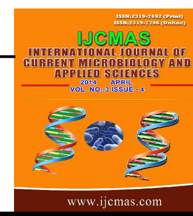

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Original Research Article

Genetic diversity analysis of sweet orange (*Citrus sinensis* osbeck) varieties/clones through RAPD markers

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A B S T R A C T

Random Amplified Polymorphic DNA (RAPD) markers were used to evaluate genetic similarity and inter relationship among twelve sweet orange varieties. Twenty random primers were screened, which produced 142 bands, of which 129 were polymorphic. The Jaccard co-efficient was used to calculate the genetic similarity indicated maximum genetic variation between Ankalamma Gudur Sathgudi and Nadimpalli Sathgudi (0.33) closely followed by Ankalamma Gudur Sathgudi and Jaffa (0.35). Jaffa and Kodur Sathgudi were genetically closer with value 0.84 followed by Himakuntla Sweet orange and Kodur Sathgudi (0.80). Unweighed pair-group method with arithmetic average (UPGMA) was used to generate the dendrogram indicated that Sathgudi Tirupati and Ankalamma Gudur Sathgudi formed one cluster and remaining varieties formed another cluster which in turn divided into two sub-clusters were Nadimpalli Sathgudi and Valentia formed first sub-cluster and Mosambi and Red blood Malta formed second sub-cluster; Jaffa and Kodur Sathgudi formed as one group and Ananthapur Sathgudi, Himakuntla Sweet orange, Valentia late and Hamlin Sweet orange did not resemble any other variety. In present study, characterization of Sweet orange varieties by RAPD has proved useful in separating all the varieties from each other. It has also provided us with primer markers that can be used to separate and distinguish each clone.

Keywords

Sweet orange; Diversity; RAPD-markers.

Introduction

Citrus is considered as one of the most important tropical fruit crop in India. There are several species of this fruit crop grown in more than 50 countries of the world and it is one of the choicest fruits having highest consumer's preference both as fresh fruit as well as for its refreshing processed juice. It plays a vital role in the fruit economy of the country next to mango and banana. However, Andhra Pradesh, Maharashtra, Karnataka, Punjab and Assam are the leading citrus growing states. Major citrus producing countries in the world are Argentina, Australia, Brazil,

China, Egypt, India, Israel, Japan, Mexico, Morocco, South Africa, Spain and United states.

India ranks sixth in the production of citrus fruit in the world. In Andhra Pradesh the important commercial Citrus fruits are Acid lime (Kagzi lime) and Sweet orange (Sathgudi). Sweet orange (*Citrus sinensis* Osbeck) var. Sathgudi is an important commercial cultivar in South India. Andhra Pradesh occupies first place in cultivation of Sathgudi Sweet orange locally called as "cheeni". The fruit is very delicious and has vitamin 'C'. Sweet orange are highly polyembryonic species, trees are medium-large with blunt-pointed leaf apex and narrowly-winged petiole. Fruits are subglobose to oval in shape, orange coloured, tight skinned with solid central core. The species is of great economic importance in world for its excellent quality. Quite a large number of cultivars, viz., Mosambi, Malta blood red, Sathgudi of India, Valencia, Pineapple , Washington Navel of the USA, Shamouti of Israel, Succari of Egypt, Dobla fina of Spain etc., are some of the commercially important cultivars.

Introduction of DNA markers based on polymerase chain reaction (PCR) technology has led to the development of several novel genetic assays that can be used for many purposes in plant genetic analyses such as cultivar identification, gene mapping and so on. 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 (Williams *et al.,* 1990). The RAPD assay has the advantages of being readily employed, requiring very small amounts of genomic DNA and eliminating the need for blotting and radioactive detection (Cipriant *et al.,* 1996). Further, it provides a fast and easy approach for taxonomic classification and cultivar typing of fruit trees.

This technique has already been used for estimation of genetic diversity and cultivar identification in many fruit crops. In citrus, RAPD technique has been used for establishment of a genetic linkage map for the virus resistance gene of *Citrus sunki* Hort. Ex. Tan. and *Poncirus trifoliate* (Cristofani *et al.,* 1999), identification of lemon mutants (Deng *et al.,* 1995), study of genetic diversity of Japanese acid lime (Abkenar and Isshiki, 2003); and study of finger printing of acid lime varieties having varied resistance to bacterial canker (Alpaa *et al*., 2010). In mandarin orange, Coletta Filho *et al.,* (1998) used RAPD for the analysis of the genetic diversity among different taxonomic groups. The Citrus RAPD markers have been used for genetic mapping (Cristofani *et al.,* 1999), identification of cultivars (Coletta Filho *et al.,* 1998), hybrids (Elisiaro *et al.,* 1999), mutants (Deng *et al.,* 1995), chimerase (Sugawara *et al.,* 2002) and phylogenetic analyses (Nicolosi *et al.,* 2000). Our research institute is working on improvement of sweet orange by cloned selection, in this, direction we collected some sweet orange clones showing good horticultural traits besides high fruit yield. However, the selection was made based on only morphological character of the tree including leaf and fruit characters. Some are look similar

barring in few characters. Therefore, the study using molecular markers was necessitated as there was no reported literature on sweet orange (*Citrus sinensis* Osbeck) varieties having tight skinned fruits. As the sweet orange is an important citrus species being grown in the state the present study was undertaken where we have applied RAPD marker technique to analyze genetic diversity among 12 sweet orange varieties collected from AICRP on Tropical Fruits (Citrus), Tirupati. The results obtained in the study are presented in the paper.

Materials and Methods

Plant material

Leaf material of 12 sweet orange varieties/clones viz., Tirupati Sathgudi, Kodur Sathgudi, Nadimpalli Sathgidi, Ananthapur Sathgudi, Ankalamma Gudur Sathgudi, Himakuntla Sweet orange, Mosambi, Jaffa, Valentia, Valentia late was collected from citrus germplasm block, AICRP on Tropical Fruits (Citrus), Tirupati and used in the study.

DNA Extraction

Genomic DNA was isolated from Leaf tissue of 12 sweet orange varieties (100mg each) following the procedure described by Murray and Thompson (1980) with slight modifications. The quality and quantity of DNA was determined by spectrophotometrically (Nano drop, Thermo) at 260nm. The final DNA concentration of each sample was adjusted to 25-50ng/µl.

DNA amplification and Gel electrophoresis

A total of 20 random primers obtained from Operon technology, USA were used for the polymorphism survey. Amplification reactions were carried out in volume of 25 µl, containing 25-50ng of genomic DNA, 10picomole of primer, 0.2mM of dNTPs, 2mM of $MgCl_2$, 1.5U of Hot Start *Taq* DNA polymerase (Fermentas). Amplifications were performed in Thermal Cycler (Corbett Research, Australia) programmed for an initial denaturation at 94ºC for 2 min, 45 cycles of 1min denaturation at 92 ºC, 1min annealing at 37^0C and 2 min extension at 72ºC, followed by final extension for 5 min at 72ºC. Amplification products were separated by electrophoresis in 1% agarose gels and stained in ethidum bromide. A photographic record was taken under UV illumination.

Scoring and Data Analysis:

The polymorphism was calculated based on the presence or absence of bands as '1' for present bands and '0' for absent ones. The '0' or '1' data matrix was created and used to calculate the genetic distance and similarity using SPSS 11.5 (SPSS Inc., Chicagao, Ill, USA). The dendrogram was constructed by using a distance matrix using the unweighted pair-group method with arithmetic average (UPGMA)

Results and Discussion

Genetic polymorphism among sweet orange varieties

The total number of marker observed among the sweet orange varieties based on the RAPD analysis with twenty primers was 142. The number of scorable bands produced per primer ranged from 2 to 12 and size of the products ranged from 300bp to 2.5kb. RAPD markers profile produced by the primers shown (Plate 1).
The maximum number of polymorphic

bands (12) obtained with OPM-12 and minimum number (2) was obtained with primers OPM-10 and OPA-04 (Table 1). The polymorphism ranged from 33.33% (primer OPA-04) to as high as 100% for 14 primers (OPA-03, OPA-07, OPA-09, OPA-10, OPA-11, OPA-12, OPA-17 and OPM-03, OPM-06, OPM-10, OPM-12, OPM-13, OPM-18, OPM-20). Average polymorphism across all the 12 sweet orange varieties was found to be 90.44%.

Genetic relationship between sweet orange varieties

Data of RAPD markers scanned from the 12 sweet orange varieties with 20 reproducible primers was used to generate similarity co-efficients. A maximum similarity value of 0.84 was observed between jaffa and kodur sathgudi varieties of sweet orange followed by Himakuntla Sweet orange and Kodur Sathgudi (0.80) where as Ankalamma Gudur Sathgudi and Nadimpalli Sathgudi were found to be genetically most diverse (0.33) (Table 2).

The cluster tree analysis (Fig. 1) showed that the sweet orange varieties were broadly divided into two main groups. The prominent outcome of this analysis is that the Tirupati Sathgudi and Ankalammagudur sathgudi formed one cluster and remaining varieties formed second cluster which in turn divided in to three sub-cluster where the first sub-cluster constituted of Nadimpalli Sathgudi and Valentia; the second sub-cluster had Mosambi and Red blood Malta; Jaffa and Kodur Sathgudi formed third sub-cluster while Ananthapur Sathgudi, Himakuntla Sweet orange, Valentia late and Hamlin sweet orange did not resemble any other variety/clone used in the study.

The present study disproved the study of, Natividade *et al.,* (2000) extracted genomic DNA from sweet orange *(Citrus sinensis* Osbeck) varieties from the germplasm collected from Centro de citricultura invio moreira, Insituto agronomico, Cordeiropolis, sp, Brazil representatives of each sweet orange group. They used RAPD to characterize and detect polymorphisms among the sweet orange varieties. In spite of phenotypic differences, no polymorphism was detected so they concluded that RAPD analysis is not applicable in differentiation of Sweet orange varieties.

Das *et al.,* (2004) estimated the genetic diversity of 25 phenotypically elite, nucellar clones of Mandarin orange *(Citrus reticulate* Blanco), selected from seven locations in three geographically separate states of the north eastern Himalayan region of India by using RAPD markers. They reported that 15 decamer primers generated 106 randomly amplified DNA fragments that were used as the genetic markers. The squatted Euclidean distance measurement showed 65% maximum and 11% minimum genetic dissimilarity between the plants selected. They are classified into two major clusters, having 15 and 10 plants. Each plant of one location was distributed with in the two major clusters. Further, this study confirms the existence of wide genetic diversity of Mandarin orange plants in the north eastern Himalayan region of India that can be used in Mandarin orange plant improvement programmes. Evaluation of genetic similarity among 35 Mandarin accessions, including l0 Species and 7 hybrids was done by Coletta Filho *et al.,* (1998). They used one ocamer and twenty two decamer primers which resulted in 109 total bands, 45 of which were polymorphic. Jaccard Coefficient was used to calculate genetic similarity, and

UPGMA to generate the dendrogram. The RAPDs obtained were sufficient to generate some accession-specific markers, using Tanakas or Webber's systematic units, these accessions were clustered into several groups. The genetic similarity within the Mandarin group was high (Gj=0.77), and this suggested that cultivated Mandarins had a narrow genetic base. The genetic similarity of Mandarins to other true citrus species (*Citron* [*C.medica L*] and Pummelo [*C. grandis* Osbeck]) was much lower (minimum Gj=0.27). Hence, they propose that the Mandarin group is a single species*, C. reticulata* Blanco composed of several genetically different individuals and a great number of hybrids, rather than a large number of species as proposed by some taxonomic studies.

Alpaa *et al.,* (2010) evaluated that genetic diversity using 20 random primers through RAPD technique among the 12 acid lime clones, Balaji, RHRL-124, RHRL-122 and PKM-1 were found to be moderately resistant to bacterial canker. Correlation of RAPD data with canker disease incidence in the moderately resistant acid lime clones viz., Balaji, RHRL-124 and PKM-1 were found as one cluster and all susceptible clones formed as a second cluster. Evaluation of genetic divergence among 94 hybrids and between hybrids and parental accessions of F1 hybrids i.e. Mandarin (*Citrus reticulata* cv. Cravo) and Sweet orange (*C. sinensis* cv. Pera) was studied by Oliveira *et al.,* (2002) using RAPD marker with 102 primers. High genetic similarity among Cravo and Pera and their hybrids was verified, showing a casual distribution from the hybrids in relation to the parents, but in intermediary positions. Principal component analysis showed little applicability in the study of hybrid genetic divergence. The hybrids and parents were classified, based on the genetic similarity, using the Tocher optimization method. Ratanadaros (1987) collected a total of 328 citrus accessions from 32 provinces during 1981-83 from Kamphaengsaen campis at Kasetsart University and reported wide genetic variation has been observed in pummelo (*C. grandis [C. maxima*]), Citron (*C. medica*)*,* Mandarin (*C. reticulata*), King mandarin (*C. sinensis*) and Lime (*C. aurantifolia*), all of which are believed to have originated in Thailand and neighbouring countries of southeast Asia.

In addition to RAPD marker, Novelli *et al.,* (2000) used microsettelite marker (AG- repeats) to evaluate 31 cultivars of sweet orange from an enrichment library of genomic DNA of sweet orange cv. Pera (*Citrus sinensis*). They reported that microsatellite or simple sequence repeats (SSRs) have been suggested as ideal for studies in cultures of vegetative propagation and as value markers for mapping in several species. Cristofani *et al.,* (2003) characterized and evaluated lemon germplasm by using microsatellites in many genetic studies because they are co-dominant and more polymorphic than other PCR based markers. They used 16 pairs of primers of microsatellite regions having AG/TC, GT/CA, TCA/AGT, TTAC/AATG repeats obtained from *Citrus sinensis* cv. Pera genomic DNA was evaluated in 20 accessions of lemon. From the 16 pairs of primers evaluated, 7 detected polymorphism in cultivars Eureka, Interdonato, Lunario, Messina, Monachello NC, Quatre saisons, Vangassay and Vicosa, when compared with Amalfitano sfuzato, Amber, Armstrong, Eureka Km 47, Femminello siracusa, Femminello santa Tereza 1,

Table.1 DNA bands amplified and polymorphism generated in 12 sweet orange varieties using 20 RAPD markers

S.No	RAPD Primer	Sequence (5'-3')	Total Bands	Polymorphic Bands	% of polymorphism
1	OPM-01	GTTGGTGGCT	5	4	80.00
2	OPM-03	GGGGGATGAG	9	9	100.00
3	OPM-04	GGCGGTTGTC	10	8	80.00
4	OPM-06	CTGGGCAACT	9	9	100.00
5	OPM-10	TCTGGCGCAC	2	2	100.00
6	OPM-12	GGGACGTTGG	12	12	100.00
7	OPM-13	GGTGGTCAAG	5	5	100.00
8	OPM-18	CACCATCCGT	5	5	100.00
9	OPM-20	AGGTCTTGGG	5	5	100.00
10	OPA-01	CAGGCCCTTC	9	8	88.89
11	OPA-02	TGCCGAGCTG	5	3	60.00
12	OPA-03	AGTCAGCCAC	7	7	100.00
13	OPA-04	AATCGGGCTG	6	2	33.33
14	OPA-05	AGGGGTCTTG	9	6	66.67
15	OPA-07	GAAACGGGTG	10	10	100.00
16	OPA-09	GGGTAACGCC	5	5	100.00
17	OPA-10	GTGATCGCAG	9	9	100.00
18	OPA-11	CAATCGCCGT	7	7	100.00
19	OPA-12	TCGGCGATAG	5	5	100.00
20	OPA-17	GACCGCTTGT	8	8	100.00
		Total	142	129	
		Average	7.10	6.45	90.44

Femminelo santa Tereza 2, Lisbon seedless, Monachello (OC), Nostralle, *S. continella* and Sicilian. The size of the amplified products ranged from 144 to 294 bp.

Variation within species can also be assayed using the molecular marker PCR-RAPD (Welsh and Mc Elelland 1990; Williams *et al.*, 1990) in which arbitrary short oligonucleotide primers, targeting unknown sequences in the *nemore*, are used to generate amplification products that often show size polymorphisms within species. RAPD analysis after the possibility of creating polymorphism without any prior knowledge of the DNA sequences of the organism investigated. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species if sufficient numbers of primers are screened. The method is economic for screening large number of samples. However, some researchers are critical of the poor reproducibility of RAPD pattern. The reproducibility within a laboratory has been reported to be assumely satisfactory (Tommerup *et al.*, 1995). However, inter laboratory comparison of RAPD patterns may not always be applicable since the RAPD patterns can be influenced by many technical factors (Penner *et al.*, 1993).

Table.2 Jaccard similarity coefficient of 12 sweet orange varieties based on polymorphism obtained with 20 random primers

Variety	Sathgudi Tirupati	Kodur Sathgudi	Nadimpalli Sathgudi	Ananthapur Sathgudi	Ankalamma gudur Sathgudi	Himakuntla sweet orange	Mosambi	Jaffa	Valentia	Valentia late	Red blood malta	Hamlin sweet orange
Tirupati Sathgudi	1.00											
Kodur Sathgudi	.508	1.00										
Nadimpalli Sathgudi	.456	.511	1.00									
Ananthapur Sathgudi	.490	.576	.536	1.00								
Ankalammagudur Sathgudi	.585	.353	.330	.356	1.00							
Himakuntla sweet orange	.474	.800	.551	.597	.351	1.00						
Mosambi	.569	.698	.537	.584	.404	.669	1.00					
Jaffa	.492	.849	.589	.597	.350	.752	.706	1.00				
Valentia	.418	.489	.670	.509	.286	.515	.536	.575	1.00			
Valentia late	.470	.725	.584	.592	.325	.711	.625	.720	.534	1.00		
Red blood malta	.543	.688	.516	.484	.410	.633	.707	.695	.481	.628	1.00	
Hamlin sweet orange	.491	.707	.567	.561	.369	.692	.571	.740	.530	.649	.623	1.00

Fig.1 Dendrogram generated using UPGMA method illustrating the genetic diversity relationships among 12 varieties of sweet orange.

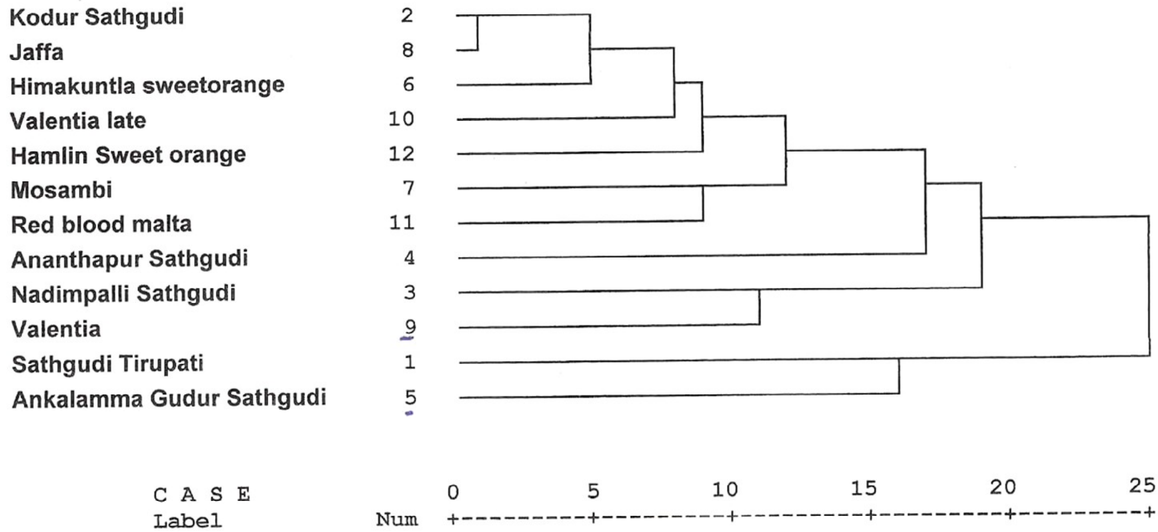
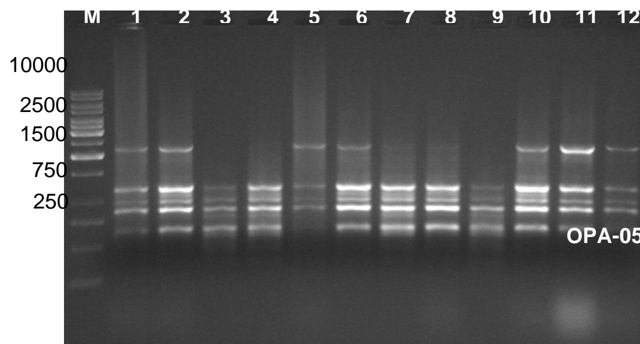
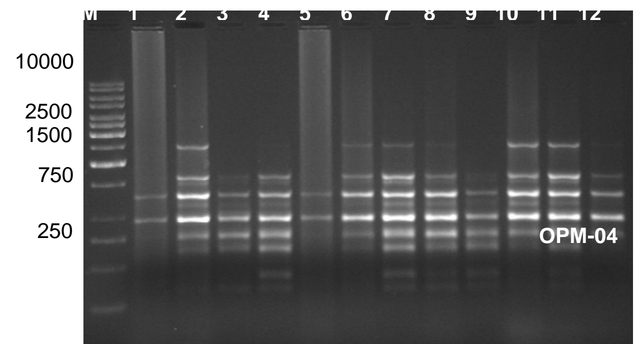


Plate.1 RAPD marker profies of 12 varieties of sweet orange generated by primers OPA-05 and OPM-04 in 1% agarose gel.





1. Sathgudi Tirupati
2. Kodur Sathgudi
3. Nadimpalli Sathgudi
4. Ananthapur Sathgudi
5. Ankalamma Gudur Sathgudi
6. Himakuntla Sweet orange
7. Mosambi
8. Jaffa
9. Valentia
10. Valentia late
11. Red blood Malta
12. Hamlin sweet orange

Out of 20 primers screened for amplification of DNA of sweet orange varieties. All primers gave reproducible and scorable bands with high percentage of polymorphism.

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, Duncan *et al.*, (1993) were also able to identify heterogenity with in groups of genotypes which originates in the same location. In present study, characterization of sweet orange varieties by 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.

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