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Research Article

MOLECULAR BIODIVERSITY STUDIES IN WILD AND CULTIVATED MEMBERS OF THE BANANA FAMILY, *MUSACEAE*

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ABSTRACT
Nineteen diverse genotypes from the family *Musaceae* including banana cultivars, seed bearing wild species, a γ-irradiation derived mutant and ensets were collected from different parts of India. These were subjected to DNA level genetic variability and classificatory analyses. Twenty four of the sixty RAPD primers (40 %) tested produced informative banding patterns. PCR amplification of total genomic DNA from 19 *Musaceae* genotypes yielded 487 bands, of which 461 were polymorphic. Thirteen primers exhibited 100 % polymorphism. The DNA band profiles revealed that the genotypes studied maintained substantial distances from each other and there were no repeat entries. The dendrogram constructed could be divided into five main clusters classifying *Musa* cultivars, seed bearing wild types and the genotypes with identical ploidy in separate groups. Both the *Ensete* species (*E. glaucum and E. superbum*) formed a unique cluster which unambiguously supported the known morphological classification of the *Musaceae* family in 2 genera viz; *Ensete* and *Musa*. The present studies could successfully recognize a set of RAPD primers useful in classification of diverse *Musaceae* family genotypes matching their known taxonomic status. The collected germplasm accessions can serve as valuable breeding materials for a banana genetic improvement programme employing conventional as well as latest bio-molecular techniques and technologies.

Key Words: *Musaceae, Banana, Plantain, Enset, RAPD, Polymorphism, Genetic Distance, Cluster Analysis*

INTRODUCTION

An economically important family, *Musaceae*, encompasses two genera viz; *Musa* (representing wild and cultivated bananas) and *Ensete* (representing the ensets) (Stover and Simmonds, 1987; Heslop-Harrison and Schwarzacher, 2007). Whereas bananas & plantains are well known as the third most important tropical fruit crop of the world (CIRAD, 2010), the ensets too are immensely valued species - especially for a drought prone country like Ethiopia. To quote, enset is presently the main crop of a sustainable indigenous African system, which ensures food security in a country that is severely food deficient (Negash, 2001; Birmeta *et al.*, 2004); and for Ethiopia, *E. ventricosum* is a staple food of almost 10 million people (Pijls *et al.*, 1995) which also was used to help ward off famine in Ethiopia (Mestel, 1994). In view of significant banana-biodiversity erosion having already taken place and the threat of extinction to the existing banana cultivars (Kulkarni *et al.*, 2006) and wild species, the need to collect, conserve and characterize the existing banana diversity (Kulkarni *et al.*, 2002) followed by their incorporation in crop improvement scheme becomes a priority; be those the cultivated/wild bananas or the ensets.

The requirement of accurately characterizing the genetic diversity can be fulfilled by employing either of the DNA marker techniques available today. Among various multi-locus DNA-fingerprinting techniques, the randomly amplified polymorphic DNA (RAPD) technique provides a cost and labor-effective means to rapidly and simultaneously assess the genetic variability across many loci (Call *et al*., 1998; Pillay *et al.*, 2001; Weising *et al.*, 2005; Senthil Kumar and Gurusubramanian, 2011). Due to these advantages, RAPDs have popularly been used to detect genetic variations within *Musaceae* family (Kaemmer *et al.*, 1992; Howell *et al.*, 1994; Bhat and Jarret, 1995; Damasco *et al.*, 1996; Kulkarni *et al.*, 1999; Pillay *et al.*, 2001; Birmeta *et al.*, 2002; Birmeta *et al.*, 2004; Ray *et al.*, 2006; Jain *et al.*, 2007; Venkatachalam *et al.*, 2007; Sheidai *et al.*, 2008; Brown *et al.*, 2009; Purohit *et al.* 2012).

A programme was initiated to collect, conserve and characterize the cultivated and wild banana germplasm useful for banana improvement work in the future. The findings of the experiments to assess the extent of

DNA level genetic diversity using RAPD markers, within a set of 19 cultivated and wild genotypes from *Musaceae* family are described herein.

MATERIALS AND METHODS

Plant Materials

The plant tissues from Bhabha Atomic Research Centre and Shivaji University's banana germplasm collection, (including seed bearing wild species, cultivars and a γ-irradiation derived mutant) maintained either as *in vitro* cultures or as seed germinated plants in earthen pots were used in the present studies.

Genomic DNA Extraction

The tender leaf tissue (from young and emerging leaf roles after discarding the fibrous mid-rib portion) of the 19 genotypes (Table 1) was harvested for the purpose of genomic DNA extraction. The collected tissues of banana genotypes were thoroughly washed with sterile distilled water, blot-dried and weighed. Genomic DNA was extracted by using the 'GenEluteTM Plant Genomic DNA Mini prep kit' (Sigma, USA). Quantification and purity estimation of the DNA was based on the spectrophotometric measurement as described by Sambrook *et al.* (1989).

Primer Selection and PCR Reaction

To generate genotyping profiles, sixty random decamer oligonucleotides belonging to the series OPA, OPAB and OPAG (Operon Technologies Ltd.) were used in this study (Table 2). PCR reaction was carried out in an Eppendorf DNA thermal master cycler. Each 25 µl reaction mixture contained 1X Taq DNA polymerase buffer with 15 mM $MgCl_2$, 0.75 U Taq DNA polymerase (BRIT, India), 100 µM of each dNTP (Genei, India), 0.4 µM RAPD primer and 50 ηg template DNA. The polymerase chain reactions were performed using following parameters: Initial denaturation at 94 ºC (4 min), 45 cycles of 94 ºC (30 sec), 36 ºC (1 min) and 72 ºC (2 min) with final extension at 72 ºC for 10 min.

Gel Electrophoresis

For analysis of the PCR amplified DNA, the products were visualized with ethidium bromide by electrophoresing on a 1.6 % agarose gel in 1X TAE buffer, (Sambrook *et al.*, 1989). Molecular weight markers of 100 bp and 1 kb ladder (Genei, India) were also loaded on each gel. The banding patterns viewing on UV transilluminator and digital image recording were done using a gel documentation system (Syngene, USA).

Data Analysis

A negative control (without DNA template) was included in all PCR reactions. For data analysis, the RAPD profiles were visually screened to identify both monomorphic and polymorphic bands. Each amplification product identifiable after electrophoresis was considered as a DNA marker and was scored across all samples. RAPD bands were scored by binary method as present (1) or absent (0) and blank as (9). Percent polymorphism was calculated as number of polymorphic bands / total number of bands x 100.

The dataset of all samples and reproducible bands were used to calculate pair-wise similarity coefficients by simple matching (SM) coefficient method using SIMQUAL programme. The matrix of similarity coefficient was subjected to unweighted pair group method with arithmetic mean (UPGMA) to generate a dendrogram using average linkage procedure. All the numerical analyses were performed using the computer program NTSYS-pc, version 2.1 (Exeter Software, New York).

RESULTS AND DISCUSSION

As a component of a research programme to collect, conserve and characterize the cultivated and wild *Musaceae* family germplasm, the DNA level genetic diversity amongst 19 cultivated and wild genotypes (Table 1) with differing ploidy and genomic status was evaluated using RAPD primers, and the results obtained are discussed ahead.

Primer Screening and RAPD Profiles

Sixty decamer random primers (series OPA, OPAB and OPAG, Table 2) were initially screened and tested for their capability to yield reproducible banding patterns with the template DNA. Figure 1 symbolically depicts the RAPD band profiles produced by the primer OPAB 20. Primers varied in their efficacy to yield reproducible banding patterns with template DNA.

Table 1: *Musaceae* family genotypes used in the study

Sr. No.	Name	Abbreviation	Type	Ploidy / Genome
Stoloniferous wild *Musa* species				
1	*Musa* species (Chandrapur)	MCH	Seed bearing wild	Diploid / NA
2	*Musa* species (Delhi University)	MDU	Seed bearing wild	Diploid / NA
3	*Musa* species (Udhampur)	UDM	Seed bearing wild	Diploid / NA
4	*Musa balbisiana*	MB	Seed bearing wild	Diploid / BB
5	*Musa ochracea*	MO	Seed bearing wild	Diploid / NA
6	*Musa rosacea*	MR	Seed bearing wild	Diploid / NA
7	*Musa velutina*	MV	Seed bearing wild	Diploid / NA
Stoloniferous cultivated *Musa* genotypes				
8	Bankel	BAN	Cultivated	Triploid / ABB
9	Basrai	BAS	Cultivated	Triploid / AAA
10	Basrai30Gy mutant	BAS30G	Cultivated mutant	Triploid / AAA
11	Binbondi	BIN	Cultivated	NA
12	Grand Naine	GN	Cultivated	Triploid / AAA
13	Matti	MAT	Cultivated	Diploid / AA
14	Mhasvad	MH	Cultivated	NA
15	Rajeli	RJ	Cultivated	Triploid / AAB
16	Safed Velchi	SV	Cultivated	Diploid / AB
17	Velchi	VEL	Cultivated	NA
Non-stoloniferous *Ensete* species				
18	*Ensete glaucum*	EG	Seed bearing wild	Diploid / NA
19	*Ensete superbum*	ES	Seed bearing wild	Diploid / NA

NA - No reliable information available

Table 2: List and sequences of the decamer-primers employed

Sr. No.	Primer	Sequence (5′ to 3′)	Sr. No.	Primer	Sequence (5′ to 3′)
1	OPA-01	CAGGCCCTTC	31	OPAB-11	GTGCGCAATG
2	OPA-02	TGCCGAGCTG	32	OPAB-12	CCTGTACCGA
3	OPA-03	AGTCAGCCAC	33	OPAB-13	CCTACCGTGG
4	OPA-04	AATCGGGCTG	34	OPAB-14	AAGTGCGACC
5	OPA-05	AGGGGTCTTG	35	OPAB-15	CCTCCTTCTC
6	OPA-06	GGTCCCTGAC	36	OPAB-16	CCCGGATGGT
7	OPA-07	GAAACGGGTG	37	OPAB-17	TCGCATCCAG
8	OPA-08	GTGACGTAGG	38	OPAB-18	CTGGCGTGTC
9	OPA-09	GGGTAACGCC	39	OPAB-19	ACACCGATGG
10	OPA-10	GTGATCGCAG	40	OPAB-20	CTTCTCGGAC
11	OPA-11	CAATCGCCGT	41	OPAG-01	CTACGGCTTC
12	OPA-12	TCGGCGATAG	42	OPAG-02	CTGAGGTCCT
13	OPA-13	CAGCACCCAC	43	OPAG-03	TGCGGGAGTG
14	OPA-14	TCTGTGCTGG	44	OPAG-04	GGAGCGTACT
15	OPA-15	TTCCGAACCC	45	OPAG-05	CCCACTAGAC
16	OPA-16	AGCCAGCGAA	46	OPAG-06	GGTGGCCAAG
17	OPA-17	GACCGCTTGT	47	OPAG-07	CACAGACCTG
18	OPA-18	AGGTGACCGT	48	OPAG-08	AAGAGCCCTC
19	OPA-19	CAAACGTCGG	49	OPAG-09	CCGAGGGGTT
20	OPA-20	GTTGCGATCC	50	OPAG-10	ACTGCCCGAC
21	OPAB-01	CCGTCGGTAG	51	OPAG-11	TTACGGTGGG
22	OPAB-02	GGAAACCCCT	52	OPAG-12	CTCCCAGGGT
23	OPAB-03	TGGCGCACAC	53	OPAG-13	GGCTTGGCGA
24	OPAB-04	GGCACGCGTT	54	OPAG-14	CTCTCGGCGA
25	OPAB-05	CCCGAAGCGA	55	OPAG-15	CCCACACGCA
26	OPAB-06	GTGGCTTGGA	56	OPAG-16	CCTGCGACAG
27	OPAB-07	GTAAACCGCC	57	OPAG-17	AGCGGAAGTG
28	OPAB-08	GTTACGGACC	58	OPAG-18	GTGGGCATAC
29	OPAB-09	GGGCGACTAC	59	OPAG-19	AGCCTCGGTT
30	OPAB-10	TTCCCTCCCA	60	OPAG-20	TGCGCTCCTC

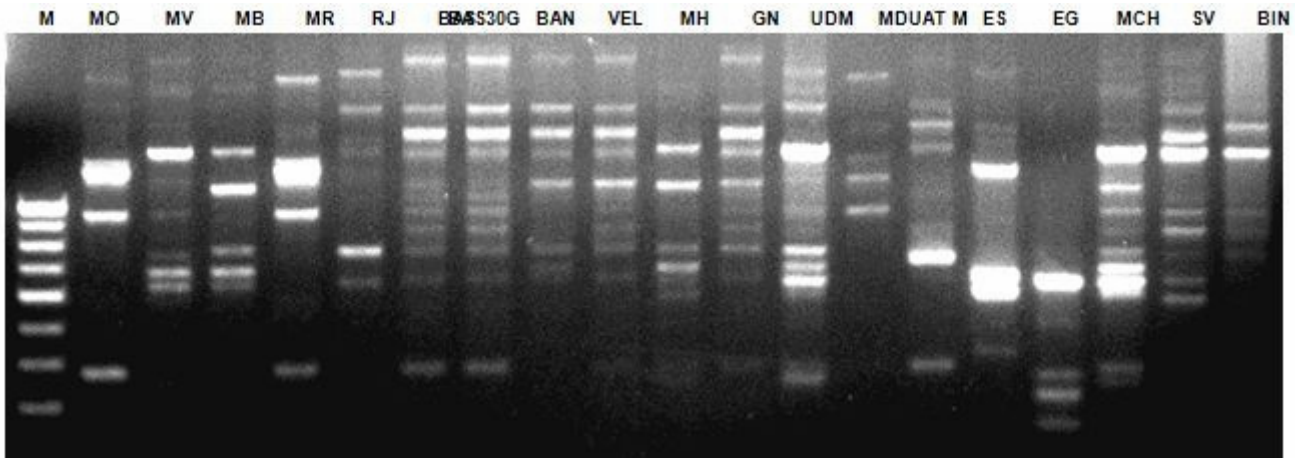

Figure 1: RAPD banding profiles of genomic DNA from different banana cultivars amplified using primer OPAB 20. Lane M represents 100 bp DNA ladder (Genei, India).

Table 3: List of the informative primers selected and degree of polymorphism obtained among 19 *Musaceae* family genotypes

Sr. No.	Primer	Primer sequence (5′-3′)	TNB*	NBP#	NMB$	Polymorphism (%)
1	OPA-04	AATCGGGCTG	31	31	0	100.0
2	OPA-06	GGTCCCTGAC	15	15	0	100.0
3	OPA-10	GTGATCGCAG	20	18	2	90.0
4	OPA-11	CAATCGCCGT	15	12	3	80.0
5	OPA-13	CAGCACCCAC	28	28	0	100.0
6	OPA-14	TCTGTGCTGG	14	12	2	85.7
7	OPA-16	AGCCAGCGAA	19	19	0	100.0
8	OPA-18	AGGTGACCGT	16	14	2	87.5
9	OPAB-02	GGAAACCCCT	19	18	1	94.7
10	OPAB-03	TGGCGCACAC	20	18	2	90.0
11	OPAB-04	GGCACGCGTT	15	8	7	53.3
12	OPAB-07	GTAAACCGCC	13	11	2	84.6
13	OPAB-08	GTTACGGACC	19	19	0	100.0
14	OPAB-12	CCTGTACCGA	24	24	0	100.0
15	OPAB-16	CCCGGATGGT	15	14	1	93.3
16	OPAB-18	CTGGCGTGTC	25	24	1	96.0
17	OPAB-20	CTTCTCGGAC	23	23	0	100.0
18	OPAG-03	TGCGGGAGTG	18	18	0	100.0
19	OPAG-04	GGAGCGTACT	20	20	0	100.0
20	OPAG-14	CTCTCGGCGA	20	20	0	100.0
21	OPAG-15	CCCACACGCA	31	31	0	100.0
22	OPAG-18	GTGGGCATAC	29	29	0	100.0
23	OPAG-19	AGCCTCGGTT	22	21	1	95.4
24	OPAG-20	TGCGCTCCTC	16	16	0	100.0

***** *TNB Total number of bands;*
[#] *NBP Number of polymorphic bands;*
[$] *NMB Number of monomorphic bands*

Twenty-four of the 60 tested primers gave reproducible PCR amplifications (Table 3) and these primers were subsequently utilized for genotyping of the 19 *Musaceae* accessions under investigation. The primers that resulted in indistinct or sub-optimal amplification products were excluded from further studies.

Table 4: A cumulative matrix of all pair-wise similarity indices among 19 *Musaceae* family genotypes

Genotypes	MO	MV	MB	MR	RJ	BAS	BAS 30G	BAN	VEL	MH	GN	UDM	MDU	MAT	ES	EG	MCH	SV	BIN
MO	1.00																		
MV	0.69	1.00																	
MB	0.70	0.86	1.00																
MR	0.92	0.68	0.69	1.00															
RJ	0.69	0.67	0.70	0.67	1.00														
BAS	0.69	0.64	0.65	0.67	0.81	1.00													
BAS30G	0.71	0.65	0.66	0.69	0.79	0.93	1.00												
BAN	0.67	0.66	0.67	0.66	0.80	0.90	0.88	1.00											
VEL	0.69	0.65	0.67	0.68	0.80	0.90	0.88	0.90	1.00										
MH	0.66	0.79	0.78	0.66	0.73	0.64	0.66	0.69	0.66	1.00									
GN	0.70	0.64	0.66	0.67	0.75	0.82	0.82	0.84	0.86	0.65	1.00								
UDM	0.65	0.77	0.75	0.65	0.65	0.61	0.62	0.63	0.61	0.76	0.63	1.00							
MDU	0.88	0.70	0.72	0.85	0.71	0.71	0.73	0.70	0.73	0.68	0.73	0.66	1.00						
MAT	0.74	0.68	0.68	0.74	0.76	0.74	0.76	0.73	0.77	0.69	0.75	0.66	0.78	1.00					
ES	0.63	0.61	0.61	0.62	0.60	0.58	0.60	0.54	0.57	0.57	0.59	0.59	0.63	0.61	1.00				
EG	0.66	0.63	0.65	0.62	0.63	0.58	0.60	0.57	0.61	0.63	0.63	0.57	0.65	0.65	0.70	1.00			
MCH	0.69	0.85	0.81	0.68	0.69	0.65	0.65	0.67	0.66	0.84	0.66	0.78	0.71	0.68	0.61	0.61	1.00		
SV	0.70	0.74	0.74	0.69	0.73	0.72	0.74	0.72	0.71	0.74	0.69	0.68	0.73	0.78	0.59	0.62	0.73	1.00	
BIN	0.71	0.75	0.74	0.70	0.74	0.72	0.73	0.71	0.71	0.75	0.67	0.68	0.72	0.77	0.60	0.62	0.75	0.93	1.00

Since the RAPD primers strongly depend on the availability of matching sequences in the genomic DNA, which is purely a matter of chance, not all the primers used can be expected to produce reliable banding patterns. In the present studies, 40 % RAPD primers were observed to generate reproducible PCR amplifications. This was in conformity with Kulkarni *et al.* (1999) who reported 45 % RAPD primers to be informative in 29 *Musa* genotypes. Birmeta *et al.* (2002) on the contrary reported only about 10 % of the primers to be informative, but while studying the diversity in a set of 111 *Ensete* accessions. Such differences could primarily be due to the contrasting genetic backgrounds of the genotypes used by different researchers.

PCR amplification of total genomic DNA from 19 *Musa* genotypes using 24 random decamer primers yielded 487 bands, out of which 461 were polymorphic. On an average each primer produced 19 bands. The RAPD profile revealed genetic polymorphism among the selected banana cultivars. The size range of the amplification products also differed with the selected primer sequence/genotype and ranged from 0.2 kb to 3.0 kb. The percent polymorphism of the informative primers (Figure 2) ranged between 53.3 % and 100 %.

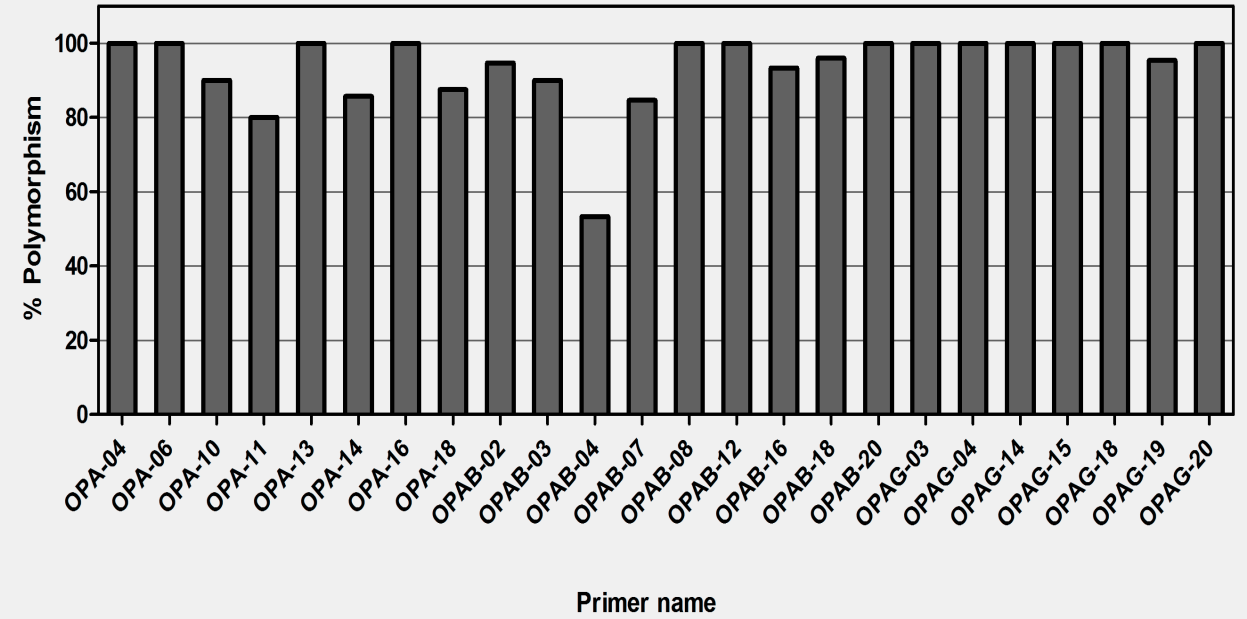

Figure 2: Percent polymorphism obtained using 24 informative RAPD primers

Among selected primers, OPAB-04 showed lowest polymorphism (53.3 %) among banana genotypes. The number of bands per amplification was primer dependent and varied from a minimum of 13 (OPAB-07) to a maximum of 31 (OPA-04 or OPAG-15). After the calculation of polymorphism percentage per primer, it was observed that thirteen primers of twenty-four exhibited 100 % polymorphisms. The average polymorphism detected in the banana cultivars was 93.8 %. Varying degrees of polymorphism have earlier been reported earlier (Kaemmer *et al.*, 1992; Howell *et al.*, 1994; Bhat and Jarret, 1995; Damasco *et al.*, 1996; Kulkarni *et al.*, 1999; Pillay *et al.*, 2001; Rekha *et al.*, 2001; Birmeta *et al.*, 2002; Thu *et al.*, 2002; Birmeta *et al.*, 2004; Jain *et al.*, 2007; Venkatachalam *et al.*, 2007; Purohit *et al.* 2012). The genotypes studied were observed to maintain substantial distances from each other and there were no repeat entries in the germplasm.

Phylogenetic Cluster Analysis

The binary data scored on the basis of presence or absence of bands was used for calculation of similarity coefficients and phlyogenetic cluster analysis (Table 4). The similarity coefficient values of cultivated and wild banana types ranged from 0.54 to 0.93. The similarity coefficient between ES (*Ensete superbum*) and

BAN (*Musa* spp.) was the lowest (0.54) mostly because both of them belong to altogether different genera. The highest magnitude of similarity index (0.93) between the cultivar BAS and its gamma-irradiation derived mutant BAS30G, and the dendrogram (Figure 3) showed that both these banana cultivars were similar to each other genetically, as compared to the rest. The earlier observation of Kulkarni *et al.* (1999) that the same two genotypes distinctly diverged from each other was possibly because they used a set of 29 genotypes within genus *Musa* which were comparatively closely related than the distantly related ones (across the genera) used in the present studies, and hence the increased magnitude of the distance separated BAS and BAS30G.

The dendrogram further indicated that the 19 genotypes studied could readily be grouped into five clusters labeled as I to V (Figure 3). The clusters I and IV contained all "stoloniferous seed bearing diploid wild *Musa species*", however, both these clusters also maintained different identities. The triploid *Musa* cultivars (RJ, BAS, BAS-30G, BAN, VEL and GN) grouped into cluster II, whereas cluster III included diploid *Musa* cultivars (MAT, SV and BIN) which again was different than cluster I containing diploid seed bearing wild banana.

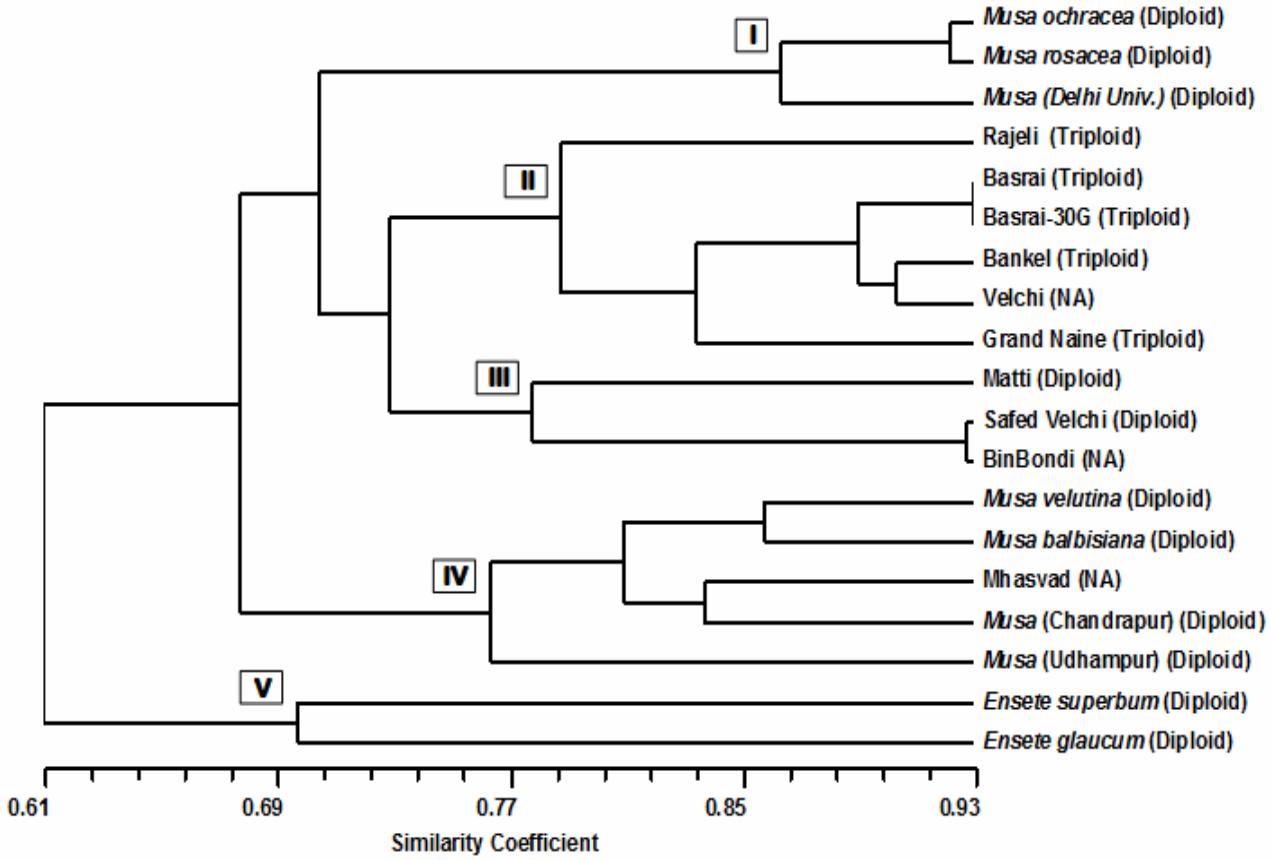

Figure 3: RAPD dendrogram showing similarity coefficients in 19 genotypes from *Musaceae* family

Although the ploidy and genome status in the case of VEL, BIN and MH are not known, based on their closeness with other members of their clusters, and also the fact that the RAPD primers employed could successfully group all the other genotypes based on their known characters (ploidy, genus and whether cultivated or wild), it can be inferred that VEL, BIN and MH must be similar to the other members of their clusters.

Both the ensets, *E. glaucum* and *E. superbum*, maintained substantial genetic distance from each other as was evident from similarity coefficient of the magnitude 0.61 between them. As expected, both these *Ensete* species also formed a divergent 'cluster V' and largely separated from other 4 clusters containing *Musa* accessions. The genus *Ensete* representing "non-stoloniferous seed bearing diploid wild types" is known to have quite different morphology than *Musa* (Cheesman, 1947; Simmonds, 1962; Westphal, 1975; Stover and Simmonds, 1987; Kulkarni *et al.*, 1997; Birmeta, 2004); and our results have once again confirmed that *Musa* (representing bananas and plantains) and *Enset* (representing ensets, the false bananas) are dissimilar genera.

To summarize, the present studies could successfully recognize a set of RAPD primers useful for detecting the genetic variability amongst and appropriately classifying a wide range of the genotypes belonging to *Musaceae* family. The wild counterparts have always been known for their broad genetic base and carry several desirable genes (Vuylsteke *et al.*, 1995). The accessions with distinct DNA profiles (such as the ones used in the present studies) are likely to contain the greatest number of novel alleles and could serve as valuable breeding materials for a banana genetic improvement programme (Novak *et al.*, 1990; Vuylsteke and Swennen, 1993; Arvanitoyannis *et al.*, 2008) employing conventional as well as latest bio-molecular techniques and technologies.

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