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Molecular Characterization and Genetic Diversity Analysis of Sweet Orange (*Citrus sinensis* L. Osbeck) Cultivars in Iraq Using RAPD Markers

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Abstract

Sweet orange (*Citrus sinensis* L. Osbeck) is one of the most important commercially cultivated fruit crops of Citrus. Genetic diversity and inter-relationship among 5 cultivars (Indian, Iraqi, Japanese, Syrian, Egyptian) of *C. sinensis* were analyzed based on RAPD markers. Six primers generated reproducible and easily storable RAPD profiles with a number of amplified DNA fragments ranging from 6 to 14 fragment bands. The total number of amplicons detected was 51, including 14 fragments unique bands with average reached 2.8 fragments / primers. While the number of polymorphic ranged from 0 to 8 with an average reached 4.4 fragments / primers with the polymorphic percentage ranged from 0% to 57.1%. While the number of monomorphic ranged from 2 to 5 fragment bands and was total of the monomorphic 15 fragments with an average reached 3 fragments / primers with the monomorphic percentage was 14.2 % to 83.3%. A maximum numbers of amplicons was amplified with primer OPS-238 reached 14 fragments while the minimum number of fragments was amplified with primer OPS-253 reached 6 fragments. The highest number of polymorphic bands reached 8 fragments was obtained with primer OPS-238 with high percentage 57.1%, while the highest number of monomorphic bands reached 5 fragments with high percentage 83.3% was obtained with primer OPS-253. RAPD markers detected genetic distance and similarity, amaximum genetic distance value was observed between Japanese (Jap) and Syrian (Syr) cultivars reached 0.530 with less similarity value reached 47%, a minimum genetic distance value was observed between sweet Iraqi (Irq) and Indian (Ind) cultivars reached 0.239 with high similarity value reached 76.1%. The similarity matrices were employed in the cluster analysis to generate a dendrogram using the UPGMA method. The cluster tree analysis showed that the sweet orange cultivars were broadly divided into two main groups A and B with similarity reached 50%. A group including individual one cultivars was Japanese. B group was divided into two sub-cluster B1 and B2 with genetic similarity reached 63%. The first sub-cluster (B1) was included two cultivars Iraqi and Indian with high genetic similarity among other cultivars reached 77%. The second sub-cluster (B2) was included two cultivars Egyptian and Syrian with genetic similarity reached 72%.

Keywords: sweet orange, genetic diversity, molecular marker, RAPD.

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1. Introduction

Citrus is one of the most economically important fruit crops of the world, belonging to the subfamily Aurantioideae of the family Rutaceae. It is widely distributed throughout the tropical and subtropical regions of the world and believed to have originated in Southeast Asia, particularly northeast India, the Malayan archipelago, China, Japan, and Australia [1, 2]. Among the cultivated species (*Citrus sinensis* L. Osbeck) sweet orange is the most important commercial fruit crop of Citrus and believed to be a hybrid between pummelo (*Citrus maxima*) and mandarin (*Citrus reticulata*) [3, 4]. It is a highly poly embryonic species, fruit pulp is used for preparing fresh juice which is rich in vitamin C and protein content and peel of the fruit is used for making perfume and soaps. Cooking oil is extracted from its seeds, Juice extracted from its leaves is used to control several diseases like ulcers, sores, etc. [5]. The use of molecular markers has been a valuable and precise strategy to identify Citrus species, cultivars and biotypes and to investigate the genetic diversity of Citrus species. Molecular marker techniques like RAPD, ISSR, RFLP, SSR, AFLP and other markers have been used for germplasm characterization, studies of genetic diversity, systematics and phylogenetic analysis [6]. Among them, random amplified polymorphic DNA (RAPD) markers have been employed most widely for characterization of plant species [7]. Most citrus species genera are diploid (2n = 2x = 18), with relatively small genomes; for instance, sweet orange (*Citrus sinensis* L. Osbeck) has a genome of about 367 Mb, nearly three times the size of the 125 Mb Arabidopsis genome [8, 9]. The conventional methods in *Citrus* cultivars identification relied on morphological features and isozymes [10]. In the cultivated citrus, sweet orange (*C. sinensis* L. Osbeck) originated as a natural hybrid between mandarin and pummel, showed low level of genetic diversity according to lots of previous studies [11-14]. The authors [15] suggested that sweet orange has a majority of its genetic makeup from mandarin and only a small proportion from pummelo. Grapefruit was reported as a hybrid of pummelo and sweet orange [16, 4, 17], and all grapefruit cultivars originated from single parent through mutations [18, 19]. It is notified that most of sweet oranges obtained by mutation from one ancestor tree, So despite of differences in morphological characters, genetic variation of sweet orange was low [20]. Using morphological traits, it is difficult to distinguish between many *Citrus* cultivars because some cultivars are distinguishable only by fruit traits and *Citrus* trees usually do not bear fruits until 3-4 years after planting. Moreover, isozyme markers can be mediated by secondary processes so that the normal patterns of expression are suppressed. Phenotypic diversity, poly embryonic, hybridization and mutations have prevented consensus on systematic classification of *Citrus* [21] and hampered *Citrus* improvement programs. The development of molecular markers based on DNA sequences has provided an ideal means for identifying genotypes, estimation of relatedness between different accessions and following inheritance of economically important characters, a wide variety of DNA-based markers have been developed in the past few years, [22]. Orange cultivars are classified into four groups: common, low acidity, pigmented and navel oranges [23 as cited in 13]. It is indicated despite the existence of substantial diversity among cultivated genotypes in respect of morphological, physiological and agronomic traits, very little DNA variation has been detected using DNA markers [13]. In Citrus, RAPD markers have been used for cultivar identification, genetic mapping, genetic diversity assessment and other breeding programs and RAPD marker have gained more attention due to the simplicity of the procedure, the low cost and the very small amount of the DNA required for analysis [12, 21, 24-29]. They notified that sweet oranges have a narrow genetic basis and that most morphological characters originated through mutations, and clonal propagation of sweet oranges is the case for the majority of citrus species [30, 14]. In paper [20] were used ISSR markers to differentiate 41 samples of orange belongs to three groups. This notified as majority of sweet orange cultivars derived from a single ancestor by mutation. However, some cultivar distinguished from others. In other study, it was found identical microsatellite profiles at 9 out of 10 SSR loci among analyzed orange cultivars and clones [31]. RAPDs have been extensively used in assessing relationships amongst various accessions of different plant species [32-35].

The objectives of the present study are to determine the genetic variability among 5 *Citrus sinensis* L. (Sweet orange) genotypes using RAPD markers and to assess the genetic relationships among these genotypes. Addition to knowledge of genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources and breeding.

2. Materials and methods

Plant material

A total of 5 sweet orange (*Citrus sinensis* L.) cultivars genotypes (Indian, Iraqi, Japanese, Syrian, Egyptian) used in this study were collected from the citrus private orchard of Babylon-Iraq.

DNA isolation

The total genomic DNA for 5 sweet orange was isolated from fully expanded leaves using the Kit. Leaf samples (300 mg) were ground to a fine powder in liquid nitrogen. DNA was extracted by using Genomic DNA Mini Kit (Geneaid, UK). The extracted DNA (200 µl) was stored at –20 °C until use. Concentration, quality and quantity of the DNA were determined by Nano drop-spectrophotometrically at λ = 260 nm. Stock DNA samples were stored at –20 °C and diluted to 20 ng uL^{-1} when in use. The analysis was conducted in the laboratory of Molecular Genetics at the university of Baghdad, genetic engineering and biotechnology institution.

PCR procedure

The RAPD primers (Table 1) were purchased from BIONEER, South Korea. A total of 6 decamer oligonucleotides of arbitrary sequence were tested for PCR amplification. AccupPower Gold Multiplex PCR premix (BIONEER, South Korea) was used to the DNA amplification with RAPD primers. The PCR were carried out in 25 µl reactions. The temperature profile for the reaction is given as: hot start at 95 °C (only at the start of reaction) for 5 min., denaturation at 95 °C for 1 min., primer annealing at 36 °C for 1 min., extension at 72 °C for 2 min. and final extension at 72 °C (only at the end of reaction) for 10 min.

DNA electrophoresis

Amplification products were separated by electrophoresis (100v for 30 min.) in 1.5 % agarose gels (1.5 mg mixed with 80 ml of TBE buffer) and stained in ethidium bromide. A photographic record was taken under the UV illumination document gel.

Data analysis

Only clear and repeatable application products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying species and cultivar were named with primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence of bands. Molecular weight of the amplified bands was estimated by using a 1 Kb DNA ladder (BIONEER, South Korea). Amplified products were analyzed by pairwise comparisons of the genotypes based on the percentage of common fragments, and a similarity matrix was calculated [36]. The 0 or 1data matrix was created and used to calculate the genetic distance and similarity using 'Simqual', a subprogram of the NTSYS-PC program (numerical taxonomy and multivariate analysis system program) [37]. A dendrogram was constructed based on the genetic distance matrix by applying an unweighted pair group method with arithmetic averages (UPGMA) cluster analysis using the MEGA (Molecular Evolutionary Genetics Analysis) version 2.0 [38].

3. Results and discussion

Figure 1 shows the results of the isolated total DNA of the leaves of the studied sweet orange cultivars manner filters and then migrated to agar gel 1.5%, electric voltage 100 V for 30 min. noting the success of the method to isolate the DNA from these sweet orange cultivars.

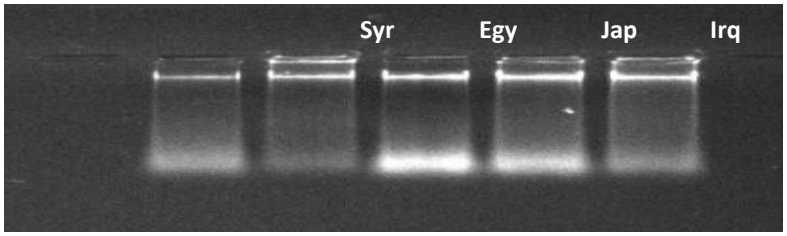

Figure 1. the isolated total DNA of the Sweet orange *C. sinensis* cultivars leaves Ind: Indian, Irq: Iraqi, Jap: Japanese, Egy: Egyptian, Syr: Syrian on agarose gel (1.5%) and electric voltage (100 V) for (30 min.)

Polymorphisms and monomorphisms detected by RAPAD markers:

One of the most important features of the RAPD technique is detecting of high levels of polymorphism and this feature has been met in present study (Fig. 2). Six primers were screened with the DNA of the 5 sweet orange cultivars genotypes. All 6 primers tested were generated reproducible and showed easily storable RAPD profiles with a number of amplified DNA fragments ranging from 6 to 14 (Table 1).

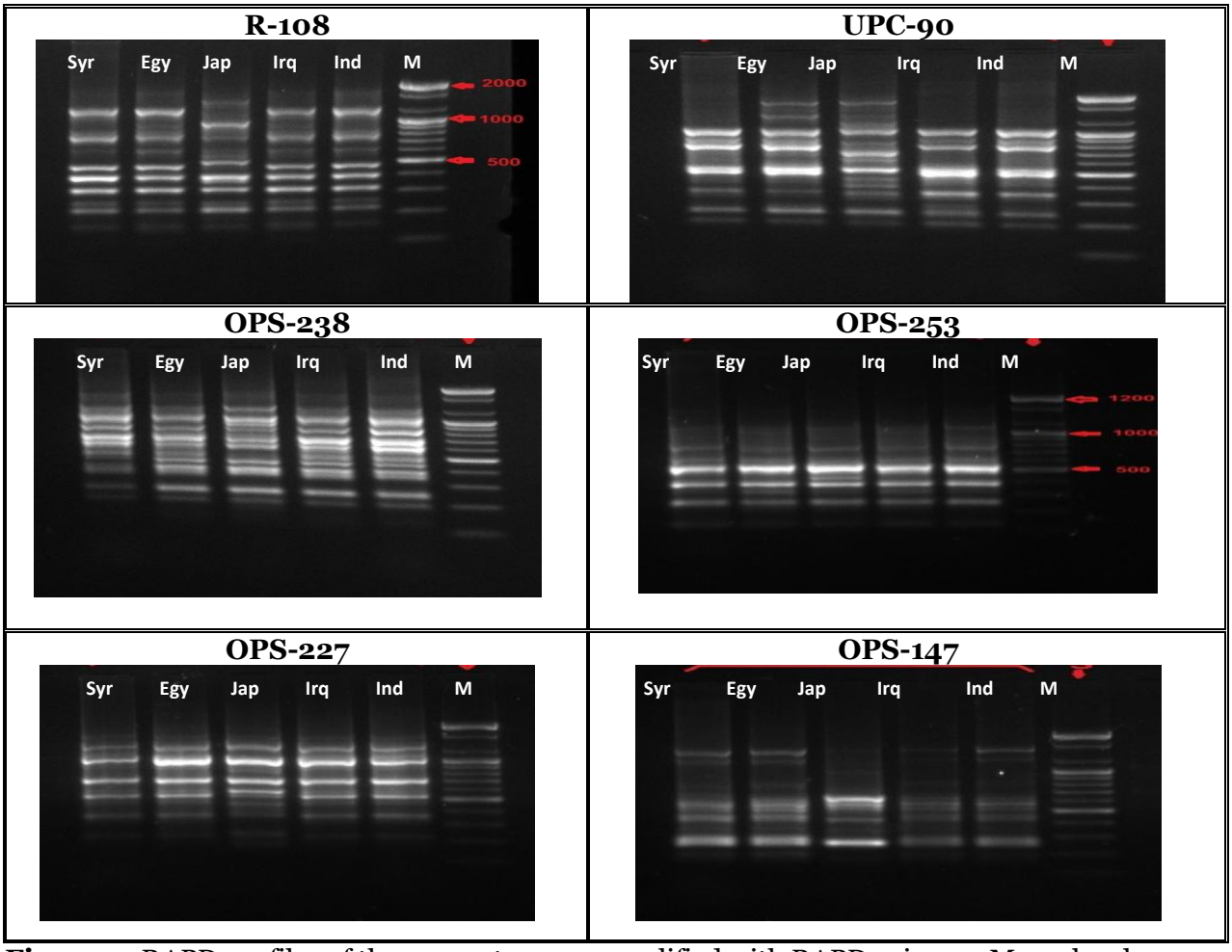

Figure 2. RAPD profiles of the 5 sweet orange amplified with RAPD primers, M: molecular weight marker, Culvers Ind: Indian, Irq: Iraqi, Jap: Japanese, Egy: Egyptian, Syr: Syrian on agarose gel (1.5%) and electric voltage (100 V) for (30 min.)

The total number of fragments produced by 5 primers was 51 with an average of 10.2 fragments / primers. The high unique fragment appeared in OPS-238 and UPC-90 primers reached 4 with high percentage of 28.5% and 30.2% respectively. While the number of polymorphic ranged from 0 to 8 with an average reached 4.4 fragments / primers with the polymorphic percentage ranged from 0% to 57.1%. While the number of monomorphic ranged

from 2 to 5 and was total of the monomorphic 15 with an average reached 3 fragments / primers with the monomorphic percentage was 14.2% to 83.3%. As shown in Table 1 a maximum numbers of amplicons was amplified with primer OPS-238 reached 14 while the minimum number of fragments was amplified with primer OPS-253 reached 6. The highest number of polymorphic bands reached 8 was obtained with primer OPS-238 with high percentage 57.1%, while the highest number of monomorphic bands reached 5 with high percentage 83.3% was obtained with primer OPS-253.

Table 1. Total number and percentage of amplicons, Unique, polymorphic, monomorphic amplicons as revealed by RAPD markers among the 5 sweet orange cultevars accessions

RAPD Primers	Primer sequences 5' to 3'	Number of Total amplified fragments	Number of Unique Fragments bands	Unique Fragments Bands Percentage (%)	Number of Polymorphic Fragments Bands	Polymorphism Fragments Percentage (%)	Number of Monomorphic Fragments Bands	Monomorphic Fragments Percentage (%)
OPS-238	TGGTGG CGTT	14	4	28.5	8	57.1	2	14.2
OPS-253	GGCTGG TTCC	6	1	16.6	0	0	5	83.3
R-157	GCTGG TTCCT	9	3	33.3	4	44.4	2	22.2
R-108	GTATTG CCCT	9	2	22.2	4	44.4	3	33.3
UPC-90	GGGGGTTAGG	13	4	30.2	6	46.1	3	23.0
TOTAL		51	14		22		15	
Average		10.2	2.8	26.16	4.4	38.4	3	35.2

When compared among sweet orange cultivars shown from RAPD marker data that high fragments number were observed in Syrian cultivar reached 39 fragments band, while the less fragments number was observed in Egyptian cultivar reached 28 fragments band (Table 2).

Table 2. Sweet orange cultivars fragments numbers RAPD markers

Genotype(cultivars)	Number of total Fragment
Ind	36
Irq	38
Jap	37
Egy	35
Syr	39

Notes: Cultivars, Ind: Indian, Irq: Iraqi, Jap: Japanese, Egy: Egyptian, Syr: Syrian.

Genetic distance and relationships among sweet orange cultivars by used RAPD markers:

Table 3 showed that data of RAPD markers scanned from 5 sweet orange cultivars with reproducible primers were used to genetic distance and similarity value co-efficient. Amiximum genetic distance value was observed between Japanese and Syrian reached 0.530 with less similarity value reached 43%. While a minimum genetic distance value was observed between Iraqi and Indian reached 0.239 with high similarity value reached 76.1% (Table 3).

Table 3. Genetic distance among sweet orange cultivars

	Ind	Irq	Jap	Egy	Syr
Ind	**0.000**	**0.239**	**0.480**	**0.286**	**0.426**
Irq	**0.239**	**0.000**	**0.5**	**0.3182**	**0.417**
Jap	**0.480**	**0.5**	**0.000**	**0.480**	**0.530**
Egy	**0.286**	**0.318**	**0.480**	**0.000**	**0.280**
Syr	**0.426**	**0.417**	**0.530**	**0.280**	**0.000**

Notes: Cultivars, Ind: Indian, Irq: Iraqi, Jap: Japanese, Egy: Egyptian, Syr: Syrian

To determine the genetic relationships among 5 sweet orange cultivars, the scoring data were used to compute the similarity matrices. These genetic similarity matrices were then used in the cluster analysis to generate a dendogram using in the cluster analysis UPGMA analysis. The cluster tree analysis (Fig. 3) showed that the cultivars were broadly divided into two main groups A and B with genetic similarity reached 50%. A group including individual one cultivar was Japanese. B group was divided into two sub-cluster B1 and B2 with genetic similarity reached 63%. The first sub-cluster (B1) was included two cultivars Iraqi and Indian with high genetic similarity among other cultivars reached 77%. The second sub-cluster (B2) was included two cultivars Egyptian and Syrian with genetic similarity reached 72%.

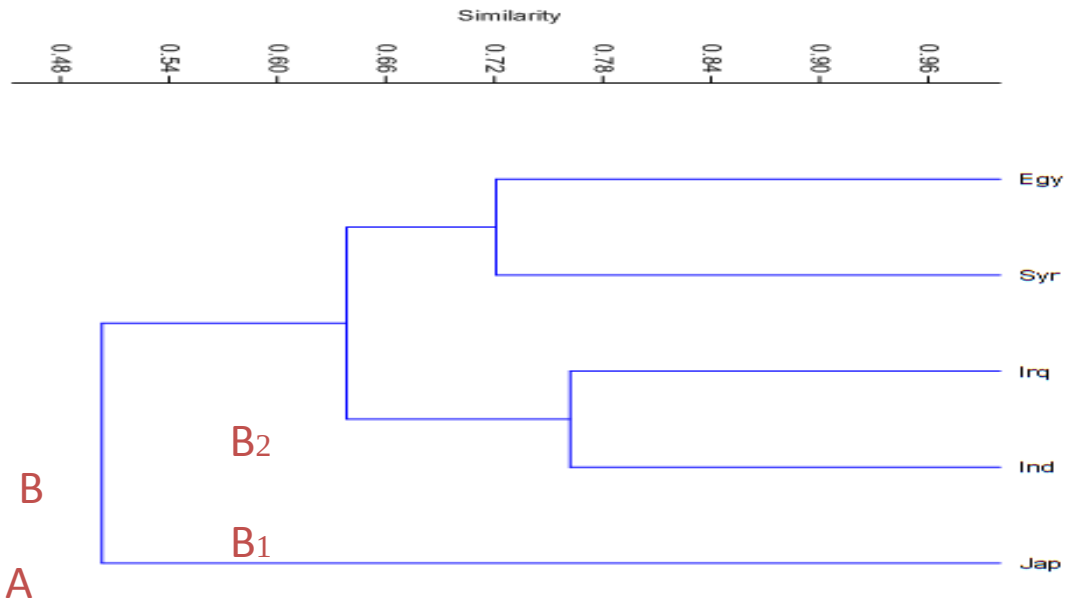

Figure 3. Dendrogram for the 5 sweet orange cultivars constructed from RAPDs data using Unweighted Pair-group Arithmetic Average (UPGMA) and similarity matrices computed according to coefficients. Cultivars, Ind: Indian, Irq: Iraqi, Jap: Japanese, Egy: Egyptian, Syr: Syrian.

The same results were also reported in other studies. The authors [16] notified variations in orange, lemon, grapefruit and lime based on mutations occurred on one ancestor tree. In paper [39] reported that it was difficult to distinguish cultivars originated mutations using isozyme markers. Low level of polymorphism in orange also found with ISSR [20], SSR [40, 13], SRAP [41]. On the other hand, no variation was found in studied oranges in some researches [42]. Orange cultivars are classified into four groups: common, low acidity, pigmented and navel oranges [23 as cited in 12]. It is indicated that despite the existence of substantial diversity among cultivated genotypes in respect of morphological, physiological and agronomic traits, very little DNA variation has been detected using DNA markers [13]. Same researchers found low level of genetic

polymorphism among 41 orange cultivars, they notified that sweet oranges have a narrow genetic basis and that most morphological characters originated through mutations, and clonal propagation of sweet oranges is the case for the majority of citrus species [30 as cited in 43]. In paper [20] were used ISSR markers to differentiate 41 samples of orange belongs to three groups, Valencia, blood and navel based on fruit traits. All of these cultivars found almost the same ISSR fingerprints. This was notified as majority of sweet orange cultivars derived from a single ancestor by mutation. However, some cultivar distinguished from others, it is explained as only a case in which replicate samples of the same cultivar from different locations had different ISSR fingerprint patterns, it is indicated that this result suggests that mutation occurred in at least one of them, although horticultural traits are not known between them [20]. In other study, it was found identical microsatellite profiles at 9 out of 10 SSR loci among analyzed orange cultivars and clones [12, 44].

4. Conclusion

In recent study carried out using large amount of orange showed that there was high level of genetic similarity in oranges [14, 41]. Similarity level of it derived from zygotic origin. Genetic similarity of all of other oranges was over 69% and some of them were identical, in this group there were many common orange cultivars and clones such as, many Japanese, Egyptian, Indian, Syrian introduced from other countries. Molecular marketing techniques may be a first step towards efficient conservation, maintenance and utilization of existing genetic diversity of sweet orange plants. This may lead further to different genetic analysis, gene mapping and ultimate improvement of the crop at genetic level. Randomly amplified polymorphic DNA (RAPD) markers are usually preferred in this kind of work as the technique is simple, versatile and relatively inexpensive and can detect minute differences [7]. The Citrus RAPD markers have been used for genetic mapping [45], identification of cultivars [21], hybrids [46], mutants [47], chimerase [48] and phylogenetic analyses [4]. Random PCR approaches are being increasingly used to generate molecular markers which are useful for taxonomy and for characterizing populations. The main advantages of these approach is that previous knowledge of DNA sequences is not required, so that any random primer can be tested to amplify any fungal DNA. RAPD primers are chosen empirically and tested experimentally to find RAPD banding patterns which are polymorphic between the isolates studied. Using PCR-RAPD, [49-51] it was also possible to identify heterogenity with in groups of genotypes which originates within the same location. In present study, characterization of sweet orange varieties by the RAPD has proved useful in separating all varieties/clones from each other. It has also provided us with primer markers that can be used to separate and distinguish each clone. In paper [15] it was suggested that sweet orange has a majority of its genetic makeup from mandarin and only a small proportion from pummelo. The demonstrated Genetic distance and Genetic relationships as revealed by RAPD markers results that nearest cultivars were Iraqi and Indian and farthest genetically were among Japanese and other cultivars. The reason is probably due to different environmental were formerly breaded on them and reflected it on genetic materials. So the environmental condition convergent may be caused semi genetic makeup (like Iraq and India).

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