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Analysis of genetic relationship between 'Bakraee' (*Citrus* sp.) and some known *Citrus* genotypes through SSR and PCR-RFLP markers

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ABSTRACT

Phylogenic relationships and genetic diversity in citrus are considered to be important in clarifying their genetic relationships, germplasm characterization and the registration of new varieties. The 'Bakraee' (*Citrus* sp.) is an unknown natural biotype which is being used widely as rootstock as well as edible fruit in some regions of Iran. In the present research work, phylogenic relationships among 'Bakraee' and some commercially important citrus varieties were investigated through SSR and PCR-RFLP molecular markers. Two UPGMA dendrograms were generated using SSR and PCR-RFLP data. SSR data showed 0.87 and 0.65 genetic similarities between 'Bakraee' and rough lemon (*Citrus jambhiri* Lush.) and sweet lime (*Citrus limettioides* Tan.), respectively. PCR-RFLP analysis demonstrated that all of the amplified fragments in 'Bakraee' are also present in rough lemon. Our study confirmed a close relationship between 'Bakraee' and rough lemon and support the possibility of rough lemon as maternal parent in the hybrid origin of 'Bakraee'.

1. Introduction

Citrus is one of the world's important fruit crops and grown in most areas with suitable climates between latitude 35°N–35°S. In Iran, citrus industry is of paramount importance (Golein and Adouli, 2011).

Variation among *Citrus* species and varieties is common due to frequent bud mutation, interspecific and intergeneric hybridization, apomixis and long history of cultivation (Scora, 1988). Tsegaye (2002) indicated that lack of knowledge about the genetic diversity of crop complicated the conservation, improvement and utilization by farmers, conservationists and breeders. Knowledge about clonal diversity allows the selection of clones prioritized for conservation, by removing duplication and optimizing genetic diversity and hence optimizing cost benefit ratio in maintaining the crop germplasm. Thus, determination of genetic diversity is the first step in proper utilization of plant resources (Graham et al., 1996). Due to existence of indigenous citrus genetic resources in Iran, it is necessary to characterize and analyze native citrus genotypes. 'Bakraee' (*Citrus* sp.) is one of the identified and collected citrus varieties which is being used extensively as rootstock and fresh fruit in southern regions of Iran. This genotype has vigorous, upright-compact canopy and rather thorn less. Leaves are medium, blunt-pointed, and light green. Flowers are large and white in color. Fruits are small-medium-sized, spherical and seedy. Rind is medium, somewhat leathery, surface nearly bumpy with prominent oil glands, easily separable and yellow to pale orange at development (Bigonah, 2011). As far as its use as rootstock is concerned, 'Bakraee' is a fast growing rootstock and induces high quality fruits in budded scion varieties. It has been observed to be tolerant to high pH in its natural habitat but it is susceptible to cold as well as foot and root rots (Fotouhi Ghazvini and Fattahi Moghadam, 2010). On the basis of morphological characteristics, it was believed that 'Bakraee' is a natural hybrid between mandarin (*Citrus reticulata* Blanco) and sweet lime (*Citrus limettioides* Tan.) (Fotouhi Ghazvini and Fattahi Moghadam, 2010).

Assessment of phylogenetic relationships using morphological markers alone has serious limitations, especially in species of a complex genus like citrus, whose taxonomy is otherwise in a chaotic state due to frequent incidences of hybridization, apomixis, polyploidy and bud mutations. Phylogenetic relationships and genetic diversity assessment in plants has now become far more simple, cost effective, reliable and reproducible, due to the advent of PCR-based DNA marker techniques such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence

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repeats (SSR), and directed amplification of minisatellite DNA (DAMD) (Weising et al., 2005). DNA-based markers have gained popularity in assessment of genetic relationship among species and cultivars. Among these markers, simple sequence repeats (SSRs) or microsatellites are especially functional for characterization of germplasm collections because they are highly polymorphic and heterozygous conserved sequences which can be used as co-dominant markers (Zane et al., 2002; Barkley et al., 2006), but they have not been widely used in citrus and only in few studies have utilized (Gulsen and Roose, 2001; Corazza-Nunes et al., 2002; Pang et al., 2003; Barkley et al., 2006).

Another marker, cytoplasmic DNA which comprises the chloroplast and mitochondrial genomes, is an interesting subject of analysis for phylogenetic reconstruction at a variety of taxonomic levels and its study became widespread once the techniques of DNA analysis were generally available (Clegg and Zurawski, 1992). Chloroplast DNA (cpDNA) is highly conserved, nonrecombinant, uniparentally inherited and of small size, and consequently quite useful in plant phylogeny and evolution studies (Olmstead and Palmer, 1994). Citrus cpDNA is strictly maternally inherited (Moreira et al., 2002).

In citrus, molecular phylogeny at various taxonomic levels has been examined in several earlier studies through application of isozymes (Herrero et al., 1996), RAPD & PCR-RFLP (Federici et al., 1998; Jena et al., 2009), RAPD, SCAR & PCR-RFLP (Nicolosi et al., 2000), AFLP (Pang et al., 2007), SSR (Barkley et al., 2006), ISSR (Kumar et al., 2010) and sequence data analysis of non-coding chloroplast DNA (cpDNA) regions (Morton et al., 2003).

In the present study, an attempt has been made to assess the SSR and PCR-RFLP polymorphisms and genetic variability in ‘Bakraee’ and few other *Citrus* species.

2. Materials and methods

2.1. Plant materials

Nineteen *Citrus* accessions comprising ‘Bakraee’ and some commercially important varieties were collected in Jiroft Agricultural Research Center intended for SSR and PCR-RFLP analysis. Candidate samples included citron, pummelo and mandarin as true ancestral species; lemon, lime, orange, grapefruit, etc. as major citrus hybrids and local or native specimens of citrus as varieties which have long history of cultivation at the southern regions of Iran. List of the accessions is presented in Table 1.

2.2. DNA extraction

From each accession, four young leaves were taken and total genomic DNA was extracted according to Murray and Thompson (1980) with minor modifications. The leaves were ground to a fine powder in liquid nitrogen and resuspended in CTAB extraction buffer (1% CTAB, 100 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.7 M NaCl, 2% sarcosyl and 140 mM 2-mercaptoethanol). The supernatant was extracted with chloroform–isoamyl alcohol (24:1), precipitated in absolute ethanol and pellet resuspended in TE containing 10 mg/ml RNase. DNA concentration was measured spectrophotometrically (Nano Drop 1000) at 260 nm and DNA templates were diluted to 12.5 ng/μl.

2.3. SSR amplification

For DNA amplification, 18 SSR primers were initially screened and finally 17 primers that produced scorable polymorphic bands were used for further analyses (Table 2). The primer sequences were obtained from http://www.plantbiology.ucr.edu/documents/Wles_of_Roose/rooselink2.html and Novelli et al. (2006), and were synthesized by Cinnagen Co., Ltd. (Iran). DNA amplification was carried out in 10 μl reactions containing 20 ng of template DNA, 0.2 mM dNTPs, 5–10 pmol forward and reverse primers which forward primer was 5′-labeled with one of the three fluorophores (6FAM, NED or HEX), 1.0 μl of 10× PCR buffer (Cinnagen, Iran), 1.5 mM of Magnesium chloride and 1 unit of *Taq* polymerase (Cinnagen, Iran). The amplification was performed in a PTC-200 Thermocycler (MJ Research, MA, USA), with reaction conditions programmed as initial pre-denaturation at 95 °C for 5 min followed by 38 cycles of denaturation at 95 °C for 1 min, annealing at optimized temperature (45–63.5 °C) for 30 s, and extension at 72 °C for 1 min. A final 7 min extension at 72 °C followed the completion of 38 cycles. PCR products were detected on an ABI prism 3130xl Genetic Analyzer. To prepare samples, 3 μl of diluted PCR products were added to 6.875 μl of formamide and 0.125 μl of GenSize 500HD Rox. Analysis was performed using the GeneMapper version 3.7 software (Applied Biosystems).

2.4. PCR-RFLP analysis

Three pairs of primers for cpDNA (Arnold et al., 1991; Demesure et al., 1995) were used (Table 2), and synthesized by Cinnagen Co., Ltd. (Iran). The amplification reactions were carried out using

Table 1
Plant materials utilized for SSR and PCR-RFLP analysis.

No.	Plant code	Genotype name	Cultivar or common name
1	G01	*Citrus aurantium* L.	Sour orange
2	G02	*Citrus limettioides* Tan.	Sweet lime
3	G03	*Citrus* sp.	Local lime like of Jiroft (Jirofti lime)
4	G04	*Citrus reticulata* Blanco	Local mandarin of Jiroft (Jirofti mandarin)
5	G05	*Citrus sinensis* (L.) Osbeck	Local orange of Jiroft (Jirofti orange)
6	G06	*Citrus medica* L.	Citron
7	G07	*Citrus paradisi* Macf.	Duncan grapefruit
8	G08	*Citrus maxima* Merill	Pummelo
9	G09	*Citrus limon* (L.) Burm. f.	Lisbon lemon
10	G10	*Citrus sinensis* (L.) Osbeck	Washington navel orange
11	G11	*Citrus sinensis* (L.) Osbeck	Marss orange
12	G12	*Citrus* sp.	Bakraee
13	G13	*Citrus* sp.	Local lemon of Jiroft (Jirofti lemon)
14	G14	*Citrus reticulata* Blanco	Dancy mandarin
15	G15	*Citrus junos* Sieb ex Yan.	Yuzu
16	G16	*Citrus* sp.	Darabi
17	G17	*Citrus auranifolia* (Chrism.) Swingle	Mexican lime
18	G18	*Citrus jambhiri* Lush.	Rough lemon
19	G19	*Citrus volkameriana* Tan.	Volkamer lemon