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Genetic diversity of citrus (Rutaceae) in Iraq based on random amplified polymorphic DNA (RAPD) markers

Aseel K. AL-Anbari[1,4], Nantawan Kanawapee[2], Talib A. AL-Kazragi[3], Hazim AL-Jewari[4], Athia Al-Mashhadani[4], Sahapat Barusrux[5], Pimwadee Pornpongrungrueng[2] and PiyadaTheerakulpisut[2]*

[1]Department of Biology, College of Pure Science, Diyala University, Iraq.
[2]Applied Taxonomic Research Center, Department of Biology, Faculty of Science Khon Kaen University, Thailand.
[3]Department of Biology, College of Education, Tikrit University, Iraq.
[4]Department of Biology, College of Pure Science, (Ibn- al hathem), Baghdad University, Iraq.
[5]Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand.

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Citrus is an economically important fruit crop with a long history of cultivation worldwide. A great number of varieties of citrus are extensively grown in the Middle East including Iraq for domestic consumption and exports. However, the genetic diversity of this genus in Iraq has not been reported. Therefore, the objective of this study was to evaluate genetic relationships of Iragi citrus genotypes to provide useful information for germplasm conservation and planning of breeding strategies. Twenty decamer primers were used to generate RAPD markers to evaluate genetic relationship among 16 genotypes (14 species and hybrids) of cultivated Citrus in Iraq. Based on RAPD polymorphisms, the citrus genotypes were classified into two main groups; the first consisted of citron (*Citrus medica*) and its hybrids (lime and lemon). The second group contained the remaining genotypes including three sub-groups; the first being formed of sour orange (*Citrus aurantium*), sweet orange (*Citrus sinensis*) and grapefruit (*Citrus paradisi*), the second the mandarins (*Citrus reticulata*) and the third the pummelo (*Citrus grandis*). The RAPD-based classification was consistent with previous studies based on other types of molecular markers.

Key words: Citrus, genetic diversity, Iraq, oranges, random amplified polymorphic DNA (RAPD) markers.

INTRODUCTION

Among the most important fruit crops in the world is Citrus, where the international production has reached 122 million tons (FAO, 2008). The taxonomy and phylogeny of the genus Citrus is very complicated and confusing and many hypotheses have been formulated. Two most widely accepted classifications of Citrus were proposed based on morphological traits. The Swingle system (Swingle and Reece, 1967) recognized 16 species in the genus Citrus. On the other hand, the Tanaka system (Tanaka, 1977) has identified as many as

*Corresponding author. E-mail: piythe@kku.ac.th. Tel: (+66) 89-623-1777.

162 species. Scora (1975) suggested that there are only three 'basic true species' of Citrus within the subgenus Citrus as defined by Swingle, that is, Citron (*Citrus medica*), Mandarin (Citrus reticulate Blanco) and Pummelo (*Citrus maxima* (Burm.) Merr. or Citrus grandis Osbeck). Other cultivated Citrus species within the subgenus Citrus are believed to be hybrids derived from these true species, species of the subgenus Papeda, or closely related genera. Additionally, Mabberley (2004) also indicated *Citrus australis* (Mudie) Planch. (Australian lime) and *C. australasica* F. Muell. (finger lime) as Australian wild parental species of many commercial hydrids. This idea has recently been supported by data derived from molecular markers (Barkley et al., 2006). High level of genetic variations exists among cultivated species of Citrus due to frequent bud mutations, wide sexual compatibility between Citrus genus and related genera, the long history of cultivation and the worldwide dispersion (Scora, 1988). Phylogeny and taxonomy for certain Citrus cultivars have been somewhat debatable in the past; however, results from molecular marker technologies are helping to clarify some of these relationships. A variety of DNA markers is available and has been used to study the classification of Citrus genus and phylogenetic relationships within Citrus and with related genera (Yamamoto et al., 1993; Federici et al., 1998).

Several molecular marker systems including Random Amplified Polymorphic DNA (RAPD), microsatellites (SSR), amplified fragment length polymorphism (AFLP) and inter-simple sequence repeats (ISSR) have been used to evaluate genetic diversity of various collections of Citrus and related genera in different countries. The monophyletic origin of Citrus was clearly confirmed by AFLP molecular analysis of 59 genotypes, six genera of the True Citrus Fruit Trees Group (Xie et al., 2008). Analysis based on 262 RAPD and 14 SCAR markers revealed that Fortunella is phylogenetically close to Citrus while the other three related genera (Poncirus, Microcitrus and Eremocitrus) are distant from Citrus and from each other (Nicolosi et al., 2000). However, molecular data based on two regions of chloroplast DNA supported a clade constituted by Citrus, Poncirus, Fortunella, and Microcitrus (Araújo et al., 2003). Data based on 119 RAPD and 48 SSR markers were used to classify 31 genotypes of Syrian Citrus and trifoliate orange into two main groups, the first consisted of Trifoliate orange (*Poncirus trifoliata*), and the other contained members of the genus Citrus. The Citrus genotypes were divided into 5 distinct groups; Sour orange, Mandarin, Rough lemon, Volkamer lemon and Sweet lime (EL-Mouei et al., 2011a). Furthermore, the same authors (EL-Mouei et al., 2011b) found that among the four Citrus groups, Lemon is distant from the others (Mandarins, Grapefruits and Sweet orange) and the highest genetic diversity was detected in the Mandarin and the lowest in the Grapefruit group. Several molecular studies supported *C. maxima*, *Citrus medica* and *Citrus reticulata* as the basal species of edible Citrus and identified probable hybrid origins of several commercial cultivars (Jena et al., 2009; Pessina et al., 2011; Ramadugu et al., 2013). In an attempt to identify the paternal and maternal origins of 30 accessions of cultivated Citrus, Li and Xie (2010) has employed three marker systems; the chloroplast DNA and internal transcribed spacer sequences and AFLP fingerprints. Molecular markers (SSR and mitochondrial DNA) have been used to characterize 201 accessions of Tunisian citrus rootstock germplasm and found that the clustering was generally consistent with varietal group classification and a core sample of accessions were identified for further use in a breeding program (Snoussi et al., 2012).

The objective of this study was to evaluate the genetic relationships among 16 taxa including 14 species and hybrids of Citrus cultivated in the central part of Iraq using RAPD markers. The genetic characterization of Citrus germplasm in Iraq has not yet been reported. The information obtained from this study is expected to provide a basis for future studies for characterization and preservation of agro-biodiversity of Citrus germplasm collection in Iraq, inferring the hybrid origin of species or cultivar identification among others.

MATERIALS AND METHODS

Plant materials

Sixteen taxa of Citrus (Rutaceae) representing 14 species/hybrids (Table 1) were collected during 2010-2012 from different geographical locations covering 4 Eastern provinces of Iraq (Figure 1). The collected plants were identified and herbarium specimens were prepared from the plant parts (stems, leaves, flowers and fruits) and deposited at the National Herbarium, Baghdad, Iraq.

Isolation of genomic DNA

Total genomic DNA was isolated from dry leaves of 16 taxa of Citrus species using the modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The RNA was removed by the treatment with 10 µg μl^{-1} RNase at 37°C for 30 min. The quality of the DNA were tested by staining with ethidium bromide after electrophoresis in 1% agarose gel at 100V for 45 min in 1XTBE buffer and the image was visualized with an ultraviolet transilluminator. The amount of DNA was determined by measuring the absorbance at 260 nm and the concentration was adjusted to 50 ng μl^{-1} and stored at -20°C.

RAPD analysis

Twenty different 10-mer oligonucleotide RAPD primers (Operon Technologies Inc., USA) (Table 2) were used. Each polymerase chain reaction (PCR) was carried out in a 25 µl volume containing 8 µg µl-1 template DNA, 1XPCR buffer, 1.5 mM $MgCl_2$, 0.32 mM dNTP, 1.0 µM primer and 0.16 unit μl^{-1} Taq DNA polymerase (iTaq DNA polymerase Kit). Amplification was performed in a thermal

Table 1. List of 16 *Citrus* taxa included in the study.

S/N	Species	Locality	Parentage	Common name
1	*C. aurantifolia* var. *Acidica* (Christm.) Swingle	Salah aldeen	*C. medica* x *C. Limon* X *C. micrantha*	Mexican lime
2	*C. aurantium* L.	Diyala	*C. grandis* x *C.reticulata*	Sour orange
3	*C. deliciosa* Ten.	Babel	-	Willow leaf Mandarin
4	*C. grandis* Osbeck	Baghdad	Female parent	Pummelo
5	*C. japonica* Thunb.	Salah aldeen	-	Margarita
6	*C. latifolia* Tanaka	Babel	*C. sinensis* x *C. aurantifolia*	Persian lime
7	*C. limetta* Risso	Baghdad	-	Sweet lemon
8	*C. limon* (L.) Burm.f.	Karbala	*C. medica* x *C. aurantium*	Lemon
9	*C. medica* L.	Diyala	Male Parent	Citron
10	*C. paradisi* Macfad	Baghdad	*C. sinensis* x *C. grandis*	Grape fruit
11	*C. reshni* Hort. ex Tanaka	Diyala	-	Cleopatra (Egyptian mandarin)
12	*C. reticulata* var. *Clementine* Blanco	Karbala	*C. reticulate* x *C. sinensis*	Mandarin
13	*C. sinensis* Osbeck	Baghdad	*C. grandis* x *C. reticulata*	Sweet orange
14	*C. aurantium* L. x *C. trifoliata* (L.) Raf.	Diyala	*C.aurantium* x *C. trifoliata*	Citradia
15	*C. sinensis* var. *moro* (L.) Osbeck	Diyala	*C. grandis* x *C. reticulata*	Red orange
16	*C. volkameriana* Pasq.	Babel	*C. medica* x *C. aurantium*	Volkamer lemon

cycler (Corbett Research, Australia) using the following conditions: denaturation at 95°C for 3 min; 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 40°C and 2 min extension at 72°C; and a final extension at 72°C for 7 min. The RAPD-PCR products were analyzed directly on 1.5% agarose gel in 1XTBE buffer. The DNA was stained with 0.5 mg ml^{-1} ethidium bromide, visualized and photographed under a UV transilluminator.

Data analysis

The amplified bands were scored for each RAPD primer based on the presence (1) or absence (0), on the basis of size. RAPD matrix was then analyzed using the NTSYS-pc statistical package version 2.1. The data matrix was used to calculate the genetic similarity within and among species based on Jaccard's similarity coefficients, and a dendrogram displaying relationships among the 16 genotypes of Citrus was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

RESULTS AND DISCUSSION

A total of 143 amplified RAPD bands ranging from 100 bp to 1.8 kb in size were observed from the 16 Citrus genotypes. The number of RAPD bands varied from 2 (primer OPX16) to 13 (primers OPA-04 and OPW-06), with an average of 7.15 bands per primer. One hundred and twenty-two polymorphic bands (85.15% of the total amplified bands) were obtained, with an average of 6.1 bands per primer. Some representative polymorphisms revealed by RAPD primers are presented in Figure 2.

The dendrogram showing the genetic relationships among the 16 Citrus genotypes (Figure 3) showed that Citrus species were basically divided into 2 main clusters, the first (Cluster I) consisted of citron, lime and lemon; the second (Cluster II) contained pummelo, mandarin, grapefruit, sweet orange, sour orange and sweet lemon. The two main clusters separated at the similarity value of 0.67. Similar clustering was reported by Uzun et al. (2009) who divided 83 accessions of the genus Citrus into two large groups based on sequence related amplified polymorphism markers (SRAP). The first group included citron, lemon, lime and rough lemon; and the second group consisted of pummelo, grapefruit, sour orange, mandarins, sweet oranges and their hybrids. Using nine cpDNA sequences Bayer et al. (2009) showed that Citrus contained two lineages; the largely "southern clade" contains primarily wild species from New mandarin group, the lime group and the pummello group. Recently, Luro et al. (2011) also organized 87 citrus varieties into two main groups based on single strand conformation polymorphism (SSCP). The first group contained mandarins, sour

Figure 1. Geographic localization of sampling sites of Iraqi *Citrus* in this study (No. 1- 16 = *Citrus* taxa listed in Table 1).

oranges, sweet oranges, pummelo and grapefruits; and the second group included citrons, lemons, and limes and lemon hybrids.

Furthermore, an assessment of genetic diversity and population structure of a citrus germplasm collection of 370 accessions using simple sequence repeat markers (SSR) revealed five main populations which supported the hypothesis that there are only a few naturally occurring species of Citrus and most other types of Citrus arose through various hybridization events and mutations. The ancestral groups included citron which was separated from the cluster containing mandarins, pummelos and papedas (Barkley et al., 2006).

The first major Cluster (I) which included citron (*C. medica*) as the basic true species consisted of 2 sub-clusters; IA included two genotypes, that is, Mexican lime (*Citrus aurantifolia* var. acidic) and Citrus japonica var. margarita which showed similarity coefficient of 0.79. The second sub-cluster (IB) contained lemon (*Citrus limon*) and Persian lime (*Citrus latifolia*). This sub-cluster linked with citron (*C. medica*) by similarity value of 0.78. Nicolosi et al. (2000) also placed *C. medica*, *C. aurantifolia* and *C. limon* in the same group based on RAPD and SCAR markers. Both lemons and limes were proposed to be hybrids with citron contributing most of the alleles as the male parent (Barrett and Rhodes, 1976; Federiciet al., 1998; Nicolosi et al., 2000; Barkley et al., 2006). Recently, Li and Xie (2010) analyzed plastid genomes, nuclear ITS sequences and AFLP fingerprints of 30 citrus accessions in an attempt to infer into the origin of cultivated citrus. Such detailed molecular analysis Guinea, Australia, New Caledonia, New Ireland and two (*C. indica* and *C. medica*) historically considered to have arisen from India. The “northern clade” contained most of the economically important citrus species and cultivars which can be separated into the kumquat group, the demonstrated that sour orange was the maternal and citron the paternal parent of *C. limon.*

Moreover, it was strongly supported that *C. aurantifolia* was a hybrid of Papeda (maternal parent) and citron (paternal parent). Using the combined molecular, morphological and cytometric parameters Pessina et al.

Table 2. The codes and sequences of twenty RAPD primers used for PCR amplification of genomic DNA from 16 *Citrus* genotypes.

Primer	Primer sequence (5´-3´)	AN	Size range of bands (bp)	PM	%	MM	%
OPA-04	AATCGGGCTG	13	100->1500	13	100	0	0
OPA-05	AGGGGTCTTG	6	270-1500	5	83.3	1	16.7
OPA-09	GGGTAACGCC	7	300->1500	5	71.4	2	28.6
OPA-12	TCGGCGATAG	6	250-1400	5	83.3	1	16.7
OPB-06	TGCTCTGCCC	9	250-1600	8	88.8	1	11.2
OPB-12	CCTTGACGCA	6	150-610	5	83.3	1	16.7
OPW-06	AGGCCCGATG	13	250-1400	11	84.6	2	15.3
OPW-07	CTGGACGTCA	8	250-1400	6	75	2	25
OPW-09	GTGACCGAGT	11	100-1100	11	90.9	0	0
OPW-19	CAAAGCGCTC	4	300-900	3	75	1	25
OPX-02	TTCCGCCACC	5	400-1300	5	100	0	0
OPX-03	TGGCGCAGTG	5	100-1000	4	80	1	20
OPX-07	GAGCGAGGCT	6	200-1500	5	83.3	1	16.7
OPX-08	CAGGGGTGGA	5	100-800	5	100	0	0
OPX-09	GGTCTGGTTG	7	200-900	6	85.7	1	14.3
OPX-11	GGAGCCTCAG	9	200->1500	5	75	4	25
OPX-12	TCGCCAGCCA	9	100-1000	8	88.8	1	11.2
OPX-15	CAGACAAGCC	5	400-900	5	100	0	0
OPX-16	CTCTGTTCGG	2	200-1000	2	100	0	0
OPX-17	GACACGGACC	7	250-1000	6	85.7	1	14.3
Total		143	100->1500	123	-	20	-

Total number and size range of amplified bands and the number of polymorphic and monomorphic bands obtained for each primer. AN = alleles number; PM = Polymorphic bands; MM = Monomorphic bands.

(2011) confirmed the hybrid origin of *C. limonimedica* from *C. medica* and *C. limon*.

Sweet orange, mandarin, sour orange, pummel and grapefruit nested in the same large Cluster (II). Within this group, the mandarin (*Citrus reticulata*) and pummelo *(C. grandis*) were considered the parental species and the remaining genotypes were hybrids derived from mandarin, pummelo and citron (Barett and Rhodes, 1976). This group separated into three subgroups; the orange-grapefruit, the mandarin and the pummelo. The first sub-group contained sour orange (C. aurantium), grapefruit *(C. paradisi*), two genotypes of sweet orange (*C. sinensis*), Citradia (*C. aurantium* x *C. trifoliata*) and *C. volkameriana*. Different marker systems have been used to support the clade containing sour orange, grapefruit and sweet orange. The marker systems used included RAPD and SCAR (Nicolosi et al., 2000), SRAP (Uzun et al., 2009), AFLP (Li and Xie, 2010), and SSCP (Luro et al., 2011). Barett and Rhodes (1976) suggested that sweet orange, sour orange and grapefruit were all were all hybrids of pummelo and mandarin. Molecular evidence recently confirmed that pummelo and mandarin were the maternal and paternal origins, respectively of sweet orange and sour orange. Whereas grapefruit was a hybrid of pummelo and sweet orange which acted as female and male parents, respectively (Barkley et al., 2006; Li and Xie, 2010).

The sweet orange (*Citrus sinensis*) group has high similarity coefficient value of about 0.82 indicating their close relationship. Fang et al. (1998) reported similar results while working on 41 samples of 33 cultivars, belonging to 3 sweet orange groups, that is, Valencia, blood and navel, based on fruit traits. All of these cultivars had almost the same DNA fingerprints, isozymes and RFLP profiles. The two taxa of sweet orange, *C. sinensis* and *C. sinensis* var. moro (red orange) were grouped together with similarity value of 0.84 which is the highest value among any two citrus taxa in this study. This result further supported the suggestion that these two varieties were hybrids between pummelo and mandarin (Barett and Rhodes, 1976). Analysis of chloroplast DNA demonstrated that pummelo was the maternal parent of the sweet orange (Li and Xie, 2010). The other member of orange (citradia; *C. aurantium* x C. trifoliata) was separated from the others and formed a group with C. volkameriana. This close relationship supported the suggestion that citradia was a hybrid between trifoliate orange and sour orange (Swingle and Reece, 1967) and Citrus volkameriana was a hybrid between citron and sour orange (Nicolosi et al., 2000).

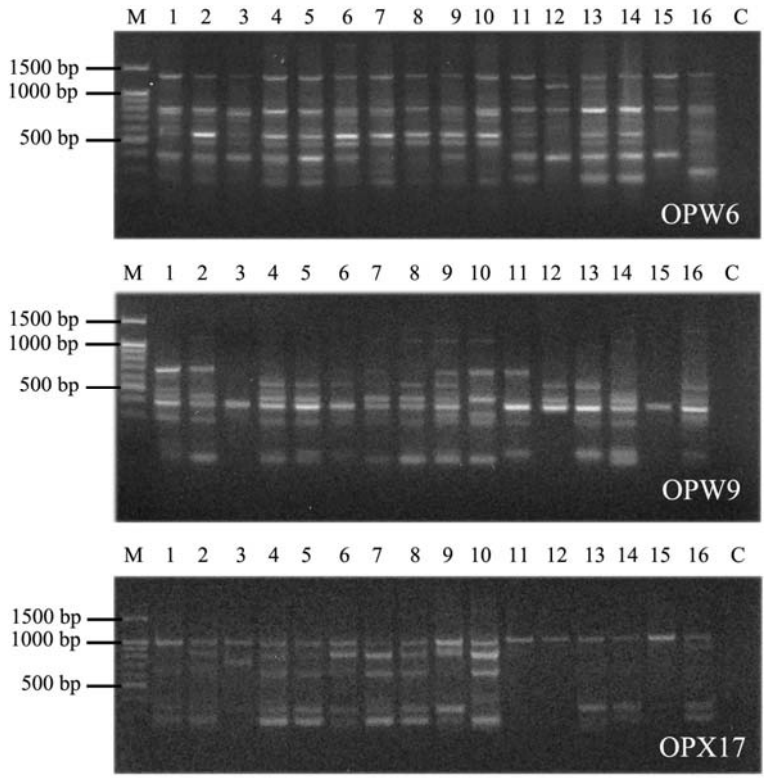

Figure 2. RAPD profiles amplified from genomic DNA of 16 *Citrus* taxa using primer OPW-06, OPW-09 and OPX-17. M = 100-bp DNA ladder, 1-16 = *Citrus* taxa described in Table 1, C = negative control without template DNA.

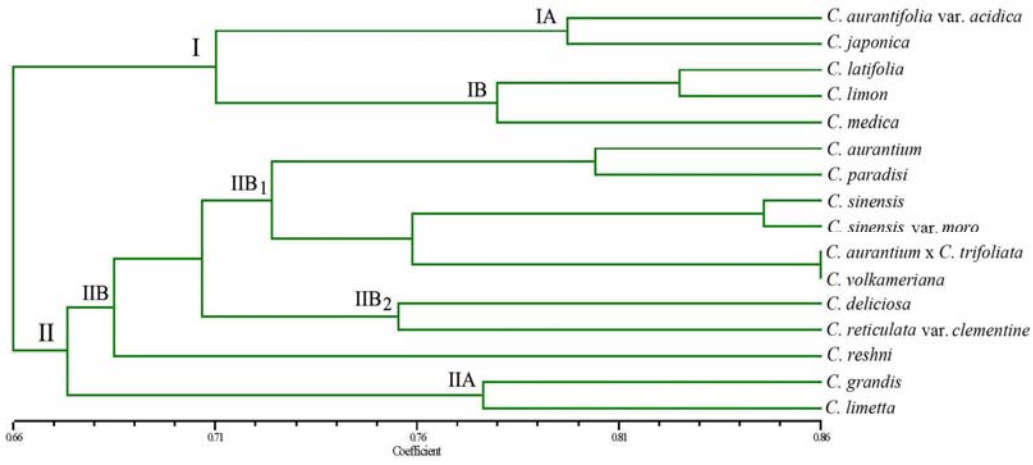

Figure 3. Dendrogram showing genetic relationships among 16 taxa of *Citrus* in Iraq.

Although, most molecular analysis placed *C. volkameriana* in the citron group, this particular type of *C. volkameriana* cultivated in Iraq seemed to have more shared alleles from sour orange than citron.

Three mandarins were included in this study, that is, *Citrus deliciosa* (willow leaf mandarin), *C. reticulata* var. clementine and *Citrus reshni* (cv. Cleopatra). In this RAPD-based analysis, willow leaf mandarin and clementine formed a group with similarity value of 0.75 which separated from the orange-grapefruit group at the

similarity value of 0.71. *C. reshni* was more distantly related and separated from the orange-mandarin group at the similarity value of 0.68. The observation that C. deliciosa was more closely related to *C. reticulata* and *C. reshni* was more distantly related was earlier shown by Filho et al. (1998) also based on RAPD markers. This molecular characterization confirmed the earlier classification by Tanaka (1954) who recognized 36 species of mandarins in five taxonomic groups; C. reticulata and *C. deliciosa* were placed in Group III whereas *C. reshni* in Group IV. The pummelo (*C. grandis*) was a member of Cluster II which was separated from all remaining genotypes. Pummelo was reported as one of the three true citrus species by Barett and Rhodes (1976) and most of the molecular studies were in agreement with this statement (Nicolosiet al., 2000; Barkley et al., 2006; Uzun et al., 2009).

Preservation of the genetic diversity of crop species throughout the world has become a major issue of international concern. Reduction in agro-biodiversity often increases vulnerability of crops to climatic stresses and diseases (Thrupp, 2000). Notably, the outbreak of citrus canker disease in Florida in 1984 leading to an eradication of twenty million citrus plants was in part due to genetic uniformity of the citrus crops (Schubert et al., 2001). Understanding of genetic diversity of citrus using both morphological and molecular data is essential forgermplasm management, planning and application of breeding programs in Iraq.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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