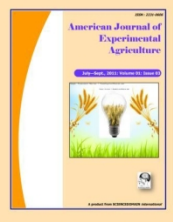

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Assessment of Genetic Variability within the Genus *Citrus* in Syria Using SSR Markers

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Authors' contributions

This work was carried out in collaboration between both authors. Author RAM did the practical work, performed the analysis, wrote the protocol and wrote the first draft of the manuscript. WC, designed the study, managed the analyses of the study, the literature searches and the discussion. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The characterization and the estimation of genetic variability between accessions belonging to *Citrus* genus using the SSR 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 SSR 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 pumelo and the fourth one 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

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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.

Keywords: Citrus; genetic variability; molecular marker; SSR, Syria.

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 pumelo (*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).

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

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

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 (300micro-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

2.3.1 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 PCR buffer, 200 µM 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, 45 cycles 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 54°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% polyacrylamide gel and stained with silver nitrate [24].

2.3.2 Data analysis

The amplification products were scored as 1 and 0 for present and absent alleles, respectively. These data were used to calculate the similarity and dissimilarity between cultivars (reflecting the genetic distance between them), using SIMGEN in the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 3.2 [25]. Means of the three accessions of each cultivars were used for clustering. The dendrogram was generated by the Unweighted Pair Group Method with Arithmetic Average (UPGMA) [26]. The genetic diversity (GD) within the genus was calculated for all loci according to the following formula of Nei [27]:

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

Where; (*n*) is the number of samples and (*p*) is the frequency 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 Pumelo. Detection of high number of polymorphic alleles is expected in the accessions of hybrid origin (7), therefore the score of alleles showed that the number of polymorphic bands possessed by such accessions ranged from 11 (in Pumelo accessions) to 19 (in Meyer accessions). The Genetic diversity (GD) was calculate and was ranged from 0.231 (Loci Org-8 and Org-10) to 0.608 (Locus Org-4). The high value of genetic diversity on a locus reflect a high level of mutations and modifications affecting this locus.

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 2. Names and sequences of SSR primers used in this study with the number of detected alleles and the genetic diversity (GD)

Loci	Primer sequences 5' to 3'	Number of detected alleles	Genetic diversity (GD)
Org-1	F: TTTGACATCAACATAAAACAAGAAAT R: TTAAAATCCCTGACCAGA	4	0.472
Org-2	F: AAAGGGAAAGCCCTAATCTCA R: CTTCCTCTTGCGGAGTGTTC	6	0.512
Org-3	F: TTCCTTATGTAATTGCTCTTTG R: TGTGAGTGTTTGTGCGTGTG	4	0.496
Org-4	F: TAAATCTCCACTCTGCAAAAGC R: GATAGGAAGCGTCGTAGACCC	5	0.608
Org-7	F: GGTGATGCTGCTACTGATGC R: CAATTGTGAATTTGTGATTCCG	4	0.205
Org-8	F: AGAAGCCATCTCTCTGCTGC R: AATTCAGTCCCATTCCATTCC	2	0.231
Org-9	F: AACACTCGCACCAAATCCTC R: TAAATGGCAACCCCAGCTTTG	3	0.447
Org-10	F: AATGCTGAAGATAATCCGCG R: TGCCTTGCTCTCCACTCC	2	0.231
Org-11	F: GCTTTCGATCCCTCCACATA R: GATCCCTACAATCCTTGGTCC	9	0.375
Org-14	F: CGCCAAGCTTACCACTCACTAC R: GCCACGATTTGTAGGGGATAG	8	0.479
Org-15	F: CGAACTCATTAAAAGCCGAAAC R: CAACAACCACCACTCTCACG	3	0.229
Org-17	F: GCCTTCTTGATTTACCGGAC R: TGCTCCGAACTTCATCATTG	5	0.485
Org-19	F: GAAAGGGTTACTTGACCAGGC R: CTTCCCAGCTGCTTGCAACAGC	4	0.498
Org-20	F: GGATGAAAAATGCTCAAAATG R: TAGTACCCACAGGGAAGAGAGC	6	0.541
Org-21	F: AGAGAAGAAACATTTGCGGAGC R: GAGATGGGACTTGGTTCATCACG	4	0.384
Org-23	F: AGGTCTACATTGGCATTGTC R: ACATGCAGZTGCTATAATGAATG	9	0.285
Org-26	F: CTTCCTCTTGCGGAGTGTTC R: GAGGGAAAGCCCTAATCTCA	6	0.510
Org-27-F87	F: ATGAAGGCTTTTTAGAGCCGAGTT R: ATAATAGGGGCCCACTTGACTTG	3	0.545
Org-28-F88	F: GTTCGCTCCACGCGATTTAT R: TGTGAAGAAAGATTTGGTGGGTTT	2	0.361
Org-30-F97	F: CTTCTTCTTCTCCTGCTCCTCCTC R: AGTGAGAAGCCAAAAACACCAAAC	5	0.561

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

Citrus group	Sweet orange	Mandarin	Lemon	Grapefruit	Pumelo
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

H obs: Values of observed heterozygosity

3.2 Species Specific Primers

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

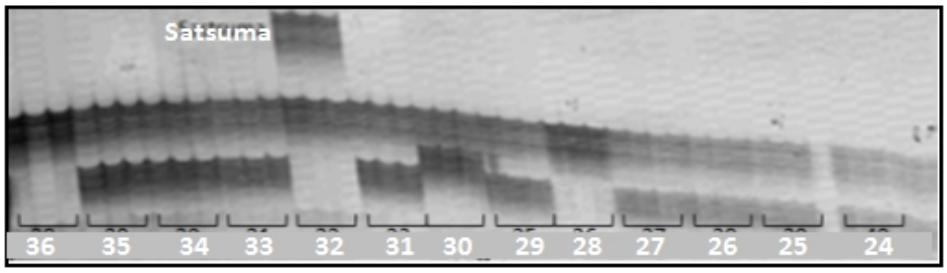

Fig. 1. Analysis of DNA by SSR primer pair (Org-3) on 6% polyacrylamide gel, showing polymorphic alleles in *Citrus* accessions and the specific allele in Satsuma (32). Number 24- 36 represent *Citrus* cultivars (refer to Table 1)

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 was specific in Satsuma from Mandarin group. The group of sweet orange could 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 pumelo, while another one found only in kumquat. Similar results were shown with the primer pairs Org-7 in the grapefruit and Pumelo 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 4. 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 Pumelo
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* Cultivars

The data obtained through the analysis of DNA by SSR primers were used to calculate the similarity and dissimilarity index, according to Nei and Li [28]. The obtained values of

dissimilarity were used to establish the dendrogram (Fig. 2), which displayed two distinct branches. The first branch contains accessions of all cultivars belonging to lemon group (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. Pumelo and grapefruit accessions formed the third cluster and the three accessions of Kumquat were formed the fourth distinct cluster.

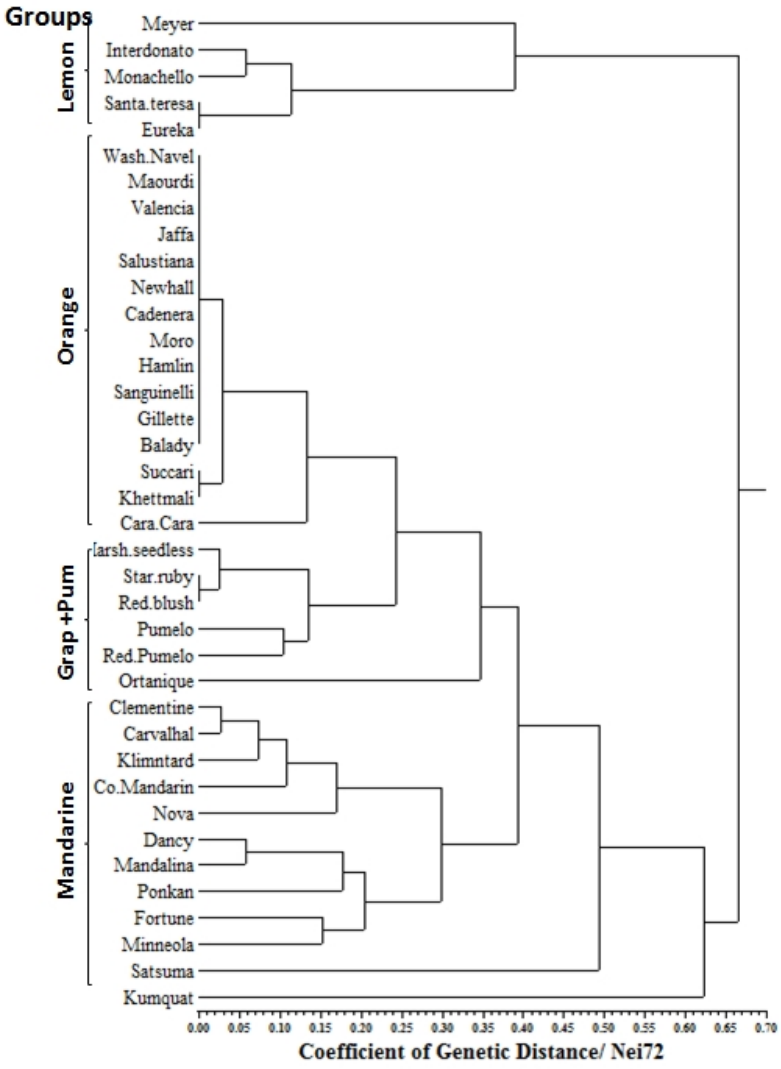

Fig. 2. Genetic relationships between 38 *Citrus* cultivars based on SSR data

4. 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 "true" species naturally occurring in the genus (mandarin and pumelo). The number of

different alleles was estimated and the heterozygosity values were calculated (Table 3). The highest number of different alleles and the highest values of heterozygosity were present in the "hybrid species". These results were in accordance with the results reported in other studies [7,9], where the *citrus* group 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 pumelo (thought to be a true *Citrus* species) 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 a 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 was very useful and informative in the characterization and estimation of genetic distance within *Citrus* genus. They were used to establish the dendrogram of genetic relationship within the genus *Citrus* (Fig. 2). All *Citrus* cultivars were clustered in two distinct branches. The first one consisted of cultivars of lemon group, while the second branch included the other cultivars. The branch containing the lemon group was very distant from the other groups and showed high level of genetic diversity among its different cultivars (GD=0.454). Three cultivars of lemon group (Meyer, Monachello and 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 proved the detection of high level of genetic diversity within the group [30,31,32].

In the second branch, four clusters were identified. The first cluster included all sweet orange cultivars and showed the highest value of genetic similarity (close to 92%), where only three SSR patterns were obtained for the 15 cultivars. They showed a small value genetic dissimilarity 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 other results [33,34,35,36]. Despite the presence of clear variations in morphological characters between orange cultivars, such leave shape, fruit size and color, [34,37] a very low genetic variability was found at molecular level with RAPD markers [20], confirming 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 contrary, other studies demonstrated the presence of high genetic diversity within sweet orange group [40,41].

In the second cluster of the dendrogram, the three cultivars of grapefruit grouped together with the two cultivars of pumelo. The cultivars of grapefruit were represented by two patterns, with a low value of genetic diversity (GD=0.0741). The two cultivars of pumelo, although they were in the same cluster with grapefruit, were separated from grapefruit accessions and were also represented by two different patterns and a low value of genetic

diversity (GD=0.121). 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]. Pumelo is considered as 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 pumelo which can explain the reason behind the presence of pumelo with grapefruit in the same cluster. Many studies have shown that pumelo 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 12 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, Swingel system seems closer to the reality than Tanaka system is.

Fortunella genus (represented by Kumquat accessions), 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.

5. CONCLUSION

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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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