

1 ASSESSMENT OF GENETIC VARIABILITY WITHIN THE 2 GENUS *CITRUS* IN SYRIA USING SSR MARKERS

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9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 ABSTRACT

Aims: The characterization and the estimation of genetic variability between accessions belonging to *Citrus* genus using the SSRs markers.

Place and Duration of Study: Laboratory of Molecular Genetic, Faculty of Agriculture, Tishreen University, Lattakia, Syria, between August 2011 to March 2013.

Methodology: 114 samples representing 4 groups of *Citrus*, obtained from the Department of *Citrus* Research in Tartous, Syria, were used in this study. DNA was extracted from young leaves and analyzed with 26 SSRs primer pairs.

Results: Six primers produced monomorphic alleles, and the other 20 primers produced 95 different alleles. The highest number of alleles (32) was detected in Lemon group while the lowest number (28 alleles) was revealed in Mandarin group. The values of genetic diversity were calculated and ranged from 0.079 in Grapefruit to 0.533 in Mandarin groups. A dendrogram based on the index of genetic distance was established and showing clear separation between *Citrus* groups where they clustered into two distinct branches. The first one containing cultivars of Lemon group, while the second one included 4 distinct clusters, one for Mandarin cultivars group, one for sweet orange group, one for Grapefruit and pummelo and the fourth for Kumquat accessions.

12 specific alleles were identified, they will be a helpful tool in the *Citrus* breeding programs.

Conclusion: The results obtained in the present work proved the utility of SSR markers for evaluating the genetic diversity and relationships between *Citrus* species maintained in the Department of *Citrus* Research in Tartous, Syria, and showed high level of genetic similarity within each cultivar and within the species of Grapefruit and of Sweet orange at the tested SSR loci.

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Key Words: *Citrus*, Genetic variability, Molecular marker, SSR, Syria.

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1. INTRODUCTION

Citrus is one of the most widely cultivated fruit trees in the world, where the global production was close to 123 million tons [1]. The taxonomy and phylogeny of *Citrus* are very complicated due to frequent bud mutations, apomixes, widely sexual compatibility between *Citrus* genus and related genera, in addition to the long history of cultivation [2].

The most widely accepted taxonomic systems for *Citrus* are the Swingle system [3] and Tanaka system [4]. There were many disagreements between these two systems, where Swingle described 16 species in *Citrus*, while Tanaka recognized 162 species. Phylogenetic analysis by Scora [5] and Barrett and Rhodes [6] suggested the existence of only three true species in *Citrus* within the subgenus *Citrus*: citron (*C. medica* L.), mandarin (*C. reticulata* Blanco) and pummelo (*C. maxima* L. Osbeck). Other accessions were originated from one or more generations of hybridization among these true species or between them and species of the subgenus *Papeda* or closely related genera. This concept has recently received a great support from various studies using molecular markers [7, 8, 9].

With the advance and the development of molecular techniques, various molecular markers have been used to evaluate phylogenetic relationships within *Citrus* and with related genera. Microsatellites, or simple sequence repeats (SSR,) are one of these markers which are highly polymorphic, and are used in a wide range of germplasm collections. In *Citrus*, SSRs were extensively exploited for genetic studies [10,11], for the assessment of genetic variability [12,13], for phylogenetic analysis [14,15], for zygotic and nucellar seedlings identification [16, 17] and for the construction of genetic maps [18,19].

Little is known about the genetic variability of Syrian *Citrus* cultivars and germplasm collections at molecular level [20, 21]. 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 their genetic relationships using molecular markers (SSRs).

2. MATERIALS AND METHODS

2.1 Plant materials:

A total of 114 samples (accessions) representing 37 cultivars (Three trees per cultivar, each tree represents an accession) belonging to the four main groups of *Citrus* genus (maintained in the Department of *Citrus* Research in Tartous, Syria), in addition to three Kumquat accessions belonging to *Fortunella margarita* (Lour.), were used in this study (Table 1).

2.2 DNA isolation:

Young leaves (3-4 weeks old) were collected and used for DNA isolation. 400 mg of fresh leaves were ground and extracted with CTAB protocol [22]. After brief air drying, DNA pellets were re-suspended in 300 µl (300 micro-liter) of 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.

2.3 PCR Amplification and Electrophoresis

SSR analysis: Twenty six SSR primer pairs derived from *Poncirus trifoliata* [23], recognizing 26 different loci (Provided by Atomic Energy Commission of Syria, AECS), were used in the analysis of DNA samples. Six of them generated monomorphic products and the other twenty were able to distinguish between samples (Table 2). The PCR reaction was composed of 35 ng of genomic DNA, 1 X of PCR buffer, 200µM of each dNTPs, 3 µM of each primer and 0.5 unit of *Taq* DNA polymerase in a total volume of 10 µl. Samples were subjected to a PCR program consisted of a cycle at 95°C for 5 min, 45cycles at 95°C for 30 Sec followed by an annealing step at 65°C for 30 Sec. with -0.7°C/cycle for 15 cycles, then

at 58°C for 30 cycles, then at 72°C for 1 min. and one cycle at 72°C for 5 min. The PCR products were separated on 6% acrylamide gel and stained with silver nitrate [24].

Table 1. List of *Citrus* cultivars used in this study.

Number assigned to cultivars.	Common Names	Species name according to Tanaka system	Groups of <i>Citrus</i> genus
1	Meyer	<i>C. meyeri</i> Tan.	Lemon
2	Interdonato	<i>C. limon</i> (L.) Burm.f.	Lemon
3	Monachello	<i>C. limon</i> (L.) Burm.f.	Lemon
4	Santa teresa	<i>C. limon</i> (L.) Burm.f.	Lemon
5	Eureka	<i>C. limon</i> (L.) Burm.f.	Lemon
6	Washington navel	<i>C. sinensis</i> (L.) Osb.	Sweet orange
7	Cara Cara	<i>C. sinensis</i> (L.) Osb.	Sweet orange
8	Gillette navel	<i>C. sinensis</i> (L.) Osb.	Sweet orange
9	Newhall navel	<i>C. sinensis</i> (L.) Osb.	Sweet orange
10	Valencia	<i>C. sinensis</i> (L.) Osb.	Sweet orange
11	Jaffa	<i>C. sinensis</i> (L.) Osb.	Sweet orange
12	Salustiana	<i>C. sinensis</i> (L.) Osb.	Sweet orange
13	Maourdi	<i>C. sinensis</i> (L.) Osb.	Sweet orange
14	Sanguinelli	<i>C. sinensis</i> (L.) Osb.	Sweet orange
15	Moro Blood	<i>C. sinensis</i> (L.) Osb.	Sweet orange
16	Hamlin	<i>C. sinensis</i> (L.) Osb.	Sweet orange
17	Cadenera	<i>C. sinensis</i> (L.) Osb.	Sweet orange
18	Balady	<i>C. sinensis</i> (L.) Osb.	Sweet orange
19	Succari	<i>C. sinensis</i> (L.) Osb.	Sweet orange
20	Khetmali	<i>C. sinensis</i> (L.) Osb.	Sweet orange
21	Mandarin common	<i>C. reticulata</i> Blanco	Mandarin
22	Mandalina	<i>C. reticulata</i> Blanco	Mandarin
23	Clementine	<i>C. clementina</i>	Mandarin
24	Nova	<i>C. clementina</i> x (<i>C. paradisi</i> x <i>C. tangerina</i>)	Mandarin
25	Carvalhal	<i>C. reticulata</i> Blanco	Mandarin
26	Dancy	<i>C. tangerina</i> Hort.ex.Tan.	Mandarin
27	Klimntard	<i>C. reticulata</i> Blanco	Mandarin
28	Fortune	<i>C. reticulata</i> Blanco	Mandarin
29	Ortanique	<i>C. sinensis</i> x <i>C. reticulata</i>	Mandarin
30	Minneola	<i>C. reticulata</i> x <i>C. paradisi</i>	Mandarin
31	Ponkan	<i>C. poonensis</i> Tan.	Mandarin
32	Satsuma	<i>C. unshui</i>	Mandarin
33	Marsh seedless	<i>C. paradisi</i> Macf.	Grapefruit
34	Star ruby	<i>C. paradisi</i> Macf.	Grapefruit
35	Red blush	<i>C. paradisi</i> Macf.	Grapefruit
36	Pummelo	<i>C. grandis</i> (L.) Osb.	pummelo
37	Red Pummelo	<i>C. grandis</i> (L.) Osb.	pummelo
38	Kumquat	<i>Fortunella margarita</i> (Lour.)	

2.4 Data analysis:

The amplification products were scored as 1 and 0 for present and absent alleles, respectively. The data matrix was analyzed and genetic distance was estimated using SIMQEND in the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 3.2 [25]. Dendrograms were generated by the Unweighted Pair Group Method with Arithmetic Average (UPGMA) and using SAHN as clustering method (26). Analysis of genetic diversity (GD) was calculated according to the following formula of Nei [27]:

$$GD = n(1 - \sum P^2) / (n - 1)$$

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

Polymorphism Information Content (PIC) was calculated according to the formula:

$$PIC = 1 - \sum_{i=1}^n P_i^2 - 2 \left[\sum_{i=1}^{n-1} \sum_{j=i+1}^n P_i^2 P_j^2 \right]$$

Where P_i = Frequency of i^{th} allele, n = Number of alleles.

3. RESULTS

3.1. Variability and genetic diversity within *Citrus* genus:

The total number of alleles produced on the 20 polymorphic loci was 95, ranging from 2 alleles/Loci Org-8, Org-28 and Org-10) to 9 alleles/locus (Loci Org-11 and Org-23), with an average of 4.75 alleles/locus (Table 2). The highest number of alleles was detected in Meyer species where a total of 32 alleles were amplified, while the lowest number (26 alleles) was detected in Pummelo. Higher number of polymorphic alleles are expected in the accession of hybrid origin (7), therefore the score of alleles showed that the number of polymorphic bands possessed by such accessions ranges from 11 (in Pummelo accessions) to 19 (in Meyer accessions). The Genetic diversity (GD) and the values of polymorphic information content (PIC) were calculated and was ranged from 0.231 and 0.233 respectively (Loci Org-8 and Org-10) to 0.608 and 0.613, respectively (Locus Org-4). The high value of genetic diversity in a locus reflect the high level of mutations affecting this locus.

Table 2 Names and sequences of SSR primers used in this study with the number of detected alleles, the genetic diversity (GD) and Polymorphism information contents (PIC).

Loci	Primer sequences 5' to 3'	Number of detected alleles	Genetic diversity (GD)	Polymorphism information contents (PIC)
Org-1	F: TTTGACATCAACATAAAACAAGAAATT R: TTAAATCCCTGACCAGA	4	0.472	0.476
Org-2	F: AAAGGGAAAGCCCTAATCTCA R: CTTCTCTTGCGGAGTGTTT	6	0.512	0.516
Org-3	F: TTCCTTATGTAATTGCTCTTTG R: TGTGAGTGTTGTGCGTGTG	4	0.496	0.5
Org-4	F: TAAATCTCCACTCTGCAAAAGC R: GATAGGAAGCGTCGTAGACCC	5	0.608	0.613
Org-7	F: GGTGATGCTGCTACTGATGC R: CAATTGTGAATTTGTGATTCCG	4	0.205	0.206
Org-8	F: AGAAGCCATCTCTCTGCTGC R: AATTCAGTCCCATTCCATTCC	2	0.231	0.233
Org-9	F: AACACTCGCACCAAATCCTC R: TAAATGGCAACCCAGCTTTG	3	0.447	0.450
Org-10	F: AATGCTGAAGATAATCCGCG R: TGCCTTGCTCTCCACTCC	2	0.231	0.233
Org-11	F: GCTTTGATCCCTCCACATA R: GATCCCTACAATCCTTGGTCC	9	0.375	0.378
Org-14	F: CGCCAAGCTTACCACTCACTAC R: GCCACGATTGTAGGGGATAG	8	0.479	0.483
Org-15	F: CGAACTCATTAAAAGCCGAAAC R: CAACAACCACCACTCTCACG	3	0.229	0.231
Org-17	F: GCCTTCTTGATTACCGGAC R: TGCTCCGAACCTTCATCATTG	5	0.485	0.489
Org-19	F: GAAAGGGTTACTTGACCAGGC R: CTTCCCAGCTGCTTGCAACAGC	4	0.498	0.502
Org-20	F: GGATGAAAAATGCTCAAAATG R: TAGTACCCACAGGGAAGAGAGC	6	0.541	0.545
Org-21	F: AGAGAAGAAACATTTGCGGAGC R: GAGATGGGACTTGTTTCATCACG	4	0.384	0.387
Org-23	F: AGGTCTACATTGGCATTGTC R: ACATGCAGZTGCTATAATGAATG	9	0.285	0.287

Org-26	F: CTTCTCTTTCGCGAGTGTTTC R: GAGGGAAAGCCCTAATCTCA	6	0.510	0.514
Org-27-F87	F: ATGAAGGCTTTTATAGAGCCGAGTT R: ATAATAGGGGCCCACTTGACTTG	3	0.545	0.549
Org-28-F88	F: GTTCGCTCCACGCGATTTAT R: TGTGAAGAAAGATTTGGTGGGTTT	2	0.361	0.364
Org-30-F97	F: CTTCTTCTTCTCCTGCTCCTCCTC R: AGTGAGAAGCCAAAAACACCAAAC	5	0.561	0.565

Genetic diversity values were estimated for each group of *Citrus* (Table 3), where the highest value was detected in Mandarin group (0.533), while the lowest one was in the Grapefruit group (0.0741).

Table 3: Values of genetic diversity in *Citrus* groups.

<i>Citrus</i> group	Sweet orange	Mandarin	Lemon	Grapefruit	Pummelo
Alleles number	29	28	32	30	26
Genetic diversity (GD)	0.179	0.533	0.494	0.079	0.121
Hobs	0.5	0.448	0.728	0.428	0.31

Hobs values of observed heterozygosity.

3.2. Species specific primers:

The comparison of alleles detected in all *Citrus* samples led to the identification of some specific alleles present in one species or group and absent in all other samples. 12 alleles were considered as specific alleles. (Table 4, Fig. 1).

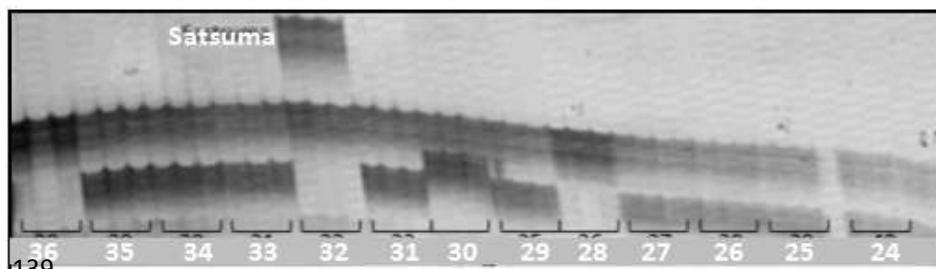


Fig. 1: Analysis of DNA by SSR primer pair (Org-3) on 6% acrylamide gel, showing polymorphic alleles in *Citrus* accessions and the specific allele in Satsuma. Number 24- 36 represent *Citrus* cultivars (refer to table 1).

The alleles produced by Org-8, Org-10 and Org-15 were able to characterize all cultivars of Lemon group. Eight alleles were detected on locus Org-23, three of them were specific, one was specific in the Lemon group, the second was specific in Meyer only (of lemon group) and the third one was specific in Ortanique (of mandarin group). Two specific alleles were detected on locus Org-20, one distinguished the grapefruit group and the other 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-97. The locus Org-11 produced 9 alleles, one of them was specific in pummelo, while another one found only in kumquat. Similar results were shown with the primer pairs Org-7 in the grapefruit and Pummelo and with Org-30-F-97 for Sweet orange. These specific alleles have great importance in breeding programs, especially to examine the success of hybridization in early stages.

Table 3.7 List of SSR species specific primers and the distinct cultivars.

Primers (Loci)	Total number of alleles	Number of specific alleles	Cultivars or Groups
Org-3	4	1	Satsuma mandarin
Org-7	2	1	Grapefruit and Pummelo
Org-8	2	1	Lemon
Org-10	2	1	Lemon
Org-11	9	1	Kumquat
Org-15	2	1	Lemon
Org-20	6	2	1 in Grapefruit, 1 in Satsuma (mandarin)
Org-23	8	3	1 in Lemon, 1 in Meyer, 1 in Ortanique
Org-30-F97	8	1	Sweet orange

3.3 Genetic relationship between Citrus accessions

The data obtained through the analysis of DNA by SSR primers were used to calculate the similarity and dissimilarity index which reflects the genetic distance between the accessions, according to Nei and Li [28]. The obtained values of dissimilarity were used to establish the dendrogram generated by UPGMA (Fig 2)

The dendrogram established using the coefficient of genetic distance showed two distinct branches. The first branch contains all accessions belonging to lemon group (accessions of Meyer, Interdonato, Monachello, Santa Teresa and Eureka), while the second one was divided into four distinct clusters. All mandarin accessions were grouped together in one cluster except Ortanique accessions which were dispersed in the second cluster between sweet orange accessions. Pummelo and grapefruit accessions formed the third cluster and the accessions of Kumquat were formed the fourth distinct cluster.

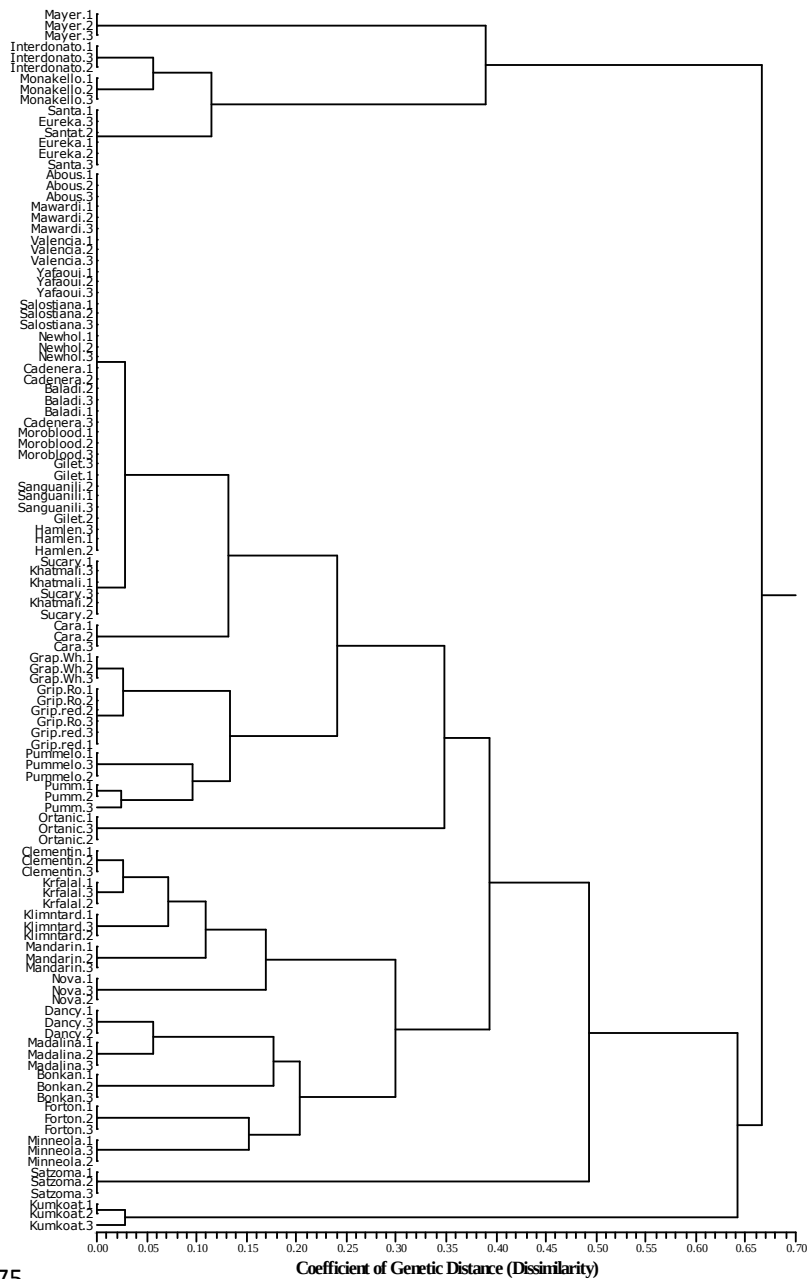


Fig. 2. Genetic relationships between *Citrus* accessions based on SSR data

DISCUSSION

The results obtained from SSR analysis allowed the distinction between cultivars of *Citrus* derived from natural hybridization between species (the majority of *Citrus* species) and the few "species" naturally occurring in the genus (mandarin and pummelo). The number of different alleles was estimated and the heterozygosity values were calculated (Table 3). The high number of different alleles and the highest values of heterozygosity were present in the "hybrid species", which is in accordance with the results obtained by other studies [7,9], where they reported that the groups of *Citrus* (thought to be hybrids) had higher proportion of heterozygous loci than the groups classified as ancestral or as *Citrus* relatives.

The lemon group (15 accessions of the five cultivars) had the highest number of different alleles (32 alleles) with the highest value of heterozygosity (0.728), while the pummelo (though it to be true *Citrus* species) from the citrus groups possessed the lowest allele number and the lowest values of heterozygosity (26 and 0.31), respectively. The high heterozygosity value (0.448) was revealed in the mandarin group, although it is considered as true species, which can be explained by the presence of high number of hybrids in this group which is also reported by Barkely *et al.* [9].

The number of common alleles shared between the different *Citrus* accessions was used to estimate the similarity percentage between the different groups. The lowest percentage (40%) was detected between lemon and mandarin groups, where the number of shared alleles was 12 out of 32 alleles revealed in lemon group. The number of common alleles detected in mandarin group was 28 and 22 of them were shared with orange group, leading to the highest percentage of similarity (77%) between the two groups.

The analysis of SSR data were very useful and informative in the characterization and estimation of genetic distance within *Citrus* genus. They were used to evaluate the genetic distance between the different cultivars and to establish the dendrogram of genetic relationship in the genus *Citrus* (Fig. 2). All *Citrus* accessions were clustered in two distinct branches. The first one consisted of all accessions of lemon group, while the second branch included the other accessions. The branch containing the lemon group was very distant from the other groups and showed high level of genetic diversity among its different accessions (GD=1.454). Three cultivars of lemon group (Meyer, Monachello, Interdonato) possessed different fingerprints, whereas Santa teresa and Eureka were identical. The results of many other studies confirmed that Lemon group was separated from the other groups, [9,29], and provided the detection of high level of genetic diversity within the group [30,31, 32].

In the second branch, four subgroups were identified. The first subgroup clustered all sweet orange accessions and showed the highest value of genetic similarity (close to 92%), where only three SSR patterns were obtained for the 45 accessions. They showed a small index of genetic distance with a low value of genetic diversity (GD= 0.179). This high percentage of similarity within orange group was expected, and was in accordance with many results [33,34,35,36]. Despite of clear variation in morphological characters between orange varieties such as leave shape, fruit size and color, [34,37] a very low genetic variability was found at molecular level with RAPD markers [20], which confirm the narrow genetic base in sweet orange. Many studies have shown that the microsatellite markers could not distinguish accessions resulting from spontaneous mutations such as sweet orange [9, 38, 39]. In contrast, other studies demonstrated the presence of high genetic diversity within sweet orange group [40, 41].

Regarding the second subgroup in our dendrogram, the three cultivars of grapefruit grouped together with two cultivars of pummelo. The cultivars of grapefruit were represented by two patterns, with a low value of genetic diversity (GD= 0.0741). The two cultivars of pummelo, although they were in the same cluster with grapefruit, were separated from grapefruit accessions and were also represented by two patterns and a low value of genetic diversity (GD=1.121). The low value of genetic diversity in grapefruit group were already reported by other studies, and due to vegetative origin, as all cultivars of grapefruit are polyembryony [30,42,43]. Pummelo is considered one of the three true species of *Citrus*, has played an important role in breeding programs, and was used as a parent for many cultivars of *Citrus*, such as oranges and grapefruit [44,45,8]. It's known that the grapefruit is derived from the hybridization between the sweet orange and pummelo which can explain the presence of its accessions with grapefruit accessions in the same cluster. Many studies have shown that pummelo had a high level of genetic diversity due to the sexual reproduction origin (monoembryony), which could be identified using molecular markers [46, 47, 48].

The highest value of genetic diversity was detected in the Mandarin group (GD= 0.533) and the cultivars were represented by 12 different patterns. All the studied cultivars grouped in the same cluster except Ortanique, which is considered as a hybrid between *C. sinensis* and *C. reticulata*, was closer to the orange group [3]. The high level of variability existing

among mandarin cultivars allowed the distinction of all cultivars used in this study, which is in accordance with numerous studies showing the same result [49, 50, 39]. These results showed that all cultivars belonging to the same group were close to each other and all are derived from one species, and from this point of view, Swingle system seems closer to the reality than Tanaka system is.

Fortunella genus, which is a close genus to *Citrus*, is nested within *Citrus* in the dendrogram, and the index of genetic distance was about 0.65. Several studies using other markers as isozymes [51], RFLP and RAPD [7] and SSR [52,14], coincided with our results and indicated that the molecular polymorphism between these two genera was lower than the morphological variations.

The results obtained in the present work showed that SSR markers were very useful for evaluating the genetic diversity and relationships between *Citrus* species maintained in the Department of Citrus Research in Tartous, Syria, and showed high level of genetic similarity between accessions of each cultivar and within the species of grapefruit and of Sweet orange at the SSR loci tested.

ACKNOWLEDGEMENTS

The authors thank Tishreen University in Lattakia and the Department of Citrus research in Tartous, in Syria, for the financial support.

AUTHORS' CONTRIBUTIONS

R. El-Mouei did the practical work, performed the analysis, wrote the protocol, and wrote the first draft of the manuscript. W. Choumane, designed the study, managed the analyses of the study, the literature searches and the discussion. All authors read and approved the final manuscript.

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