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Application of RAPD technique for molecular marking of lime (*Citrus aurantifolia*) genotypes of West Bengal

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Abstract
Citrus aurantifolia is an important fruit crop of Eastern India. The region is one of the centre of origin of the crop and harbours excellent genotypes. For genotypic documentation of the resource 12 representative plant types of West Bengal were selected for molecular analysis using 28 decamer primers. Out of which 14 primers proved useful generating 64 amplicons of which 58 were polymorphic. Primer OPB 02 and OPAT 04 very effective with highest PIC. A Dendrogram was constructed using Squared Euclidean Distance using Ward's method which reflected no apparent relation between genetic diversity and geographical distance. Some distant genotypes grouped together while genotypes from a single location fall on different clusters. 9 primers namely OPAT04, OPB10, OPAA10, OPAD10, OPM05, OPB02, OPH11, P140 and P141 were useful generating unique amplicons marking specific genotypes. The study revealed the occurrence of high level of genetic diversity and proved the efficiency of RAPD markers for exploring the same.

Keywords: *Citrus aurantifolia,* RAPD, Polymorphic, Squared Euclidean Distance, PIC, Dendrogram,

1. Introduction
Citrus is cultivated since ancient times and South East Asia is regarded as centre of origin of this crop. The Eastern India harbours a large number of lime (*Citrus aurantifolia*) genotypes that are sufficient to meet the local demand with an excess to export. The Citrus orchards are neglected and no organised attempt was made for documentation and marking of the germplasms in Eastern India. When compared to rice and pulses the fruit crops usually get a back seat in research. The fruit crop does not need much care and grows naturally as Eastern India is regarded as one of the centre of origin. The tree mainly grows in homestead gardens luxuriously and produces a high amount of fruits.

Lime (*Citrus aurantifolia*) also known as "Kagzi lebu" in Bengal [1] is rich in vitamin C and contains antioxidant with high medicinal, pharmaceutical and cosmetic values. This fruit crop also contains minerals like calcium and iron. The medicinal value of lime could be exploited for alleviation of malnutrition [2]. It works against asthma and also as antidepressant, stress relief, colds, flue, fever, nosebleeds, mouth ulcers, throat infections and boils [3].

The reproduction of Citrus is very interesting but complicated. The peculiar embryo development varies from species to species. Though the *Citrus sinensis w*hole genome sequencing has been done but this genus shows huge variation within genus and reveals unique plant type characters within species. The assessment of genetic variation and genetic relationship is important for proper utilization of germplasm resources for breeding. The genetic diversity could be utilised for analysis of quantitative trait associations. A proper fingerprinting of each unique plant type is essential to maintain the purity of a selection and also important to reduce duplication of genotypes.

The academic institutions and Agriculture Departments of India/maintain some ex-situ collections. For establishment and extension of that resources new germplasm inclusion from different region becomes impeccable. Citrus can be brought in two forms, bud wood or seed. In case of bud wood the imported specimen maintains the genetic homogeneity but increases the risk of introduction of exotic diseases. Citrus diseases are not seeds transmissible [4].

Some Citrus species produces both sexual (zygotic) and asexual (nucellar) embryos. In Citrus expansion and establishment programme this polyembryony trait could be wisely utilised by extracting the embryos and retaining those who ideally mimic the mother's molecular fingerprint [5, 6].

Molecular DNA markers were used for analysis of germplasm screening [7] cultivar identification [8] genetic diversity study [9, 10]. Till date RAPD, SSR, AFLP, SNP markers were

used for genotypic identification of different Citrus species. The molecular techniques are now crossing the boundary of diversity studies and abundantly utilised for reduction of redundancies of germplasm, gap filling of collections, correction of misleading accessions and to assess genetic drift of a population. In this paper an attempt is made to identify some decamer primers capable for genotypic identification and suitable for management of *Citrus aurantifolia* germplasms.

2. DNA Extraction & RAPD Analysis

Genomic DNA was extracted from the soft leaves of the genotypes using DNA CTAB Extraction procedure standardised by [11]. The quantity and amount of DNA were determined as described using uncut bacteriophage lambda DNA [12].

Amplification was achieved by the protocol outlined by [13] with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1.5 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10X Taq Polymerase buffer´(Bangalore Genei) , and 15 ng of decamer primers (Eurofins) in a total volume of 25 µL. The amplification was performed in a thermal-cycler (Gene Amp PCR System 9700, Applied BioSystems). Total reaction consisted of 45 cycles, each cycle comprising three steps (denaturation at 92 °C for 30 seconds; annealing at 38°C for 30 seconds; extension at 72 °C for 1 minute), with an initial denaturation at 94 °C for 30 seconds and a final extension at 72 °C for 5 minutes, followed by cooling at 4 °C. Amplification fragments were separated on 2% agarose (Merck-Genei) gels containing ethidium bromide (0.5 µg per mL of agarose) at 60 V for 6 hours in Tris Borate EDTA buffer. The gel was visualized and photographed under UV excitation using an electronic dual wave transilluminator system (Ultra.Lum Inc., USA).

RAPD analysis was carried with 28 Operon decamer primers selected by preliminary screening to give polymorphism and reproducible fragment patterns in the species using plant genomic DNA of lime genotypes and analysis of polyembryony in *Citrus reticulata*. Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra.Lum Inc., USA). The size of the fragments (molecular weight in base pairs) was estimated by using a 100-bp ladder marker (Bangalore Genei), which was run along with the amplified products. The primers that could generate differential banding patterns of the selected genotypes were noted and the profiles were compared.

3. Result & Discussion

All the lime genotypes were collected from different orchards of Nadia district. The plants were developed by air-layering and high yielding with three flushes each year. The plants are disease resistant and produce fruits with enough juice. Variation in plant type characters and physico-chemical characteristics were noticed (Table 1 & 2).

DNA isolated from *Citrus aurantifolia* leaves were used for primer screening. In total 28 decamer primers belong to the Operon series were used for RAPD analysis. After preliminary screening 14 primers yielding more than one band and strong, intense, unambiguous and reproducible DNA amplicons were selected. The list of the selected primers, their sequences, maximum number of fragments obtained and range of the size of the fragments with Polymorphic information content (PIC) were as shown in Table 3. The amplified fragments varied from 2 to 6, with average of 4.85 fragments per primer (Table 4). The size of the amplicons were in the range from 100 bp to 1000 bp. The *Citrus* genome was about 563 mbp [14] and fourteen (14) decamer primers generated sixty four (64) amplicons. Out of 64 amplicons 58 were polymorphic with a polymorphic percentage more than 90. The analysis based on 64 markers was expected to saturate the genome at a density of one marker for every 8.79 mbp, which appeared to be adequate to make meaningful statements about the diversity or relatedness among the different mandarin orange plant types [15]. Each set of PCR reaction accompanied positive and negative control. In every PCR reaction no DNA fragments were found in the negative control while similar banding patterns were found in the positive control indicating contamination-free PCR ingredients and the consistency of the protocol.

Out of the total primers 14 decamer primers proved very suitable in achieving differential banding pattern among genotypes. Primers producing single band or smeared bands were discarded from the experiment. Primer OPB02 with highest PIC value of 0.439 preceded by OPAT04 with PIC value of 0.413 were able to mark most genotypes. OPAT04, OPB10, OPAA10, OPAD10, OPM05, OPB02, OPH11and P140 showed 100% polymorphism.

This study shows resemblance with the result presented by [16] Kumar *et al*., 2013 with 6 acid lime varieties. They used RAPD technique for the analysis of the genetic diversity among six varieties of acid lime and obtained bands in the range of 2 – 6 averaging 3 bands per primer but in our study it is 4.85 much higher values. Rahman and Munsur (2009) [17] studied the genetic diversity of 40 lime accessions of Bangladesh using D2 and Principal component analysis and found wide spread variation among the segregates.

However, such effort on ecotypes of West Bengal region and other parts of India was not found in earlier reports. The results obtained in this investigation further showed the advantages of using RAPD markers to reveal genetic diversity analysis of this high value fruit crop. Out of the fourteen Operon primers 9 primers namely OPAT04, OPB10, OPAA10, OPAD10, OPM05, OPB02, OPH11, P140 and P141 produced 12 unique bands other than their normal amplifications as shown in Table 3. OPC-01 primer generated three unique bands identifying the uniqueness of plant type 2 (600 bp amplicon), 9 (450 bp amplicon) and 12 (100 bp amplicon). OPB10 generated three amplicons of 600 bp, 400 bp, 200 bp molecular weight and identified plant type 12. OPAL-04 amplified three unique amplicons of which one related to plant type 1 (850 bp) and other two related to plant type 12 (amplicon size 200 bp, 100bp respectively). OPAD-10 with a 700 bp amplicon characterized plant type 4. P-140 with a 200 bp fragment differentiated plant type 12 and with a1000 bp fragment identified plant 10.

3.1 Estimation of genetic distance among the selected plants

A Genetic Dissimilarity Matrix was calculated from the presence and absence of 64 RAPD bands obtained from 12 selected plant types by 14 primers according to Squared Euclidean Distance using Ward's. Method [18]. Ward's method means calculating the incremental sum of squares. *Half square Euclidean distance* is the only distance measure that can be used with this clustering method.In this case, the equation is

$$d^2(p,q) = (p_1 - q_1)^2 + (p_2 - q_2)^2 + \ldots + (p_i - q_i)^2 + \ldots + (p_n - q_n)^2.$$

Based on this matrix, the highest (68%) genetic dissimilarity was observed between the plants 10 and 3 both selected from Kalyani location of Nadia district, West Bengal. The lowest dissimilarity (0%) was observed between the plants 10 and 11 with 14 decamer primers, though plant type 10 had a source location Kalyani and 11 had a source location from Malda district. The apparent similarity of genotype 10 and 11collected from adjacent districts of Bengal assumes they may be duplicates. With 14 primers they showed exactly similar banding pattern. Genotype 10,11,5.1,9 and 12 plant types formed one cluster irrespective of their source of origin. Genetic diversity is generally associated with geographical diversity but the former is not necessarily directly related with geographical distribution. This indicated that the geographical and genetic distributions did not follow the same trend as found in our study and also reported by [19, 20].

Morphological traits have been extensively used to determine the relationship among plants and its varieties [21]. 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 [22].

In contrast, molecular 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. In the present study an attempt has been made to determine the extent of genetic diversity in twelve 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 related to genotypic uniqueness these 14 primers proved effective. The study indicated decamer RAPD primers are useful in screening of genotypes, analysis of cultivar affinity and to reduce duplication.

Table 1: General field characters of the 12 elite lime (*Citrus aurantifolia*) selections of West Bengal

Plant Accession no		**Lime 1**	**LIME 2**	**LIME 3**	**LIME 4**	**LIME 5**	**LIME 6**	**LIME 7**	**LIME 8**	**LIME 9**	**LIME 10**	**LIME 11**	**LIME 12**
Age of the plant(years)		6	6	6	6	6	6	6	6	6	6	6	6
Plant hight(ft)		7.5	12	14	15	16	5.5	9	7	5.5	10.5	5.5	11.5
Avg Plant width(cm)		340	315	445	440	385	475	460	415	310	380	375	290
Source of the plant		Lake hall	Lake hall	Kalyani	Ghoragacha	Ghoragacha	Ghoragacha	Ghoragacha	Lake hall	Malda	Kalyani	Kalyani	Malda
Plant type		Upright	Spreading	Drooping	Drooping	Open dome	Open dome	Open dome	Erect closed	Open dome	Open dome	Erect closed	Erect closed
Propagation method of the plant		Air layering	Air layering	Air layering	Air layering	Air layering	Air layering	Air layering	Air layering	Air layering	Air layering	Air layering	Air layering
No. of branches	Primary	2	2	2	2	2	2	2	2	3	2	2	2
	Secondary	6	8	7	13	6	8	7	13	12	5	7	5
Bearing type		Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year	Thrice in a year
Fruit colour		Yellowish green	Light green	Deep green	Yellowish green	Deep green	Light green	Deep green	Light green	Light green	Light green	Light green	Deep green
Fruit surface		Rough	Smooth	Medium	Smooth	Smooth	Rough	Medium	Medium	Medium	Medium	Medium	Rough

Table 2: Physico-chemical properties of 12 elite lime (*Citrus aurantifolia*) genotypes of West Bengal

Genotype	Fruits Wt	Juice Wt	Peel Wt	Seed Wt	Locule No	Total Seed No/Fruits	Fruit Diameter		Seed No/Locule
							HARIZONTAL	VERTICAL	
LIME1	34.433	10.495	14.62	0.413	9	6	10.9	15	0.666
LIME2	13.915	7.157	10.91	0.322	10	7	9.4	9.8	0.7
LIME3	38.983	12.348	17.892	0.717	9	13	11.6	14.9	1.444
LIME4	30.945	10.88	10.918	0.388	10	8	11.9	12.5	0.8
LIME5	32.049	10.199	10.795	0.547	11	14	12.4	12.8	1.272
LIME6	29.766	10.844	12.043	0.595	8	9	10.9	13.6	1.125
LIME7	36.424	12.417	12.595	0.011	9	4	12.9	11.1	0.444
LIME8	28.466	9.517	9.568	0.452	11	10	11.9	12.2	0.909
LIME9	23.539	4.743	9.107	0.615	12	8	10.3	11.4	0.666
LIME10	29.528	6.238	11.234	0.561	9	8	11.3	10.9	0.888
LIME11	27.459	9.249	9.294	0.429	12	11	10.5	11.6	0.916
LIME12	32.691	7.283	9.952	0.594	10	9	9.8	9.4	0.9
AVG	29.84983	9.280833	11.57733	0.470333	10	8.916667	11.15	12.1	0.894167
SD	6.489397	2.430314	2.522455	0.183849	1.279204	2.810963	1.050108	1.781725	0.278383
CV	21.74014	26.18637	21.78788	39.08917	12.79204	31.52482	9.418011	14.725	31.13319
SEM	2.052127	0.768533	0.79767	0.058138	0.40452	0.888905	0.332073	0.563431	0.088032

Table 3: Numbers and fragment, number of polymorphic fragments, Polymorphism percentage; Polymorphic Information Content obtained from 12 lime (*Citrus aurantifolia)* plant selection of West Bengal using 14 decamer random primers.

Rapd Primar	Total No Of Band	Polymorphic Band	Polymorphism %	Average Pic
OPAT04	5	5	100	0.413889
OPC 01	4	3	75	0.114583
OPB10	4	4	100	0.152778
OPAL04	5	4	80	0.180556
OPA18	2	1	50	0.243056
OPAA10	4	4	100	0.371528
OPAD10	6	6	100	0.30787
OPM05	5	5	100	0.35
OPA01	7	5	71.43	0.380556
OPB05	5	4	80	0.347222
OPB02	6	6	100	0.438889
OPH 11	3	3	100	0.342593
P 140	6	6	100	0.37963
P 141	2	2	100	0.361111

*Polymorphic information content

Table 4: Unique bands present in different plant types of *Citrus aurantifolia* as obtained by selected primers.

	Primer Name	Unique Band No	Genotype	Bandrange
RAPD	OPC O1	2	LIME 2	600BP
		3	LIME 9	450BP
		4	LIME12	100BP
	OPB 10	2	LIME12	600BP
		3		400BP
		4		200BP
	OPAL 04	1	LIME1	850BP
		4	LIME 12	200BP
		5		100BP
	OPAD 10	3	LIME4	700BP
	P 140	1	LIME10	1000BP
		5	LIME12	200BP

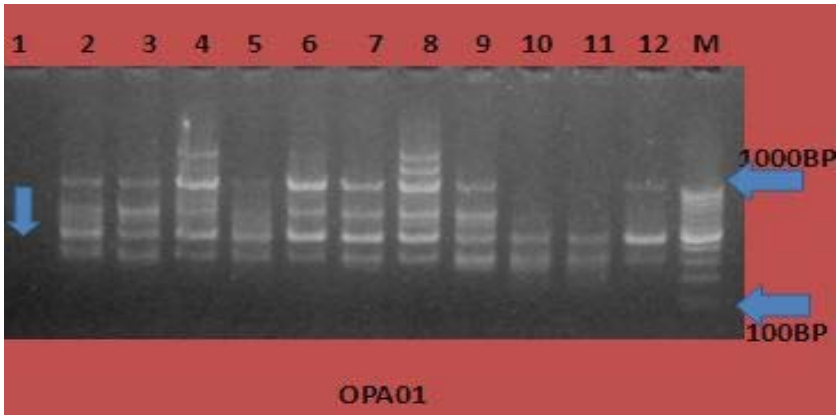

Fig 1: Showing the RAPD profile generated by decamer primer OPA01 with genomic DNA of twelve genotypes of *Citrus aurantifolia*

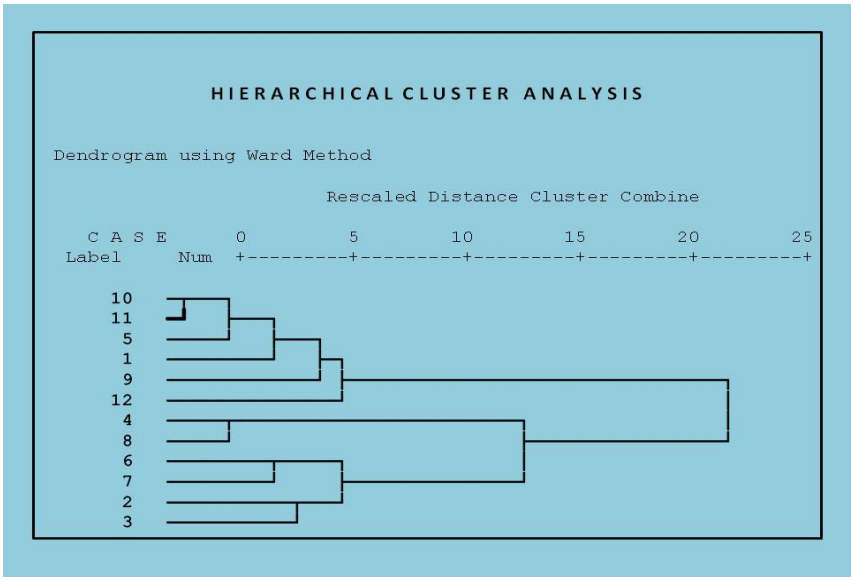

Fig 2: Dendrogram based on RAPD profile of 12 selected plants of *Citrus aurantifolia* of Mondouri Research Farm, BCKV, West Bengal

4. Conclusions

The result shows high level of genetic variation within the acid lime accession. The dendrogram reveals high level of genetic diversity with more than 90% polymorphism reclaiming the result presented by previous authors from different parts of world. Acid lime landraces are composed of different phenotype, genotype and large number of hybrid. The selected molecular markers provided sufficient information regarding the variation presented by this group of Citrus. It is a cross pollinated crops and large amount of genetic variation exists within the accessions, that can be used efficiently for gene tagging and genome mapping of favourable traits such as selection for larger fruit, high juice content, disease and insect resistance, abiotic stress tolerance etc. The result of this study provides valuable information to breeders for efficient development of new variety of acid lime, reduction of genotype duplication, orchard management etc. This is a first approach towards molecular-genotypic marking of lime genotypes in Eastern India.

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