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Research Article

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Genetic diversity and Molecular characterization of few citrus species in Visakhapatnam by RAPD markers

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Abstract

Random Amplified Polymorphic DNA (RAPD) markers were used to evaluate genetic similarity and interrelationship among 5 citrus species, including *C. aurantium, C. sinensis, C. lemon, C. aurantifolia* and *C. trifoliata*. Out of 20 decamer primers screened, 5 were selected which produced reproducible amplification; in that 54 bands were observed, of which 46 were polymorphic. The Jaccard coefficient was used to calculate the genetic similarity. UPGMA was used to generate the dendrogram which clearly separated every species distinguishably. The maximum similarity was observed to be seen between *C. aurantium* and *C. sinensis* of approximately, 0.605 and the least between *C. aurantifolia* and *C. lemon* of approximately 0.350. And same phenomenon was observed in distance matrix also.

Key words: Citrus, Random Amplified Polymorphic DNA, Genetic diversity, Similarity.

1. Introduction

Citrus is one of the most economically significant crop around the world with 122 million tones production according to citrus fruit statistics 2015 by Food and Agricultural Organization of the United nations (Citrus fruit statistics, 2015). Citrus plant is widely distributed around the world and believed to originated in tropical and subtropical regions and belongs to the family Rutaceae (Moore, 2001). India is the fifth largest country in production of citrus fruits around the world and Andhra Pradesh state is one of the major producer of citrus fruits in India with 38lakh tones production with 2,50, 000 hectares cultivation and there were an enormous diversity of Citrus genetic resources, both cultivated and wild (National Horticulture database., 2010). Sweet orange (*Citrus sinensis*) and acid lime (*Citrus aurantifolia*) are the major commercial citrus fruits and Lemons (*Citrus limon*), Pomeloes (*Citrus maxima*), and mandarins (*Citrus reticulata*) are cultivated in limited areas (CITRUS(Citrus spp)-drysrhu, 2017) in Andhra Pradesh.

Genetic variations and interrelationships among genotypes help in classifying, utilizing germplasm resources and in breeding. Molecular markers and qualitative trait associations can be identified with the determination of their genetic diversities, identification of species, cultivars and biotypes. This type of research can increase the both quantity and quality of the different economically and nutritionally important fruits' production. The origin of citrus has lead to different hypotheses. Citrus being polyembryonic, genetically heterogenous and the fact that it needs long generation time for the selection and recombination, the phylogeny and taxonomy often seems to be complex (Nicolosi *et al.,* 2000). So, the identification of genetic diversity in different citrus species is necessary to improve their production, resistance to different pathogens and diseases. Different molecular marker techniques like Random Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Regions (SCAR), Inter-simple sequence repeats (ISSR), Restriction Fragment Length Polymorphism (RFLP) etc., have been using for germplasm characterization, studies of genetic diversity, systematics and phylogenetic analysis (Weising *et al.,* 2005). These techniques were completely depends on distribution of markers, levels of polymorphism, type of markers employed in

genome (Virk *et al.,* 2001; Fernandez *et al.,* 2002). RAPD have been commonly employing technique for characterization of DNA including plant DNAs because of its low quantity of DNA requirement, simple procedure, economically low (Williams *et al.,* 1990; Abkenar and Isshiki, 2003; Maya *et al.,* 2012). In the present study, RAPD used as a molecular marker to identify the molecular similarities between *Citrus sinensis, Citrus aurantifolia, Citrus limon*, *Citrus trifoliata* and *Citrus aurantium*.

2. Materials and methods

2.1 Collection of plant materials

Five plant species (*C. aurantium, C. sinensis, C. lemon, C. aurantifolia* and *C. trifoliata*) of citrus were collected from Visakhapatnam region and identified with their morphological characters. Fresh and tender leaves were collected from these plants and allowed them for DNA extraction.

2.2 Deoxyribonucleic acid (DNA) extraction

The DNA of selected plants were extracted separately as per method Inga *et al.,* 2014 as follows. The sample fragments were homogenized in a mortar grind pestle. Then, 500µl is transferred into 2ml test tube, to this added 750µl of hexadecyl trimethyl ammonium bromide (CTAB) and 20µl mercaptoethanol. The tube was incubated at 65°C for 45min, and mixed intermittently by inverting. Subsequently, 750µl of chloroform/isoamylalchohol (24:1) was added and shaken at 100 rpm for 30 min. The tube will then centrifuged at 12000rpm for 10 min. The upper aqueous phase (containing the DNA) was transferred to a new 1.5ml tube and the step were again repeated, but centrifuged for 5min. The aqueous phase now have to be again transferred to a new 1.5 ml tube. Then, 3M NaAc (pH 4.6) and 2 vol. of 95% EtOH was used for DNA precipitation by incubating at -20°C for 1hr. The precipitate centrifuged again at 12000 x g for 10min, supernatant was discarded, DNA was remained at bottom of test tube as pellet and again 750µl of 70% EtOH was added and centrifuged for 5 min. Again, the ethanol will poured off, the tube centrifuged for a few seconds, and the remaining liquid was removed with a pipette. The tube will placed horizontally in the fume hood (with the cap open) for 30 min and finally added 100µl of EB buffer to the tube.

2.3 Random Amplified Polymorphic DNA analysis

After estimating the quantity of the DNA, the samples were adjusted to a concentration of 50 ng/µL. A total of 20 RAPD primers were selected and used for amplification (Table 1). The RAPD reactions were performed with the following concentrations: genomic DNA (50 ng), Buffer 1X, MgCl2 (2mM), dNTPs (0.1mM), primer (0.4 µM), Taq DNA polymerase (1 unit), and distilled water in a final volume of 25µl. Amplification was performed in a Biorad Mini thermal cycler with the following program: one cycle of 95ºC for 5 minutes, forty cycles of 95ºC for 30 seconds, 36ºC for 1min and 72ºC for 2min, followed by a final cycle of 5min at 72ºC. The amplification products were subjected to electrophoresis on a 2% agarose gel. The gel was stained with 5mg/ml ethidium bromide solution. The amplified fragments were visualized under UV light and photographed using a Biorad XR® digital photo documentation system.

Table 1. Primers used for amplification of candidate DNA barcodes.

Locus	Primer Name	Primer Sequence
	OPA-01	CAGGCCCTTC
	OPA-02	TGCCGAGCTG
	OPA-03	AGTCAGCCAC
	OPA-04	AATCGGGCTG
	OPA-05	AGGGGTCTTG
	OPA-06	GGTCCCTGAC
	OPA-07	GAAACGGGTG
	OPA-08	GTGACGTAGG
	OPA-09	GGGTAACGCC
	OPA-10	GTGATCGCAG
RAPD	OPA-10	GTGATCGCAG
	OPC-06	GAACGGACTC
	OPC-07	GTCCCGACGA
	OPC-08	TGGACCGGTG
	OPC-09	CTCACCGTCC
	OPC-10	TGTCTGGGTG
	OPE-01	CCCAAGGTCC
	OPE-02	GGTGCGGGAA
	OPE-03	CCAGATGCAC
	OPE-04	GTGACATGCC
	OPE-05	TCAGGGAGGT

2.4 Data analysis

RAPD Amplification profiles of 5 species were compared with each other and bands of DNA

fragments scored manually as (1) or (0) depending on the presence or absence of a particular band. The data was analyzed using DENDROUPGMA online software. This programmer was used to calculate Jaccard's coefficient.

3.0 Results and Discussion

A quantitative analysis of the mentioned citrus samples were done at an absorbance of 260nm, which gave the concentrations of the total genomic DNA as represented in the Table 2. The highest amount of genomic DNA was observed to be 951µg/ml in *C. sinensis* and the least in 58 µg/ml in *C. aurantium*. These genomic DNA samples that were analyzed when subjected through 2% agarose gel electrophoresis.

Table 2. Quantification of DNA sample of *C. sinensis, C. aurantifolia, C. limon*, *C. trifoliata* and *C. aurantium.*

Sample	Absor-bance at 260nm	Volume of Distilled water (µl)	Sample dilution (µl)	Concent-ration of DNA (µg/ml)
1	0.116	90	10	58
2	1.902	90	10	951
3	0.681	90	10	340.5
4	0.485	90	10	242.5
5	0.818	90	10	409

In Citrus, a wide variety of DNA based markers has been used in order to study their genetic variation as well as phylogenic and taxonomic relationship among different genera, and one of them is Random Amplified Polymorphic DNA(RAPD) analysis (Federici *et al.,* 1998). These molecular studies have provided some insight to Citrus phylogeny and the species concept was generally supported.

Among 20 RAPD primers used in this study, five primers shown consistent and repeatable amplification those are OPE-4, OPC-9, OPA-4, OPA-2, OPA-2 and OPA-9 (Table 3). The maximum number of amplified DNA bands (18) were observed in OPC-9. However, 100 percent polymorphism was observed in OPE-4 and OPA-9 primers. The minimum amount of polymorphism among five RAPD primers was observed in OPA-4 of about 62.5%. Some unique bands were also observed by these primers. Maximum number of 5 bands were shown, unique to OPA-2; and the least (1) that is unique to OPC-9.

Table 3. RAPD primers that has shown repeatable amplification, total number of bands, polymorphic bands and unique bands and percentage of polymorphism.

Primer	Total number of bands	Total number of polym-orphic bands	% of polym-orphism	Unique bands
OPE-04	12	12	100	3
OPC-09	18	16	88.8	1
OPA-04	8	5	62.5	3
OPA-02	12	9	75	5
OPA-09	4	4	100	2

There were similar experimental works using RAPD markers on distinguish and clustering genotypes in different citrus species. In the studies of Coletta Filho *et al.,* and Malik *et al.,* 250 amplifies bands were found for 25 RAPD primers, they were pretty consistent with 212 RAPD band generated using 23 primers among 25 mandarin accessions (Coletta Filho *et al.,* 1998) and 83% usual polymorphism was found among 18 citrus species (Malik *et al.,* 2012).

The studies of Aseel *et al.,* (2014) to observe genetic diversity of citrus cultivars in Iraq including some genotypes of present study based on RAPD markers indicates that produced 143 amplified RAPD bands were 100bp to 1.8kb in size from 16 citrus genotypes. OPX16 primer produced 2 bands, OPA04 and OPW-06 produced 13 bands, with an average of 7.15 bands per primer.

Similarity (Table 4) and Distance matrices (Table 5) were constructed using Unweighted Pair Group of Method (UPGMA) using Jaccard's coefficient. The maximum similarity was observed to be seen between *C. aurantium* and *C. sinensis* of approximately, 0.605 and the least between *C. aurantifolia* and *C. lemon* of approximately 0.350 and same phenomenon was observed in distance matrix also. The dendrogram (Figure 1) constructed using the 0-1 matrix revealed that, selected five

plants species were initially divided into two groups, *C. trifoliata* and *C. lemon* were in one group with same distance and *C. aurantium*, *C. sinensis* and *C. aurantifolia* in another group which further divided into two groups, where *C. aurantium* and *C. sinensis* in one clad and *C. aurantifolia* in single clad.

Aseel *et al.,* (2014) revealed that, the 16 Citrus genotypes showed that, all species were basically divided into Cluster-I which, consisted of citron, lime and lemon; Cluster-II which 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 stated by Uzun *et al.,* (2009) who separated 83 accessions of the Citrus genus into two large groups based on sequence related amplified polymorphism markers (SRAP). 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. Luro *et al.,* (2011) also segregated 87 citrus varieties based oon single strand conformation polymorphism (SSCP) into two main groups. The first group contained souroranges, mandarins, pummelo, sweet oranges, and grapefruits; and the second group included lemons, citrons, lemon hybrids and limes.

Table 4. Similarity matrix constructed with Jaccard's coefficient.

	C. aurantium	*C. sinensis*	*C. lemon*	*C. aurantifolia*	*C. trifoliata*
C. aurantium	1	0.605	0.432	0.475	0.475
C. sinensis		1	0.391	0.556	0.429
C. lemon			1	0.350	0.500
C. aurantifolia				1	0.463
C. trifoliata					1

Table 5. Distance matrix based on Jaccard's coefficient.

	C. aurantium	*C. sinensis*	*C. lemon*	*C. aurantifolia*	*C. trifoliata*
C. aurantium	0	0.395	0.568	0.525	0.525
C. sinensis		0	0.609	0.444	0.571
C. lemon			0	0.650	0.500
C. aurantifolia				0	0.537
C. trifoliata					0

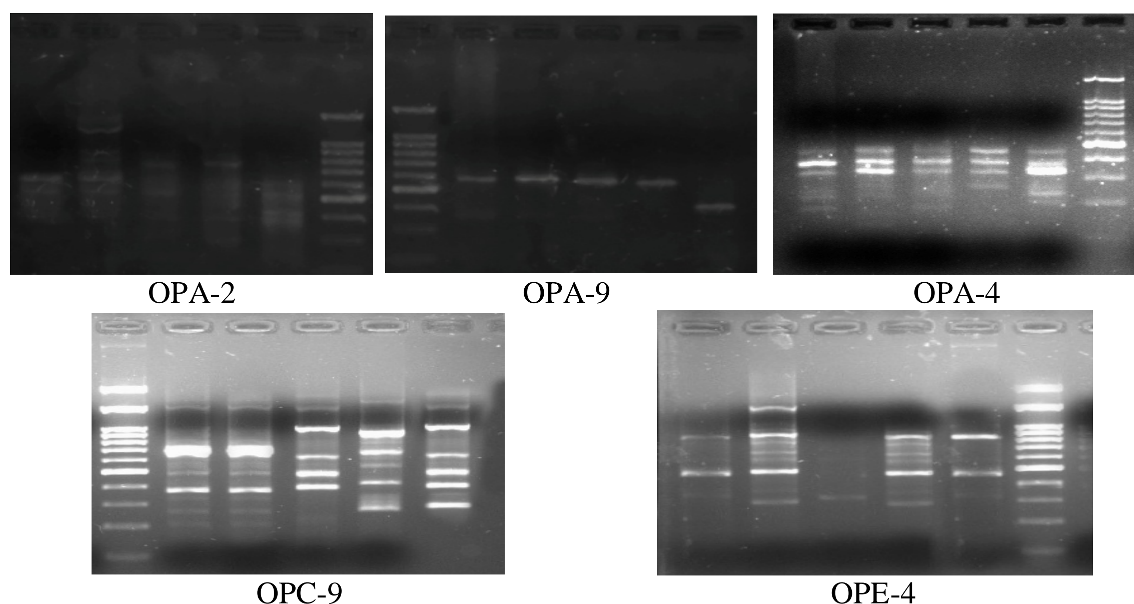

Figure 1. RAPD profiling with five primers for the selected citrus species.

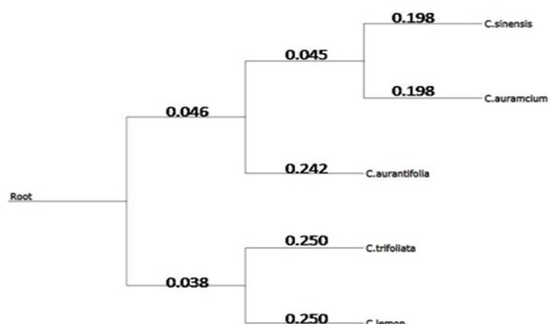

Figure 2. Dendrogram of citrus genotypes, generated by UPGMA clustering analysis.

In contrast to these results, Natividade *et al.,* 2000 and Novelli *et al.,* 2000 did not observe polymorphisms among the cultivars of *C. sinensis* based on RAPD and microsatellites markers. Further supports the view that a majority of *C. sinensis* cultivars derived from a single ancestor through somatic mutation (Hodgson, 1967). It was revealed that somatic mutations may be one of the sources of variability in Citrus species for the moderate level of polymorphisms, in spite of the high morphological variability. The present study results, proved to be useful for germplasm characterization and diversity analysis in Citrus cultivars and be able to manipulate genetic determinants of economically important traits to improve their productivity and resistance to pests in India.

Conflict of interest

We have none to declare.

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