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Variation and intraspecific relationships in Indian wild *Musa balbisiana* (BB) population as evidenced by random amplified polymorphic DNA

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Abstract

Sixteen collections of the wild *Musa* species, *Musa balbisiana* Colla collected from different regions of India were studied for their intraspecific relationships using random amplified polymorphic DNA (RAPD) markers. Out of 80 primers screened, 34 primers produced reproducible bands and four primers among them showing polymorphic bands were used. In all, 43 DNA fragments were amplified averaging 10.75 per primer. Of these, 31 amplified fragments showed polymorphism (averaging of 7.75 per primer). The extent of polymorphism (74.6%) has indicated the existence of considerable variation at the DNA level within the species. The 16 accessions were clustered into four as against seven clusters obtained through morphotaxonomic characterization. The inter relationships based on geographical origin in comparison with molecular characterization have been discussed.

Introduction

Banana is a major fruit crop grown in more than 120 countries and forms the basis of staple food for millions in the developing economies. Banana (*Musa* spp.) belongs to the family *Musaceae* and order *Scitaminae* with two genera, *Musa* and *Ensete*.

Present day bananas have largely evolved from two wild species, *Musa acuminata* Colla and *M. balbisiana* Colla. Originally they were seedy, non-pulpy and non-edible growing in forest clearings. Evolution of edible bananas was initiated with the *M. acuminata* types which were found distributed mainly in the South East Asian countries. Human interventions and movement brought *M. acuminata* into Indian subcontinent where it introgressed with the hardy wild *M. balbisiana* endemic to North Eastern India. This resulted in the development of earliest bispecific types with *M. acuminata* contributing to A genome and *M. balbisiana* contributing to B genome. Differential combinations of these two wild species resulted in the development of a broad spectrum of genomic groups ranging from diploids to tetraploids (AA, AB, BB, AAA, AAB, ABB, AABB, ABBB etc.) Development of triploidy is considered as an important step in the process of evolution. Till recently, only *M. acuminata* and *M. balbisiana* were thought to be the progenitors of present day bananas, but identification of *M. schizocarpa* N.W. Simmonds and *M. textilis* Née represented by S and T genomes in the cultivated and wild types, has added a new dimension to the evolution theory (Carreel 1994).

India is recognized as one of the major centres of *Musa* origin and diversity at the global level along with South East Asian countries and Papua New Guinea. Genus *Musa* has more diversity for *acuminata* in South East Asian countries including Thailand, Malaysia, Indonesia etc. while India has more diversity for *balbisiana* species along with Philippines (Simmonds 1959, 1962). The diversity is spread across North Eastern states, Western Ghats, Eastern Ghats and Andaman and Nicobar Islands (Singh and Uma 2000).

Recently, molecular marker techniques, such as RFLP, RAPD, and AFLP are being used for clonal identification, linkage mapping, population diversity studies, taxonomy and evolutionary studies, plant breeding and in diagnostics. In banana, isozyme polymorphism has been studied in 70 *Musa* cultivars for which seven enzyme systems were found suitable for cultivar identification (Bhat et al. 1992a, b). The RAPD technique has been successfully used to distinguish diverse *Musa* germplasm (Kaemmer et al. 1992; Howell et al. 1994; Bhat and Jarret 1995). Howell et al. (1994) identified 116 amplification products using nine primers. But the study on variation and intraspecific relationships exclusively among wild *balbisiana* types is very limited.

M. balbisiana was given priority among those defined by the genetic improvement working group of PROMUSA to strengthen the creation of new varieties resistant to pest and diseases. Special address has been made to prospect new wild and land races of *M. balbisiana* and to evaluate the existing diversity in South and South East Asia (Anonymous 2002). Thus, the present work was undertaken, to collect and study the genetic diversity of *M. balbisiana* in India.

The study was undertaken, (i) To standardize RAPD primers for *balbisiana*, (ii) to assay the amount of similarity and diversity present between and within the genus, and (iii) to study the inter relationships among the *balbisiana* collections.

Materials and methods

Many explorations were conducted to collect the diversity for *M. balbisiana* in various states of India. The North Eastern states including Assam, Tripura, Bihar and Southern states like Karnataka, Tamil Nadu and Kerala were explored. A total of 16 *M. balbisiana* types were collected and maintained in the NRCB field genebank for studying the genetic diversity and phylogeny analysis through RAPD markers (Table 1). Of these 16 accessions, two were collected from the secondary sources for which original passport data about their exact place of collection is not available.

Table 1. Test types of wild *balbisiana used in RAPD analysis.*

Sample No.	IC No.	Name of the sample	Source of the sample
1	250471	Borkal Baista (BORB)	Kahikuchi, Assam
2	250902	Sasra Bale (SASBL)	Western Ghats of Karnataka
3	250548	Bhimkol (BIMK)	Pusa, Bihar
4	250897	Attikol (ATIK)	IIHR, Hessaraghatta, Karnataka
5	250520	Manguthamng (MANG)	Diphu, Assam
6	250486	Nendrapadathi (NENP)[a]	Assam (collection from secondary sources)
7	250961	*Musa balbisiana* (MBAL)	Yercaud, Tamilnadu
8	250511	Athiakol 1 (ATI1)	Guwahati, Assam
9	251051	Athiakol 2 (ATI2)	Guwahati, Assam
10	251008	Elavazhai 1 (ELAV1)	Western Ghats of Kerala
11	251050	Bhimkol 2 (BIMK2)	Dispur, Assam
12	250460	Bhimkol 3 (BIMK3)	Dispur, Assam
13	250464	Athiakol 3 (ATI3)	Jorhat, Assam
14	250500	Manohar (MANO)	Jorhat, Assam
15	250899	Bacharia Malbhog (BAMOL)	Karnataka (collection from secondary sources)
16	250620	Elavazhai 2 (ELAV2)	Western Ghats of Kerala

IC – Indigenous Collection.

[a]A commercial cultivar also exists with the same name 'Nendrapadathi' (AAB-Pome, dessert type). Nendrapadathi, for a wild *Musa balbisiana* could probably be a wrong naming of a clone at the collection areas (Uma and Sathiamoorthy 2002).

DNA extraction and RAPD analysis

Genomic DNA was extracted with CTAB as described by Gawal and Jarret (1991) with minor modifications. The purified DNA was diluted to 10 ng mL^{-1} for PCR amplification. The protocol described by Williams et al. (1990) was followed for RAPD analysis with four random primers (OPA11, OPB04, OPC04 and OPD03) out of 80 standardized primers from Operon Technologies Inc. (Alameda, CA). The optimum RAPD PCR amplification reaction mixture was a total of 25 μL containing 50 ng gDNA, 200 μM each of all four dNTPs, 0.2 μM of a single 10-mer primer, 10× PCR buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin), and 1 U of Taq DNA polymerase (GENEI), India.

DNA amplification was performed in a DNA Eppendorf gradient thermal cycler. The thermal cycles used were: 40 cycles of 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 15 min followed by a slow cooling to 4 °C. The amplified products were subjected to electrophoresis in 1.5% agarose gel using 1× TAE buffer. The gels were stained with ethidium bromide.

Visualization and documentation of bands in agarose gel

The banding patterns were documented in Alpha Innotech Image analyzer. The molecular weight of the amplified fragments was calculated by comparing the molecular weight of the marker with the help of the software, Alpha Imager Version-4.0.

Data analysis

PCR amplified products from individual plants were scored as either presence (1) or absence (0). Analysis of similarity matrix within the NTSYS program (Rohlf 1990), using the unweighted pair group method with arithmetic averages (UPGMA), was used to determine the values of genetic distance. The genetic distance was calculated as the percentage of total number of bands scored that were clearly different between each pair of accessions. The results from each accession were analyzed by the UPGMA clustering method to produce a dendrogram. Morphotaxonomical traits were also analyzed using the same software.

Results and discussion

Standardization of primers

The sixteen accessions were tested with 80 random primers and 34 produced reproducible bands. Of these, only 4 primers (OPA11, OPB04, OPC04 and OPD03, Table 2) produced more than 5 polymorphic bands and they were used in the study.

Diversity analysis

Unlike *M. acuminata*, *M. balbisiana* has limited diversity and distribution. The reports of diversity for *M. balbisiana* is mainly from India and Philippines (Sotto and Rabara 2000). Sporadic occurrence of *M. balbisiana* is mentioned from Malaysia, Thailand, Bangladesh and Srilanka etc. The 16 accessions of *M. balbisiana* were subjected to both morphotaxonomic and molecular characterization using RAPD markers. In the morphotaxonomic characterization, a total of 98 traits were studied, of which the wild *M. balbisiana* showed distinctive variations for 16 traits (Table 3). They were considered for scoring and further subjecting them to NTSYS analysis.

Table 2. Primer code, amplified products, and polymorphism in *Musa balbisiana* accessions.

S. No.	Primer code	Nucleotide sequence from 5′–3′	Amplified bands	Polymorphic bands	Polymorphism[a]
1.	OPA11	CAATCGCCGT	16	11	68.75
2.	OPB04	GGACTGGAGT	11	6	54.55
3	OPC04	CCGCATCTAC	8	7	87.5
4	OPD03	GTCGCCGTCA	8	7	87.5
TOTAL			43	31	298.3
AVERAGE			10.75	7.75	74.6

[a] Polymorphism = (polymorphic bands/amplified bands) × 100.

Table 3. Traits used for morphotaxonomic characterization.

S. No.	Main traits	Classifications
1.	Pseudostem colour	a. Dark green b. Yellowish green c. Ash coated dark green
2.	Height of the pseudostem	a. <4.0 m b. >4.0 m
3.	Nature of leaves	a. Erect b. Drooping
4.	Colour of male bud	a. Maroon b. Purple brown
5.	Shape of male bud	a. Lacerate b. Rounded
6.	Size of male bud	a. Medium b. Large
7.	Nature of male axis	a. Barren b. Persistent bracts
8.	Position of male axis	a. Pendulous b. Angular
9.	Colour of free tepal	a. With pink shade b. Opaque white
10.	Length of peduncle	a. Short ($\leq$ 30 cm) b. Long ($\geq$ 30 cm)
11.	Bunch shape	a. Cylindrical b. Irregular
12.	No. of hands	a. 3–5 b. >5
13.	Wax coating on the fruit	a. Profuse b. Mild
14.	Nature of the tip of the fruit	a. Blunt b. Gradual peak c. Conspicuous peak
15.	Size of the fruit	a. Short and stout b. Elongated c. Short and not stout
16.	Nature of seeds	a. Hard and black in colour b. Soft and brown in colour

Cluster analysis using morphological traits

Morphotaxonomic characterization of the 16 accessions using 98 traits (IPGRI–INIBAP/CIRAD descriptor 1996) was carried out. Of these, 16 distinctive traits (Table 3) were considered for diversity evaluation, which exhibited a total of 34 classifications. Depending on the presence or absence of a particular classified trait, the individual accessions were scored as (1) or (0). The data obtained in the matrix form was subjected to NTSYS analysis. The dendrogram obtained and the clustering pattern observed is indicated in Table 4.

Cluster analysis using molecular characterization

Genetic variation

Totally 43 DNA fragments were amplified by four primers with an average of 10.75 fragments per primer (Table 2). The number of amplified products with OPD03 and OPA11 were 8 and 16, respectively. Among the 43 amplified bands, 31 showed polymorphism with an average of 7.75 fragments per primer. In case of OPB04 and OPC04, the amplified products varied from 8 to 11.

The average polymorphism exhibited by all the 4 primers was 74.6 per cent, indicating that there was considerable variation at the DNA level within the *balbisiana* accessions.

The four primers generated accession-specific and monomorphic bands. Primer OPA11 produced five monomorphic bands and 11 polymorphic bands. Primer OPB04 produced five monomorphic bands and six polymorphic bands. The OPD03 primer generated one monomorphic band and seven polymorphic bands. One monomorphic and seven polymorphic bands were generated with OPC04 primer. Here the result indicates that these amplified products are unique to the test accessions and could be used for their identification.

Table 4. Formation of clusters using distinct morphological traits and molecular characterization.

Cluster	Morphotaxonomic members	Molecular members
Cluster 1	Bhimkol, Borkal Baista, Manguthamng	Borkal Baista, Bacharia Malbhog
Cluster 2	Athiakol 1, Bhimkol 2, Bhimkol 3, Sasra Bale	Sasra Bale, Athiakol 1, Bhimkol 2, Bhimkol 3 Bhimkol, Manguthamng, Elavazhai 2 Nendrapadathi, Manohar Athiakol 2, Elavazhai 1, Athiakol 3
Cluster 3	*Musa balbisiana*	*Musa balbisiana*
Cluster 4	Attikol	Attikol
Cluster 5	Bacharia Malbhog	—
Cluster 6	Athiakol 2, Athiakol 3, Elavazhai 1, Elavazhai 2	—
Cluster 7	Manohar, Nendrapadathi	—

Genetic similarity

The genetic similarity (GS) estimated was found to range from 0.68 to 1.0 among the 16 wild *balbisiana* accessions. The GS within these was greater than 0.85, such as SASBL and BIMK2 (1.0), BIMK3 (1.0), ELAV2 (0.80), ATI1 (0.95). The dendrogram analysis showed that they have a close relationship with morphotaxonomic characterization. The results obtained from RAPD and morphotaxonomic characterization exhibited almost 88% similarity. From this observation, it was identified that the primer OPA11 worked excellent in *Musa balbisiana* types (Figure 1).

Cluster analysis with RAPD markers

The clustering pattern obtained through RAPD markers was compared with the clustering pattern of morphotaxonomic characterization. Results of molecular characterization (Figure 2) indicated that the distinct clustering of Borkal Baista and Bacharia Malbhog with 35% dissimilarity. But between them, the dissimilarity was as high as 30%. Borkal Baista, an unique accession with specific traits like huge male bud with male axis extending geotropically even upto 2.0 m.

Accession Attikol is distinct from all *balbisiana* types with 38% dissimilarity with other test accessions. This was collected from Karnataka and has distinct features like very short and stout fruits and more than 95% germinability, short and less robust unlike other wild *balbisiana*. (A similar result of clustering separately was also noticed in morphotaxonomic characterization).

Second cluster is the major group encompassing 13 test accessions (Table 4), which were otherwise spread into three microclusters. Within this Athiakol 1 is grouped with Sasra Bale with more than 10% dissimilarity. Bhimkol 2, Bhimkol 3 exhibited 100% similarity, suggesting their synonymous status.

Bhimkol and Mangutamng grouped together with less than 5% dissimilarities and exhibited

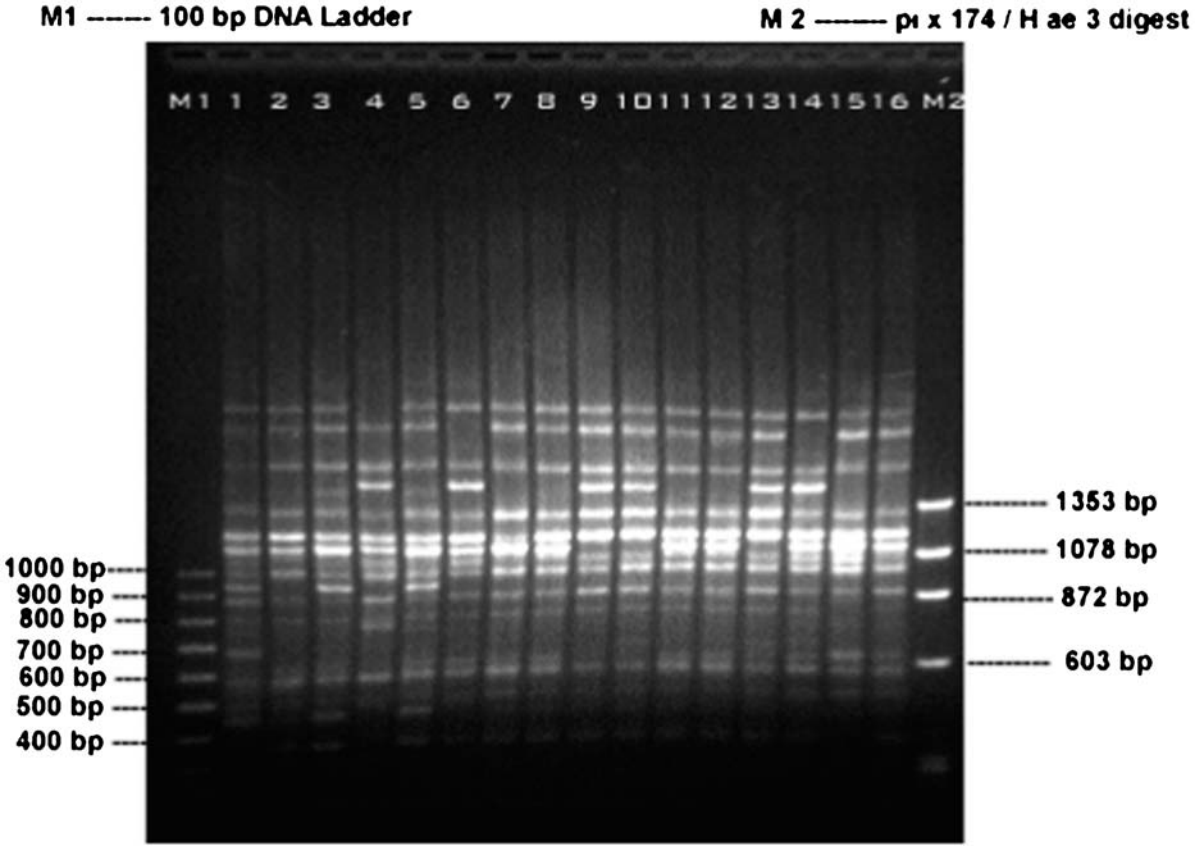

Figure 1. RAPD profile for Indian wild *Musa balbisiana* diploids with primer OPA 11.

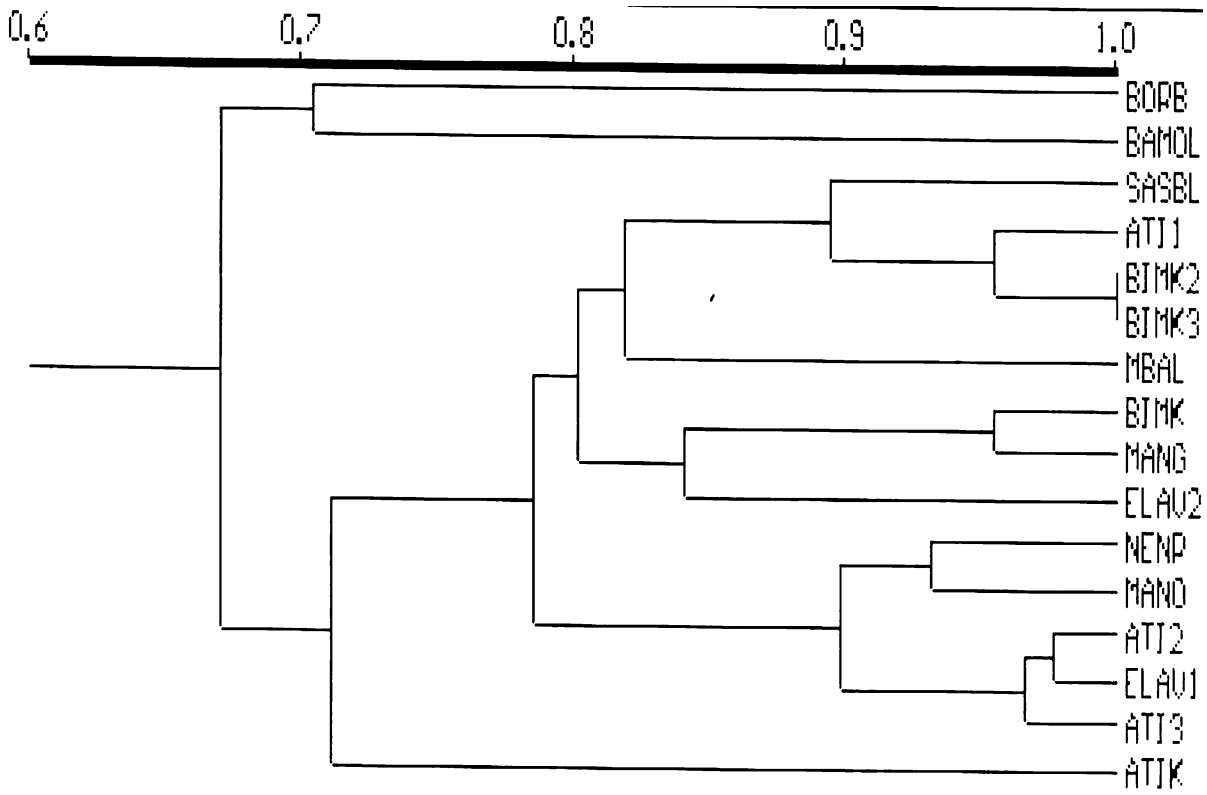

Figure 2. UPGMA dendrogram of genetic relationships among Indian wild *Musa balbisiana* diploids based on RAPD analysis.

relatedness with Elavazhai 2, expressing more than 85% similarities.

Athiakol 2 and Athiakol 3 which are otherwise (also morphologically similar) grouped together and they exhibited relatedness of 90% with Nendrapadathi and Manohar.

The results of comparison between clustering pattern obtained through morphotaxonomic and molecular characterization exhibited 88% of congruity. Bhimkol and Mangutamang of cluster 1 in morphotaxonomic characterization have been shifted to cluster 2 in molecular characterization. The accession Bacharia Malbhog, an unique member of cluster 5 in morphotaxonomic characterization grouped along with Borkal Baista of cluster 1 in molecular characterization. Bacharia Malbhog is the slender most accession among the *balbisiana* types. This accession is early to shoot and very shy suckering in nature. Attikol and *M.balbisiana* clustered independently in both the grouping patterns. In the present study, results of RAPD characterization are analogous to morphotaxonomic characterization to a greater extent. Morphotaxonomic characterization grouped the 16 test accessions into seven clusters while only four clusters from molecular characterization. Though there appears relatively more phenotypic variation, the genotypic expression seems to be much restricted.

Conclusion

The 16 test clones used in the present study shared only 10 names like Bhimkol 1, Bhimkol 2, and Bhimkol 3 etc. Though these accessions collected from different locations shared the congruous name, they were not synonyms. The geographic distribution of the test accessions had an effect on clustering, in other words, most of the accessions from the same geographic location like the test clones from Assam (cluster 2) were grouped together with an exception of Sasra Bale from Karnataka. Both collections from Kerala namely Elavazhai 1 and Elavazhai 2 grouped together along with those from North Eastern states in Cluster 2.

The present characterization result of RAPD goes with morphotaxonomic characterization for

clustering of most of the test types and fail only in some specific clusters. In general, characterization using RAPD strengthens diversity analysis in *Musa* accessions but needs refinement using more random primers for more specific results and also using AFLP markers.

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