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Genetic diversity and differentiation analysis among wild antecedents of banana (*Musa acuminata* Colla) using RAPD markers

S Mukunthakumar*, P Padmesh, P S Vineesh, Reby Skaria, K Hari Kumar and P N Krishnan

Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695 562, India

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Musa acuminata Colla, the wild progenitor of banana, has a long evolutionary history intervened by human activity. Forty three accessions of *M. acuminata* ssp. *burmannica*, collected from 3 wildlife sanctuaries of Agastyamalai Biosphere Reserve of Western Ghats, India were studied for their genetic diversity and differentiation using random amplified polymorphic DNA (RAPD) markers. The results indicated a relatively high level of genetic diversity in *Musa* accessions at the species [Nei's gene diversity (H)=0.47; Shannon information index (I)=0.66)] as well as population level (H=0.42; I=0.61). A relatively high degree of genetic differentiation was also observed among the populations (G_{ST}=0.30). Moreover, analysis of molecular variance (AMOVA) showed the existence of vast (81%) genetic variation within the populations. The Mantel test revealed a significant correlation (Rxy=0.54; P<0.001) between the geographic distance and the genetic distance of these populations. The genetic variability among these accessions suggests that the management for the conservation of the genetic diversity in *M. acuminata* should aim at preserving each and every accession at Agastyamalai Biosphere Reserve.

Keywords: AMOVA, conservation, genetic diversity, Mantel test, *Musa acuminata* ssp. *burmannica*

Introduction

The genus *Musa* is represented by 70 species, which are categorised into two sections, *viz.*, Musa and Callimusa, and distributed between India and the Pacific, as far North as Nepal and extending to the northern tip of Australia[1]. Most of the present day bananas have been evolved from two wild ancestors, *M. acuminata* with 'AA' genome and *M. balbisiana* with 'BB' genome belonging to section Musa. They are generally seedy, non-pulpy and non-edible, and grow in the forests. *M. acuminata* is the most widespread of the species and originated in southeast Asia[2]. It is further divided into 9 subspecies[3], of them only 3 subspecies, *viz.*, *M. acuminata* ssp. *banksii, M. acuminata* ssp. *burmannica* and *M. acuminata* ssp. *burmannicoides*, are reported from India[4]. Despite being a progenitor, *M. acuminata* subspecies assume significance as they are precious genetic resources for disease resistance against devastating pathogens[5-7]. Therefore, such fertile sources are highly desirable for genetic improvement studies. However, all the available crops are sterile and parthenocarpic in nature, which makes the conventional breeding strategies difficult and cumbersome.

The discontinuous distribution and dwindling population due to habitat destruction are serious threats to the existing genetic diversity in subspecies of *M. acuminata*. Therefore, conservation of these wild subspecies and promotion of their conservation for improvement of edible varieties are the need of the time. The knowledge of native wild *Musa* subspecies is a wealth, but not much information is available on the subject. Moreover, information on the genetic diversity and phylogenetic relationships within the South Indian banana germplasms are scarcely availble. Recent collecting expeditions in northern India and Malaysia suggest that other poorly known or unexplored areas of diversity are likely to harbour other agronomically interesting characteristics[8]. Since genetic characterization is essential for scientifically sound germplasm conservation, it has become essential to document the genetic diversity within the available wild *Musa* germplasm[9-10].

Genetic markers are widely used either alone or in combination with morphological markers to obtain more consistent information on existing genetic diversity with a number of species. Randomly amplified polymorphic DNA (RAPD) markers could represent an interesting source of polymorphic markers as it harbours considerable length variation between individuals and is extremely abundant in

*Author for correspondence:
E-mail: mukunthakumar@gmail.com

eukaryotic genomes. RAPD is a cost effective DNA marker system, which has been successfully employed to detect variation at the intergeneric[11], inter-specific[12], intraspecific[13-14], intervarietal[15-16]and at the cultivar levels[17]. In the present study, different wild accessions of *M. acuminata* ssp. *burmannica* from 3 wild life sanctuaries (Neyyar, Peppara & Shendurney) of Agasthyamalai Biosphere Reserve (ABR) of Western Ghats, India were analyzed for their genetic diversity and differentiation through RAPD. ABR (8-21°N latitude, 73-77°E longitude) is one of the richest endemic centers of the Western Ghats forming a compact block of hilly range towards the southern most end of the Western Ghats.

Materials and Methods

Sample Collection

A total of 43 individuals of wild *Musa* from the remote and humid forest regions of 3 wildlife sanctuaries of Neyyar, Peppara and Shendurney of ABR were collected for the present study (Table 1). Identification of the collected wild *Musa* specimens was done using the descriptor based on the morphological characteristics provided by International Network for the Improvement of Banana and Plantain (INIBAP)[18] and *Musa*logue-INIBAP[2]. The collected *Musa* accessions were maintained in Banana Conservatory of the Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, India for future studies.

DNA Extraction and RAPD Amplification

Total genomic DNA from the young leaves was isolated following the modified Murray and Thompson method using CTAB[19]. After ethanol precipitation, DNA was resuspended in 100 µL of 1× TE buffer (pH 8.0). DNA was quantified spectrophotometrically by taking the absorbance at 260 nm using Biophotometer (Eppendorf, Hamburg). Of 20 primers, 10 primers produced amplicons and were selected for genetic diversity analysis by taking into consideration the repeatability, sharpness and intensity of bands (Table 2). The RAPD assay was carried out in 25 µL reaction mixture containing 0.5 µL dNTPs (0.2 mM), 2.5 µL 10× polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100), 0.5 µL Taq DNA polymerase (1.0 U/µL) (Finnzymes, Helsinki, Finland), 1 µL primer (15 pmol) and 50 ng of genomic DNA. The amplification was performed in a thermal cycler (Eppendorf-ESP-S, Hamburg) with oil free operation. After the initial cycle of 2 min at 94°C, 2 min at 36°C and 2 min at 72°C, 38 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C were performed. The last cycle was followed by final extension of 7 min at 72°C. Amplified products were resolved on 1.4% agarose gels (1× TBE) and visualized by ethidium bromide (EtBr) staining.

The intensity of fluorescence varied within and among the *Musa* populations studied. Reproducible RAPD products were scored as presence (1) or absence (0) of a fragment. Dice coefficient of similarity defined as 2a/(2a+u), where "a" is the number of positive matches and "u" is the number of non matches, was computed using the WINDIST software[20] for genetic similarity (GS) matrix preparation. The populations from which the samples were taken for the present analysis were assumed to be in Hardy-Weinberg equilibrium implying that the population is at random mating. Inter and intrapopulation diversity was analyzed using various parameters.The software program POPGENE v. 1·3[21] was used to obtain the genetic diversity parameters, observed number of alleles (*na*), effective number of alleles (*ne*), and expected heterozygosity (H_S). Genetic diversity measures (H_T, total gene diversity; G_{ST}, degree of gene differentiation) were tested using Nei's[22]gene diversity statistics. The Shannon[23] diversity index was calculated as $I=-\Sigma P_i \log_2 P_i$, in which P_i represents the frequency of the given RAPD fragments. This index was then used to measure the total diversity (H_T) and the mean intra-population diversity (H_S). Then the proportion of diversity (G_{ST}) among populations was calculated as $G_{ST}=(H_T-H_S)/H_T$. The gene flow estimate (Nm)[24] was calculated as $Nm=(1-G_{ST})/4G_{ST}$, where *Nm* is the number of migrants per generation.

The genetic variation among the natural populations was also estimated from the analysis of molecular variance (AMOVA) using GeneAlex 6[25], in which the total genetic variance was partitioned into 'among populations' and 'within populations'. The significance level of this F statistic analogue was determined by 999 random permutations. Further, Mantel test with 999 permutations was conducted with the same software to determine the extent of correlation, if any, between genetic distance (GD) and geographical distance (in Km) between populations[26]. In order to examine the genetic relationship among the populations, a UPGMA (unweighted pair group method using arithmetic average) phenogram was constructed based on GD matrix using MEGA 5.05[27].

Table 1—List of *M. acuminata* samples with details of collection site and location coordinate

Pop ID	Wildlife sanctuaries	Location	Altitude (m)	Site coordinate
1	Peppara Wildlife Sanctuary	Bonacaud	474	N8 42.342 E 7718.294
2		Bonacaud	340	N8 42.250 E 77 18.197
3		Valipara near Bonacaud	775	N8 41.972 E77 10.224
4		Valipara near Bonacaud	862	N8 41.821 E77 10.472
5		Valipara near Bonacaud	978	N8 41.743 E77 10.467
6		Valipara near Bonacaud	826	N8 41.137 E77 10.902
7		Sooryakanthi near Bonacaud	798	N8 41.122 E77 10.869
8		Sooryakanthi near Bonacaud	849	N8 40.711 E77 10.807
9		Sooryakanthi near Bonacaud	787	N8 40.735 E77 10.799
10		Sooryakanthi near Bonacaud	943	N8 40.671 E77 10.866
11		Sooryakanthi near Bonacaud	846	N8 40.800 E77 10.882
12		Sooryakanthi near Bonacaud	900	N8 41.367 E77 11.027
13		Sooryakanthi near Bonacaud	829	N8 41.276 E77 10.858
14		Sooryakanthi near Bonacaud	1072	N8 41.378E77 11.065
15		Sooryakanthi near Bonacaud	1051	N8 41.477E77 11.081
16		Sooryakanthi near Bonacaud	1049	N8 41.325E77 11.282
17		Sooryakanthi near Bonacaud	1037	N8 41.461E77 11.024
18		Sooryakanthi near Bonacaud	961	N8 41.591E77 10.810
19		Sooryakanthi near Bonacaud	1094	N8 41.363E77 11.266
20		Sooryakanthi near Bonacaud	1097	N8 41.332E77 11.245
21		Sooryakanthi near Bonacaud	1107	N8 41.313E77 11.239
22		Sooryakanthi near Bonacaud	1144	N8 41.155E77 11.202
23		Pokkirimotta near Bonacaud	1101	N8 41.061E77 11.159
24		Agastyamala	584	N8 38.601E77 12.138
25		Agastyamala	580	N8 38.597E77 12.146
26		Agastyamala	593	N8 39.179E77 11.345
27	Neyyar Wildlife Sanctuary	Neyyar	354	N8 31.034E77 13.555
28		Neyyar	446	N8 31.102E77 13.238
29		Neyyar	472	N8 31.055E77 13.114
30		Neyyar	245	N8 33.251E77 14.083
31		Neyyar	452	N8 31.220E77 12.406
32	Shendurney Wildlife Sanctuary	Pandimotta	789	N8 49.172 E77 11.488
33		Pandimotta	726	N8 49.275 E77 11.580
34		Pandimotta	722	N8 49.203 E77 11.530
35		Pandimotta	686	N8 48.543 E77 11.498
36		Pandimotta	700	N8 48.477 E77 11.478
37		Pandimotta	910	N8 49.211 E77 12.059
38		Pandimotta	884	N8 49.269E77 12.117
39		Pandimotta	468	N8 48.331 E77 11.119
40		Pandimotta	472	N8 48.493 E77 11.195
41		Kannampallimedu	616	N8 57.309 E 77 09.450
42		Kannampallimedu	704	N8 57.234 E 77 10.064
43		Kannampallimedu	533	N8 56.574 E 77 10.015

Results and Discussion

The collected wild *Musa* accessions were identified using the descriptor based on the morphological characteristics provided by INIBAP and *Musa*logue-INIBAP and all of them have been identified as *M. acuminata* ssp. *burmannica.*

Evaluation of phylogenetic relationships is an essential element of germplasm characterization and conservation, and needed to establish effective breeding programmes. In the present study, 43 accessions were tested with 20 random primers. Of these, only 10 primers produced more than

6 polymorphic bands and were used in the study (Table 2). Of 104 bands generated, 13 (12%) were found to be shared by individuals of all the 43 accessions. The remaining 91 fragments were found to be polymorphic (87.5%). On an average, the primers generated 10.4 amplicons and 9.1 polymorphic bands per primer. While majority of the primers produced 100% polymorphism, the average polymorphism exhibited by all the 10 primers was 87.5%, indicating that there was considerable variation at the DNA level within the collected accessions.

The Nei's genetic diversity (H) was observed in the range 0.41-0.44 (Table 3). The wild *Musa* showed high levels of genetic diversity at the species level (H=0.47, I=0.66, *ne*=1.89, *na*=2.0). Among the three populations, Peppara populations showed maximum genetic diversity (H_T=0.44, H_S=0.17, I=0.63), whereas the Neyyar populations showed the lowest diversity (H_T=0.41, H_S=0.12, I=0.59).

The coefficient of genetic differentiation G_{ST} was found to vary from 0.28 to 0.32 at the population level with mean value of 0.30, while at the species level it exhibited a mean value of 0.39. The level of gene flow (Nm), which refers to number of individuals entering into the population in a generation, was found to be 0.77 at the species level, while it was little >1 (1.17) at the population level. Neyyar population showed highest level of gene flow (1.23) and correspondingly low levels of G_{ST} (0.28). The AMOVA analysis showed significant genetic differentiation (P<0.001) among the *Musa* populations. Of the total genetic diversity, 19% was partitioned among populations and 81% within the populations (Table 4). The Mantel test attributed moderate and significant positive correlation between matrices of genetic and geographic distances (Rxy=0.547; P<0.001) with 999 permutations.

The similarity matrix developed using the POPGENE 1.3 software showed similarity index ranging from 0.32-0.98 with mean value of 0.68. To further reveal the relationships among wild *M. acuminata* accessions collected from the 3 wildlife sanctuaries of ABR, UPGMA of MEGA 5.05 was used to generate a phenogram based on Nei's genetic distances (Fig. 1). The grouping of the 43 accessions of *Musa* in the present investigation showed two major clusters, namely, Cluster-I and Cluster-II, comprising of 23 and 20 accessions, respectively. The geographic distribution of the test accessions had an effect on clustering; in other words, most of the accessions from the same geographic location were grouped together.

Genetic variation in plants has proven valuable in plant conservation and management, and for identification of populations, species and sub-species[28]. A lack of knowledge about the genetic structure of these populations may result in the differential harvest of the populations that will ultimately have a drastic and long-term effect[29]. Jain *et al*[15] have reported that RAPD is reliable, rapid and inexpensive screening method to discriminate the

Table 2—List of primers and their sequence used for RAPD analysis of 43 accessions of *M. acuminata*

No.	Primer	Sequence (5′ – 3′)	Total bands	Polymorphic bands	%
1	S 62	GTGAGGCGTC	7	7	100
2	S63	GGGGGTCTTT	14	12	85.7
3	S66	GAACGGACTC	9	8	88,8
4	S68	TGGACCGGTG	8	8	100
5	S70	TGTCTGGGTG	10	10	100
6	S71	AAAGCTGCGG	9	9	100
7	C73	AAGCCTCGTC	11	10	90.9
8	C75	GACGGATCAG	12	10	83,3
9	C76	CACACTCCAG	13	8	61.5
10	S80	ACTTCGCCAC	11	9	81.8
Total bands			104	91	87.5
Mean per primer			10.4	9.1	

Table 3—Summary of genetic diversity data in *M. accuminata*

Diversity indices	Wild *Musa* populations			Species
	Peppara	Neyyar	Shendurney	
H	0.44	0.41	0.43	0.47
I	0.63	0.59	0.61	0.66
G_{ST}	0.32	0.28	0.29	0.39
Nm	1.08	1.23	1.19	0.77
H_T	0.44	0.41	0.43	0.47
H_S	0. 17	0. 11	0.14	0.14
na	2.00	1.98	2.00	2.00
ne	1.81	1.73	1.78	1.89

H, Nei's gene diversity at population level; I, Shannon index of genetic diversity; G_{ST}, Degree of genetic differentiation; Nm, Estimate of gene flow; H_T, Total gene diversity; H_S, Diversity within population; *na*, Observed number of alleles; *ne*, Expected number of alleles.

Table 4—Analysis of molecular variance (AMOVA) in *M. acuminata* accessions

Source	df	SS	MS	Estimated variance	% variance	P value
Among Pops	8	125.99	15.70	1.57	19%	< 0.001
Within Pops	34	233.60	6.87	6.87	81%	< 0.001

AMOVA analysis: df, Degrees of freedom; SS, Sum of squares; MS, Mean sum of squares; P, Probability value.

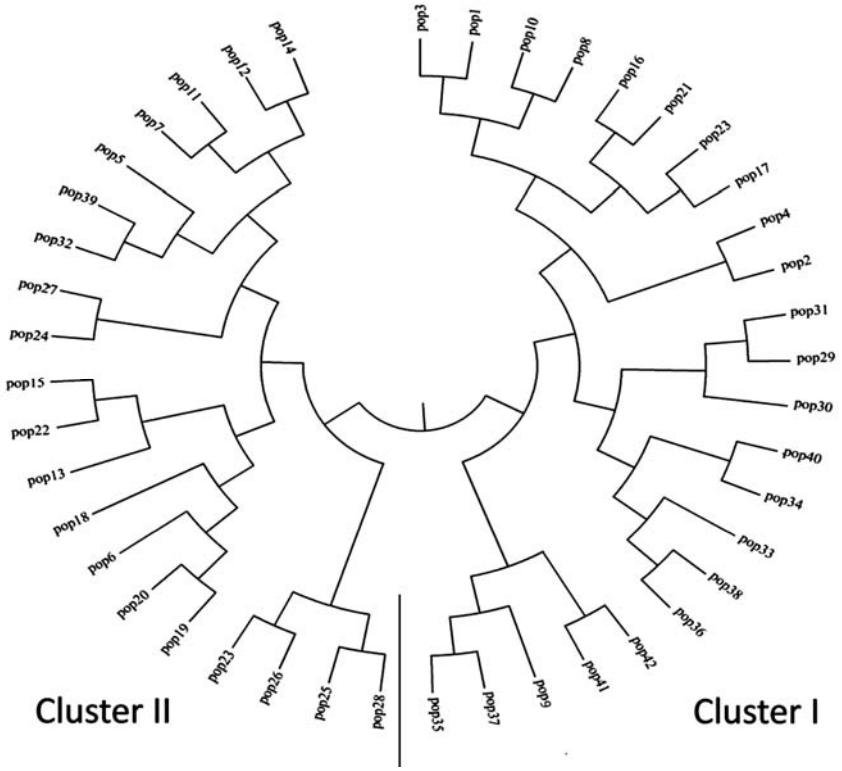

Fig. 1—Phenogram based on UPGMA analysis of *M. acuminata* accessions. [The accession numbers are enlisted in Table 1].

Musa genotypes. The level of genetic diversity observed corroborates with the wide range of morpho-variability observed in banana populations, which are native of southern India[30]. The present study with H_T=0.47 and I=0.66 at species level shows higher genetic diversity compared to those reported in *M. balbisiana* (H_T=0.24, I=0.36)[31]. This could be probably because, unlike *M. acuminata* where several subspecies has been reported, no subspecies has been identified in *M. balbisiana*. In the present study, the value of observed (*na*) and expected number of alleles (*ne*) ranged from 1.98 to 2.00 and 1.73 to 1.81, respectively. This is slightly lesser than earlier reports (*na*=2.5 alleles per primer) for diploid cultivars of AA genome[10]. This difference may be attributed to the different marker system (STMS) used in the study and also due to the inherent genetic differences in the cultivars, resulted as a consequence of years of selective breeding.

The present results of Mantel statistics have shown significant correlation between genetic distance among populations with the geographical distance (Rxy=0.547; P< 0.001), which might have resulted due to the gene flow. The gene flow has been a leading factor during genetic differentiation of species, especially in the moment when the species was faced with a strong biological selection against environmental change of habitat[32]. *M. acuminata* ssp. *burmannica* present within ABR are usually distributed in disturbed habitats strongly influenced by human activities and wild animals. However, the correlation between its genetic and geographic distance at population level suggests that some natural dispersal mechanism may also be involved in the distribution of species.

The high level of genetic diversity and genetic differentiation maintained within populations of wild *Musa*, despite its low gene flow is an interesting phenomenon that entails detailed studies with accessions from other centres of diversity in India. Our study reveals high genetic variation among the wild *Musa* accessions of three wildlife sanctuaries belonging to ABR and emphasizes the need for conservation and propagation assisted rehabilitation, and selection of the natural populations of *Musa* for future studies. Therefore, as part of long-term conservation strategy, efforts should be made to increase the effective population size and thereby accelerate the gene flow among populations. These efforts are absolute necessity to maintain and perhaps

to increase the existing natural variation in this underexplored taxa. It would be worthwhile studying the diversity of *Musa* in these regions, and future molecular analyses should include taxa of wild *Musa* from the whole Western Ghats.

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