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Analysis of Genetic Variation among Different Banana Cultivars

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***Abstract* - The banana *(Musa spp.)* is considered as an important crop plant due to its high economic value as good dietary source. Banana is the rich source of carbohydrates and vitamins, particularly vitamins B; it is also a good source of potassium, phosphorus, calcium and magnesium. Here, we analyzed the genetic relationship of four different banana varieties that are cultivated in south India. Random amplified polymorphic DNAs (RAPDs) fingerprinting of these banana varieties (Grand Naine, Nendran, Poovan and Red Banana) were carried out by five decamer primers (OP-5, OP-6, OP-7, OP-8,OP-11) led to DNA amplification. The distances in the dendrogram constructed by UPGMA (Unweighted pair group method Arithmetical Means) were compared with the genetic distances between genotypes pairs to calculate the cophenetic correlation. The genetic variation between Grand naine and Nendran was found to be 0.89 nucleotide. Similarly the Poovan is getting 0.94 nucleotide variation from the above one and Red is getting 0.97 nucleotide at the highest from all. The results presented here also contribute to narrowing the gaps in our current understanding of the migration path of bananas and the emergence of secondary centers of diversity. Cultivated bananas *(Musa spp.)* are mostly diploid or triploid cultivars with various combinations of the A and B genomes inherited from their diploid ancestors Musa acuminata Colla. and *Musa balbisiana* Colla. respectively.**

***Keywords: Musa Spp,* Random Amplified Polymorphic DNAs, Decamer Primers, UPGMA, *Musa Acuminata* Colla and *Musa balbisiana* Colla**

I. Introduction

The plant Banana and plantains *(Musa spp.),* the forth most important fruit crop in the world, are vegetatively propagated crops with great economic important in tropical and sub tropical countries. The crop encompasses a range of diploids, triploids and tetraploids. Cheesman (1948) first suggested that cultivated bananas originated from intra and interspecific hybridization between the two wild diploid species *Musa acuminata* Colla. and *Musa balbisiana* Colla., each contributing the A and B genomes respectively.

According to this system, cultivated dessert and East-African highland bananas are classified as AAA whilst plantains are AAB. There exist other genome combinations, for example ABB and ABBB. They occur naturally or are produced by artificial hybridization (Stover and Simmonds, 1987). The main genomic groups are AA, AAA, AAB and ABB, although AB, AAAB, AABB, and ABBB are also possible (Stover and Simmonds, 1987). Many pests and diseases have significantly affected banana production.

A phenomenon called somaclonal variation being a very serious problem in the banana tissue culture industry resulting in the production of undesirable plant off-type (Phillips *et al.,* 1994; Cullis 1992). Somaclonal variation at DNA level included gene methylation changes, DNA rearrangements and alterations in copy number (Brar *et al.,* 1998). The accumulation of recurrent somatic mutations followed by human selection for their tasty fruit led to great phenotypic diversity amongst plantain and cooking bananas in the region (De Langhe 1969).

Random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990; Pillay *et al.,* 2000;) is a technique which allows one to easily back taxon names with a molecular identification system in the form of a barcode inherent to the plant. RAPD based fingerprinting has been successfully applied to the characterization of diverse Musa germplasms (Onguso *et al.,* 2004), analysis of Musa breeding populations (Crouch *et al.,* 1999) and detection of somaclonal variants (Grajal-Martin *et al.,* 1998).

II. Review Of Literature

María Del Carmen Vidal., (2000) obtained Somaclonal variant CIEN BTA-03 resistant to yellow Sigatoka from a susceptible banana clone (Williams clone), by increasing the production of adventitious buds using 6-Belcilaminopurine at high concentrations. This somaclone has exhibited yellow Sigatoka resistance in the field for five consecutive years of asexual reproduction.

Roux, N.S (2001) analysed that the use of in vitro cultures for induction of mutations in Musa spp. could be the method of choice if several steps of the mutation induction process could be optimised. The following aspects were investigated: (a) the plant material used; (b) the determination of an optimal mutagenic dose for physical or chemical mutagens; (c) the dissociation of chimerism; and (d) the application of an early mass screening method for the selection of useful mutants with improved characters. The proposed new methodology, which will speed up the mutation-induction process and improve its efficiency, may revolutionize the use of mutations in Musa spp., especially at a time when there is an increased interest in collecting mutants to understand gene structure and function.

Hautea, D.M *et al.,* (2002) induced mutation in the two most popular Philippine banana cultivars by gamma and fast neutron irradiation of in vitro shoot-tip cultures. Promising clones were selected and evaluated further using molecular markers. The RAPD, SSR and AFLP techniques were successfully established under local conditions and found useful. RAPD and AFLP markers showed sufficient polymorphism for genotype discrimination, but SSR and AFLP markers are more highly reproducible.

Molina, C.M. *et al.,* (2006) carried out intensive research on the most important fungal banana pathogens, *Mycosphaerellafijiensis* and M. musicola, which are still major causes of loss in banana-growing regions worldwide. Their initial efforts focused on the development of polymorphic locus-specific SSR markers for *M. fijiensis and M. musicola.* This technique, along with other PCR-based DNA fingerprinting techniques such as DNA Amplification Fingerprinting (DAF) and Selective Amplification of Microsatellite Polymorphic Loci (SAMPL), allowed to carry out a comparative survey.

Venkatachalam Lakshmanan *et.al.,* (2007) developed a large number of micropropagated plantlets of banana, *Musa acuminata* var. Nanjanagudu Rasabale (NR), from axillary shoot bud explants over 10 years ago were screened for genetic variation, if any, using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeats) markers. Of the 4000 *in vitro* plantlets, 11 were used for screening that involved shoot cultures with distinct variation in morphological characteristics (morphotypes). Similarly, the mother maintained in the field was also subjected for genetic analysis.

Pankaj Kumar Jain *et al.,* (2007) analyze the genetic relationship of four different banana varieties that are cultivated in south India. Random amplified polymorphic DNAs (RAPDs) fingerprinting of these banana varieties (Grand Naine, Red Banana, Nendran and Rasthali) carried out by three primers (OPA-19, OPB-18, OPD-16) led to DNA amplification. 43.47% of the amplification products were monomorphic (common to all the genotypes), whereas 30.43% were unique, but only 26.08% revealed the relationship between these genotypes.

III. Aim and Objective

The major emphasis of this study was to fingerprint asses the genetic diversity among the varieties of banana. This study involves:

1. To isolation good quality DNA from banana leaves and to estimate the genetic diversity and genetic relatedness among five varieties.
2. To develop protocol for the isolation of PCR amplification DNA from commercial cultivars of banana leaves.

All the laboratory investigations were made at the SPIC Agro Biotech Centre, Coimbatore.

IV. Materials & Methods

A. Isolation of Genomic DNA

DNA, the genetic material can be isolated from prokaryote and Eukaryotic cells by phenol chloroform extraction method gives fairly, in-fact pure DNA. The principle involves breakage of cell to release DNA and subsequent treatment with detergents and enzymes to degrade most of the contaminating proteins. The digest is deproteinized by successive: phenol chloroform: isoamyl alcohol extractions and the DNA is recovered by ethanol precipitation. The basic steps involved in this method are cell lysis followed by deproteinization and recovery of DNA.

1. Materials Required

Leaf samples, Eppendorf tubes, Lysis buffer, TE buffer, Phenol: Chloroform: Isoamyl alcohol, Ice cold ethanol.

2. Sample - Plant Material

The mother plant collected from SPIC Agro farm, Coimbatore, India.

3. Sample Preparation

The fresh and greenish leaves were collected from the plants, which are taken into the lab through brown covers. The leaf material was first washed with water to remove the dust particles. Then the leaves were wiped with Ethanol to remove the microorganism and air dried. The plant materials were taken in the air tightened plastic bags and stored at 4^0C.

4. Stock Solutions

Table I Concentration Of Stock Solutions

S.No.	Chemicals	Concentration
1	Tris Hcl (Ph-8)	1M
2	EDTA (Ph-8)	0.5M
3	Phenol:Chloroform:Isoamyl alcohol	25:24:1
4	Ethanol	70%
5	TE Buffer (Ph-8)	10mm Tris Hcl + 1mm EDTA

Table II Composition Of Homogenization Buffer

Preparation of Homogenization buffer: (10ml)	
Tris	0.12 gm
SDS	0.2 gm
EDTA	0.08 gm
Nacl	0.1 gm
Distilled water	10ml

Table III Composition of Lysis Buffer

Preparation of lysis buffer: (10ml)	
Tris	0.12 gm
Glucose	0.18 gm
Nacl	0.1 gm
EDTA	0.08 gm
Distilled water	10 ml

5. Procedure

1. 0.2g of leaves were taken and grinded it in mortar and pestle then 2ml of homogenized buffer was added to it and grinded the sample for 2min.
2. 2ml of lysis buffer was added to it and incubated the mixture in room temperature for 5min. The tubes were centrifuged at 8000 rpm for 10min.
3. 1ml of supernatant was transferred to a fresh centrifugation tube and added 500 µl of phenol, 480 µl of chloroform and 20 µl of iso amyl alcohol.
4. The tubes were gently mixed by inverting and centrifuged at 10000 rpm for 10min.
5. The aqueous layer was collected in separate Eppendorf tubes and 1ml of chill ethanol was added and then incubated at -4°C for 5 min. Then the tubes were centrifuged at 12000 rpm for 10min. The supernatant was discarded and the pellet (DNA) was dissolved in 100µl of TE buffer.

6. Materials Required

1 XTAE buffer, Agarose, Ethidium Bromide (10mg/ ml), Tracking dye.

7. Procedure

1. 1.5% Agarose was prepared by adding 1.5g of agarose to 100ml of 1 X TAE buffer. The mixture was warmed in a flask at 100°C in a water bath until the Agarose was dissolved. The Agarose solution was cooled until its temperature reached 50°C and 10 µl of Ethidium bromide was added to the Agarose.
2. The gel tray was tapped on the two sides. The comb was placed in to the notched end of the tray. The contents were poured is to the tray and allowed to set.
3. After solidification of gel, the comb was removed and the tape was also removed. The tray was placed on a raised platform within the apparatus in such a way that the sample wells were close to the negative black electrode.
4. 1 X TAE buffer was poured in to the gel tank such a way that the gel was fully immersed in the buffer.
5. 10 µl of sample DNA and 5µl of 4 X Gel loading buffer were mixed properly and it was loaded in Agarose gel. The power supply was turned on at 100volts
6. The gel was removed from platform and transferred to the UV transilluminator for visualization and analyzed using gel documentation.

8. Materials Required

Standard DNA, Unknown DNA sample, Saline citrate solution, Diphenyl amine reagent, UV spectrophotometer, Pipettes, Tissue paper.

9. Procedure

1. 50 - 250µl of standard DNA were taken in a series of tubes marked S1 to S5 and 10µl of sample DNA were taken in three separate test tubes. In all the tubes, the total volume was made up to 1000µl with distilled water.
2. 1000µl of distilled water was taken as blank and 5ml of diphenyl amine reagent was added in all the tubes.

3. The tubes were kept in a boiling water bath for 10min and the absorbance was read at 600nm. A standard graph was constructed by plotting OD along X – axis and quantity of DNA along Y – axis. Concentration of DNA was calculated.

4. The DNA was quantified by using UV spectrophotometer and we measured the OD.

B. Polymerase Chain Reaction

If a pair of oligunucleotide can be designed to be complementary to a target DNA molecule such that they can be extended by a DNA polymerase towards each other, then the region of the template bounded by the primers can be greatly amplified by carrying out cycles of denaturation, primer annealing and polymerization. The process is known as the polymerase chain reaction (PCR). PCR allows the production of more than 10million copies of a target DNA sequence from only a few molecules.

1. Materials Required

Sample (Template), PCR tubes, Pipettes, Sterile tips

2. Reaction Mixture

Forward primer, Reverse primer, dNTPs, Taq polymerase, 10 X assay buffer, Nuclease free water.

3. Procedure

The solution were gently vortexed and briefly centrifuged after thawing. All the contents were added in a thin walled PCR tube.

TABLE IV COMPOSITION OF REACTION MIXTURE

S. No.	Reagent	Quantity for 15 µl reaction mixture
1	Template DNA	3
2	Taq DNA polymerase	2
3	d NTP's Mix	3
4	Forward primer	1.5
5	Reverse primer	1.5
6	10 X assay buffer	1
7	Nuclease free water	0

The total volume was made up to 12 µl using sterile nuclease free water. The samples were vortexed and centrifuged briefly to collect all drops from walls of tube. The three step PCR was followed:

The denaturation temperature was set to 94°C for 1min, annealing 54°C for 1min 30 second and extension 72°C for 2min. The samples were placed in a thermal cycler and started the PCR. The steps were repeated for 35 cycles. The amplified expected PCR product was checked by Agarose gel electrophoresis.

C. Construction of Dendrogram Based on Statistical Analysis

The amplified bands were scored as 1 and 2 based on band presence and absence, respectively. Size of amplified bands were estimated using gel pro analyzer software. The binary data set was used to calculate the pairwise Dice similarity index (Dice, 1945) and to assemble the corresponding similarity matrix. The matrix obtained was used to generate a dendrogram using the UPGMA method (Unweighted pair group method Arithmetical Means). The distances in the dendrogram were compared with the genetic distances between genotypes pairs to calculate the cophenetic correlation. All the analyses were performed with the aid of the 2010 version of the Lab Image Bio Imaging platform – Pc computer program.

V. RESULTS AND DISCUSSION

A.Collection of Plant Samples

The different banana plants were collected from SPIC agriculture farm for isolation of genomic DNA and for the somaclonal variants.

Fig 1 Plant Samples

B. Isolation of Genomic DNA

The genetic material from the plant sources were isolated using phenol chloroform extraction method and confirmed by Agarose gel electrophoresis.

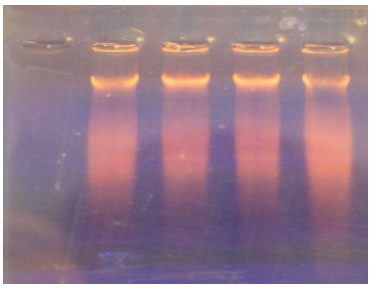

Fig. 2 Visualization of genomic DNA

Table V Results For Diphenyl Amine Method

S. No.	Plant Species	Quantity of DNA in mg
1	Grand Naine	0.32
2	Nendran	0.23
3	Poovan	0.27
4	Red	0.19

A set of 10 random primers were used for the amplification of DNA. PCR were performed according to the protocol of William *et al.,* 1990. All primers yielded maximum amplified product with all banana species. The primer amplified DNA products from each banana species generating reproducible band pattern. These band differentiations were analysed by loading the PCR product in 1.5% Agarose gel as shown in figure.

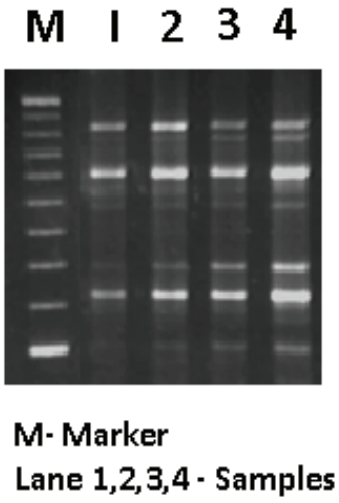

Fig. 3 Visualization of amplified DNA

Fig. 4 Marking of bands in amplified DNA

Table VI Bands Value For Lane 1

Lane 1	Distance (Pixel)	Rf	Area	Raw Volume	Band %
1	16	0.107	84	17941	8
2	21	0.141	63	16894	7.54
3	29	0.195	126	30247	13.49
4	43	0.289	105	24908	11.11
5	53	0.356	84	19812	8.84
6	66	0.443	126	28000	12.49
7	81	0.544	126	28615	12.77
8	91	0.611	105	23871	10.65
9	110	0.738	63	15041	6.71
10	138	0.926	126	18824	8.4

Table VII Bands Value For Lane 2

Lane 2	Distance (Pixel)	Rf	Area	Raw Volume	Band %
1	30	0.201	80	15620	15.22
2	51	0.342	64	7886	7.68
3	59	0.396	64	14897	14.51
4	66	0.443	32	9569	9.32
5	95	0.638	64	16415	15.99
6	110	0.738	96	18258	17.79
7	128	0.859	80	19998	19.48

Table VIII Bands Value For Lane 3

Lane 3	Distance (Pixel)	Rf	Area	Raw Volume	Band %
1	30	0.201	126	19842	15.08
2	52	0.349	90	9156	6.96
3	59	0.396	90	20213	15.36
4	66	0.443	108	25319	19.24
5	95	0.638	72	17259	13.12
6	110	0.738	90	13897	10.56
7	127	0.852	108	25900	19.68

Table IX Bands Value For Lane 4

Lane 4	Distance (Pixel)	Rf	Area	Raw Volume	Band %
1	27	0.181	34	8235	9.88
2	30	0.201	68	16642	19.96
3	52	0.349	85	11345	13.6
4	62	0.416	51	13473	16.16
5	91	0.611	51	11243	13.48
6	105	0.705	51	5806	6.96
7	129	0.866	68	16645	19.96

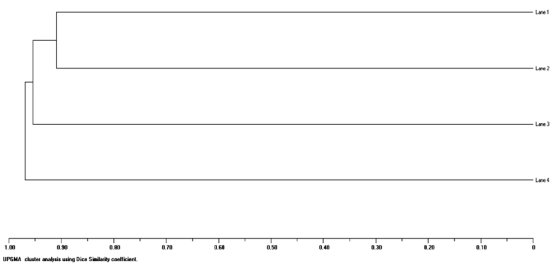

Fig. 5 Dendrogram constructed by UPGMA method

TABLE X BANDS VALUE FOR LANE 6

Lane 5	Distance (Pixel)	Rf	Area	Raw Volume	Band %
1	26	0.174	38	7483	7.68
2	30	0.201	95	19881	20.4
3	52	0.349	76	9373	9.62
4	67	0.45	133	28881	29.64
5	97	0.651	95	15801	16.21
6	105	0.705	76	6255	6.42
7	132	0.886	38	9779	10.03

VI. CONCLUSION

This study helps to detect studyseveral polymorphic bands. Few of the primers gave band pattern that were identical or had difference too small to provide information of genetic diversity. They could have distributed in the 4 banana species. From the band patterns the phylogenetic tree was constructed. The genetic variation between Grand naine and Nendran was found to be 0.89 nucleotide. Similarly the Poovan is getting 0.94 nucleotide variation from the above one and Red is getting 0.97 nucleotide at the highest from all.

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