# MOLECULAR CHARACTERIZATION AND GENETIC DIVERSITY OF SOME EGYPTIAN *CITRUS* CULTIVARS USING RAPD AND ISSRs MARKERS

## LAMYAA M. SAYED[1] AND M. F. MAKLAD[2]

1. *Department of Genetics, Faculty of Agriculture, Ain shams University Shoubra El-Kheima, Cairo, Egypt*

2. *Department of Horticulture, Faculty of Agriculture, Ain shams University, Shoubra El-Kheima, Cairo, Egypt*

*C*itrus L. genus includes many of the most important fruit crops in the world, such as orange varieties, lemons, tangerine, mandarins, grapefruits and others (Mabberley, 2008). Since ancient times, *Citrus* fruits have been used on a large scale as food, medical fields, ornamental properties and cosmetic (Dugo and Giacomo, 2002). Initially, based on morphological and geographical data, *Citrus* spp. taxonomy was found exclusively (Moore, 2001). However, according to the biochemical and morphological traits, an advanced suggest that there are only three 'true' *citrus* cultivars, i.e., mandarin (*C. reticulata* Blanco), pummelo (*C. Maxima* L. Osbeck), and citron (*C. medica* L.). Other mentioned cultivated spp. theorized to be hybrids derived as apomictically perpetuated biotypes (Barrett and Rhodes, 1976; Scora, 1988).

In the past two decades, studies of gene discovery, molecular genetics, genetic diversity, molecular breeding and population genetics based on the use of molecular markers and became routine and revolutionized biology. The rapid developments in the field of molecular genetics courses, a various techniques to be used in studying DNA polymorphisms for the selection of desired parents for improvement of cultivars through breeding programs (Whitkus *et al*., 1994; Karp *et al*., 1996; 1997a&b). The genetic diversity of a crop is fundamental to be known, to its improvement, providing a basis for selection of superior parental combination (Schlotterer, 2004).

Recently, DNA-based markers have gained popularity in genetic studies trough cultivars and cultivars. Similarly, in *Citrus*, molecular markers have been implemented in large-scale for germplasm studies, gene mapping, genomic characterization, and assessment of intra- and intergenetic variation. Randomly Amplified Polymorphic DNA (RAPD, Luro *et al.*, 1995; Higashi *et al*., 2000) and Inter-Simple Sequence Repeats (ISSRs, Fang and Roose, 1997; Bornet and Branchard, 2001; Pradeep-Reddy *et al.*, 2002) are more common in use among various molecular techniques, because of the combination of their analytical power and relative simplicity. The purpose of the present study was to determine the genetic rela-

tionship of some *Citrus* cultivars, including different families through RAPD and ISSRs molecular marker based PCR techniques.

## MATERIAL AND METHODS

### Plant materials

Eleven commercially important *Citrus* cultivars represented five species as shown in Fig. (1): six mandarin, four Clementine and one tangerine cultivars listed in Table (1) were used in this study. The trees were fifteen years old. The date of fruit maturity was recorded for each cultivar when fruits reached the maturity stage and become saleable. At harvest time, samples of 10 fruits from each cultivar were taken in determining some physical and chemical properties :

1. Fruit color score was determined by using a color chart (Robert, 1938)
2. The average number of seeds per fruit was counted.
3. T.S.S in juice was measured by hand refract meter; the total acidity percentage was determined according to the Official Methods of Analysis (A.O.A.C, 1990) and T.S.S/acidity was calculated as ratio between T.S.S and acid percentage.

### DNA extraction and PCR-based molecular markers

The collected leaf samples were immediately stored in liquid nitrogen until DNA extraction. Total genomic DNA of each genotype was extracted from young leaves using the modified cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The extracted DNA was solubilized and diluted to a final concentration of 30 ng/μL with 1X TE buffer and stored at -20°C until use.

Seventeen RAPD primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-07, OPA-08, OPA-09, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14, OPA-15, OPA-16, OPA-17 and OPA-18) listed in Table (2) and Ten ISSRs primers (844A, 17898A, 17899B, HB-08, HB-09, HB-10, HB-11, HB-13, HB-14 and HB-15) listed in Table (3) were used. PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The reaction mixture of 25 μl consisted of 50 ng of genomic DNA, 1 U of Taq DNA polymerase, 2.5 μl of 10X PCR amplification buffer, 0.2 mM dNTP, 10 p moles each of the primers and 1.5 mM $MgCl_2$.

Amplification for the RAPD and ISSRs-PCR was done by initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 45 second, annealing temperature of primers was 37-44°C for 45 second, extension at 72°C for 2 minute and the final extension was conducted at 72°C for 10 minutes.

### Gel analysis and phylogenetic relationships

Each variable RAPD and ISSRs bands were considered as a locus, so that every locus had two alleles and scored as present (1) or absent (0). For data analysis,

only polymorphic, reproducible, and clear-cut bands were kept. Phylogenetic relationships were estimated using NTSYSpc 2.01b software using the unweighted pair-group method using arithmetic averages; UPGMA (Rohlf, 2000).

## RESULTS AND DISCUSSION

### *Fruiting measurement*

Data in Table (4) shows the date of fruit maturity and some physical and chemical properties as well as peel color, average no. of seeds per fruit and T.S.S/acid ratio of the 11 *Citrus* cultivars. As for the dates of fruit maturity, some cultivars were very early in maturity date during (October) such as Nour Clementine, Fedela Clementine and Spinosa Clementine. On the other hand, Kara mandarin was considered to be late in the date of beginning of fruit maturity (January). Fedela Clementine, Sunburst mandarin and Fine Clementine induced the fruit color score Tangerine orange 9/1 (Dark orange color) compared to the other cultivars which color ranged from yellow and light orange to orange color .

Fedela Clementine, Kara mandarin, Spinosa Clementine, Seedless mandarin and Fine Clementine cultivars didn't contain any seeds in own fruits which were considered seedless fruit,  but the other cultivars were deferent in the average of seeds per fruits between high number such as Balady mandarin and Sunburst mandarin which ranged between (10-25 seeds per fruits for both cultivars) these results is in harmony with (Stephen *et al*., 1993)

who found that seed numbers will vary depending upon cross-pollination in Sunburst Tangerine but will generally average between 10 to 20 seeds per fruit.  But the other cultivars contain the low no. of seeds per fruit. T.S.S/acid ratios were nearly value between all cultivars under study. Mandarin cultivars recorded the highest value of T.S.S/acid ratio followed by Clementine cultivars, Fine Clementine recorded the lowest value compared to the all cultivars under study.

### *Identification based on DNA analysis*

Several powerful marker techniques are currently available for genetic analysis of both plant and animal species. The choice of the most appropriate technique for a specific study is not obvious and depends principally on the purpose of the research and the biology and genetic structure of the cultivars. Therefore, comparisons are needed in order to decide which technique is most appropriate for the issue being examined (Biswas *et al*., 2010). In this study, two of the most widely adopted marker techniques; RAPD and ISSRs were examined. In order to determine their utility in the discriminating and establishing genetic relationships among *Citrus* relatives, several approaches were followed (Siragusa *et al*., 2006; Biswas *et al*., 2010; Tripolitsiotis *et al.*, 2013).

### *Randomly amplified polymorphic DNA (RAPD) analysis*

Random Amplified Polymorphic DNA (RAPD-PCR) technique is simple, fast and sensitive. It requires no prior

knowledge of the DNA sequence and can amplify a large number of DNA fragments for the reaction. Table (5) listed the total 446 amplified fragments across the 11 *Citrus* cultivars which exhibited by the 17 random primers and there were 91 polymorphic bands (20.40%). Figure (2) showed an example using primer OP-17, the lowest number of RAPD amplified fragments was detected for primer OP-A13 that showed 7 fragments, while the highest number was 49 fragments with primer OP-A15. Primers OP-A13 gave the lowest percentage of polymorphism (28.57%), while primer OP-A05, OP-A08, OP-A09, OP-A11 and OP-A18 produced the highest percentage of polymorphism (100%) as shown in Table (5).

At the level of RAPD molecular markers, the 17 primers showed 327 fragments as a unique marker between positive and negative for each cultivar. The total numbers of amplified and polymorphic fragments generated by each primer and the specific markers for the 11 *Citrus* cultivars are shown in Table (6). For these reasons many fruit tree crops have been successfully fingerprinted using RAPD markers especially *Citrus* cultivars (Baig *et al.*, 2009; Leng *et al.*, 2012; Sun *et al.*, 2012). Similarity indices using NTSYSpc 2.01b software, pair-group method UPGMA (Rohlf, 2000) exhibited the highest degree was 57% between *Balady mandarin* (B.M.) and *Seedless mandarin* (Se.M.), whereas the lowest degree of similarity was 40% between *Fedela Clementine* (Fe.C.) and *Sunburst mandarin* (S.M.) (Table 7). The phyloge-

netic relationship divided the 11 cultivars into two main classes; each class was divided into two subclasses. Class I involves; *Nour Clementine*, *Fedela Clementine*, *Kishu seedless mandarin* and *Spinosa Clementine* obtained in Subclass I and *Balady mandarin*, *Seedless mandarin* also *Minneola tangelo* into subclass II. Whilst class II involves; *Willow leaf mandarin* and *Fina Clementine* obtained in Subclass I and *Kara mandarin* with *Sunburst mandarin* in subclass II as appeared in Fig. (3).

### Inter - simple sequence repeats (ISSRs) analysis

Among molecular markers, ISSRs represent an easy and widely adopted system, since their use does not require any prior information about target sequences and their efficiency and reproducibility are ensured (Fang and Roose, 1997; Bornet and Branchard 2001; Pradeep-Reddy *et al.*, 2002). Ten primers were used. Figure (4) shown an example using primer HB-15. The lowest number of ISSRs amplified fragments was detected for primer HB-14 that showed 19 fragments, while the highest number was 40 fragments with primer HB-10. The lowest percentage of polymorphism (81.48%) was given by primer HB-15, while primer 844A, HB-09, HB-11 and HB-14 produced the highest polymorphism (100%) (Table 8). ISSRs were successfully used to characterize *Citrus* germplasm (Scarano *et al.*, 2002; Shahsavar *et al.*, 2007; Uzun *et al.*, 2009) and in particular to distinguish among cultivars belonging to *Citrus* cultivars

(Fang and Roose, 1997; Uzun *et al.*, 2009) as well as in other cultivars (Terzopoulos *et al.*, 2005; Chen *et al.*, 2008; Lu *et al*., 2009). From 304 total amplified fragments, there were 211 specific markers between positive and negative for each cultivar with 78 polymorphic fragments (25.7%) of polymorphism (Table 9). Analysis with NTSYSpc 2.01b software, pair-group method UPGMA detected that the highest degree of similarity was between *Kara mandarin* and *Fedela Clementine* (51%), whereas the lowest degree of similarity (34%) between *Balady mandarin* and *Sunburst mandarin* (Table 10). The phylogenetic relationship divided the 11 cultivars into two main classes, each one of them was divided into two subclasses. Class I involves; (*Nour Clementine*) and (*Fedela Clementine* and *Kishu seedless mandarin*) and (*Seedless mandarin* with *Minneola tangelo* and *Kara mandarin*) and *Willow leaf mandarin* were collected in subclass I, whilst Subclass II contain *Spinosa clementine* and *Balady mandarin*. Class II involves; *Sunburst mandarin* and *Fina Clementine* as appeared in Fig. (5).

*Molecular genetic markers related to some fruiting traits*

In this study, as shown in Table (11), some RAPD and ISSRs markers (10-mer) may be linked to some fruiting traits such as beginning of fruit maturity (RAPD primer OPA-16 - 2278 bp. and ISSRs primer 844A - 385 bp), peel color (ISSRs primer 17895A - 1178 bp), average no. of seeds per fruit (RAPD primer OPA-10 -

1088 bp) and T.S.S/ acid ratio (RAPD primer OPA-7 - 909 bp). DNA marker-assisted selection was established by Ismail (2003) and Maklad (2012) in which genetic markers for some mango cultivars are likely to be useful to cultivars identification and to detect linkages with agriculturally important trait. The results from both techniques, showed that the average of polymorphism percentage through the 11 *Citrus* cultivars was higher using ISSRs than RAPD markers and the phylogenetic relationship was more reliable, indicate that ISSRs molecular marker for fingerprinting, mapping and diversity study of *Citrus* and its relatives. These results confirmed the usefulness of ISSRs-PCR analysis to detect the genetic variability between cultivars which agreed with who demonstrated that ISSRs markers are a valuable method for detecting genetic variability among rice varieties and for rapidly identifying cultivars (Siragusa *et al.*, 2006; Biswas *et al.*, 2010; Tripolitsiotis *et al.*, 2013).

## SUMMARY

*Citrus* L. genus includes several of the most important world's fruit crops, such as oranges, lemons, limes, mandarins, grapefruits, pummelos and kumquats. In this respect, the present study was to determine the genetic relationship of some *Citrus* species, including six mandarin, four Clementine and one tangerine cultivars through RAPD and ISSRs based PCR molecular marker. The obtained results showed that, the 11 tested *Citrus* cultivars were highly similar at the DNA level, and

exhibited using the 17 and 10 random primers, 91 and 78 polymorphic bands (20.40% and 25.7%, respectively) in both RAPD and ISSRs molecular markers, respectively. The highest degree of similarity when use RAPD molecular marker was between Balady mandarin and Seedless mandarin (57%) whereas the lowest degree of similarity (40%) between Fedela Clementine and Sunburst mandarin. But the highest degree of similarity between Kara mandarin and Fedela Clementine (51%) whereas the lowest degree of similarity (34%) between Balady mandarin and Sunburst mandarin when use ISSRs technique. In this study some RAPD and ISSRs markers may be linked to some fruiting traits such as beginning of fruit maturity (RAPD primer OPA-16 - 2278 bp and ISSRs primer 844A - 385 bp), peel color (ISSRs primer 17895A - 1178 bp), average no. of seeds per fruit (RAPD primer OPA-10 - 1088 bp) and T.S.S/ acid ratio (RAPD primer OPA-7 - 909 bp). The results from both techniques, showed that the average of polymorphism percentage through the 11 *Citrus* cultivars was higher using ISSRs than RAPD markers and the phylogenetic relationship was more reliable, indicate that ISSRs molecular marker for fingerprinting, mapping and diversity study of *Citrus* and its relatives. These results confirmed the usefulness of ISSRs-PCR analysis to detect the genetic variability between cultivars which agreed with who demonstrated that ISSRs markers are a valuable method for detecting genetic variability among rice varieties and for rapidly identifying cultivars.

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Table (1): Abbreviation, common and scientific name of five *Citrus* species represented by 11 cultivars used in this study.

| Abbreviation | Common Name | Scientific Name (Swingle system) |
|---|---|---|
| Mandarin | | |
| K.M. | Kishu seedless mandarin | *Citrus kinokuni mukakukishu* |
| B.M. | Balady mandarin | *Citrus reticulata* |
| Se.M. | Seedless mandarin | *Citrus reticulate* |
| W.M. | Willow leaf mandarin | *Citrus reticulata* Blanco - *Citrus deliciosa* Ten. |
| Ka.M. | Kara mandarin | *Citrus reticulata* Blanco |
| S.M. | Sunburst mandarin | *Citrus reticulata* Blanco RUTACEAE |
| * Clementine | | |
| N.C. | Nour clementine | *Citrus clementina* hort. ex Tanaka |
| Fe.C. | Fedela clementine | *Citrus clementina* |
| S.C. | Spinosa clementine | *Citrus deliciosa* var. *tangarina* |
| F.C. | Fina clementine | *Citrus deliciosa* var. *tangarina* |
| ** Tangerine | | |
| M.T. | Minneola tangelo | *Citrus* × tangelo - J.W. Ingram & H.E. Moore, 1975 |

* Clementine (Citrus × Clementine) is a hybrid between a Mediterranean Citrus × delicious and a sweet orange,

** Tangerine (*Citrus tangerina*) is an orange-colored *Citrus* fruit that is closely related to, or possibly a type of; mandarin orange (*Citrus reticulata*).*Citrus tangerina* is considered a separate species. Under the Swingle system, tangerines are considered to be a group of mandarin (*C. reticulata*) varieties. While tangerines genetically resemble mandarins, the genetics are still not thoroughly studied.

Table (2): Names, sequences and GC% for the 17 random primers used in RAPD-PCR technique.

| Primer names | Sequences | GC% |
|---|---|---|
| OPA-01 | 5′- CAG GCC CTT C –3′ | 70 |
| OPA-02 | 5′- TGC CGA GCT G –3′ | 70 |
| OPA-03 | 5′- AGT CAG CCA C –3′ | 60 |
| OPA-04 | 5′- AAT CGG GCT G –3′ | 60 |
| OPA-05 | 5′- AGG GGT CTT G –3′ | 60 |
| OPA-07 | 5′- GAA ACG GGT G –3′ | 60 |
| OPA-08 | 5′- GTG ACG TAG G –3′ | 60 |
| OPA-09 | 5′- GGG TAA CGC C –3′ | 70 |
| OPA-10 | 5′- GTG ATC GCA G –3′ | 60 |
| OPA-11 | 5′- CAA TCG CCG T –3′ | 60 |
| OPA-12 | 5′- TCG GCG ATA G –3′ | 60 |
| OPA-13 | 5′- CAG CAC CCA C –3′ | 70 |
| OPA-14 | 5′- TCT GTG CTG G –3′ | 60 |
| OPA-15 | 5′- TTC CGA ACC C –3′ | 60 |
| OPA-16 | 5′- TTC CGA ACC C –3′ | 60 |
| OPA-17 | 5′- GAC CGC TTG T –3′ | 60 |
| OPA-18 | 5′- AGG TGA CCG T –3′ | 60 |

Table (3): Codes, sequences and GC% for the 10 ISSRs primers used in a ISSRs-PCR technique.

| Primer code | Sequences | GC% |
|---|---|---|
| 844A | 5′- CTC TCT CTC TCT CTC TAC –3′ | 50.0 |
| 17898A | 5′- CAC ACA CAC ACA AC –3′ | 50.0 |
| 17899B | 5′- CAC ACA CAC ACA GG –3′ | 57.1 |
| HB-08 | 5′- GAG AGA GAG AGA GG –3′ | 57.1 |
| HB-09 | 5′- GTG TGT GTG TGT GG –3′ | 57.1 |
| HB-10 | 5′- GAG AGA GAG AGA CC –3′ | 57.1 |
| HB-11 | 5′- GTG TGT GTG TGT CC –3′ | 57.1 |
| HB-13 | 5′- GAG GAG GAG GC –3′ | 72.7 |
| HB-14 | 5′- CTC CTC CTC GC –3′ | 72.7 |
| HB-15 | 5′- GTG GTG GTG GC –3′ | 72.7 |

Table (4): Date of fruit maturity and some fruit properties; as well as peel color, average number of seeds per fruit and T.S.S /A ratio of the 11 *Citrus* cultivars.

| Cultivars | Date of fruit maturity | Peel color | Average no. of seeds/ fruit | T.S.S/A ratio |
|---|---|---|---|---|
| Kishu seedless mandarin | November | Orpiment orange 10/2 Orange color | 0 | 10.7 |
| Balady mandarin | First week of Dec. to last week of Feb. | Orange buff 507/1 Light orange color | 12 - 25 | 13.0 |
| Seedless mandarin | December to February | Orange buff 507/1 Light orange color | 0 | 13.0 |
| Willow leaf mandarin | December to February | Orpiment orange 10/2 Yellow to orange color | 18 | 13.0 |
| Kara mandarin | January | Orpiment orange 10/2 Yellow to orange color | 1 - 15 | 11.6 |
| Sunburst mandarin | Half of November till end of December | Tangerine orange 9/1 Dark orange color | 10 - 20 | 11.8 |
| Nour Clementine | October to December till January | Orange buff 507/1 Orange color | 1 - 2 | 10. 6 |
| Fedela Clementine | October to November | Tangerine orange 9/1 Dark orange color | 0 | 10.7 |
| Spinoza Clementine | October | Orange buff 507/1 Light orange color | 0 | 10.6 |
| Fine Clementine | November | Orpiment orange 10/2 Yellow to dark orange color | 0 | 8.7 |
| Minneola tangelo | December to February | Tangerine orange 9/1 Bright deep orange color | 0 - 15 | 11.6 |

Table (5): DNA amplified bands and polymorphism% generated with the 11 *Citrus* cultivars using 17 RAPD primers.

| Primer | Monomorphic bands | Unique bands | Polymorphic bands | Polymorphism% | Total |
|---|---|---|---|---|---|
| OPA-01 | 4 | 10 | 3 | 76.47 | 17 |
| OPA-02 | 2 | 3 | 5 | 80.00 | 10 |
| OPA-03 | 4 | 6 | 1 | 63.63 | 11 |
| OPA-04 | 2 | 10 | 6 | 88.88 | 18 |
| OPA-05 | 0 | 33 | 5 | 100.00 | 38 |
| OPA-07 | 1 | 13 | 0 | 92.85 | 14 |
| OPA-08 | 0 | 22 | 6 | 100.00 | 28 |
| OPA-09 | 0 | 25 | 5 | 100.00 | 30 |
| OPA-10 | 2 | 23 | 2 | 92.59 | 27 |
| OPA-11 | 0 | 13 | 4 | 100.00 | 17 |
| OPA-12 | 1 | 19 | 5 | 96.00 | 25 |
| OPA-13 | 5 | 2 | 0 | 28.57 | 7 |
| OPA-14 | 1 | 36 | 10 | 97.87 | 47 |
| OPA-15 | 2 | 38 | 9 | 95.91 | 49 |
| OPA-16 | 2 | 33 | 9 | 95.45 | 44 |
| OPA-17 | 2 | 21 | 10 | 93.93 | 33 |
| OPA-18 | 0 | 20 | 11 | 100.00 | 31 |
| Total | 28 | 327 | 91 | 20.40% | 446 |

Table (6): Number of amplified fragments and specific markers of the 11 *Citrus* cultivars based on RAPD-PCR analysis using 17 primers.

| Primers | TAF | PF | N.C. AF | N.C. SM | Fe.C. AF | Fe.C. SM | K.M. AF | K.M. SM | S.C. AF | S.C. SM | B.M. AF | B.M. SM | Se.M. AF | Se.M. SM | M.T. AF | M.T. SM | W.M. AF | W.M. SM | Ka.M. AF | Ka.M. SM | S.M. AF | S.M. SM | F.C. AF | F.C. SM | TSM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OPA-01 | 17 | 3 | 6 | 1+ | 6 | - | 5 | 1+ | 6 | 1+ | 5 | 1+ | 5 | - | 5 | 1+ | 5 | - | 6 | 2+ | 6 | 2+ | 5 | 1+ | 10 |
| OPA-02 | 10 | 5 | 6 | - | 6 | - | 6 | - | 5 | - | 6 | - | 6 | 1+ | 5 | - | 7 | 1+ | 6 | 1+ | 6 | - | | | 3 |
| OPA-03 | 11 | 1 | 4 | - | 4 | - | 5 | - | 5 | - | 5 | 1+ | 4 | - | 7 | 3+ | 4 | - | 5 | 1+ | 5 | 1+ | 4 | - | 6 |
| OPA-04 | 18 | 6 | 4 | - | 5 | 1+ | 3 | - | 4 | - | 4 | 2+ | 3 | - | 3 | 1+ | 3 | - | 5 | 2+ | 5 | 2+ | 5 | 2+ | 10 |
| OPA-05 | 38 | 5 | 5 | 3+ | 5 | 2+ | 1 | - | 4 | 3+ | 1 | 1+ | 7 | 7+ | 4 | 3+ | 1 | 1+ | 5 | 5+ | 5 | 4+ | 6 | 4+ | 33 |
| OPA-07 | 14 | 0 | 2 | 1+ | 2 | 1+ | 2 | 1+ | 3 | 2+ | 1 | - | 2 | 1+ | 3 | 2+ | 3 | 2+ | 1 | - | 2 | 1+ | | | 13 |
| OPA-08 | 28 | 6 | 3 | 2+ | 3 | 1+ | 3 | 3+ | 4 | 4+ | 3 | - | 4 | 2+ | 4 | 2+ | 2 | - | 2 | 2+ | 4 | 4+ | 3 | 2+ | 22 |
| OPA-09 | 30 | 5 | 3 | - | 8 | 8+ | 5 | 3+ | 5 | 2+ | 3 | - | 3 | - | 4 | 3+ | 1 | - | 5 | 4+ | 3 | 2+ | 4 | 3+ | 25 |
| OPA-10 | 27 | 2 | 3 | 1+ | 5 | 2+ | 5 | 2+ | 6 | 3+ | 4 | 1+ | 4 | 2+ | 5 | 2+ | 5 | 3+ | 4 | 2+ | 5 | 3+ | 5 | 2+ | 23 |
| OPA-11 | 17 | 4 | 2 | 1+ | 2 | 2+ | 3 | 2+ | 2 | 2+ | 2 | 1+ | 2 | 1+ | 2 | 2+ | 2 | - | 2 | 1+ | 2 | 1+ | 2 | - | 13 |
| OPA-12 | 25 | 5 | 4 | 2+ | 4 | 2+ | 3 | 1+ | 4 | 1+ | 3 | 2+ | 4 | 2+ | 2 | - | 9 | 7+ | 3 | 1+ | 2 | - | 4 | 1+ | 19 |
| OPA-13 | 7 | 0 | 5 | - | 5 | - | 5 | - | 5 | - | 5 | - | 5 | - | 5 | - | 7 | 2+ | 5 | - | 5 | - | 5 | - | 2 |
| OPA-14 | 47 | 10 | 8 | 4+ | 8 | 5+ | 7 | 3+ | 8 | 5+ | 6 | 5+ | 5 | 4+ | 4 | 3+ | 4 | 1+ | 6 | 2+ | 5 | 2+ | 7 | 2+ | 36 |
| OPA-15 | 49 | 9 | 4 | 1+ | 5 | 2+ | 7 | 3+ | 8 | 4+ | 11 | 8+ | 8 | 5+ | 10 | 6+ | 9 | 3+ | 10 | 3+ | 8 | 3+ | 6 | - | 38 |
| OPA-16 | 44 | 9 | 6 | 2+ | 7 | 4+ | 6 | 3+ | 7 | 3+ | 6 | 2+ | 7 | 2+ | 8 | 6+ | 8 | 3+ | 6 | 2+ | 8 | 3+ | 6 | 3+ | 33 |
| OPA-17 | 33 | 10 | 5 | 2+ | 5 | 1+ | 6 | 3+ | 7 | 4+ | 8 | 2+ | 7 | 1+ | 6 | - | 5 | 2+ | 6 | 3+ | 5 | 2+ | 5 | 1+ | 21 |
| OPA-18 | 31 | 11 | 5 | 3+ | 3 | 2+ | 4 | 2+ | 4 | 1+ | 6 | 2+ | 4 | 1+ | 5 | 3+ | 6 | 2+ | 3 | - | 6 | 1+ | 6 | 3+ | 20 |
| Total | 446 | 91 | 23 | | 33 | | 27 | | 35 | | 28 | | 28 | | 37 | | 28 | | 32 | | 31 | | 25 | | 327 |

TAF = Total amplified fragments  PF = Polymorphic fragments for each primer       AF = Amplified fragments
SM = Specific markers including either the presence or absence of a fragment  p = (+) ve
TSM = Total number of specific markers

Table (7): Similarity indices among the 11 *Citrus* cultivars based on RAPD-PCR using 17 primers.

| | N.C. | Fe.C. | K.M. | S.C. | B.M. | Se.M. | M.T. | W.M. | Ka.M. | S.M. | F.C. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| N.C. | 1.00 | | | | | | | | | | |
| Fe.C. | 0.544 | 1.00 | | | | | | | | | |
| K.M. | 0.464 | 0.428 | 1.00 | | | | | | | | |
| S.C. | 0.466 | 0.433 | 0.512 | 1.00 | | | | | | | |
| B.M. | 0.471 | 0.410 | 0.455 | 0.434 | 1.00 | | | | | | |
| Se.M. | 0.452 | 0.405 | 0.449 | 0.429 | 0.557 | 1.00 | | | | | |
| M.T. | 0.443 | 0.410 | 0.440 | 0.398 | 0.447 | 0.429 | 1.00 | | | | |
| W.M. | 0.455 | 0.407 | 0.465 | 0.407 | 0.433 | 0.453 | 0.407 | 1.00 | | | |
| Ka.M. | 0.430 | 0.422 | 0.415 | 0.409 | 0.410 | 0.417 | 0.410 | 0.444 | 1.00 | | |
| S.M. | 0.449 | 0.402 | 0.420 | 0.414 | 0.428 | 0.422 | 0.415 | 0.425 | 0.500 | 1.00 | |
| F.C. | 0.462 | 0.427 | 0.446 | 0.414 | 0.440 | 0.422 | 0.427 | 0.475 | 0.463 | 0.481 | 1.00 |

Table (8): DNA amplified bands and polymorphism% generated in 11 *Citrus* cultivars using 10 ISSRs primers.

| Primer | Monomorphic bands | Unique bands | Polymorphic bands | Polymorphism% | Total |
|---|---|---|---|---|---|
| 844A | 0 | 21 | 15 | 100.00 | 36 |
| 17898A | 1 | 31 | 4 | 97.22 | 36 |
| 17899B | 1 | 26 | 11 | 97.36 | 38 |
| HB-08 | 4 | 18 | 4 | 84.61 | 26 |
| HB-09 | 0 | 28 | 6 | 100.00 | 34 |
| HB 10 | 1 | 28 | 11 | 97.50 | 40 |
| HB 11 | 0 | 20 | 7 | 100.00 | 27 |
| HB 13 | 3 | 9 | 9 | 85.71 | 21 |
| HB 14 | 0 | 13 | 6 | 100.00 | 19 |
| HB 15 | 5 | 17 | 5 | 81.48 | 27 |
| Total | 15 | 211 | 78 | 25.7% | 304 |

Table (9): Number of amplified fragments and specific markers of the 11 *Citrus* cultivars based on ISSRs-PCR analysis using 10 primers.

| Primers | TAF | PB | N.C. | | Fe.C. | | K.M. | | S.C. | | B.M. | | Se.M. | | M.T. | | W.M. | | Ka.M. | | S.M. | | F.C. | | TSM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | AF | SM | |
| 844A | 36 | 15 | 5 | 1+ | 4 | 1+ | 6 | 2+ | 5 | 2+ | 7 | 4+ | 3 | 1+ | 5 | 1+ | 6 | 3+ | 1 | 1+ | 5 | 2+ | 7 | 3+ | 21 |
| 17898A | 36 | 4 | 3 | 2+ | 4 | 2+ | 6 | 5+ | 4 | 2+ | 7 | 6+ | 2 | 1+ | 4 | 1+ | 7 | 4+ | 2 | 1+ | 6 | 4+ | 5 | 3+ | 31 |
| 17899B | 38 | 11 | 8 | 4+ | 4 | 1+ | 8 | 4+ | 9 | 4+ | 9 | 2+ | 4 | 1+ | 5 | 1+ | 8 | 4+ | 5 | 1+ | 4 | - | 7 | 4+ | 26 |
| HB-08 | 26 | 4 | 8 | 3+ | 10 | 3+ | 9 | 2+ | 6 | 1+ | 8 | 2+ | 4 | - | 7 | 1+ | 6 | 2+ | 7 | 1+ | 6 | 1+ | 7 | 2+ | 18 |
| HB-09 | 34 | 6 | 2 | 2+ | 1 | - | 4 | 2+ | 4 | 4+ | 4 | 3+ | 3 | 2+ | 5 | 1+ | 6 | 4+ | 5 | 4+ | 5 | 3+ | 4 | 3+ | 28 |
| HB-10 | 40 | 11 | 7 | 5+ | 6 | 3+ | 7 | 1+ | 7 | 6+ | 6 | 3+ | 2 | - | 7 | 3+ | 5 | 2+ | 5 | 1+ | 6 | 2+ | 7 | 2+ | 28 |
| HB-11 | 27 | 7 | 4 | 2+ | 4 | 1+ | 4 | 2+ | 6 | 4+ | 4 | 2+ | 3 | - | 5 | 2+ | 5 | 1+ | 1 | 1+ | 4 | 3+ | 6 | 2+ | 20 |
| HB-13 | 21 | 9 | 7 | 2+ | 5 | - | 8 | 2+ | 7 | 1+ | 7 | 1+ | 4 | - | 6 | - | 7 | - | 5 | - | 6 | 1+ | 6 | 2+ | 9 |
| HB-14 | 19 | 6 | 6 | 2+ | 5 | 1+ | 5 | 1+ | 6 | 2+ | 5 | 2+ | 1 | 1+ | 6 | 2+ | 3 | - | 2 | - | 5 | 1+ | 5 | 1+ | 13 |
| HB-15 | 27 | 5 | 11 | 3+ | 10 | 2+ | 11 | 2+ | 9 | 1+ | 12 | 2+ | 8 | - | 10 | 2+ | 8 | - | 10 | 2+ | 7 | 2+ | 10 | 1+ | 17 |
| Total | 304 | 78 | | 26 | | 14 | | 23 | | 27 | | 27 | | 6 | | 14 | | 20 | | 12 | | 19 | | 23 | 211 |

TAF = Total amplified fragments   PF = Polymorphic fragments for each primer        AF = Amplified fragments
SM = Specific markers including either the presence or absence of a fragment   p = (+) ve
TSM = Total number of specific markers

Table (10): Similarity indices among the 11 *Citrus* cultivars based on ISSRs-PCR technique using 10 primers.

| | N.C. | Fe.C. | K.M. | S.C. | B.M. | Se.M. | M.T. | W.M. | Ka.M. | S.M. | F.C. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| N.C. | 1.00 | | | | | | | | | | |
| Fe.C. | 0.491 | 1.00 | | | | | | | | | |
| K.M. | 0.419 | 0.512 | 1.00 | | | | | | | | |
| S.C. | 0.419 | 0.483 | 0.412 | 1.00 | | | | | | | |
| B.M. | 0.415 | 0.443 | 0.438 | 0.470 | 1.00 | | | | | | |
| Se.M. | 0.400 | 0.460 | 0.392 | 0.412 | 0.447 | 1.00 | | | | | |
| M.T. | 0.446 | 0.478 | 0.453 | 0.423 | 0.403 | 0.489 | 1.00 | | | | |
| W.M. | 0.443 | 0.439 | 0.419 | 0.387 | 0.400 | 0.442 | 0.479 | 1.00 | | | |
| Ka.M. | 0.442 | 0.500 | 0.450 | 0.434 | 0.429 | 0.468 | 0.505 | 0.462 | 1.00 | | |
| S.M. | 0.365 | 0.430 | 0.344 | 0.359 | 0.341 | 0.364 | 0.456 | 0.400 | 0.433 | 1.00 | |
| F.C. | 0.416 | 0.444 | 0.409 | 0.409 | 0.421 | 0.429 | 0.452 | 0.416 | 0.449 | 0.475 | 1.00 |

Table (11): Performance of different the 11 *Citrus* cultivars against four fruit characters.

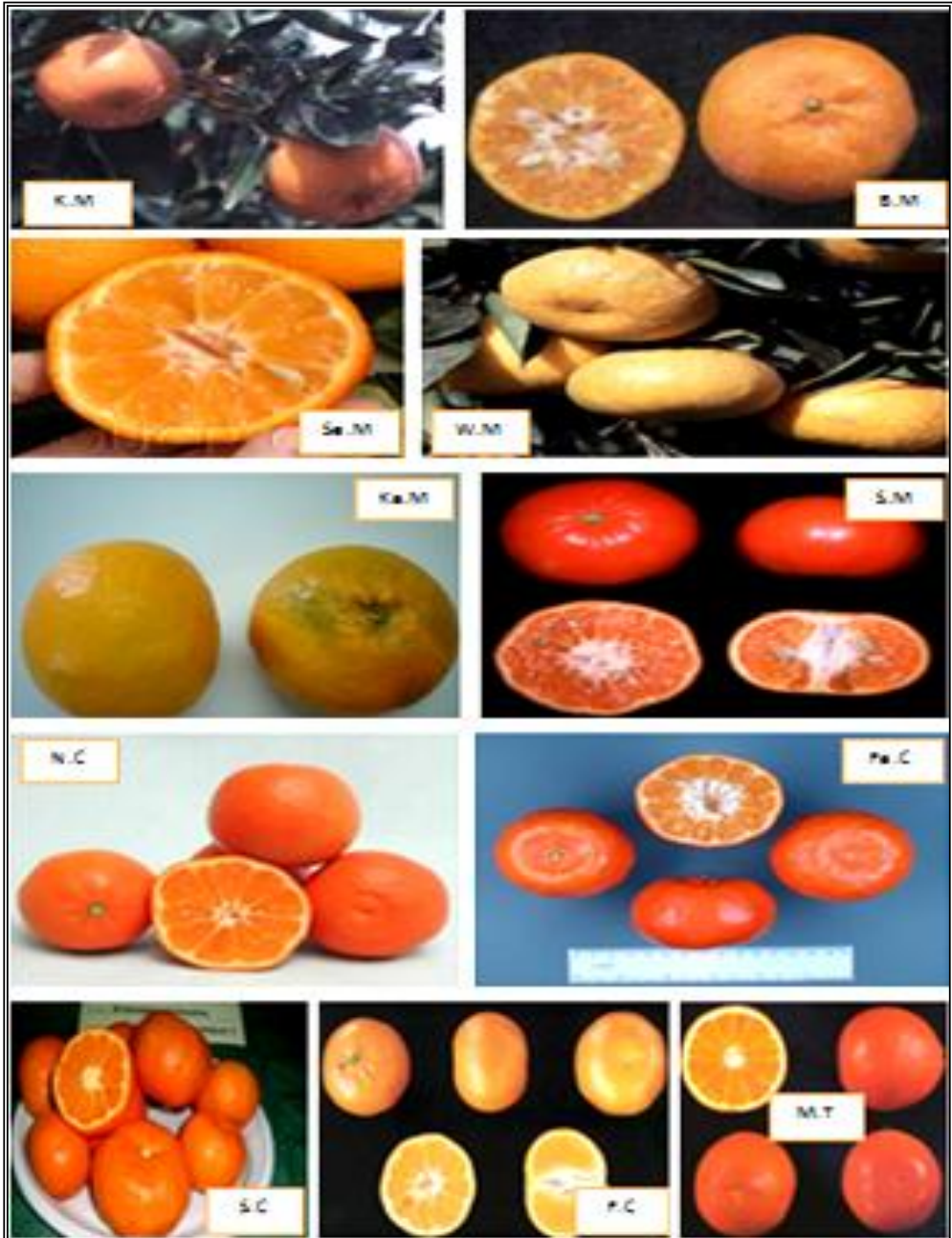| Fruit characters | Genotype | Cultivars | | | | | | | | | | | Marker linked | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N.C. | Fe.C. | K.M. | S.C. | B.M. | Se.M. | M.T. | W.M. | Ka.M. | S.M. | F.C. | Primer | MW |
| Date of fruit maturity | Early | + | + | - | + | - | - | - | - | - | - | - | OPA-16 | 2278 |
| | | | | | | | | | | | | | 844A | 385 |
| Peel color | Dark orange | - | + | - | - | - | - | - | - | - | + | + | ISSR 17898A | 1178 |
| Average no. of seeds per fruit | Not found | - | + | + | + | - | + | - | - | - | - | + | OPA-10 | 1088 |
| T.S.S /A ratio | Low | - | - | - | - | - | - | - | - | - | - | + | OPA-7 | 909 |

Fig. (1): Phenotype of the 11 *Citrus* cultivars, K.M. (*Kishu seedless mandarin*) - B.M. (*Balady mandarin*) - Se.M. (*Seedless mandarin*) - W.M. (*Willow leaf mandarin*) -- Ka.M. (*Kara mandarin*) - S.M. (*Sunburst mandarin*) - N.C. (*Nour clementine*) - Fe.C. (*Fedela clementine*) - - S.C. (*Spinosa clementine*) - F.C. (*Fina clementine*) - M.T. (*Minneola tangelo*). [The available *Citrus* cultivars in the breeding citrus program, 2006 Dr. Salama Eid Salem Shreif, Horticulture Research Institute]
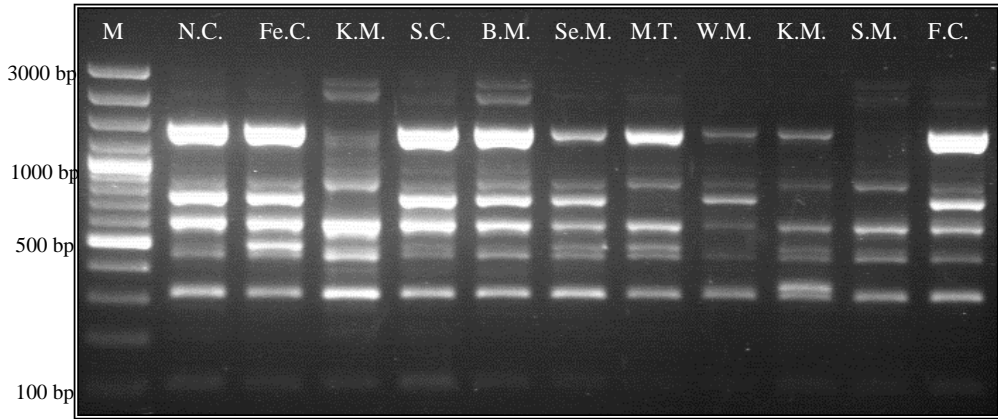
Fig. (2): RAPD profiles as detected for the 11 *Citrus* cultivars using primer OPA-17; M = 100 bp blus DNA ladder and names of species (Table 1).
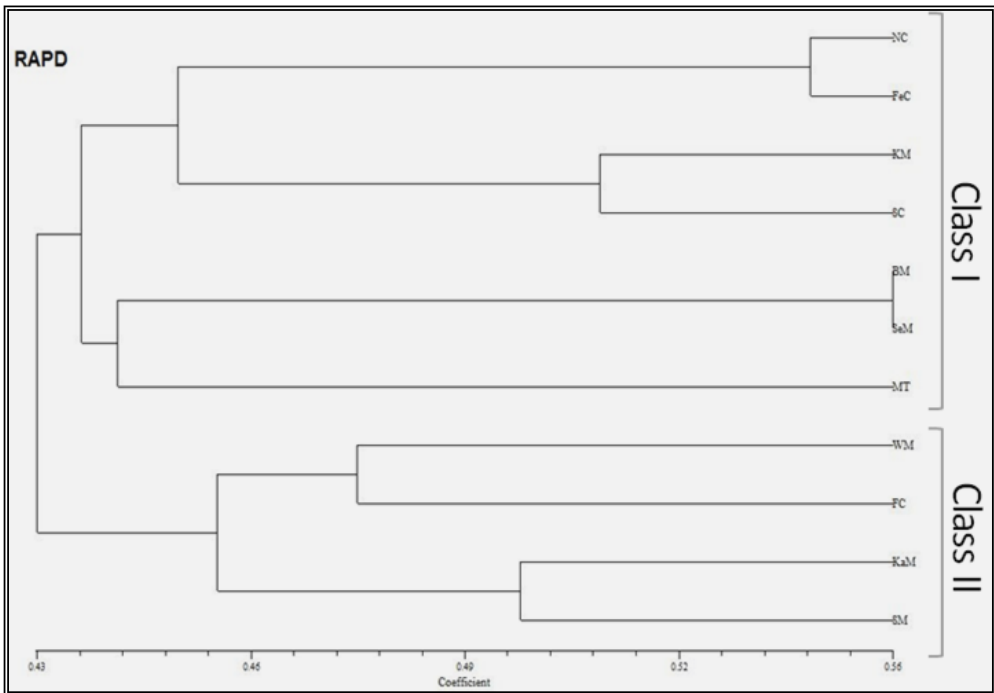


Fig. (3): Phylogenetic relationships between the 11 *Citrus* cultivars, according to RAPD-PCR technique using NTSYSpc 2.01b software.
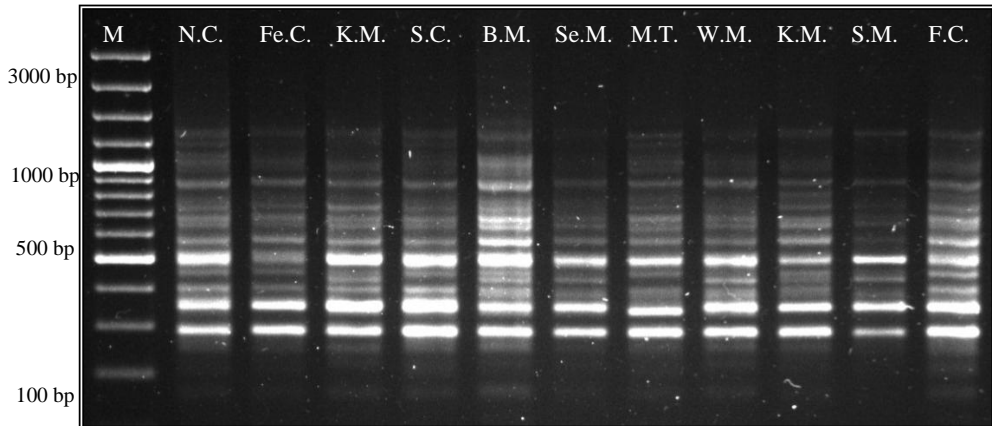
Fig. (4): ISSRs profiles as detected for the 11 *Citrus* cultivars using primer HB-15; M= 100 bp blus DNA ladder and names of species (Table 1).
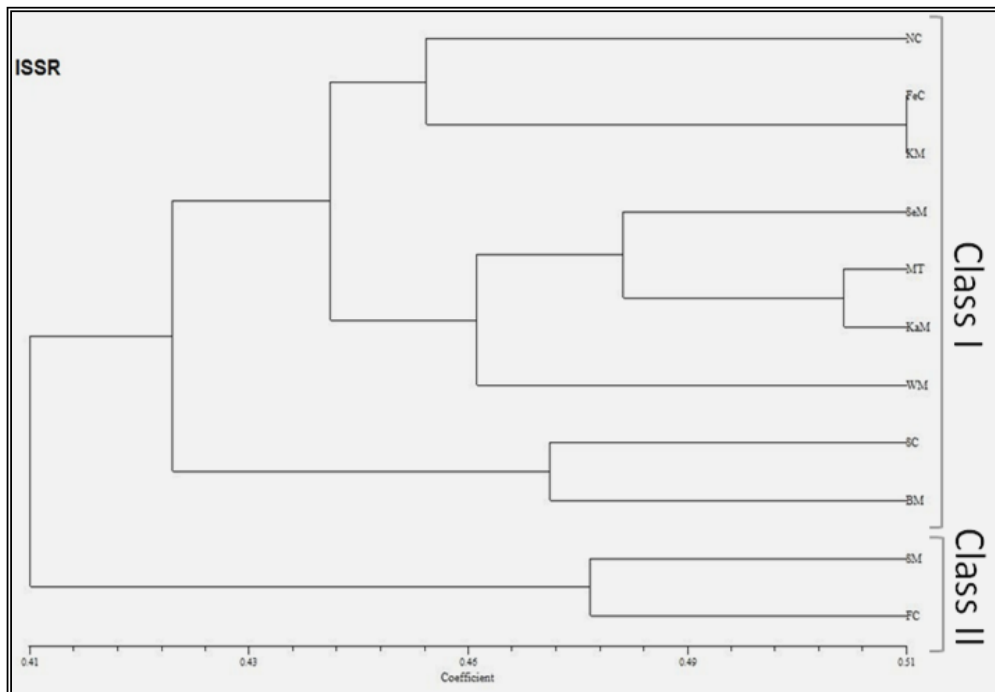


Fig. (5): Phylogenetic relationships between the 11 *Citrus* cultivars, according to ISSRs-PCR technique using NTSYSpc 2.01b software.