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Evaluation of Genetic Diversity in Citrus Genotypes by IRAP Molecular Marker

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ABSTRACT: It is important to identify the genetic diversity and phylogenic relationships for achieving desirable citrus cultivars. In present investigation IRAP markers were used to determine genetic diversity among 29 citrus genotypes. From 5 used primers, 49 polymorphic bands were amplified. Primers IRAP-1 and IRAP-2 (with 15 and 7 amplified bands), produced maximum and minimum polymorphic bands, respectively. Similarity among samples was calculated using NTsys software and Jaccard coefficient. Rang of similarity was 0.34-0.90 based on polymorphic bands with average of 0.65. Cluster analysis has been done based on Jaccard's similarity matrix and the UPGMA method. Cluster analysis has divided citrus genotypes into five separate groups. According to the similarity matrix results, the lowest similarity (0.34) was belonged to Dansi mandarin and Pommelo and the highest similarity (0.90) was belonged to unknown natural types G74 and Siavaraz orange 3 (G6). In current research Pommelo and mandarin confirmed as true species of citrus in distinct cluster.

Keywords**:** citrus, Cluster analysis, genetic diversity, IRAP, similarity matrix

INTRODUCTION

Citrus is one of the most important fruit crops in the world. Citrus species are diploid (2 n=18) trees with hesperidium fruits, and seeds often with two or more nucellar embryos which are genetically identical to the seed parent. Nucellar embryony (a type of apomictic reproduction) has very important consequences regarding evolution, breeding and the culture of citrus fruit trees (Asins et al., 1999).

Citrus taxonomy and phylogeny, based on morphology and geography are very complicated, controversial and confusing (Jannati et al., 2009). This led to major controversy on systematics of species within the Citrus subgenus (Moore, 2001). Two dissimilar classifications schemes have been developed and adopted; the Swingle system that recognizes 16 species (Swingle and Reece, 1967) and the Tanaka taxonomy that superfluously splits and identifies 162 species in the genus (Tanaka, 1977). However, advanced studies based on biochemical and morphological traits, suggests that there are only three 'true' species, i.e. citron (C. medicaL.), mandarin (C. reticulataBlanco), and pummelo (C. maximaL. Osbeck). Other mentioned cultivated Citrus spp. theorized to be hybrids derived as apomictically perpetuated biotypes (Barrett and Rhodes, 1976; Scora, 1988). Therefore, use of molecular markers has more advantages than that of morphologically based phenotypic characterization, because molecular markers are generally unaffected by external impact (Uzun and Yesiloglu, 2012).

(Asins et al., 1999) investigated the presence of copia-like retrotranposons in citrus. They found that these elements were quite abundant throughout the citrus genome and very heterogeneous for the rt domain. Polymorphisms based on copia-like elements (RFLPs and IRAPs) have been found distinguishing groups of varieties within Citrus sinensis (Asins et al.,1999),Citrus clementine (Breto et al., 2001) and Citrus lemon (Bernet et al., 2003). Moreover, polymorphisms based on these elements are more abundant than those based on primers of random sequence or simple sequence repeats (Breto et al., 2001). (Wei ,2007) used IRAP and REMAP markers to estimate phylogenetic relationship among 24 Citru cultivars.

Therefore little is known about the genetic variability of the Iranian citrus. The objective of present study was to assess genetic diversity and relationship of some important citrus genotypes using IRAP marker.

MATERIALS AND METHODS

A total of 29 cultivars of citrus were collected from Iranian Citrus Research Institute, located at Tonekabon city, Mazandaran Province, Iran and used for morphological and molecular studies (Table 1).

Table1. Plant materials utilized for IRAP analysis

Plant code	Local name	Scientific name	Plant code	Local name	Scientific name
G1	Sour Orange	Citrus aurantium	G61	unknown Natural Type	Citrus sp.
G2	Mars Orange	Citrus sinensis	G63	unknown Natural Type	Citrus sp.
G3	Tomson Orange	Citrus sinensis	G65	unknown Natural Type	Citrus sp.
G4	Siavaraz orange 1	Citrus sinensis	G67	unknown Natural Type	Citrus sp.
G5	Siavaraz orange 2	Citrus sinensis	G70	unknown Natural Type	Citrus sp.
G6	Siavaraz orange 3	Citrus sinensis	G71	unknown Natural Type	Citrus sp.
G7	Siavaraz orange 4	Citrus sinensis	G72	unknown Natural Type	Citrus sp.
G8	Moallemkoh (Natural Type)	Citrus sp.	G73	unknown Natural Type	Citrus sp.
G9	Shelmohalleh(Natural Type)	Citrus sp.	G74	unknown Natural Type	Citrus sp.
G10	Atabaki Mandarin	Citrus reticulata	G76	unknown Natural Type	Citrus sp.
G11	Unshu Mandarin	Citrus unshiu	G78	unknown Natural Type	Citrus sp.
G12	Dansi Mandarin	Citrus reticulata	G79	unknown Natural Type	Citrus sp.
G13	Bami Mandarin	Citrus reticulata	G80	unknown Natural Type	Citrus sp.
G14	Mahali Mandarin	Citrus reticulata			
G15	Clemantin Mandarin	Citrus clementina			
G16	Pomelo	Citrus grandis			

DNA isolation

Total genomic DNA was isolated from fully expanded leaves using the CTAB (hexadecyltrimethylammonium-bromide) method (Murray and Thompson, 1980) with few modifications. The DNA concentration was determined spectrophotometrically (Nano Drop 1000) at 260 nm. The extracted DNA was diluted to 20 ngµL^{-1} and stored at -20°C for PCR amplification.

IRAP analysis

The IRAP analysis was performed according to the method developed by Biswas et al., (2011). The reactions were carried out in 20 µl volumes in a tube using six primers, (Sinagene, Iran). Each reaction tube contained 20 ng templates DNA, 2.5 mM MgCl2, 0.2 mM of dNTPs, and 2 µL of 1xTaq DNA polymerase buffer, 0.3 mM primer and 1 units of Taq DNA polymerase (Sinagene, Iran).

Amplification was performed in a DNA thermal cycler (Biorad Thermal Cycler MJ Research, Inc, USA), using the following conditions: 94ºC for 2 min; 35 cycles at 94ºC for 30 s, 59-60ºC for 30 s and 72ºC for 1 min; final extensions at 72ºC for 10 min. PCR products were resolved on 2% agarose gel in 1xTAE buffer. The DNA was stained with 0.5 mg/mL ethidium bromide, visualized and photographed under a UV transilluminator. Electrophoretic profile was visualized under UV radiation and photographed with a UV transilluminator. The sizes of DNA fragments were estimated by comparison with standard ladder (1kb; fermentase, Germany).

Statistical analysis

Presence or absence of each band was scored with one and zero for thirteen primers. Then Zero-one matrix was prepared. The total number of bands and polymorphic bands for each primer was calculated with using Total lab software and the percents of polymorphism were calculated using the formula (number of polymorphic bands / total bands). Polymorphism Information Content (PIC) was calculated for dominant markers that the allelic

relationship between their bands was unclear with the formula PIC=Σ [2fi (1-fi)]. Dice similarity matrix was obtained using the software NTSYS-pc 2/02 (Rulf, 1998) and UPGMA cluster analysis was performed. Cophenetic matrix was calculated to evaluate the adaptation of cluster analysis to the data. Similarity matrices were compared with the cophenetic matrix and cophenetic correlation coefficients were calculated (Peakal and Smouse, 2006).

The results of analyzing 29 genotypes of citrus using IRAP marker showed that among the total of 56 scored bands, 49 bands, equivalent to 88.88% were polymorphic. maximum numbers of the bands were belonged to the primers IRAP-1 and the minimum numbers of the bands were belonged to the primer IRAP-5 (Table). The PIC values for the 5 primers ranged from 0.18 to 0.27, with an average of 0.22 (Table 2). The maximum amount of PIC was belonged to primer IRAP-1 (0.27) and the minimum amount of PIC was belonged to primer IRAP-2 (0.18) (Table 2).

Table2. Statistical analysis and results of genetic diversity of 29 genotypes of citrus

Row	Primer Name	Primer Sequence	Total Band	Polymorphic Band	% polymorphism	PIC
1	IRAP-1(LCC)	TCCGATGGCCATGATTTACTC	15	15	100	0.27
2	IRAP-2(LCB)	GGACCTATTTGCCAATGCT	11	7	63.6	0.18
3	IRAP-3(SSCB)	GGCTTGGATCGCTTGGAGGC	11	9	81.81	0.26
4	IRAP-4(SSGB)	AGTACGTCATTGCCTGTCCG	10	10	100	0.21
5	IRAP-5(SSCC)	ATCTCCCATTTCCGACCACT	9	8	88.88	0.22
	Mean		11.2	9.8	86.85	0.22

In order to classify genotypes based on RAPD data, Dice, Jacquard and simple matching similarity coefficient were calculated. After comparing the correlation of the matrices of similarity, each matrix of similarity was used to draw clusters based on UPGMA algorithms, simple connection and complete connection. Cophenetic coefficient was calculated for every cluster. This coefficient shows the amount of similarity between similarity matrix and the cluster. Then the greater number in comparison between the coefficient matrix and cophenetic matrix indicating better fitting for the cluster and similarity matrix (Nei, 1972). Accordingly, jaccard similarity coefficient and UPGMA algorithms were chosen as the most compatible clustering algorithm and similarity coefficient (Table 3).

Table 3. Cophenetic coefficients obtained of algorithms with similarity coefficient

	UPGMA algorithm	Simple connection algorithm	Complete connection algorithm
Dice similarity coefficient	0.844	0.763	0.720
Jacquard similarity coefficient	0.857	0.847	0.740
Simple matching similarity coefficient	0.804	0.763	0.720

A similarity matrix was calculated using IRAP data according to Jaccard coefficient (Jaccard ,1908). Similarity dandrogram was constructed using the UPGMA cluster analysis (Figure 3). Cophenetic correlation between ultrametric similarities of tree and similarity matrix was found to be high ($r= 0.85$, $P < 0.01$), suggesting that the cluster analysis strongly represents the similarity matrix. The genotypes studied had similarity values ranging from 0.34 to 0.90.

The results of similarity matrix showed that the highest genetic similarity (0.90) was existed between the genotypes of unknown natural types G63 and Siavaraz orange 3 and the lowest genetic similarity (0.34) was observed between the genotypes of Pommelo and Dansi mandarin. An UPGMA dandrogram was generated by IRAP data and the similarity (0.65) for all genotype pairs was used as a the clusters cut off value (Fig. 1). From this dandrogram, 29 genotypes could be classified into five classes (A, B, C, D and E).

Considering the dandrogram (Fig. 3), cluster A, included sour orange (Citrus aurantium L.) (G1). The cluster B, the largest group, consisting genotypes of unknown natural types (G61, G63, G65, G67, G70, G71, G72, G73, G74, G76, G78, G79 and G80), siavaraz oranges (G4, G5, G6 and G7), Tamson orange (G3) and Mars orange (G2). Within this cluster, the genotypes unknown natural types G63 and siavaraz oranges3 (G6) showed 0.90 genetic similarity.

Moreover, a high level of genetic similarity (0.77) was reported within the sweet orange cultivar based on RAPD markers (Malik et al., 2012). However, Novelli et al. 2000 did not observed polymorphisms among cultivars of C. sinensis based on RAPD and microsatellites markers. Similarly, (Fang and Roose ,1997) also reported low genetic variation among cultivars of C. sinensis based on ISSR markers.

The cluster C with two subclusters, C1 including Clementin mandarin (G15) and unshiu mandarin (Citrus unshiu) (G11). C2 including Dansi mandarin (G12), local mandarin (G14), Bami mandarin (G13) and Atabaki mandarin (G10). Mandarins are one out of three citrus types that (Barrett and Rhodes ,1976) proposed it as true species. (Coletta Filho et al., 1998) reported very narrow genetic base of mandarin group using RAPD marker and suggested that the mandarin group as a single species C. reticulata.

Cluster D, included Shelmohalleh (Natural Type) (G9). Pommelo (G16) was in group E separately, that showed a little similarity in comparison with the other genotypes. Pummelo was reported as one of the three true citrus species by(Barrett and Rhodes ,1976) and most of subsequent studies were in agreement with this statement (Federici et al., 1998; Nicolosi et al., 2000; Barkley et al., 2006; Uzun et al., 2009).

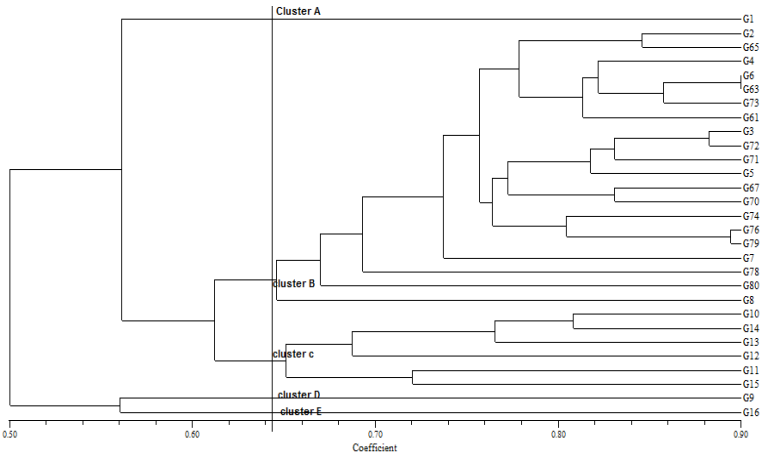

Fig 1. Dandrogram generated using UPGMA, showing relationships between 29 citrus genotypes, using IRAP data

The present study suggested that retrotransposon based fingerprinting methods are useful tool for rapid characterization of citrus and its related genera. This approach could be effciently employed for conservation and management of citrus germplasm genetic resource

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