	.608	.580	.641	.627	.684	.871	.508	.657	.723	.824	.795	
19. Fort. marigarata	.626	.559	.494	.552	.540	.549	.524	.495	.577	.563	.649	.814	.567	.704	.810	.744	.734	.800

Table (8): Similarity indices among the 19 Citrus Taxa based on RAPD and ISSR-PCR analyses.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Blood Orange																		
2. White khallili	0.947																	
3. Trifoliata org.	0.894	0.933																
4. Sour org.	0.909	0.928	0.924															
5. Naval org.	0.897	0.911	0.927	0.947														
6. Balady org.	0.894	0.911	0.898	0.902	0.905													
7. Succari org.	0.870	0.886	0.873	0.877	0.880	0.962												
8. Tanarief org.	0.877	0.908	0.895	0.889	0.888	0.950	0.956											
9. Jaffa org.	0.893	0.878	0.879	0.887	0.881	0.940	0.937	0.959										
10. Egyption Jaffa	0.903	0.830	0.879	0.888	0.891	0.923	0.921	0.938	0.967									
11. Balady mandarine	0.859	0.882	0.863	0.867	0.851	0.870	0.859	0.894	0.891	0.887								
12. Valencia org.	0.862	0.880	0.861	0.857	0.854	0.867	0.870	0.882	0.884	0.889	0947							
13. Lemon	0.838	0.856	0.851	0.856	0.930	0.850	0.839	0.855	0.858	0.843	0.891	0.893						
14. Grapefruit	0.856	0.849	0.854	0.864	0.847	0.843	0.828	0.844	0.870	0.841	0.874	0.881	0.945					
15. Lime	0.850	0.849	0.843	0.863	0.842	0.838	0.824	0.839	0.851	0.846	0.882	0.918	0.902	0.899				
16. Santra Clement	0.844	0.852	0.857	0.872	0.855	0.851	0.831	0.842	0.858	0.849	0.896	0.903	0.870	0.898	0.911			
17. Sel. Malawy Clem.	0.860	0.859	0.864	0.884	0.856	0.842	0.828	0.843	0.860	0.845	0.893	0.905	0.877	0.901	0.914	0.929		
18. Fortunella Japonica	0.838	0.827	0.841	0.825	0.814	0.858	0.870	0.872	0.892	0.875	0.888	0.918	0.882	0.890	0.888	0.892	0.894	
19. Fort. marigarata	0.834	0.814	0.809	0.817	0.797	0.837	0.845	0.842	0.867	0.858	0.870	0.900	0.874	0.881	0.889	0.865	0.866	0.945

Table (9): AFLP profiles of the three *Citrus* species with four combinations.

No.	MS	Combination I			No.	MS	Combination II		
		Balady orange	Balady mandarin	Lime			Balady orange	Balady mandarin	Lime
1	1747	1	1	1	1	1600	1	1	1
2	1616	1	1	1	2	1480	0	0	1
3	1383	0	0	1	3	1434	0	0	1
4	1279	0	0	1	4	1334	1	1	1
5	1230	0	1	1	5	1240	1	1	1
6	1183	0	1	1	6	893	1	1	1
7	1138	1	0	0	7	773	1	1	1
8	1095	1	1	0	8	577	1	1	1
9	974	1	0	0	9	517	1	0	0
10	937	0	0	1	10	418	1	1	1
11	867	0	0	1	11	431	1	1	1
12	713	0	0	1	12	415	1	1	1
13	635	0	0	1	13	386	1	1	1
14	587	1	1	1	14	334	1	1	1
15	465	0	0	1	15	289	1	1	1
16	447	1	1	1	16	249	0	1	1
17	382	0	1	1	17	232	0	1	1
18	368	1	1	1	18	186	0	1	0
19	340	0	0	1	19	180	1	0	0
20	280	1	0	0	20	167	1	1	0
21	249	0	1	1	Total		15	16	16
22	213	0	1	0					
23	203	1	1	1					
Total		10	12	18					

No.	MS	Combination III			No.	MS	Combination IV		
		Balady orange	Balady mandarin	Lime			Balady orange	Balady mandarin	Lime
1	2396	0	0	1	1	1341	1	0	0
2	1744	0	1	1	2	1302	0	1	0
3	1616	0	1	1	3	1246	0	0	1
4	1497	0	1	1	4	1122	1	1	1
5	1190	1	1	1	5	1089	0	1	0
6	1145	1	1	1	6	1058	1	0	0
7	1102	0	0	1	7	1027	1	1	1
8	1061	1	1	1	8	997	0	0	1
9	1021	1	1	1	9	885	1	0	0
10	946	1	1	1	10	834	1	1	1
11	910	0	1	0	11	677	1	1	1
12	843	0	1	0	12	658	0	1	1
13	781	1	1	1	13	620	0	1	1
14	723	0	1	0	14	584	1	0	0
15	597	1	1	1	15	534	1	1	1
16	553	1	1	1	16	519	0	1	1
17	533	0	0	1	17	474	1	1	1
18	493	1	0	0	18	447	0	1	1
19	475	0	1	1	19	434	0	1	0
20	363	1	1	1	20	409	0	1	1
21	267	1	0	0	21	385	0	1	1
22	238	1	0	0	22	352	1	1	1
Total		12	16	16	Total		11	16	15

Table (10): Cultivar-specific AFLP markers of the three *Citrus* species with four combinations

Primers Combinations			Balady orange		Balady mandarin		Lime	
	TAF	PF	AF	SM	AF	SM	AF	SM
Combination I	23	17	10	3	12	1	18	8
Combination II	20	8	15	2	16	1	16	2
Combination III	22	13	12	3	16	2	16	3
Combination IV	22	15	11	4	16	3	15	2
Total	87	53	48	12	60	7	65	15

Table (11): Similarity indices among the three *Citrus* Taxa based on AFLP using four combinations

Citrus Taxa	Combination I		Combination II		Combination III		Combination IV	
	Balady orange	Balady mandarine	Balady orange	Balady mandarine	Balady orange	Balady mandarine	Balady orange	Balady mandarine
Balady orange								
Balady mandarine	0.636		0.839		0.643		0.519	
Lime	0.429	0.667	0.774	0.875	0.643	0.813	0.538	0.839

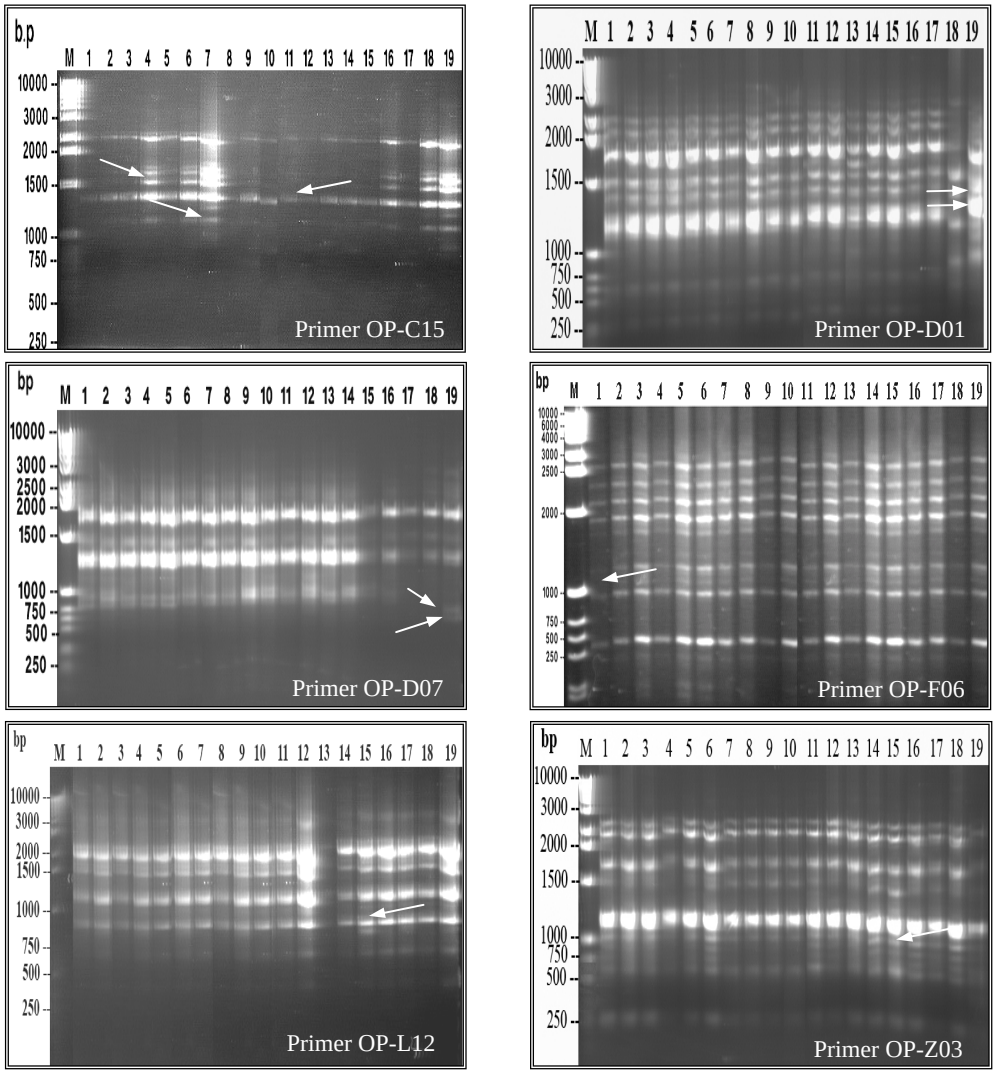


Fig. (1): RAPD profiles of the 19 *Citrus* species amplified with six primers.

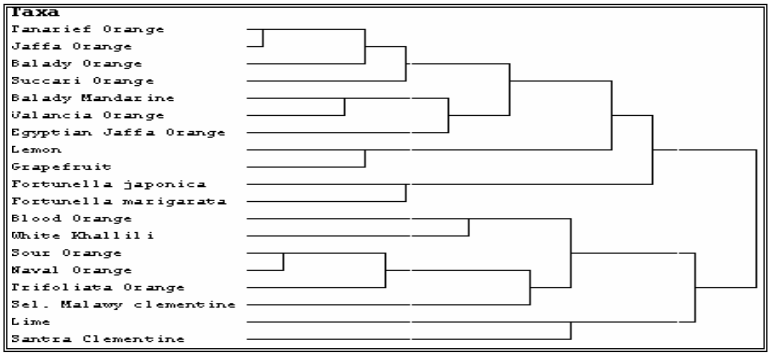


Fig. (2): Dendrogram for the genetic distances relationships among the 19 *Citrus* taxa based on similarity indices data of RAPD analysis.

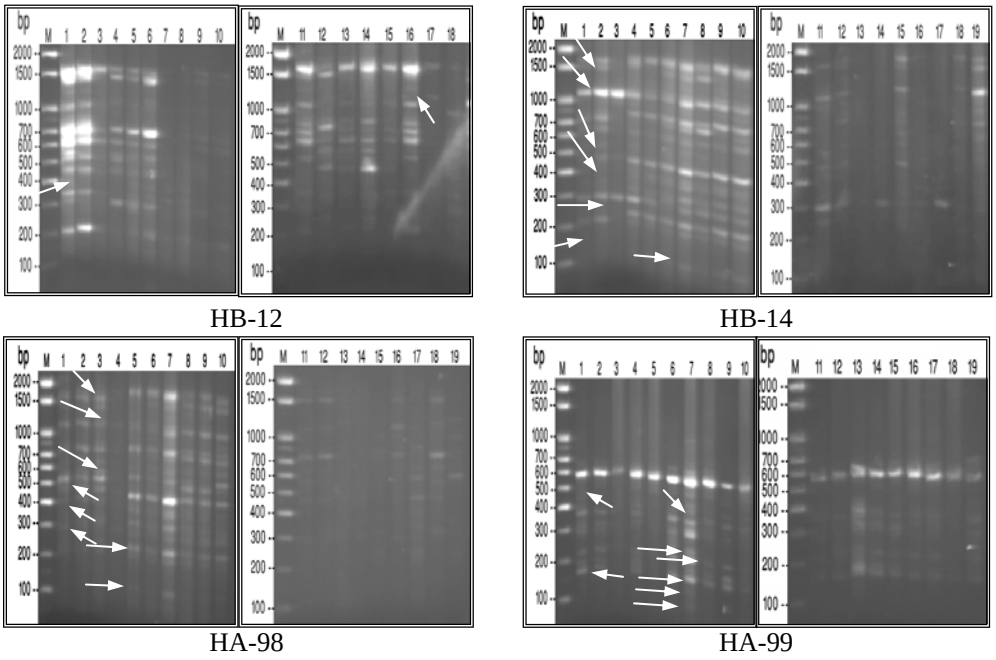


Fig. (3): ISSR profiles of the 19 *Citrus* species with different primers.

Fig. (4): Dendrogram for the genetic distances relationships among the 19 *Citrus* taxa based on similarity indices data of ISSR analysis.

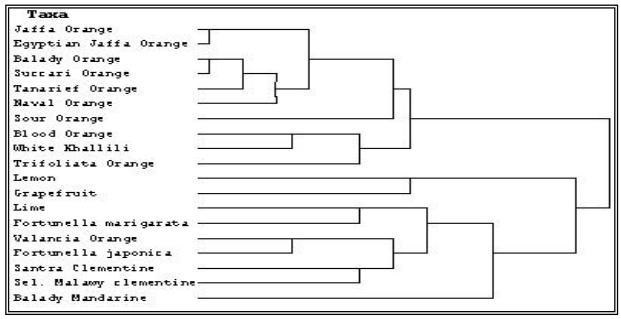
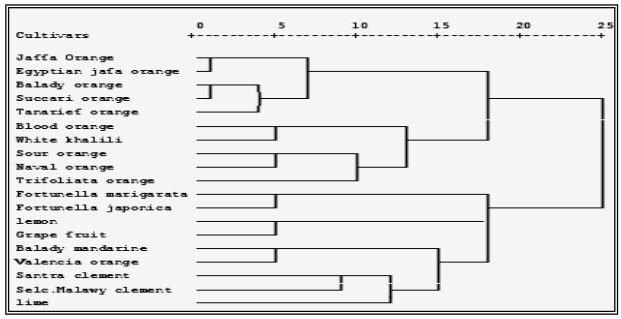


Fig. (5): Dendrogram for the genetic distances relationships among the 19 *Citrus* taxa based on similarity indices data of combined identification based on RAPD and ISSR-PCR analyses.



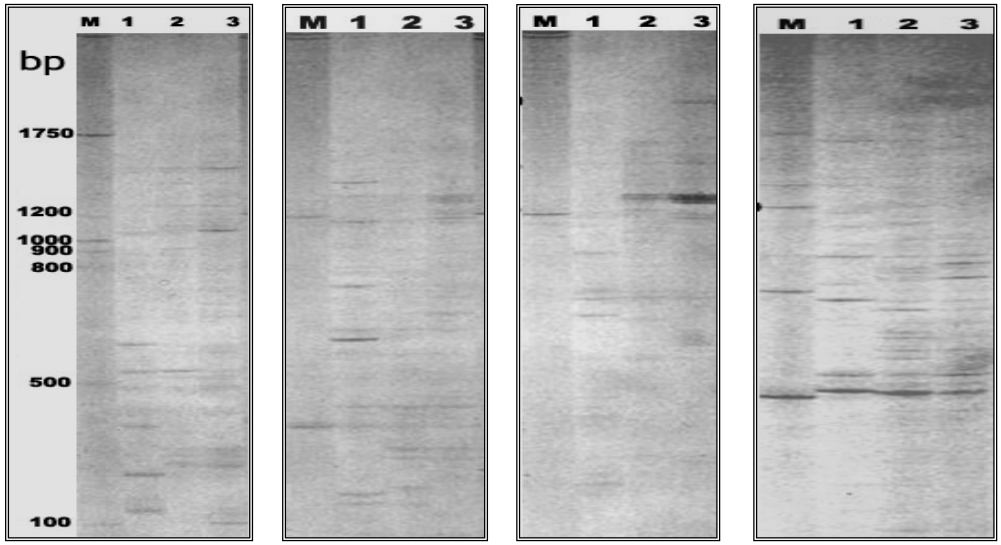


Fig. (6): AFLP profiles of the three *Citrus* species.
1- Balady orange 2- Balady mandarim 3- Lime M = marker

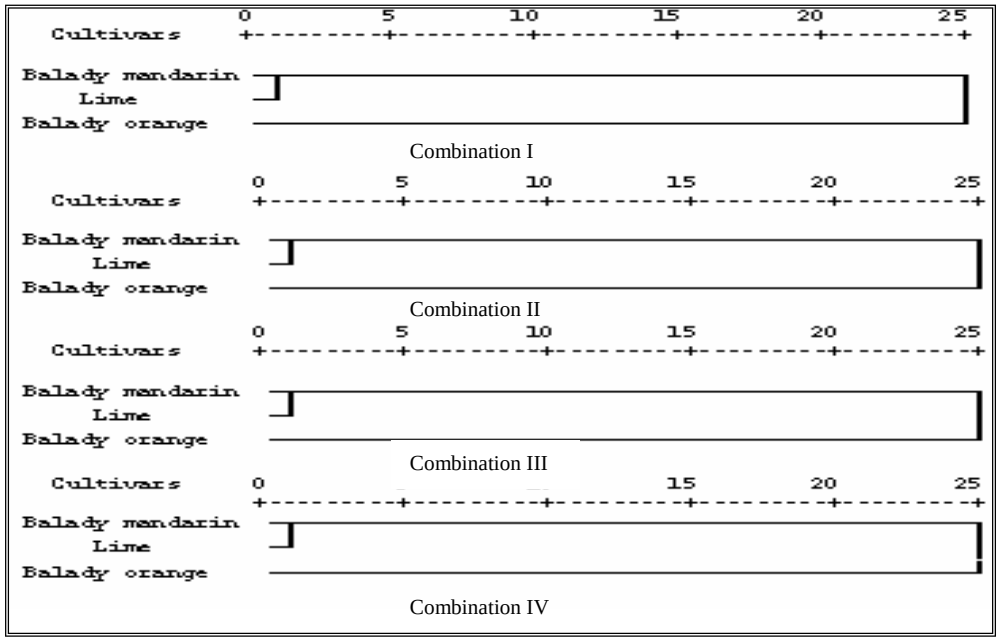


Fig. (7): Dendrograms for the genetic distances relationships among the three *Citrus* taxa based on similarity indices data of AFLP analysis.