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MOLECULAR AND PHYSIOCHEMICAL STUDIES OF FIFTEEN ORANGE (*CITRUS SINENSIS* L.) CULTIVARS

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ABSTRACT

Molecular fingerprints, phylogenetic relationships and physicochemical characteristics of 15 orange (*Citrus sinensis* L.) cultivars were studied. The inter simple sequence repeats (ISSR) technique with seven primers was used. The dendrogram constructed based on 66 amplification products generated by the ISSR classified the cultivars into two major clusters. The first cluster included the Blood orange cultivar only, whereas the second comprised the rest of the cultivars with different degrees of genetic similarities. The highest phylogenetic relationship was noted between the two citrus accessions, Mouzambique and Roja, with 96% similarity. On the other hand, a genetic similarity of 53% was noted between the Balady orange and the Blood orange. Based on these findings, it could be concluded that the seven ISSR primers were effective to differentiate the orange cultivars under investigation. The physicochemical parameters of fruit juice included vitamin C concentration, pH, percentages of total soluble solids, and titratable acidity. The Hamlin, Centrial and Roja orange cultivars had the highest concentration of vitamin C (60.67, 58.33 and 57.14 mg/100 ml^{-1}, respectively). On the contrary, Tanneriffe cultivar showed the lowest vitamin C concentration (26.55 mg/100 ml^{-1}). The Tunisi cultivar had the highest pH of 6.443 in contrast to Mafred, Roja and Valencia accessions with pH vales of 3.317, 3.32 and 3.29. Tunisi and Balady orange cultivars exhibited the highest percentage of total soluble solids (T.S.S) of 12.3 and 12.07%, respectively. Whereas Valencia and Mafred showed the least T.S.S expressed by 8.83 and 8.27%, respectively. Roja cultivar recorded the highest (1.397%) titratable acidity. In contrast, the Succari, Tunisi and Khalili red accessions demonstrated the least titratable acidity of 0.05, 0.09 and 0.093 %, respectively.

INTRODUCTION

The exploration, collection, classification, conservation, characterization, evaluation, documentation and dissemination of information to PGR national and international programmes for its current and future use are among the objectives of the National Gene Bank and Genetic Resources (NGBGR) of Egypt. In addition, the NGBGR focuses on the indigenous and locally adapted cultivars for their contained useful genetic variation and their rapid rate of disappearing through replacement by high yielding varieties.

In Egypt, citrus is one of the most important subtropical fruit crops for their unique flavor and nutritional values. In 2005, about 2,797,600 MT of citrus fruits were harvested from an area of 141,358 ha under citrus crop. During 2001-2005, the total production of oranges and mandarins was about 88% of the total citrus production. However, the Egyptian exports

of oranges and mandarins declined from 258,331 MT in 2001 to 127,643 and 169,520 MT in 2002 and 2003, respectively. In realizing the importance of maintaining a large diversity of citrus adapted cultivars, and ensuring their healthy growth and desirable fruit quality, the NGBGR started a national program to survey, characterize and evaluate different citrus species grown in Egypt.

The use of molecular markers has been valuable to facilitate the breeding of citrus species. Techniques like RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) were used in germplasm characterization, estimation of genetic diversity, phylogenetic analysis and systematics (Fang *et al*., 1998; Federici *et al*., 1998; Nicolosi *et al*., 2000).
Both of the Inter-Simple Sequence Repeats (ISSRs) and the Simple Sequence Repeats (SSRs) are used as useful genetic markers. The advantage of the ISSRs marker over the SSRs is that it does not require prior knowledge of genome sequence and uses primers that are anchored at the 5' or 3' end of a repeated region and extends into the flanking region. Thus, the ISSRs analysis allows amplification of the genomic segments between inversely oriented repeats (ISSRs).In general, fruit and vegetable crops contain many different bioactive compounds with antioxidant properties such as vitamin C (ascorbic acid), vitamin E, carotenes and polyphenol compounds (Slaterry *et al*, 2000; Fernandez, 2004). The chemical composition of orange juice has been widely studied (AFNOR, 1996; AIJN, 1996). Orange juice is known as one of the nutritional sources rich of carotenes, pro-vitamin A and vitamin C (Gardner *et al*., 2000; Gil-Izquierdo *et al*., 2001). Vitamin C is an important natural antioxidant that may inhibit the development of major oxidative human conditions (Omaye and Zhang, 1998).
The objectives of the present work were fingerprinting, detection of phylogenetic relationships using ISSR markers, and to characterize some physiochemical characteristics of 15 orange cultivars maintained as germplasm collection at the Moshtohor Faculty of Agriculture, Kalyoubia governorate.

MATERIALS AND METHODS

Fifteen orange cultivars (*Citrus sinensis* L.) were collected by the horticulture department at National Gene-Bank and Genetic Resources (NGBGR) of Egypt (Table 1).

Table-1. The fifteen orange cultivars under the present study

No.	Cultivar's name	No.	Cultivar's name
1	Balady orange	9	Mezazie
2	Blood orange	10	Mouzambique
3	Centrial	11	Roja
4	Hamlin	12	Succari
5	Jaffa	13	Tanneriffe
6	Khalili Red	14	Tunisi
7	Khalili White	15	Valencia
8	Mafred		

DNA extraction: Five to six healthy young leaves of each cultivar were randomly collected and used for DNA extraction. About 100 mg of young leaf material was collected from different trees of each variety. DNA was extracted according to CTAB method of Lassner *et al*. (1989). DNA concentration was estimated with a spectrophotometer and by gel analysis. PCR reactions were conducted using seven ISSR primers. Names and sequences of these primers are listed in table (2).

Table-2. List of primers used, their sequence, melting temperature and annealing temperature.

No.	Primer code no.	Sequence	Tm	Ta
IS-1	17898B	$(CA)_6GT$	43.4	49-40
IS-2	17899B	$(CA)_6GG$	46.3	51-42
IS-3	17898A	$(CA)_6AC$	43.4	49-40
IS-4	HB-15	$(GTG)_3GC$	40.0	45-36
IS-5	814	$(CT)_8TG$	53.7	58-49
IS-6	17899A	$(CA)_6AG$	43.4	49-40
IS-8	HB-8	$(GA)_6GG$	52.61	57-48

The amplification reaction was carried out in 25 µl reaction volume containing 1x PCR buffer, 1.2 mM MgCl2, 0.2 mM dNTPs, 50 pmol primer, 1 u Taq DNA polymerase (ABgene) and 50 ng template DNA. Temperature cycling was performed on MJ Research PTC-200 thermal cycler. The amplification profile consisted of initial denaturation of the template DNA at 94ºC for 4 min, followed by 10 cycles of 94ºC for 45s, touchdown one-degree decrement for annealing temperature started with 5ºC above Tm for each primer for 30s and 72ºC for 2 min. followed by 25 cycles of 94ºC for 45s, last annealing temperature for 30s (Table 2) and 72ºC for 2 min and final extension of 72 for 5min. The amplification products were visualized in an ultraviolet transilluminator, after horizontal electrophoresis in 2.2% agarose gel, using the TBE 1x buffer, the being stained with ethidium bromide.

Physicochemical analyses: The physiochemical characteristics used to characterize and discriminate the 15 orange cultivars were based on those previously prescribed for citrus by the International Plant Genetic Resources Institute (IPGRI).

PH: Ten grams of orange juice was blended with 20 ml de-ionized water. The pH of the resultant solution was measured with a cyberscan pH meter.

Titratable acidity: The solution prepared to measure the pH was titrated with 0.1 M NaOH to pH 8.1, monitoring with an electrode pH meter. Results were expressed as grams of citric acid per Kilogram fresh weight (FW).

Total Soluble Solids (T.S.S): Total soluble solids were measured using orange juice samples with an Atago digital refractometer (PAL-1, Tokyo, Japan). Results are reported as %.

Determination of ascorbic acid (vitamin C): Vitamin C was determined using the High Performance Liquid Chromatography (HPLC). A volume of 50 ml of orange juice was homogenized with 40 ml of an extraction solution (30 $g.1^{-1}$ meta-phosphoric acid + 80 $g.1^{-1}$ % acetic acid). The resulting mixture was filtered under suction and adjusted up to 100 ml with distilled water. Samples were filtered through a 0.45 µm membrane filter and duplicates of 20µm for each extract were analyzed by the HPLC. Separation of the ascorbic acid was performed by the HPLC using a Hypersil BDS C8 (5 µm) stainless steel column (250 mm x 4.6 mm) (Thermo Electron, United Kingdom). The solvent system used was an isocratic gradient of a solution 70% Buffer (0.85 % v/v H_2SO_4 in 17.5 mM KH_2PO_4, pH 1.8) and 30% Methanol. The flow rate was fixed at 1.5 ml/min. A UV- vis detector was set at 245

nm; chromatographic data and UV-vis spectra were collected, stored and integrated using a chromostar light software. The calibration curve was built with one concentration level using an ascorbic acid standard solution (100 mg ml^{-1} in a solution 30 g 1^{-1} meta-phosphoric acid + 80 g 1^{-1} % acetic acid) (Sanchez-Moreno *et al*, 2003). Results are expressed as milligrams of ascorbic acid per 100 ml juice.

Data analysis: For the molecular studies, ISSR bands were scored as present (1) or absent (0) for all orange varieties and then to estimate the relationships among the examined accessions, similarity matrix was estimated by pair wise comparisons of the cultivars based on the percentage of common fragments. Each band was assumed to represent a unique genetic locus. The molecular results were analyzed using the Phoretix 1D Pro software from nonlinear Dynamics. Dendrogram was generated by the Unweighted Pair Group Method with Arithmetic Average (UPGMA).

Results of the physiochemical analyses were statistically analyzed using one way ANOVA at $P\leq 0.05$ to verify differences among treatment means (Waller and Duncan, 1969). Cluster Analysis (ClA) was performed to examine the natural linkage of the varieties on the basis of the information provided by the analyzed variables. The procedure applied was an agglomerative hierarchic classification. Euclidean distances and the un-weighted pair group method based on arithmetic averages (UPGMA) were used in the clustering (Romesburg, 1984).

RESULTS AND DISCUSSION The seven ISSR primers detected 64 amplification fragments, varying from 6 (IS-8) to 13 (IS-3) fragments per primer, and ranged from 207 to 1518 base pair in size. All the primers revealed polymorphisms among the 15 cultivars ranging from 62.5% for primer (IS-8) to 100% for primer (IS-1). The overall polymorphism for the seven primers across all 15 cultivars was 79%.

The estimates of the genetic-distance ranged from 53% for the least distance related cultivars (Balady orange and Blood orange), to 96% for the most closely related cultivars (Mouzambique and Roja). The rest of the genetic similarity index between each pair is shown in Table (3).

The Dice ISSR-based coefficients of genetic similarity among the 15 cultivars of orange resulted in a dendrogram (Fig.1), which comprised two clusters. The first cluster includes the Blood orange cultivar only. While the second one is divided into two groups; the first group includes three cultivars (Khalili white, Balady orange and Mezazie). The second group is subdivided into two subgroups where one comprised the cultivar Khalili red only, and the other consists of the rest of cultivars. The dendrogram represent the relationship among the 15 orange cultivars and gave an idea about their descending pattern.

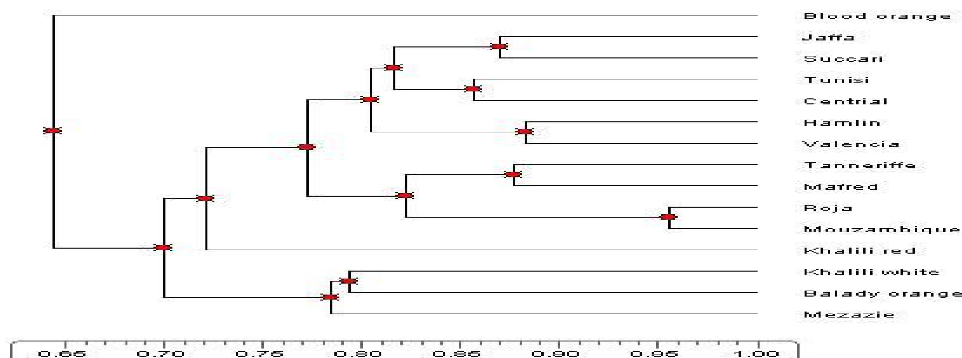
It is concluded that PCR-based molecular markers are useful for the analysis of genetic diversity in horticultural and field crop species (Wolf *et al.* 1995; Torres *et al.* 1993; Debener *et al.* 1996; Swoboda and Bhalla 1997). The present investigation clearly demonstrated that ISSR PCR could distinguish all of the orange cultivars.

Table 3. Genetic similarity index values between pairs of 15 orange cultivars based on ISSR.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.00														
2	0.83	1.00													
3	0.78	0.82	1.00												
4	0.75	0.80	0.96	1.00											
5	0.72	0.88	0.83	0.84	1.00										
6	0.77	0.81	0.85	0.85	0.87	1.00									
7	0.72	0.74	0.75	0.75	0.81	0.88	1.00								
8	0.63	0.65	0.71	0.75	0.74	0.79	0.81	1.00							
9	0.70	0.75	0.78	0.79	0.78	0.80	0.82	0.86	1.00						
10	0.67	0.74	0.77	0.78	0.83	0.85	0.81	0.81	0.84	1.00					
11	0.61	0.66	0.69	0.69	0.74	0.76	0.72	0.71	0.72	0.83	1.00				
12	0.59	0.64	0.70	0.73	0.76	0.72	0.73	0.72	0.70	0.76	0.79	1.00			
13	0.65	0.61	0.63	0.67	0.67	0.66	0.67	0.69	0.70	0.76	0.79	0.78	1.00		
14	0.65	0.69	0.78	0.82	0.75	0.80	0.76	0.82	0.79	0.87	0.75	0.77	0.71	1.00	
15	0.61	0.57	0.66	0.66	0.59	0.65	0.69	0.71	0.69	0.66	0.53	0.60	0.67	0.73	1.00

(1) Khalili Red, (2) Mafred, (3) Mouzambique, (4) Roja, (5) Tanneriffe, (6) Valencia, (7) Hamlin, (8) Centrial, (9) Tunisi, (10) Succari, (11) Balady orange, (12) Mezazie, (13) Khalili White, (14) Jaffa and (15) Blood orange.

Figure 1: Dendrogram demonstrating the relationships among fifteen orange cultivars based on seven primers of ISSR.



The physicochemical parameters of the orange juice were shown in Table-4. The highest significant pH of 6.443 was measured in the Tunisi cultivar whereas the least ones of 3.290, 3.317 and 3.320 were measured in the Valencia, Mafred and Roja, respectively (Table 4). The rest of cultivars showed intermediate values of pH. The titratable acidity in the cultivars studied ranged from 1.397 % for the Roja cultivar to 0.093, 0.090 and 0.05 in Khalili red, Tunisi and Succari ones, respectively. It is noticed that the highest titratable acidity of juice was almost correlated with the lowest pH (Table 4). The present study also demonstrates that the highest concentration of the total soluble solids (~12 %) was measured in the Tunisi and Balady orange cultivars, whereas the lowest one (~8 %) was tested in Mafred and Valencia (Table 4). The highest content (mg. 100 ml^{-1}) of ascorbic acid was measured in the Hamlin (60.67), Centrial (58.33) and Roja (57.14) cultivars, whereas the lowest one was observed in Tanneriffe (26.55).

Table 4: The physicochemical characteristics of juice of the 15 orange cultivars.

No.	**Variety**	**pH**	**Titratable acidity (%)**	**Total Soluble solids (%)**	**Ascorbic acid (mg/100ml)**
1	Balady orange	3.603 i	1.083 c	12.07 ab	40.91 c
2	Blood orange	3.933 f	0.8600 e	10.80 d	35.90 de
3	Centrial	4.010 e	0.6300 g	9.800 ef	58.33 a
4	Hamlin	3.563 i	0.9500 d	9.933 ef	60.67 a
5	Jaffa	3.840 g	1.007 d	10.80 d	35.00 ef
6	Khalili Red	5.757 b	0.09333 h	9.833 ef	47.64 b
7	Khalili White	4.267 c	0.7867 f	11.07 cd	31.56 f
8	Mafred	3.317 j	1.100 c	8.267 h	35.19 def
9	Mezazie	3.630 i	0.7967 ef	9.233 fg	39.38 cd
10	Mouzambique	3.603 i	0.9867 d	9.833 ef	35.99 de
11	Roja	3.320 j	1.397 a	10.53 de	57.14 a
12	Succari	6.2 b	0.05 h	11.6 bc	40.83 c

13	Tanneriffe	4.10 d	0.8033 ef	11.60 bc	26.55 g
14	Tunisi	6.443 a	0.09000 h	12.30 a	42.77 c
15	Valencia	3.290 j	1.220 b	8.833 gh	40.90 c

Means followed by the same letter (s) within the same column are not significantly different ($P \leq 0.05$; the Duncan's Multiple Range Test).

Table-5: Physiochemical dissimilarity index values between pairs of 15 orange cultivars based on the physiochemical characteristics studied.

	1	2	3	4	5	6	7	8	9	10	11
1	0.000										
2	5.186	0.000									
3	2.835	5.535	0.000								
4	17.571	22.450	17.744	0.000							
5	19.869	24.785	20.110	2.407	0.000						
6	6.053	0.917	6.403	23.352	25.683	0.000					
7	7.473	11.945	7.059	10.842	13.238	12.853	0.000				
8	9.435	4.362	9.503	26.798	29.138	3.484	16.211	0.000			
9	6.879	2.714	7.229	23.206	25.536	2.595	12.826	4.690	0.000		
10	3.234	3.829	3.852	18.960	21.299	4.661	8.579	8.057	4.325	0.000	
11	5.404	1.033	5.834	22.340	24.674	1.406	11.878	4.654	1.789	3.445	
12	16.301	21.254	16.658	1.737	3.617	22.148	9.915	25.607	22.069	17.817	
13	14.386	9.389	14.447	31.832	34.165	8.498	21.244	5.044	9.299	13.058	
14	3.545	7.509	2.090	15.949	18.298	8.384	5.499	11.510	9.199	5.416	0.000
15	3.251	5.420	4.183	17.482	19.804	6.244	7.337	9.659	5.739	1.660	5.173

(1) Balady orange, (2) Blood orange, (3) Succari, (4) Centrial, (5) Hamlin, (6) Jaffa, (7) Khalili Red, (8) Khalili White, (9) Mafred, (10) Mezazie, (11) Mouzambique, (12) Roja, (13) Tanneriffe, (14) Tunisi and (15) Valencia

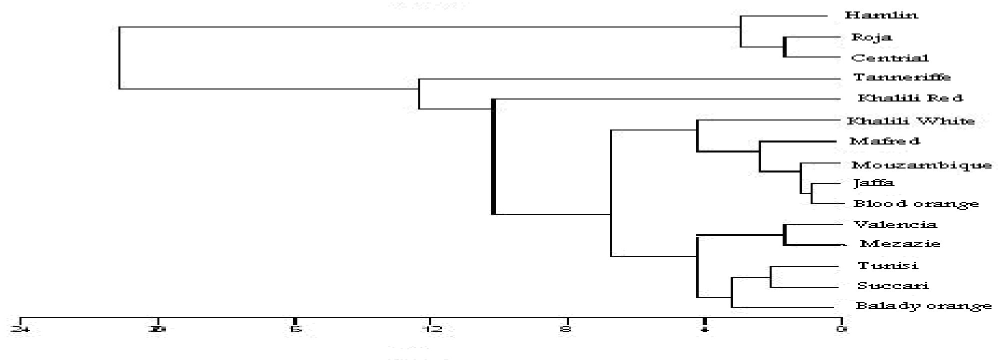


Figure 2: Dendrogram illustrating the relationships between 15 orange cultivars according to their physicochemical dissimilarity characteristics.

The dissimilarity among orange cultivars under investigation is presented in Table-5. The least average dissimilarity (0.917) was scored between cultivars Blood orange and Jaffa, while the highest average dissimilarity (34.165) was scored between Hamlin and Tanneriffe cultivars.

A dendrogram illustrating the relationships among the 15 orange cultivars according to the physicochemical characteristics is presented in Figure-2. As demonstrated, the dendrogram is composed of two main clusters; the first includes Hamlin, Roja and Centrial cultivars. The other cluster is divided into four groups. The first group includes only the cultivar Tanneriffe, whereas the second one comprises the cultivar Khalili Red. The third group contains five cultivars; the Khalili White, Mafred, Mouzambique, Jaffa and Blood orange, whereas cultivars Valencia, Mezazie, Tunisi, Succari and Balady orange comprised the fourth group. The environment greatly influences the content of some compounds involved in the formation of physicochemical characteristics under the study (Paterson *et al*., 1991). Therefore, the pattern of the dendrogram resulted from the physiochemical characteristics under the present investigation did not match that of the dendrogram resulted from the molecular study.

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