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Genetic Variability and Phylogenetic Relationships among Egyptian Citrus Cultivars Using SSR Markers

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Abstract In the present study, ten Microsatellite or SSR (Simple Sequence Repeats) primer pairs were used to detect genetic variability and relationships among nine Egyptian citrus cultivars; six cultivars of sweet orange namely, Shamuti, Navel, Red Khalily, Succari, Valencia and Balady, in addition to cultivars of Sour Orange, Common Mandarin and Lime. Out of the ten primer pairs; seven primers generated clear patterns and produced alleles ranging from 1 to 8 with a total of 37 alleles. The genetic similarity values among studied citrus cultivars ranged from 0.050 to 0.524. The highest similarity value (0.524) was observed between the two sweet orange cultivars; Red Khalily and Succari, followed by 0.500 between two other sweet orange cultivars (Balady and Navel). The lowest similarity coefficient (0.050) was detected between Lime (*C. aurantifolia*) and Valencia orange (*C. sinensis*). From the dendrogram tree, citrus cultivars were grouped into two main clusters. The first cluster included Valencia orange while the second one was divided into three sub-clusters. Sour Orange and Lime were separated into the first and second sub-clusters, respectively, whereas Common Mandarin and the remaining sweet orange cultivars (Shamuti, Navel, Red Khalily, Succari, and Balady) were clustered together in the third sub-cluster. The genetic variability detected in Egyptian citrus germplasm could be useful in future breeding programs in order to genetic improvement of Egyptian citrus and produce new cultivars with commercial importance.

Introduction

Citrus is one of the most important fruit crops in the world and sweet orange accounts for about 70% of total world citrus production. In Egypt, citrus is considered the main fruit in terms of area and production with over 3,730,685 tonnes (FAO, 2011). Sweet orange is divided into four main groups; common or round cultivars, low acidity, pigmented and Navel orange (Hodgson., 1967). The four groups are cultivated in Egypt, but little is known about the genetic variability within and among Egyptian Sweet orange cultivars and other citrus species.

The taxonomy and phylogeny of the genus *Citrus* is very complex and confusing; mainly due to genetic heterogeneity of the genus, sexual compatibility between *Citrus* and related genera, high frequency of bud mutations, long history of cultivation and wide dispersion (Scora, 1988; Nicolosi *et al.*, 2000;

Jannati *et al.*, 2009). In the past, citrus was classified based on morphological and geographical data or biochemical techniques such as isozymes. Differentiation of cultivars through morphological features is not sufficient and inaccurate. Since citrus shows variability in its morphological traits such as size and shape of canopy, spines, fruit size and color, ripening season and number of seeds per fruit, etc (Orford *et al.*, 1995). The most widely accepted taxonomic systems for Citrus are those of Swingle and Reece (1967) and Tanaka (1977), they recognized 16 and 162 species, respectively. Later, phylogenetic analysis by Scora, 1975; Barrett and Rhodes, 1976; Fang *et al.*, 1993 proposed that there are only three basic true species of citrus within the subgenus *Citrus*; *C. medica* L., *C. reticulata* Blanco and *C. maxima* L. Osbeck. Other cultivated

species within *Citrus* are believed to have originated from hybridization among these true species or with closely related genera or by natural mutations. Recently, this assumption has gained support by various biochemical and molecular studies (Nicolosi *et al.*, 2000; Asadi Abkenar and Isshiki, 2003; Baig *et al*., 2009; Federici *et al.*, 1998; Uzun and Yesiloglu, 2012)

Use of molecular markers has more advantages than that of morphological and chemical properties used in characterization of citrus species. Genetic markers are stable, detectable in all tissues, and independent of environmental conditions or production practices. Moreover, it is possible to compare genotypes at any time of the year. Simple sequence repeats (SSR) or microsatellites markers are co-dominant, multi allelic, highly polymorphic genetic markers and they were found to be appropriate for genetic diversity studies. The SSR markers were used to diversity assessments studies of citrus by a number of investigators (Corazza-Nunes *et al.*, 2002; Golein *et al*., 2005*;* Jannati *et al.*, 2009**;** Amar *et al.*, 2011; Singh *et al.*, 2011; Snoussi *et al.*, 2012). Specially, Biswas *et al.* (2011) and Amar (2012) found that SSR exhibited relatively higher levels of polymorphism among *Citrus* species than that of other molecular techniques such as, amplified fragment length polymorphism (AFLPs), Sequence-specific amplification polymorphisms (SSAPs), and sequence-related amplified polymorphism (SRAP).

The present study aimed to use SSR markers to evaluate genetic variability and phylogenetic relationships among six important Egyptian sweet orange (*C. sinensis*) cultivars namely, Shamuti, Navel, Red Khalily, Succari, Valencia and Balady and three other *Citrus* species; Sour Orange (*C. aurantium*), Common Mandarin (*C. reticulate*) and Lime(*C. aurantifolia*)

Materials and Methods

Plant materials

A total of six important Egyptian sweet orange cultivars (Shamuti, Navel, Red Khalily, Succari, Valencia and Balady orange) and three other *Citrus* species (Sour Orange, Common Mandarin and Lime) were used in this study. Fruit shape, size and color, season of maturation and any other special characteristics for these cultivars according to Egyptian Ministry of Agriculture and Land Reclamation, 2003 are presented in Table 1. All plant materials were collected from a citrus orchard at Kafr El-Sheik Governorate, Egypt.

Table 1: List of the nine studied citrus cultivars and its fruit description.

No.	Common name	Scientific name (Swingle system)	Fruit description (shape, size, peel color, season of ripening & any other special characters)	
1	Sour orange	*Citrus aurantium* (L.)	round- medium, orange, midseason, resistance of Phytophthora.	
2	Common Mandarin	*Citrus reticulata* Blanco	Round, medium to small, orange, midseason, Alternate bearing, easy peeling.	
3	Lime	*Citrus aurantifolia* Christm	Round, small, yellow to green, early to midseason, high acidity 7-9%.	
Sweet oranges				
4	Shamuti	*Citrus sinensis* (L.) Osbeck	Oval Oblate, medium to large, orange, midseason.	
5	Navel	*Citrus sinensis* (L.) Osbeck	round with Navel, large, orange, early to midseason.	
6	Red Khalily	*Citrus sinensis* (L.) Osbeck	Round, medium, orange, mid to late season, red pulp and juice.	
7	Succari	*Citrus sinensis* (L.) Osbeck	Round, medium to small, orange, early season, acidless.	
8	Valencia	*Citrus sinensis* (L.) Osbeck	Round, medium, orange, late season.	
9	Balady	*Citrus sinensis* (L.) Osbeck	Round, medium to small, orange, midseason, heavy seeds.	

DNA isolation

Total genomic DNA was isolated from young leaves using a standard CTAB (Cetyl-tetramethyl ammonium bromide) protocol as described by Murray and Thompson (1980).

SSR analysis

Ten microsatellite primer pairs were used in this study (Table 2). PCR amplifications were performed in a 20 µl reaction volume; each reaction contained 1.0 µl template DNA (50ng); 0.10 µl Taq DNA polymerase (5U/ µl) promega , 4 µl of 5X buffer, 1 µl of 10 mM of each of the four dNTPs, 1.0 µl of 1mM forward and reverse primers. The volume was brought up to 20 µl by autoclaved double distilled water. The amplification protocol consisted of 5 min of initial denaturation at 94C° followed by 35 cycles each cycle consisted of 40 sec at 94°C; 40 sec at lower annealing temperature of the primer from 50 up to 68°C; 1 min at 72°C, and a final extension step of 10 min at 72°C (Plaschke *et al.*, 1995).

Table 2: Forward, reverse primer sequences and repeat motif for 10 SSR primer pairs used in the study.

Locus	Forward sequence	Reverse sequence	Repeat motif
CiBE0039	CCTGACATCCAGACAAGG	AGCCTCCAGAATCACAGTC	(AT)9
CiBE0105	GCAGTAAAGAGAATAAGAACAGA	GGCAAAGCACAATAATAGAGA	(AAT)5
CiBE0214	TACTTGTGAGACCCTAACTGG	CGTTGTGGAAGGAATAATGT	(AT)9
CiBE0246	ATTTGAGTTGTGTTGAGGTTG	CGGTGACGAAGAGTATGATT	(GGT)4
CiBE0447	CACAAAGAGAGTAACCCACAA	CGTCAAGAAGAGAGAATGATG	(TTC)14
CiBE0473	AGGGAGACCATTTGAGACTT	CGTGATTATTTAGAGAGAACCC	(AGAGA)2
CiBE0591	AAGAACTCCGTTGGGTTT	ACTCCGAATCCTCTCATT	(GAA)7
CiBE0733	TCTAAGTTGGTTGGGAGTT	TCTTTATGATTGTATTTGATGGA	(AATTA)2
CiBE0914	GGGCTCAGTTCTTCTCTACTC	GCATTAGGCTTCTCTCATACC	(TTA)15
CiBE1137	GGACATTATCTTCTTCTTCTCCT	ACATACTCATTACCCACCAAA	(CTT)8

Data analysis

Only clear amplification products were scored as 1 for presence of bands and 0 for absent one. The specific bands for citrus cultivars and species were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Similarity matrix was performed using SPSS program version 10 and cluster analysis was conducted to produce a dendrogram using unweighted pair-group method with arithmetic average (UPGMA). Similarity and dendrogram tree

were performed using SPSS program version 10. Polymorphic information content (PIC) were calculated according to Anderson *et al*., (1993) using the following simplified formula:

$$PIC_i=1-\sum p^2_{ij}$$

Where p_{ij} is the frequency of the j^{th} allele for marker i^{th} summed across all alleles for the locus.

Results and Discussion

Out of ten SSR primer pairs used, seven primers CiBE0039, CiBE0105, CiBE0214, CiBE0246, CiBE0447, CiBE0473 and CiBE0591 generated clear banding patterns with high polymorphism; only CiBE0473 primer pair showed a monomorphic band. A total of 37 bands were obtained from the seven SSR primers with the nine Egyptian citrus cultivars. The polymorphic information content (PIC) ranged from 0.61 for CiBE0591 to 0.76 for CiBE0214 and CiBE0246. (Fig.2 and Table.3). The number of bands ranged from one band for primer CiBE0473, to 8 bands for primers CiBE0447 which produced the highest number of polymorphic bands (8 bands). On the other hand, primers CiBE0039, CiBE0105 and CiBE0246 generated only five polymorphic bands. The mean percentage of polymorphic loci was 97.3%, which indicates a high level of polymorphism. This level of polymorphism depends on the degree of diversity between the genotypes under study and the efficiency of the primers used to detect the genetic polymorphism. This level of polymorphism was higher than the values observed in previous citrus studies, such as Asadi Abkenar and Isshiki., (2003) who obtained 70.4% of polymorphism in their study of genetic diversity between Japanese citrus species and cultivars using RAPD markers. In addition, Ahmed., (2012) had reported 58.3% polymorphism with RAPD markers in Egyptian citrus cultivars.

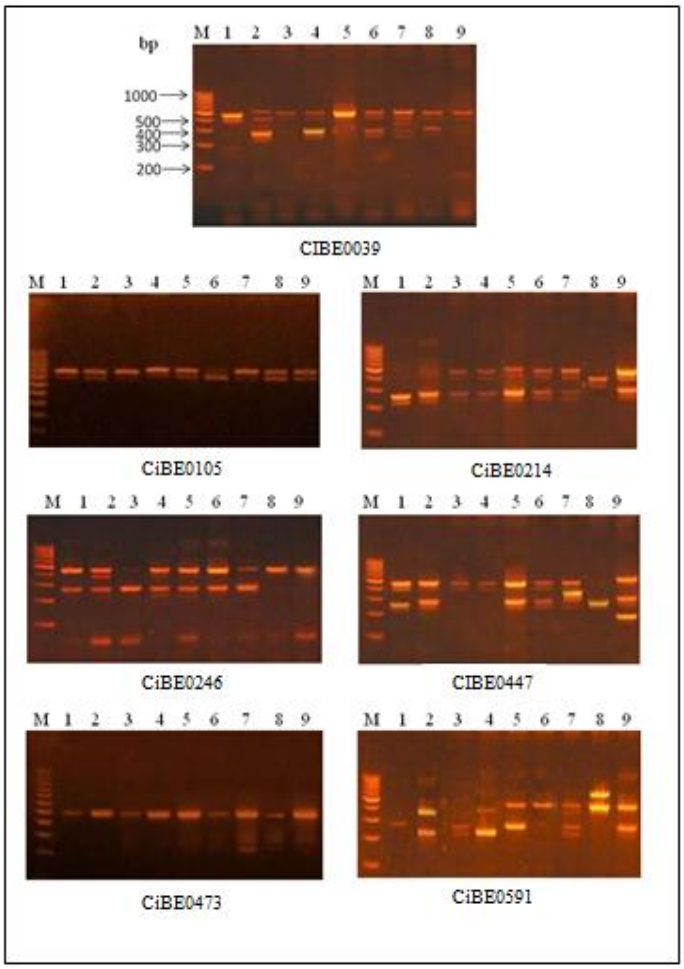

Fig. 2: SSR profiles as detected by seven primer pairs for nine citrus cultivars. Lanes from 1 to 9 represented Sour orange, Mandarin, Lime, Shamuti, Navel, Red Khalily, Succari, Valencia and Balady orange.

Table 3: Total number of bands, size range (bp), polymorphism (%) and mean PIC generated in nine citrus genotypes using 7 SSR primer pairs.

Primer code	Total bands	Size range (bp)	Polymorphic bands	Polymorphism (%)	Mean PIC
CiBE0039	5	250-650	5	100	0.6407
CiBE0105	5	540-740	5	100	0.6160
CiBE0214	7	223-507	7	100	0.7667
CiBE0246	5	331-528	5	100	0.7667
CiBE0447	8	288-644	8	100	0.6420
CiBE0473	1	380	0	0	0.6420
CiBE0591	6	238-700	6	100	0.6173
total	37		36	97.3	

SSR data were used to estimate the genetic similarities and the phylogenetic relationship among the nine Egyptian citrus cultivars. A similarity coefficient matrix among all studied cultivars is presented in Table 4. Similarity values among the nine citrus cultivars ranged from 0.050 to 0.524. The highest similarity index (0.524) was observed between Red Khalily and Succari orange, followed by the similarity index between Navel and Balady orange (0.500), whereas the lowest one (0.050) was recorded between Lime (*C. aurantifolia*) and Valencia orange (*C. sinensis*). Genetic distances among sweet orange cultivars (Shamuti, Navel, Red Khalily, Succari, and Balady orange) were relatively small with similarity coefficient ranged from 0.320 to 0.524. The high similarity among sweet oranges proves a narrow genetic base between sweet orange cultivars. So that the observed morphological polymorphism could be associated with somatic mutations. This result have been confirmed in several previous studies by a number of authers such as Luro *et al.*, 1995; Orford *et al.*, 1995; Fang and Roose, 1997; Novelli *et al.*, 2000; Golein *et al.*, 2005 and Novelli *et al.*, 2006. On the other hand, Valencia orange had the highest genetic distance compared to the other sweet orange cultivars. While, Shamuti and Valencia orange were found to be the most genetically diverse cultivars compared to each other with a similarity index of 0.083

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Table 4: Similarity matrix among nine Egyptian citrus cultivars based on SSR primer pairs.

	Sour orange	Mandarin	Lime	Shamuti	Navel	Red Khalily	Succari	Valencia
Mandarin	0.208							
Lime	0.278	0.182						
Shamuti	0.400	0.476	0.471					
Navel	0.300	0.318	0.353	0.474				
Red Khalily	0.286	0.364	0.263	0.450	0.421			
Succari	0.240	0.214	0.273	0.320	0.348	0.524		
Valencia	0.091	0.227	0.050	0.083	0.200	0.316	0.318	
Balady	0.174	0.250	0.263	0.381	0.500	0.474	0.455	0.250

The UPGMA dendrogram based on the similarity index separated the nine citrus cultivars into two main clusters (Fig.1). Valencia orange (the only late season cultivar) was the most divergent separated in the first cluster while the second cluster consisted of all remaining cultivars. These results are in an agreement with those reported by Mabberley (1997) who reported that International Code of Nomenclature for Cultivated Plants, published in 1995(Art. 4.1) Valencia in a separate group as: Citrus `Valencia` (Sweet Orange Group), where the history of a particular cultivar is unknown or unclear. Also these results are supported by Amar (2012) that Valencia was separated from the other sweet orange cultivars. The second cluster was divided into three sub-clusters. Cultivars of Sour Orange and Lime were separated into the first and second sub-clusters, respectively, whereas the Common Mandarin and all remaining sweet orange cultivars; Shamuti, Navel, Red Khalily, Succari, and Balady, were clustered together in the third sub-cluster. Common Mandarin and Shamuti orange clustered together with similarity index of 0.476. Shamuti orange was placed closer to Mandarin than to Sweet orange group. The obtained result revealed that Shamuti cultivar may have been produced as a hybrid between Mandarin and another parent. This could be explained by its origin, where it is known that sweet orange characteristics seem to come from a cross between mandarin (*C. reticulata* Blanco) and pummelo (*C. grandis* L. Osbeck) **(**Barrett and Rhodes,1976; Nicolosi *et al.*, 2000 and Li *et al.*, 2010).

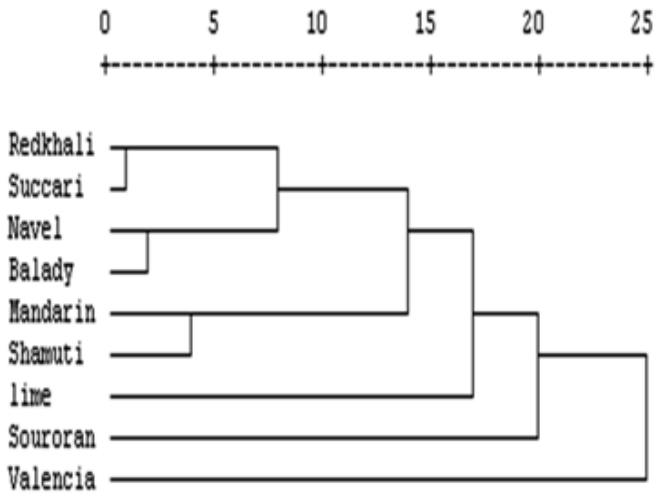

Fig. 1: Genetic diversity among nine Egyptian citrus cultivars obtained by Jaccard Similarity Coefficients and clustered by UPGMA (unweighted pair-group method with arithmetic averages) using seven SSR primer pairs.

The comparison of amplified fragments produced by SSR primers revealed the presence of some specific DNA bands, which are distinguished among citrus cultivars and species. Five primers gave unique bands with a specific citrus genotype as shown in (Table 5). It was observed that the three primers; CiBE0105, CiBE0214 and CiBE0591, gave unique bands (540, 338 and 700 pb, respectively) for Valencia orange which were absent in other citrus cultivars and species under study. Primers CiBE0105 and CiBE0214 generated unique amplified bands (740 and 223 pb) to Sour orange. Primer CiBE0246 gave two specific bands for Mandarin. Thus, it was able to distinguish among Mandarin and other species (Sour orange, Lime and sweet orange). Primers CiBE0214, CiBE0246 and CiBE0447 generated a specific band with molecular weight 300, 331 and 288 pb, respectively for Succari, Shamuti and Balady orange, respectively. Therefore, these primers are very useful in a breeding program since they could be used in marker-assisted selection and to differentiate between *Citrus* species. Moreover, these markers allowed the distinction between very close citrus cultivars; for instance Succari from Red Khalily and Balady from Navel. Our results are in harmony with the results of El-Mouei *et al.*, (2011) using different *Citrus* rootstocks collected from the Research Department in Tartous, Syria. Where it proved that SSR markers were very useful and informative in the differentiation and estimation of genetic diversity within and among the different *Citrus* cultivars.

Table 5: List of SSR primers producing unique bands for specific citrus genotype.

cultivar	Primer revealing Unique bands (band size as base pairs)
Sour orange	CiBE0105(740), CiBE0214(223)
Mandarin	CiBE0246(362), CiBE0246(450)
Shamuti	CiBE0246(331)
Succari	CiBE0214(300)
Valencia	CiBE0105(540), CiBE0214(338), CiBE0591(700)
Balady	CiBE0447(288)

In conclusion, we have confirmed that using SSR markers detect considerable levels of genetic variability in Egyptian citrus germplasm that can be used for many purposes such as genetic mapping, identification of some close cultivars, establishing marker-assisted breeding programs. Thus, the identification of similar groups among cultivars could be useful in selecting the appropriate parents to

be used in artificial crosses or mutations to be used to produce new cultivars with commercial importance, and in the genetic improvement of Egyptian citrus for both the quantity and quality properties.

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الاختلافات الوراثية والعلاقات التطورية بين اصناف الموالح المصرية باستخدام واسمات SSR

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استخدم في هذه الدراسة عشرة بوادئ SSR لتحديد درجة الاختلاف والعلاقات الوراثية بين تسعة من اصناف الموالح المصرية تضم ستة من اصناف البرتقال الحلو (الشاموتي وابوسرة والخليلي الاحمر والسكري والفالنشيا و البلدي) بالاضافة الي النارنج واليوسفي البلدي والليمون المالح. من بين العشرة بوادئ المستخدمة سبعة بوادئ فقط اظهرت نتائج واضحة حيث تراوح عدد الاليلات الناتجة ما بين واحد الي ثمانية اليلات باجمالي ٣٧ اليل. تفاوتت درجة الاختلاف بين اصناف الموالح التسعة موضع الدراسة من 0.050 الي ٠.٥٢٤. وقد لوحظ ان اعلي قيم للتماثل الوراثي (٠.٥٢٤) كانت بين اصناف البرتقال الحلو (الخليلي الاحمر والسكري) يليها ٠,٥٠٠ بين صنفين اخرين من البرتقال الحلو وهما البرتقال البلدي وابوسرة. وظهرت اقل درجة تشابة (٠.٠٥٠) بين الليمون المالح والبرتقال الفالنشيا. وقد اظهرت شجرة النسب انقسام اصناف الموالح الي مجموعتين رئيسيتين تضم الاولي البرتقال الفالنشيا منفردا، بينما تنقسم الثانية الي ثلاثة تحت مجموعات: حيث انفصل النارنج والليمون المالح في اول وثاني تحت مجموعة علي الترتيب بينما اشتملت تحت المجموعة الثالثة على اليوسفي البلدي وباقي اصناف البرتقال الحلو(الشاموتي والبسرة والخليلي الاحمر والسكري والبلدي). يمكن الاستفادة من الاختلافات الوراثية التي تم تحديدها داخل اصناف الموالح المصرية في برامج التربية المستقبلية بهدف التحسين الوراثي للموالح وانتاج اصناف ذات اهمية تجارية.