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GENETIC DIVERSITY ANALYSIS OF ACID LIME (*CITRUS AURANTIFOLIA* SWINGLE) CULTIVARS

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ABSTRACT
Acid lime (*Citrus aurantifolia* Swingle) is an important commercial fruit crop, cultivated in our country. High variation of acid lime fruits are observed in existing varieties due to crossing within the other acid lime accessions. Determination of genetic variation is important to the plant breeders for development of high yielding variety. Therefore, an attempt has been made to study the genetic diversity of 6 acid lime varieties, maintained in our orchard. Ten Random amplified polymorphic DNA (RAPD) primers were used to assess the genetic diversity of acid lime. The average genetic similarity level among the 6 varieties was 60.5%, separated four major cluster groups. Total of 76 alleles of which 35 were polymorphic detected by ten primers RAPD markers. The Jaccard coefficient was used to calculate the genetic similarity. UPGMA was used to generate the dendrogram which clearly showed that the six acid lime varieties formed four clusters. Among the different clusters, the cluster size varied from 2 (cluster I and III) to 1 (cluster II, IV). The cluster I consisted of PKM -1 and Vikram. The cluster II consisted of Saisarbathi. The cluster III consisted of Pramalini and Tenali. The cluster IV consisted of Kasipentla. Among the cultivars, Pramalini and Tenali showed nearly 92% similarity followed by PKM-1 and Vikram which showed nearly 80% similarity. Kasipentala showed nearly 58% similarity, and Saisarbathi nearly 46% similarity. RAPD are highly polymorphic and more informative for the assessment of genetic diversity of acid lime varieties.

INTRODUCTION

Citrus in India is grown in 0.48 million ha area with a total production of 4.27 million tonnes. The most important commercial citrus groups or cultivars in India are the mandarin (*Citrus reticulata* Blanco) followed by sweet orange (*Citrus sinensis* Osbeck) and acid lime (*Citrus aurintifolia* Swingle) sharing 41, 23 and 23% of the respectively. India is the largest producer of acid lime in the world (Chadha, 2002). It is generally grown under both tropical and subtropical climatic conditions in the plains and up to 1200 MSL. In India, it is commercially cultivated in the states of Maharastra, Andra Pradesh, Karnataka, Tamil Nadu, Gujarat and Bihar. In Tamil Nadu, it is widely cultivated under rainfed and irrigated conditions in the districts of Dindigul, Trichy, Tirunelveli, Virudhunagar, Ramanathapuram, Madurai, Theni etc., in an area about 1,060 ha with a production of about 4,400 tonnes per annum (Anon., 2003).

Genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources and breeding. Without determining the diversity reliably, it would not be possible to identify molecular markers or qualitative trait associations. Moreover the viability and purity of rootstocks can be analyzed through the utilization of fingerprints based on molecular markers. This process can increase both quantity and quality of fruit production. Citrus is an economically important fruit crop, ranking almost as high as in world production and trade. The phylogeny and taxonomy of citrus fruit are complex, confusing and controversial due to the genetic heterogeneity of the genus, as well as its polyembryonic nature and the long generation time needed to carry out selection and recombination (Swingle, 1946; Nicolosi *et al*., 2000). Therefore, analysis of the genetic diversity of citrus fruit is crucial. To this end, DNA markers are being widely used in studying polymorphism between species or in populations. The application largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism and reproducibility of products (Virk *et al*., 2001; Fernandez *et al*., 2002). Techniques using Random Amplified Polymorphic DNA (RAPD) markers are simple, fast, and sensitive. They require no prior knowledge of the DNA sequence and can amplify a large number of DNA fragments for reaction. The introduction of DNA markers based on the 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 analysis such as cultivar identification and gene mapping. RAPD markers that result from the PCR amplification of genomic DNA fragments using short oligonucleotide (usually 10-mers) of arbitrary sequence as primers (William *et al*., 1990) provide a fast and easy approach for taxonomic classification and cultivar-typing of fruit trees. In citrus, RAPD markers have been used for genetic diversity analysis (Abkenar and Ishhiki, 2003; Mariniello *et al*., 2004; Campos *et al*., 2005; Novelli *et al*., 2006; Shaaban *et al*., 2006; Shahsavar *et al*., 2007; Hvarleva *et al*., 2008), chimeras (Sugawara *et al*., 2002) and phylogenetic analysis (Nicolosi *et al*., 2000). DNA fingerprinting using PCR-based markers is very important for breeding and taxonomy of citrus. No DNA-based markers approach to the study of citrus has been attempted even with a large area under cultivation in India. In this study we used RAPD markers to characterize the acid

lime genotypes. The objectives of the study were to achieve a better understanding of genetic variation and to investigate their inter-relationship. In India collection and conservation of citrus species/ types started long back in the mid of 19th century. During the past, collection and conservation were primarily made for the quality of fruits. The current research efforts are addressed to collection of gene pool with distinct desirable traits, which can be utilized for improvement of cultivars. The great genetic diversity is under serious threat of rapid extinction or depletion of the germplasm mainly due to population pressure and farmers preference (Singh. 2004).The present study was undertaken to evaluate certain important cultivars of acid lime (*Citrus aurantifolia* Swingle).

MATERIALS AND MATHODS

Plant material

A total of 6 acid lime genotypes used in this study were collected from the citrus orchard of Horticultural college and Research Institute, Periyakulam, Tamilnadu, India.

DNA isolation

Total genomic DNA was isolated from fully expanded leaves using the CTAB (hexadecyltrimethylammonium-bromide) method (Murray and Thompson, 1980) with few modifications. Briefly, 2g of leaves were ground in liquid nitrogen to a fine powder. The powder was added to 6 mL of extraction buffer (100 mM Tris–HCl pH 8.0, 20mM EDTA, 1.4 M NaCl, 2% (wv-1) CTAB, 2–mercaptoethanol 2% and 1% (wv-1) PVP and incubated at 65ºC for 30 min. The DNA was extracted with chloroform – octanol (24: 1). The DNA was washed with 70% ethanol and dissolved in 100-400μL of TE (10mM Tris–HCl pH 8.0, 1 mM EDTA and 0.2mg mL-1 RNase). The DNA concentration was determined spectrophotometrically at 260nm. Stock DNA samples were stored at –20ºC and diluted to 20ng uL-1 when in use.

PCR procedure

The RAPD primers were purchased from Operon Technologies Alameda, CA, USA. A total of 40 decamer oligonucleotides of arbitrary sequence were tested for PCR amplification. The basic protocol reported by William *et al*. (1990) for PCR was performed in a total volume of 12.5μL, containing 25ng of template DNA, 0.4 μM of single primer, 0.6 U Taq DNA polymerase (Bangalore Genei, India), 0.20μM of each dNTP, 1.5mM MgCl2, 10 mM Tris–HCl, and 50 mM KCl. DNA amplification was carried out in a PTC- 10096V Thermocycler (MJ Research, Inc, USA) and the thermal cycler conditions for PCR reactions were an initial denaturation cycle of 1 min and 30 s at 94ºC was followed by 45 cycles comprising 1 min at 94ºC, 1 min at 36ºC and 2 min at 72 ºC. An additional cycle of 7 min at 72ºC was used for final extension. Amplification products were separated by electrophoresis (8.3 V cm-1) in 1.5% agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination.

Data analysis

Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying species and cultivars were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using "Simqual" a subprogram of the NTSYS-PC program (numerical taxonomy and multivariate analysis system program) (Rohlf, 1993). The genetic associations between accessions were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the primers (Jaccard, 1908). The dendrogram was constructed by using a distance matrix using the unweighed pairgroup method with arithmetic average (UPGMA) sub-program of NTSYS-PC.

RAPD - PCR analysis

DNA from six acid lime cultivars was amplified using a set of 10 RAPD primer pairs OPA-02, OPG-02, OPG-05, OPO-12, OPA-07, OPB-05, OPA-09, OPH-15, OPA-17 and OPH-20. The RAPD primers which are located near to were selected in the present study. Those RAPD primers which amplified the present set of germplasm were used for diversity analysis after optimization of PCR conditions. Finally 10 primers which showed good amplification were selected.

RESULTS AND DISCUSSION

Cluster based on dendrogram

The similarity values obtained for each pair wise comparison of RAPD markers among the six acid lime cultivars were used to construct dendrogram based on Jaccard's coefficient and the results are presented (Fig. 1). Total of 76 alleles of which 35 were polymorphic detected by ten RAPD markers. The six acid lime cultivars formed 4 clusters at nearly 60.5% similarity levels. Among the different clusters, the cluster size varied from 2 (cluster I and III) to 1 (cluster II, IV). The cluster I consisted of PKM -1 and Vikram. The cluster II consisted of Saisarbathi. The cluster III consists of Pramalini and Tenali. The cluster IV consisted of Kasipentla. Among the cultivars, Pramalini and Tenali showed nearly 92% similarity followed by PKM-1 and Vikram which showed nearly 80% similarity. Kasipentala showed nearly 58% similarity, and Saisarbathi nearly 46% similarity. On drawing a vertical line in the dendrogram (Fig. 1) along the point corresponding to a distance of 0.92 all the six acid lime cultivars got divided into 4 clusters. All the cultivars of distance 0.32 came under single cluster. At a distance of 0.90 all the cultivars formed single stand six clusters. At a distance of 0.92 Tenali and Pramalini formed single cluster, Whereas at a distance of 0.82 single cluster was formed by PKM – 1 and Vikram. At a distance of 0. 58, Pramalini, Tenali and Kasipentla came under single cluster. At a distance of 0.46 PKM – 1, Vikram and Saisarbati were found to form single cluster. Among the six acid lime cultivars, Saisarbati had maximum genetic diversity.

Molecular characterization

Morphological traits have been extensively used to determine the relationship among plants and its varieties (Ortiz *et al*., 1998). However, morphological markers do not often reflect genetic relationships because of their interaction with the environment epistasis and the largely unknown genetic control of the traits (Smith and Smith, 1998). In contrast, molecular

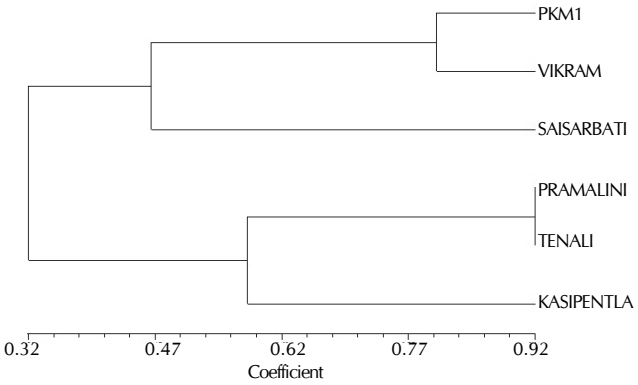
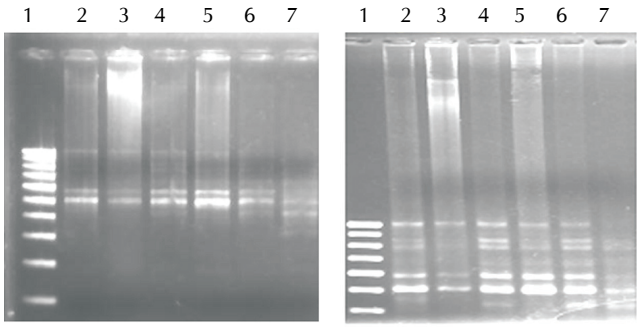


Figure 1: Dendrogram for Acid lime based on marker data



Lane 1 - Ladder (100bp)
Lane 2 Pkm1
Lane 3 - Saisarbathi
Lane 4 - Pramalini
Lane 5 - Vikram
Lane 6- Tenali
Lane 7 - Kasipentla

Lane 1 - Ladder (100bp)
Lane 2 Pkm1
Lane 3 - Saisarbathi
Lane 4 - Pramalini
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Lane 6- Tenali
Lane 7 - Kasipentla

Figure 2: RAPD marker analysis in acid lime cultivars

markers are not influenced by environment or developmental stage of a plant making them ideal for genetic relationship studies. Random amplified polymorphic DNA (RAPD) is one of the widely used molecular markers for identifying varieties at the genotypic level. It can help to overcome the complications arising in morpho – anatomical characterization. RAPD analysis has been successfully used to identify the generic diversity in a number of crop plants (Reiter *et al.,* 1992). In the present study an attempt has been made to determine the extent of genetic diversity in six acid lime cultivars, based on RAPD markers making use of arbitrary primers to amplify random DNA sequences in the genome.

In order to identify promising primer for identifying RAPD markers analysis 10 DECAMER for primers were screened. Only 10 primers out of the all primers yielded amplification products. Total number of bands ranged from 1 to 6 (Fig. 2). The primers OPA-02, OPA-07 and OPA-09 yielded polymorphic products and others showed no sequence complimentary to this primer in the DNA. In the present study a total number of 76 bands were generated with an average of 3 bands per primer with 35 were polymorphic and others were monomorphic. Finally, 3 primers viz., OPA-02, OPA-07, and OPA-09 for RAPD analysis were identified based on the number of polymorphic bands obtained. Five random primers were identifying in 7 local citrus accessions (Shaaban *et al.,* 2006). Studied on *Brassica sp* (Demeke *et al*., 1992) indicated that the minimum 17 primers were necessary to obtain a stable classification of related species. However, (Bhat and Jarret, 1995) suggested that the number of polymorphism might be more important than the number of primers for the generation of a stable phenogram. They also suggested that the number of polymorphism required to generate a stable phenotic analysis would vary with the plant material under investigation and the sequences that are amplified.

Ten promising primers used in the present study which yielded 35 polymorphic scorable bands with on average of 17.5 bands per primers. The amplification product ranged from 100 – 700 bp. The number of bands resolved for amplification was primers dependent and varied form 2 – 6. In a similar study carried for the assessment of genetic diversity in 57 *Musa* genotypes at NBPGR, New Delhi, Bhat and Jarret 1995, reported that 49 promising primers yielded 605 scorable bands with an average of 12.35 bands per primers in a similar study. High level of genetic diversity in acid lime varieties has been reported by previous studies and composed of different phenotype, genotype and large number of varieties. This variation allows identifying the different cultivars with molecular markers. Molecular marker may provide information on the history and biology of cultivars, but it does not necessary to reflect what may be observed in morphological traits (Metais *et al*., 2000).

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