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Molecular Characterization and Genetic Diversity in Genus *Citrus* from Syria

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ABSTRACT

The genetic variability and the relationship between and within four main groups of the *Citrus* genus (Lemon, Mandarin, Grapefruit & Sweet orange) were investigated using molecular markers. Thirty one cultivars, represented by 93 tree samples, obtained from the germplasm collection maintained in the Department of *Citrus* Research in Tartous, Syria, were used in this study. DNA was analyzed by 10 Operon primers and 11 SSR primer pairs. Specific markers allowed the distinction between and within four *Citrus* groups were identified. The highest value of genetic diversity was detected in Mandarin group (0.513) while the lowest was in the Grapefruit group (0.074). The genetic relationships between different species were discussed.

Key Words: *Citrus*; Genetic diversity; Molecular marker; Syria

INTRODUCTION

Citrus is one of the most important fruit crops in the world, where the international production has reached 122 million tons (FAO, 2008). Currently two commonly classifications of *Citrus* are used, the Swingle system (Swingle & Reece, 1967) who recognized 16 species in the genus *Citrus* and Tanaka system (Tanaka, 1977), which recognizes 162 species in the genus *Citrus*. Scora (1975) and Barrett and Rhodes (1976) suggested that there are only three 'basic' true species of *Citrus* within the subgenus *Citrus* as defined by Swingle: Citron (*C. medica* L.), Mandarin (*C. reticulata* Blanco) and Pummelo (*C. maxima* L. Osbeck). Other cultivated *Citrus* species within the subgenus *Citrus* are believed to be hybrids derived from these true species, species of the subgenus *Papeda*, or closely related genera. This idea has recently supported by data derived from molecular markers (Federici *et al*., 1998; Nicolosi *et al*., 2000; Barkley *et al*., 2006).

High level of genetic variations existing among *Citrus* cultivated species due to frequent bud mutations, widely sexual compatibility between *Citrus* genus and related genera, apomixes and the long history of cultivation and the wide world dispersion (Scora, 1988). Phylogeny and taxonomy for certain *Citrus* cultivars have been somewhat debatable in the past; however, results from molecular marker technologies are helping to clarify some of these relationships. A variety of DNA markers is available and has been used to study the classification of *Citrus* genus, and phylogenetic relationships within *Citrus* and with related genera. Some of these markers are based on molecular hybridization (Yamamoto *et al*., 1993; Federici *et al*., 1998) and others are based on Polymerase Chain Reaction (PCR) technique (Nicolosi *et al*., 2000; Breto *et al*., 2001; Gulsen & Roose, 2001). The Random Amplified Polymorphic DNA (RAPD) and the microsatellite markers (Simple sequence repeats SSR) are widely used in different studies. For example, RAPD markers have been used for analysis of genetic diversity in *Citrus* (Coletta Filho *et al*., 1998; Das *et al*., 2004), for characterization of *Citrus* hybrids in breeding programs (Bastianel *et al*., 1998), for identification of *Citrus* lemon (Mariniello *et al*., 2005) and for phylogenetic analysis (Machado *et al*., 1996; Nicolosi *et al*., 2000). Microsatellite, or simple sequence repeats SSR, were extensively exploited for genetic studies (Zane *et al*., 2002). They have been employed for phylogenetic studies in *Citrus* (Pang *et al*., 2003; Barkley *et al*., 2006), for the assessment of genetic variability (Corazza-Nunes *et al*., 2002; Golein *et al*., 2005) and for the construction of genetic maps (Cristofani *et al*., 2003; Kijas *et al*., 1995).

However, the genetic variability in Syrian *Citrus* cultivars and germplasm collections has not been fully characterized. Therefore, the objectives of the present study were focused on the characterization of a group of *Citrus* species maintained in the Department of *Citrus* Research in Tartous, Syria and the evaluation of the genetic relationships between them using molecular markers.

MATERIALS AND METHODS

Plant materials: A total of 93 samples representing 31 cultivars of the four main groups of *Citrus* genus, three trees

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per cultivar, were used in this study (Table I). Young leaves (3-4 weeks old) were collected from the fields of Citrus Research Department in Tartous, Syria.

DNA isolation: Fresh young leaves (200 mg) were ground with 1 mL of preheated (65°C) extraction buffer (2% hexadecyltrimethylammonium bromide [CTAB: Sigma H-5882], 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). Mixtures were incubated at 60°C for 30 min. then extracted with chloroform-isoamyl alcohol (24:1). Aqueous phases containing DNAs were separated by centrifugation for 10 min., 4000 t/min, at room temperature, then transferred to 2 mL clean tubes. Nucleic acids were precipitated by adding 2/3 of their volumes of cold isopropanol. Nucleic acids were then washed twice with 76% ethanol, 10 mM ammonium acetate. After brief air drying, DNA pellets were resuspended in 300 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and kept at -20°C until use.

The analysis was conducted in the laboratory of Molecular Genetic in the Faculty of Agriculture, Tishreen University, Lattakia, Syria.

PCR Amplification and Electrophoresis

RAPD markers: Thirty eight decamer primers (Operon Technologies Inc.) were tested on 31 DNA samples (One sample from each cultivar). Ten of them (OPB-04, OPB-20, OPE-13, OPF-15, OPK-03, OPK-09, OPM-08, OPR-05, OPR-20, OPS-09) were selected to be used for Random Amplified Polymorphic DNA (RAPD) analysis. The selection was based on the specificity and the reproducibility of amplified products.

Each PCR reaction contained 15 ng of DNA template, 200 µM of dNTPs, 15 pg of Operon primer, 1 X of PCR buffer with 1.5 mM of $MgCl_2$ and one unit of *Taq* DNA polymerase, in a final volume of 15 µL. PCR program conditions consisted of 94°C for 5 min, then 35 cycles of 94°C for 1 min followed by 37°C for 1 min and 72°C for 3 min and one cycle at 72°C for 5 min. Electrophoresis was carried out on 1.5% agarose gel and stained with 0.5 mg L^{-1} of ethidium bromide.

SSR markers: Eleven SSR primer pairs were used in the analysis of the 93 DNA samples (Table II). The PCR amplifications were conducted in a total volume of 10 µL solution containing 35 ng of genomic DNA, 1 X of PCR buffer, 200 µL M of dNTPs, 3 µM of each primer and 0.5 unit of *Taq* DNA polymerase. PCR programs used for the amplification consisted of a cycle of 95°C for 5 min, 45 cycles of 95°C for 30 s followed by annealing step at 65°C for 30 s with -0.7°C/cycle for 15 cycles, then at 54°C for 30 cycle, then at 72°C for 1 and one cycle at 72°C for 7 min. The PCR products were separated on 6% acrylamide gel and stained with silver nitrate (Bassam & Caetano-Anollés, 1993).

Data analysis: The amplification products were scored as 1 and 0 for present and absent bands or alleles, respectively. The data matrix was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 3.2 (Rohlf, 1993). Dendrograms were generated by the Unweighted Pair Group Method with Arithmetic Average (UPGMA) (Sneath & Sokal, 1973). Analysis of genetic diversity (GD) was calculated according to the following formula of Nei (1987):

$$GD = n(1 - \Sigma p^2)/(n - 1)$$

Where (n) is the number of samples and (p) is the frequency of one allele.

Gene diversity was calculated as following:

$$H = (1 - \Sigma p_{ij}^2)$$

Where (p_{ij}) is the frequency of the jth allele generated with the primer i (Weir, 1990).

RESULTS

Variability and genetic diversity within *Citrus* genus: The amplification of 93 samples with 10 Operon primers produced a total of 57 different fragments (bands). Out of them, 36 were polymorphic. The number of fragments amplified per primer varied from 4 (OPE-13, OPF-15, OPM-08) to 8 (OPK-09), with an average of 5.7 fragments per primer. The highest number of polymorphic bands was detected by OPK-09 (7 polymorphic bands), while the lowest one was detected with OPF-15 and OPM-08 (2 polymorphic bands). The percentage of polymorphism detected by these primers ranged from 25% (OPE-13) to 87.5% (OPK-09) (Table III).

The highest number of polymorphic fragments was detected in Meyer, where 41 fragments were obtained. Out of them, 23 were polymorphic. The lowest number was detected in Mandalina and Ponkan, where a total of 29 fragments were amplified and 10 of them were polymorphic. In many cases, the samples belonging to the same cultivar produced the same RAPD pattern, as in Meyer, Maourdi and Marsh seedless grapefruit (Fig. 1).

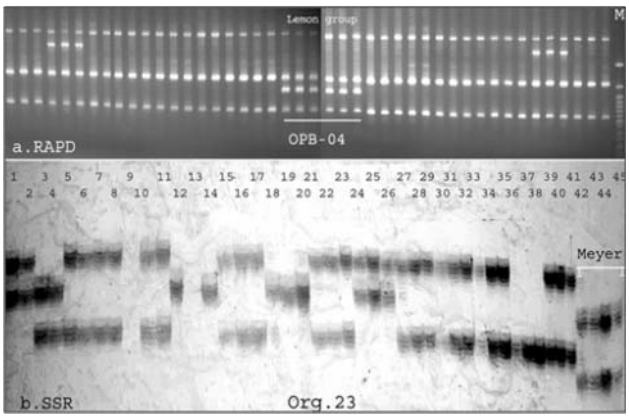
For SSR analysis, eleven SSR primer pairs were used in the analysis of 93 DNA samples. Out of them, 7 revealed polymorphic loci, while 4 showed monomorphic alleles on the concerned loci. The total number of alleles produced on the 7 loci was 31 (the four monomorphic loci were excluded from the analysis), ranging from 2 (Loci Org-8 & Org-10) to 8 (Locus Org-23) alleles per locus, with an average of 4.42 alleles/locus. The highest number of alleles was detected in the species Meyer, where the seven primers resulted in a total of 14 amplified products, of which 8 were polymorphic. The lowest number was detected in the sweet orange group, where a total of 10 amplified products were obtained with Khettmali, Balady and Succari, out of which 4 alleles were polymorphic. The value of polymorphic information content (PIC) was ranged from 0.176 (Org-8, Org-10) to 0.554 (Org-4).

Table I: List of *Citrus* species and cultivars used in this study with their common and scientific names

Common Names (Cultivars)	Species name according to Swingle system (1967)	Groups of *Citrus* genus
Meyer	*C. limon* (L.) Burm.f.	Lemon
Interdonato	*C. limon* (L.) Burm.f.	Lemon
Monachello	*C. limon* (L.) Burm.f.	Lemon
Abussoura (Washington navel)	*C. sinensis* (L.) Osbeck	Sweet orange
Maourdi	*C. sinensis* (L.) Osbeck	Sweet orange
Valencia	*C. sinensis* (L.) Osbeck	Sweet orange
Yafaoui	*C. sinensis* (L.) Osbeck	Sweet orange
Cadenera	*C. sinensis* (L.) Osbeck	Sweet orange
Moroblood	*C. sinensis* (L.) Osbeck	Sweet orange
Newhall navel	*C. sinensis* (L.) Osbeck	Sweet orange
Sanguinelli	*C. sinensis* (L.) Osbeck	Sweet orange
Gillette navel	*C. sinensis* (L.) Osbeck	Sweet orange
Hamlin	*C. sinensis* (L.) Osbeck	Sweet orange
Balady	*C. sinensis* (L.) Osbeck	Sweet orange
Salustiana	*C. sinensis* (L.) Osbeck	Sweet orange
Khettmali	*C. sinensis* (L.) Osbeck	Sweet orange
Succari	*C. sinensis* (L.) Osbeck	Sweet orange
Mandarin common	*C. reticulata* Blanco	Mandarin
Mandalina	*C. reticulata* Blanco	Mandarin
Clementine	*C. reticulata* Blanco	Mandarin
Nova	*C. reticulata* Blanco	Mandarin
Carvalhal	*C. reticulata* Blanco	Mandarin
Dancy	*C. reticulata* Blanco	Mandarin
Klimntard	*C. reticulata* Blanco	Mandarin
Fortune	*C. reticulata* Blanco	Mandarin
Ortanique	*C. reticulata* Blanco	Mandarin
Ponkan	*C. reticulata* Blanco	Mandarin
Satsuma	*C. unshui* Marc	Mandarin
Marsh seedless	*C. paradisi* Macf.	Grapefruit
Star ruby	*C. paradisi* Macf.	Grapefruit
Red blush	*C. paradisi* Macf.	Grapefruit

Fig. 1: Analysis of DNA samples and detection of specific markers with

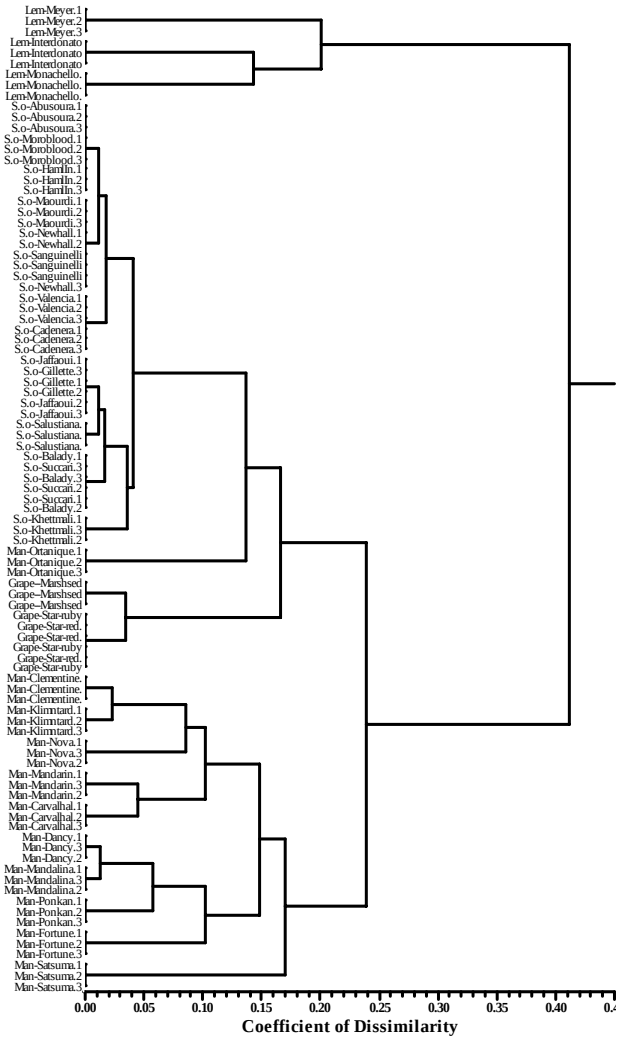
a. RAPD primer. b. SSR primer. M. stands for a molecular size marker



Species specific primers: The comparison of amplified products produced by RAPD primers revealed the presence of some specific primers allowing the differentiation between groups, species and cultivars. The analysis of the samples with OPB-04, OPF-15, OPR-05 showed many fragments amplified in all samples but each of these three primers produced a specific band, which was present only in Lemon group. On the other hand, the two groups, Lemon

Fig. 2: Dendrogram of genetic distance based on RAPD and SSR data in *Citrus* genus

Man stands for Mandarin group, Grape stands for Grapefruit, S.o stands for sweet orange, Lem stands for Lemon



and Grapefruit, were sharing one specific fragment amplified by the primer M08, which was absent in the other samples. Similar situations were reported with other primers, where they were able to produce specific fragments in other groups (Fig. 1, Table III).

The SSR primers also allowed the detection of specific alleles present in some cultivars or groups and absent in the others (Table III). Each of the two primer pairs (Org-8 & Org-10) generated only two alleles. One was present in cultivars belonging to the Lemon group and the other allele was present in the cultivars of the other groups. These primer could be called "Lemon specific primers". Similar results were detected with the primer Org-20 in the Grapefruit and with Org-30-F-98 for Sweet orange.

Genetic diversity and relationships within the genus *Citrus*: The value of genetic diversity was estimated for each group (Table IV). The highest value of genetic diversity was detected in Mandarin (0.513), while the lowest

Table II: Names and sequences of SSR primers used in this study and the value of gene diversity and polymorphic information content (PIC)

Primers Loci	Primer sequences 5' to 3'	Total no. of alleles detected	H*	PIC*
Org-3	TTCCTTATGTAATTGCTCTTTG TGTGAGTGTTTGTGCGTGTG	4	0.493	0.497
Org-4	TAAATCTCCACTCTGCAAAAGC GATAGGAAGCGTCGTAGACCC	4	0.549	0.554
Org-8	AGAAGCCATCTCTCTGCTGC AATTCAGTCCCATTCCATTCC	2	0.174	0.176
Org-10	CGCCAAGCTTACCACTCACTAC GCCACGATTTGTAGGGGATAG	2	0.174	0.176
Org-12	TCAACACCTCGAACAGAAGG CCCACATGCTAGCACAAAGA	1	0	0
Org-18	GACAACATCAACAAACGCAAGAGC AAGAAGAAGAGCCCCCATTAGC	1	0	0
Org-22	GGTACTGATAGTACTGCGGCG GCTAATCGCTACGTCTTCGC	1	0	0
Org-24	GCACCTTTTATACCTGACTCGG TTCAGCATTTGAGTTGGTTACG	1	0	0
Org-20	GGATGAAAAATGCTCAAAATGTAGTACCCACAGGGAAGAGAGC	6	0.497	0.501
Org-23	AGGTCTACATTGGCATTGTC ACATGCAGZTGCTATAATGAATG	8	0.486	0.490
Org-30-F-98	CTTCTTCTTCTCCTGCTCCTCCTC AGTGAGAAGCCAAAAACACCAAAC	5	0.531	0.536

H*: Gene Diversity PIC*: Polymorphic Information Content

one was in the Grapefruit group (0.0741). Data from RAPD and SSR markers were used to create three matrixes, one with RAPD data, one with SSR data and the last one for combined results from RAPD and SSR markers. The matrixes were used to estimate the genetic similarity between the samples (Nei & Li, 1979). The three dendrograms established showed similar clustering, where all studied species were clustered into two main branches (Fig. 2). The first one contained the three cultivars (Meyer, Monachello, Interdonato) belonging to the Lemon group. The second branch consisted of two main clusters, where all cultivars related to Mandarin were regrouped together, except for Ortanique samples, which were dispersed with the cultivars belonging to Sweet orange in the second group. Three cultivars of grapefruit were clustered in the same branch (Fig. 2).

Although the two dendrograms (based on RAPD data alone & on SSR data alone) showed similar clustering and distribution of species (data not presented), the dendrogram based on SSR data showed lower variation between cultivars comparing to the dendrogram based on RAPD data, especially within the Sweet orange group. The little differences existed between the two dendrograms were at the level of the distribution of cultivars inside the same group.

Table III: List of RAPD and SSR specific primers and number of amplified products

RAPD primers	Total no. of fragments	Specific fragments	Cultivars and groups
OPB04	5 (fragments)	1	Lemon
OPF15	4 (fragments)	1	Lemon
OPR05	7 (fragments)	1	Lemon
OPR05	7 (fragments)	1	Grapefruit
OPM08	4 (fragments)	1	Lemon and Grapefruit
OPS09	8 (fragments)	1	Mandarin and Sweet orange
OPB20	7 (fragments)	1	Lemon
OPB20	7 (fragments)	1	Sweet Orange and Ortanique
OPB20	7 (fragments)	1	Mandarin group except Ortanique
OPF15	4 (fragments)	1	Meyer (from Lemon Group)
SSR Primers	**N# of alleles**	**Sp. alleles**	**Cultivars and Groups**
Org-8	2 (Alleles)	1	Lemon
Org-10	2 (Alleles)	1	Lemon
Org-20	6 (Alleles)	1	Grapefruit
Org-20	6 (Alleles)	1	Satsuma mandarin
Org-23	8 (Alleles)	1	Ortanique
Org-23	8 (Alleles)	1	Lemon
Org-23	8 (Alleles)	1	Meyer (from Lemon Group)
Org-30-F-98	5 (Alleles)	1	Sweet orange

Table IV: Values of genetic diversity in the four groups of *Citrus*

Citrus groups	Sweet orange	Mandarin	Grapefruit	Lemon
Genetic diversity (GD)	0.158163	0.512397	0.074070	0.454321

DISCUSSION

RAPD and SSR markers were used to characterize and analyze the genetic variability between 93 samples from 31 cultivars belonging to the four main *Citrus* groups, Sweet orange, Mandarin, Lemon and Grapefruit. Among the loci analyzed by 11 SSR primer pairs, only 7 were polymorphic and some of them showed specific alleles that are able to identify some species or cultivars. The alleles produced by Org-8 and Org-10 were able to characterize all cultivars of Lemon group. The locus Org-23 possessed many alleles, among them one distinguishes the Lemon group, another was only present in Meyer cultivar (Lemon group) and a third specific allele was present only in Ortanique (Mandarin group). On the locus Org-20, six different alleles were detected. One of them was characteristic of Grapefruit group and another one was specific in Satsuma from Mandarin group. The group of Sweet orange can be identified by a specific allele on the locus Org-30-F-98. These specific alleles are very useful in breeding programs aiming to improve the *Citrus* genus when these species are used as parents in the cross. It was observed that some primers were able to identify particular genotypes in many other studies. Zerihum *et al*. (2009) identified some SSR markers producing specific alleles for particular genotypes of sweet orange and mandarin.

The RAPD markers were also very informative. The ten RAPD Operon primers, used in the analysis of our samples allowed the differentiation among species and the identification of some cultivars. They could also be used as marker assisted selection in the citrus breeding programs.

Similar results were obtained by Nicolosi *et al*. (2000) and Abkenar and Isshiki (2003), where RAPD markers allowing the distinction of some citrus genotypes were identified. Baig *et al*. (2009) has also identified 3 specific RAPD markers in Satsuma (Mandarin) only. Our results proved the feasibility of using molecular markers to distinguish between and within the different *Citrus* groups.

The analysis of molecular data showed high level of genetic similarity within the analyzed cultivars, while different levels of genetic diversity were detected within and between the four *Citrus* groups. These results were illustrated in the dendrogram established with the results of SSR and RAPD markers, which was divided into 2 distinct branches. One consisted of Lemon group cultivars and the second included the other three groups. The branch contained the Lemon group was very distant from the other groups and showed high level of genetic diversity among its different cultivars (GD= 0.454). Our results on Lemon group confirmed those of other studies showing the ability of the SSR and RAPD markers in the detection of polymorphism among lemon varieties, the evaluation of genetic relationships and the detection of high level of genetic diversity (Fang & Roose, 1997; Luro *et al*., 2001; Mariniello *et al*., 2005). In the second branch, three distinct clusters were also identified. The cluster of Sweet orange showed the highest level of genetic similarity, where the 14 cultivars analyzed were regrouped in seven close groups (the maximum index of genetic distance was about 0.05) with a value of genetic diversity of 0.158. The high level of similarity within the Sweet orange group was expected as it was showed in many other studies, when many cultivars were analyzed with minisatellite, ISSR, RFLP and SSRs (Orford *et al*., 1995; Fang & Roose, 1997; Novelli *et al*., 2000; Novelli *et al*., 2006). The use of RAPD markers also achieved a similar conclusion, where Targon *et al*. (2000) reported that RAPD markers were not suitable for differentiation of Sweet orange genotypes. In contrary, some studies demonstrated the presence of genetic diversity and the detection of difference between Jaffa and Blood red cultivars from Sweet orange group (Baig *et al*., 2009), between the Navel cultivars, Newhall and Gillette (Fang & Roose, 1997) and within sweet orange genotypes (Shaaban *et al*., 2006).

The three cultivars belonging to Grapefruit group were similar and were represented in 2 different patterns. They showed a small index of genetic distance with a low value of genetic diversity (GD= 0.0741). The high level of genetic similarity within the group is in accordance with the results of Fang and Roose (1997), who reported that among seven cultivars of Grapefruit, only one was deviated from the others. Corazza-Nunes *et al*. (2002) also studied the genetic variability in grapefruits and pummelos using RAPD and SSR markers and found that the majority of grapefruit accessions showed a narrow genetic base. The high level of similarity in Grapefruit group supported the hypothesis that the majority of Grapefruit cultivars were derived from the same ancestral tree by mutations (Gmitter, 1995). In our dendrogam of *Citrus*, the Grapefruit group was placed closer to the Sweet orange than to Mandarin group and this is in accordance with Swingle (1946), Scora (1975) and Barrett and Rhodes (1976), who believed that the grapefruit was produced from a cross between *C. grandis* × *C. sinensis* (Sweet orange).

The highest level of genetic diversity existed in the Mandarin group (GD= 0.512) where the index of genetic distance between different cultivars was about 0.38 and the 11 cultivars were represented by 11 different patterns. Ten cultivars from Mandarin group were regrouped in one cluster while the only cultivar, Ortanique, was placed closer to Sweet orange and Grapefruit than to Mandarin group. This situation could be explained by its origin, where it is known that Ortanique is a hybrid between the Sweet orange (*C. sinensis*) and the Mandarin (*C. reticulata*) (Swingle 1946).

High level of genetic diversity in Mandarin group has been previously reported by several studies using isozymes (Torres *et al*., 1982), RFLP and RAPD (Federici *et al*., 1998) and ISSR (Fang *et al*., 1998). These studies proposed that Mandarins constitute only one species C. *reticulata* composed of different phenotypes, genotypes and a large number of hybrids. The high level of variations existing between Mandarin cultivars allowed the identification of the different cultivars with molecular markers. Our results showed that the level of genetic diversity detected between the Mandarin cultivars was higher than the level of genetic diversity between the cultivars of the other groups. The Mandarin group was divided into two sub groups and the index of genetic similarity ranged from 1 (100% similarity) between Dancy and Mandalina to 0.86 between Satsuma and Clementine. This information is consistent with the observation made by Swingle and Reece (1967) that Mandalina is a Lebanese cultivar and it seems very similar to Dancy. Nicolosi *et al*. (2000) have reported, using RAPD and SCAR markers, that all the Mandarin cultivars were grouped together in one cluster. They also mentioned that Dancy and Bonkan were very closely linked and clearly separated from Clementine and Satsuma so that they were placed in another cluster. This finding was confirmed in our study.

In conclusion, the use of RAPD and SSR markers in our study allowed us to compare all cultivars present in the Department of Citrus Research in Tartous, Syria. They proved the high level of genetic similarity within each cultivar and within the species of Grapefruit and Sweet orange. The identification of molecular markers specific in some cultivars will become useful and efficient tools in the breeding programs of *Citrus*.

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