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Genetic Diversity Assessment of Some Citrus Species Cultivated in Iraq Based on RAPD Molecular Marker.

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Abstract

Genotyping of six Citrus accessions was carried out using RAPD marker .The genetic variability among the six Citrus accessions was estimated using six decamer RAPD primers. Five primers generated reproducible and easily storable RAPD profiles with a number of amplified DNA fragments ranging from nine to 12 ,the primer OPA – 17 was negative results . The total number of amplicons detected was 50, including unique bands reached seven polymorphism were 34,this represents a level of polymorphism of 68.33% and an average number of 6.8 polymorphic bands per primer. a maximum numbers of amplicons was amplified with primer OPA-18 reached 12 while the minimum number of fragments was amplified with primers OPA-12 and OPN-16 reached 9 respectively. The highest number of polymorphic bands reached 8 were obtained with primer OPN-16 and OPA-01respectively, while the highest number of monomorphic bands reached 3 was obtained with primer OPA-12 and OPS-147 with percentage reached 33.33% and 30 respectively. RAPD markers detected genetic distance and similarity , a maximum genetic distance value was observed between sour orange (SO) and sweet orange (OR) reached 0.578 with less similarity value reached 42%, a minimum genetic distance value was observed between sweet orange (OR) and mandarin (MA) reached 0.316 with high similarity value reached 69% . The similarity matrices were employed in the cluster analysis to generate a dendrogram using the UPGMA method. The cluster tree analysis showed that the citrus species were broadly divided into two main groups A and B. A group including two species were troyercitronge (TC) and sour orange (SO) with genetic similarity reached 56%. B group was divided into two sub-cluster B1 and B2 with genetic similarity reached 51%. The first sub-cluster (B1) including only citrone(CI) species but the second sub-cluster (B2) divided into two groups(B2A and B2B),the first group (B2A) included sweet orange (OR) and mandarin (MA) with high genetic similarity reached 69%, and second group (B2B) included volkamer lemon (VL) species only.

Key words: Citrus, Genetic Diversity, Molecular marker, RAPD.

Introduction

Citrus is one of the most widely cultivated fruit in the world, Genus *Citrus* belongs to family Rutaceae and subfamily Aurantioideae(. Dugo and Di Giacomo, 2002). Most major production areas are far removed from the original areas which is south eastern Asia(Webber *et al*, 1967). Citrus is the most produced fruit in the world with over the 126 million tons of production (FAO, 2011). It is widely grown in most areas with suitable climates tropical, subtropical, and borderline subtropical/temperate (Kahn *et al*., 2001). Genetic variability in citrus is considered to be the result of many factors, such as hybridization, mutation and type of reproduction (mostly apomictic)(The Citrus and Date Crop Germplasm Committee, USA,CDCGC, 2004). The low intraspecific diversity found in cultivated species such as sweet orange, sour orange, citron, troyercitronge and other citrus species contrasts with the high variability of agriculturally important traits such as ripening period and color and size of fruits (Novelli*et al*., 2006). The genus *Citrus* L. belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family (Webber, 1967). The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of tribes Clauseneae and Citreae is composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae and Merrillinae, and Citreae has Triphasiinae, Citrinae and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesiclesin the fruit. This subtribe contains three groups; primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella and Citrus (Swingleand Reece, 1967). Most of genus including Citrus belongs to subfamily Aurantioideae originated from Monsoon regions and expand from West Pakistan to China, India islands, Northwest Australia, New Guinea (Baik, 2009). Citrus taxonomy and phylogeny are very complicated, controversial and confusing, mainly due to sexual compatibility between Citrus and related genera, the high frequency of bud mutations and the long history

different plant species (Ahmed, 1999; Nebauer*et al*., 2000; Besnard*et al*., 2001; Iruela*et al*., 2002). In citrus, RAPD markers have been used for genetic diversity analysis (Abkenar and Ishhiki, 2003; Mariniello et al., 2004; Campos *et al*., 2005; Novelli*et al*., 2006; Shaaban*et al*., 2006; Shahsavar*et al*., 2007; Hvarleva*et al*., 2008). DNA fingerprinting using PCR-based markers is very important for breeding and taxonomy of citrus. DNA-based markers approach to the study of citrus has been attempted even with a large area under cultivation in Iraq. In this study we used RAPD markers to characterize the citrus genotypes. The objectives of the study were to achieve a better understanding of genetic variation and to investigate their inter-relationship among some citrus cultivars.

Materials and Methods

Plant material

A total of six citrus genotypes were collected from the citrus orchard of Karbala station, used in this study (SO)sour orange *C. aurantium*, (OR)sweet orange *C. sinensis*, (TC)Troyer citrange*C. sinensis* X *P. trifoliate*,(MA) mandarin *C. reticulate*, (CI) citron *C. medica* and ,(VL)Volkamer lemon (*C. volkameriana*).

DNA isolation

Total genomic DNA was isolated from fully expanded leaves using the Kit,leave 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 DNA were determined by Nano drop-spectrophotometrically at 260 nm. The analysis was conducted in the laboratory of Molecular Genetic in 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 six decamer oligonucleotides of arbitrary sequence were tested for PCR amplification. AccupPower Gold Multiplex PCR premix(BIONEER \South Korea) was used to DNA amplification with RAPD primers and the thermal cycler conditions for PCR reactions were an initial denaturation cycle of 1 min and 30 s at 94 °C was followed by 45 cycles comprising 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. An additional cycle of 7 min at 72 °C was used for final extension.

DNA Electrophoresis

Amplification products were separated by electrophoresis (100 V) for (30 minutes) in 1.5% agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination.

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

of cultivation and wide dispersion (Nicolosi*et al*., 2000). The conventional methods in *Citrus* cultivars identification relied on morphological features and isozymes (Hvarleva*et al*, 2008). Citrus taxonomy was based on mainly morphological and geographical data in the past and many classification systems have been formulated, two of these systems suggested by Swingleand Reece (1967) and Tanaka (1977) have been the most widely accepted. The number of recognized species is the major difference between two systems. Swingle recognized 16species in the genus Citrus, whereas Tanaka (1977) recognized 162 species. Scora (1975) and Barrett and Rhodes (1976) suggested that there are only three 'basic' true species of Citrus within the subgenus Citrus as follow: citron (*C. medica*L.), mandarin (*C. reticulata*Blanco), and pummelo (*C. maxima* L. Osbeck). Later, Scora (1988) added *C. halimi*as another true species. Other cultivated species within Citrus were derived from hybridization between these true species or closely related genera followed, mainly, by natural mutations. (Federici*et al*., 1998; Nicolosi*et al*., 2000; Barkley *et al*., 2006; Uzun*et al*., 2009). Elucidating relationships, taxonomy, and diversity is important for developing breeding strategies, conserving biodiversity, and improving breeding efficiency. Also understanding genetic variability in citrus is critical for characterizing germplasm, controlling genetic erosion and the registration of new cultivars (Herrero *et al*., 1996; Barkley *et al*., 2006). The genetic features found in Citrus favor the construction of genetic maps, Citrus has a small genome (1C = 0.62 pg) (Guerra, 1984 and Barrett, 1985), is diploid with a small number of chromosomes (n = 9) (Soost and Cameron, 1975) and is highly polymorphic. Regarding to germplasm management molecular characterization has a number of applications such as relationships between accessions, characterizing newly acquired germplasm, monitoring shifts in population genetic structure in heterogeneous germplasm, exploiting associations among traits of interest and genetic markers and genetic enhancement (Ebtsam, 2013). Different types of molecular markers have been used to obtain genetic linkage maps. Among these, random amplified polymorphic DNA (RAPD) markers are the most common because the technique is easy, inexpensive, uses a low amount of genomic DNA, and produces markers that are highly polymorphic and that represent the whole genome (Shahsavar*et al*, 2007 and Botstein *et al*., 1980). RAPD markers are detected by the random amplification of genomic DNA fragments of different sizes through the polymerase chain reaction (PCR) (Williams *et al*., 1990). 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.. RAPDs have been extensively used in assessing relationships amongst various accessions of

arithmetic averages (UPGMA) cluster analysis using the MEGA (Molecular Evolutionary Genetics Analysis) version 2.0 (Kumar *et al*., 2001).

Results and Discussion

Figure (1) showed the results of isolated total DNA of the leaves of the studied olive cultivars manner filters and then migrated to agar gel 1.5 %, voltage 100 V for 30 minutes noting the success of the method to isolate DNA from this Citrus verities.

approximate size of the amplified fragment in base pairs. Amplified products were analyzed by pairwise comparisons of the genotypes based on the percentage of common fragments, and a similarity matrix was calculated (Nei and Li, 1979). 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) (Rohlf, 1993). A dendrogram was constructed based on the genetic distance matrix by applying an unweighted pair group method with

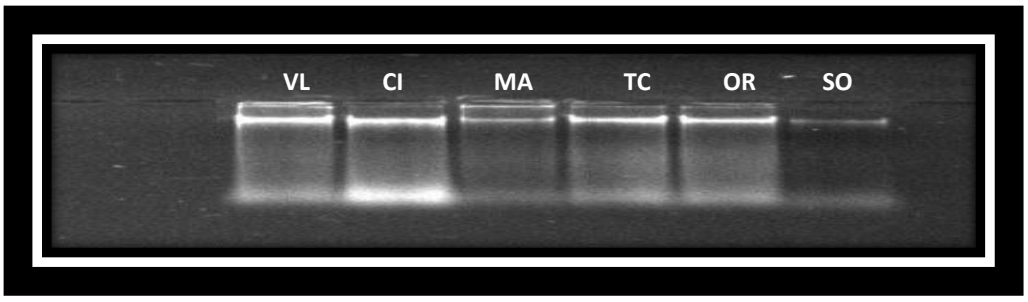
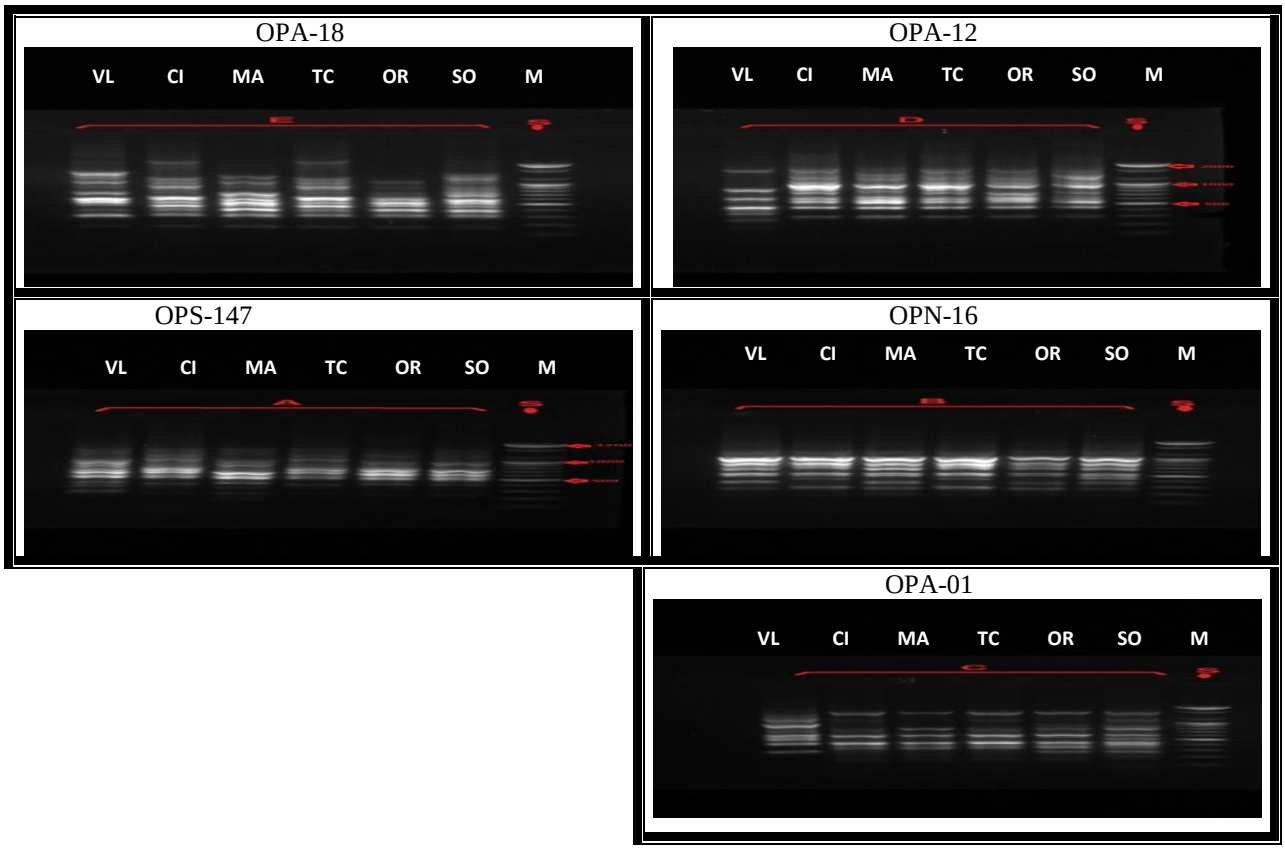


Figure (1) represents the genomic DNA of the Citrus leaves (SO)sour orange *C. aurantium*, (OR)sweet orange *C. sinensis*, (TC)Troyer citrange*C. sinensis* X *P. trifoliate*,(MA) mandarin *C. reticulate*, (CI) citron *C. medica* and ,(VL)Volkamer lemon (*C. volkameriana*) on agarose gel (1.5%) and voltage (100 V) for (30 minutes).

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**).**

Polymorphisms and monomorphisms detected by RAPAD markers :



Fig(2)RAPD profiles of the 6 Citrus genotypes amplified with RAPD primers,M:molecular weight marker (SO)sour orange *C. aurantium*, (OR)sweet orange *C. sinensis*, (TC)Troyer citrange*C. sinensis* X *P. trifoliate*,(MA)

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mandarin *C. reticulate*, (CI) citron *C. medica* and ,(VL)Volkamer lemon (*C. volkameriana*) on agarose gel (1.5%) and voltage (100 V) for (30 minutes).

total of the monomorphic 5 with an average reached 1 fragments / primers with the monomorphic percentage was 0% to 11.11%.As shown in table(1) a maximum numbers of amplicons was amplified with primer OPA-18 reached 12 while the minimum number of fragments was amplified with primers OPA-12 and OPN-16 reached 9 respectively. The highest number of polymorphic bands reached 10 was obtained with primer OPS-147, while the highest number of monomorphic bands reached 11.11 was obtained with primer OPA-12.

Six primer were screened with the DNA of the 6 citrus genotypes . among the 6 primer tested primers , 5 primers generated reproducible and easily storable RAPD profiles with a number of amplified DNA fragments ranging from 9 to 12 (table 1) . the primer OPA – 17 was negative results. The total number of fragments produced by 5 primers was 50 with an average of 10 fragments/ primers. While the number of polymorphic ranged from 8 to 10 with an average reached 9 fragments/ primers with the polymorphic percentage ranged from 75% to 100%.While the number of monomorphic ranged from 0 to 3 and was

Table(1) Total number of amplicons, polymorphic, monomorphic amplicons, and percentage of monomorphism, polymorphism as revealed by RAPD markers among the 6Citrus 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 (%)
OPA-18	AGGTGACCG T	12	3	25	7	58.33	2	16.66
OPA-12	TCGGCGATAG	9	2	22.22	4	44.44	3	33.33
0PS-147	AGCTGCAGCC	10	0	0	7	70	3	30
OPN-16	AAGCGACCTG	9	0	0	8	88.88	1	11.11
OPA-01	CAGGCCCTTC	10	1	10	8	80	1	10
TOTEL		50	7		34		10	
Average		10	1.4	11.44	6.8	68.33	2	13.55

observed in sweet orange (OR), troyercitronge (TC) and mandarin (MA) reached 31fragments band respectively, while the less fragments number was observed in citron (CI) reached 28 fragments band (table 2).

Citrus species fragments numbers RAPD markers:

When compered among citrus species shown from RAPD marker data that high fragments number were

Table(2) Citrus species fragments numbers RAPD markers

Genotype	Number of total fragment
SO	31
OR	32
TC	32
MA	32
CI	28
VL	30

orange (OR) reached 0.578 with less similarity value reached 42%. While a minimum genetic distance value was observed between sweet orange (OR) and mandarin (MA) reached 0.316 with high similarity value reached 69% (table 3).

Genetic distance among citrus species:

Data of RAPD markers scanned from 6 citrus species with reproducible primers were used to genetic distance and similarity value co-efficient.Amiximum genetic distance value was observed between sour orange (SO) and sweet

Table (3)genetic distance among citrus species.

	SO	OR	TC	MA	CI	VL
SO	**0.000**	**0.578**	**0.440**	**0.512**	**0.537**	**0.524**
OR	**0.578**	**0.000**	**0.512**	**0.316**	**0.537**	**0.488**
TC	**0.440**	**0.512**	**0.000**	**0.4**	**0.462**	**0.488**
MA	**0.512**	**0.316**	**0.4**	**0.000**	**0.5**	**0.369**
CI	**0.537**	**0.537**	**0.462**	**0.5**	**0.000**	**0.432**
VL	**0.524**	**0.488**	**0.488**	**0.369**	**0.432**	**0.000**

(SO)sour orange *C. aurantium*, (OR)sweet orange *C. sinensis*, (TC)Troyer citrange*C. sinensis* X *P. trifoliate*,(MA) mandarin *C. reticulate*, (CI) citron *C. medica* and ,(VL)Volkamer lemon (*C. volkameriana*)

including two species were troyercitronge (TC) and sour orange (SO) with genetic similarity reached 56%. B group was divided into two sub-cluster B1 and B2 with genetic similarity reached 51%. The first sub-cluster (B1) including only citrone(CI) species but the second sub-cluster (B2) divided into two groups(B2A and B2B),the first group (B2A) included sour orange (OR) and mandarin (MA) with high genetic similarity reached 69%, and second group (B2B) included volkamer lemon (VL) species only

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Genetic relationships as revealed by RAPD markers used dendograms:

To determine the genetic relationships among 6 citrus genotype ,the scoring data were used to compute the similarity matrices.These genetic similarity matrices were then used in the cluster analysis to generate adendogram using in the cluster analysis UPGMA analysis. The cluster tree analysis (Fig.3) showed that the citrus species were broadly divided into two main groups A and B. A group

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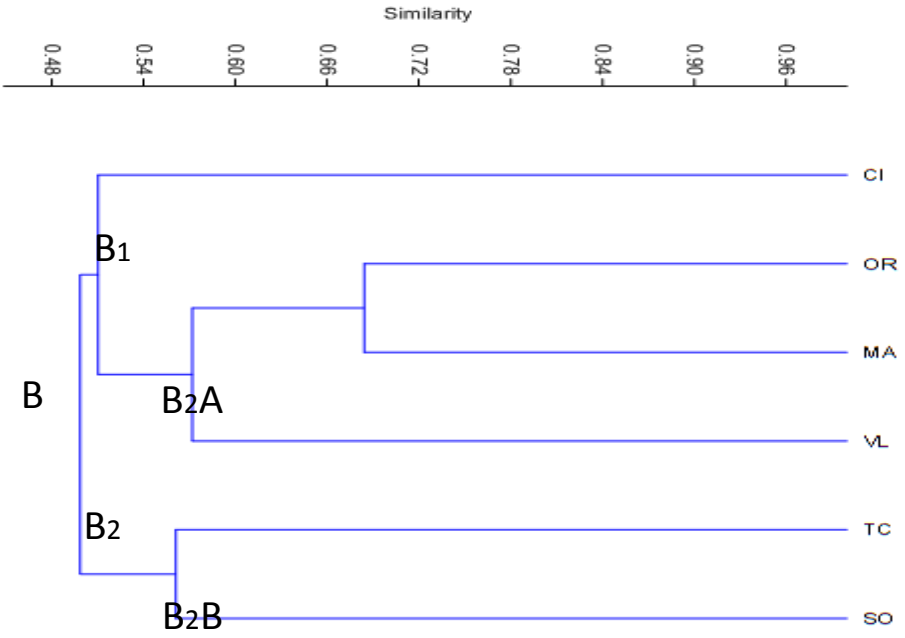

Fig. (3): A lrogram for the 6 Citrus genotypes constructed from RAPDs data using Unweighted Pair-group Arithmetic Average (UPGMA) and similarity matrices computed according to coefficients.(SO)sour orange *C. aurantium*, (OR)sweet orange *C. sinensis*, (TC)Troyer citrange*C. sinensis* X *P. trifoliate*,(MA) mandarin *C. reticulate*, (CI) citron *C. medica* and ,(VL)Volkamer lemon (*C. volkameriana*)

The results obtained from RAPD analysis allowed the distinction between species of *Citrus* derived from natural hybridization between species (the majority of *Citrus* species) and the few "true" species naturally occurring in the genus (mandarin). The number of analysis of RAPD data was very useful and informative in the characterization and estimation of genetic distance within *Citrus* species. They were used to establish the dendrogram of genetic relationship within the genus *Citrus* (Fig. 3).

The results showed that high genetic distance was between sour orange and sweet orange and low genetic distance was between sweet orange and mandarin and with high similarity .This results were consistent withUzun*et al.*(2009) where they noticed that sweet orange, mandarin, sour orange, pummelo and grapefruit nested in same large group in previous study. While this group separated two subgroup at similarity level of 0.64. The first subgroup included sweet oranges, mandarins and sweet oranges were separated from mandarins at 0.78. Parental sweet orange tree was a hybrid of pummelo and mandarin (Scora, 1975; Barrett and Rhodes, 1976), which was later supported by Nicolosi*et al.* (2000). Barkley *et al.* (2006) suggested that sweet orange has a majority of its genetic makeup from mandarin and only a small proportion from pummelo.The citrus fingerprint data generated has revealed many genotype specific primers (Table 1,2) which have potential to be used further in cultivar identification and classification. These results can be further used to manipulate genetic determinants of horticulturally important traits and to characterize the basis of productivity of citrus.

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