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Diversity studies in ecotypes of banana (*Musa* spp.) using molecular markers and D^2 analysis

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ABSTRACT

The present study was aimed at analyzing the genetic diversity of promising banana ecotypes grown in Kerala. Twenty eight ecotypes of banana were collected from different parts of Kerala. DNA isolated from these was used for RAPD analysis. Six most-promising primers viz., OPA-01, OPA-03, OPA-13, OPB-04, OPB-10 and OPB-12 were used. These yielded 46 scorable bands with an average of 7.66 bands per primer. RAPD data were analyzed statistically and a dendrogram was constructed. Twenty three characters were observed in the twenty eight banana ecotypes and were statistically analyzed as per the method proposed by Mahalanobis (D^2). From RAPD dendrogram, it was found that the banana clones clustered into fourteen groups at a distance of 0.200. At a distance of 0.250, 8 out of 12 Nendran (AAB group) ecotypes formed a single cluster at the same distance. Among Palayankodan (AAB group) ecotypes PKNNR, Pisang Ceylon, Motta Poovan, Chandra Bale and Palode Palayankodan grouped together and formed a single cluster. Attu Nendran, Monthan, Robusta, Koonoor Ethan, Ilavazha and Vellapalayankodan formed individual clusters and had maximum genetic divergence. Among diploid clones, Ilavazha (BB group) had maximum genetic divergence. Among triploid clones, Attu Nendran, Robusta, Koonoor Ethan and Vellapalayankodan showed maximum genetic divergence. Among Nendran (AAB group) ecotypes, Attu Nendran and Koonoor Ethan revealed maximum genetic divergence. Among Palayankodan (AAB group) ecotypes, Vellapalayankodan recorded the highest genetic divergence. In D^2 analysis too, a similar trend was observed.

Key words: Banana, ecotypes, RAPD, molecular markers, D^2 analysis, genetic divergence

INTRODUCTION

Banana (*Musa* spp.) is one of the most important fruit crops of India, next only to mango. It is cultivated in tropical and subtropical regions of the world. India is the largest producer of banana, contributing 19.71% of the global production and a total production of 19.19mt from 0.565 mha area (Singh, 2007). Banana is the most important fruit crop of Kerala and one of the oldest cultivated fruit crops. In Kerala, several landraces are available and cultivated in different parts, known by different local names, being a highly evolved crop. Banana cultivars have a number of synonyms, resulting in a somewhat confused taxonomical status. Simmonds (1962) concluded that present day cultivars evolved by hybridization of two ancestral parents, *Musa acuminata* and *M. balbisiana*, which are considered as the main contributors of A and B, genomes respectively. All cultivars are classified into various genomic groups such as AA, AAA, AB, AAB and ABB based on morphological scoring (Stover and Simmonds, 1987). Morphological characterization has been a major tool for classifying banana cultivars into different genomic groups. Efforts have also been made to classify bananas and plantains using quantitative traits. Variability and genetic divergence among Indian bananas were studied by Valsalakumari *et al* (1985). Molecular techniques have proved to be a powerful tools and have led to understanding genetic relationships among banana cultivars. Such studies are reported by Howell *et al* (1994), Bhat and Jarret (1995), Rekha *et al* (2001), Kahangi *et al* (2002) and Soni (2010) in *Musa* and Singh *et al* (2003) and Rai and Mishra (2005) in mango. Among various molecular characterization techniques, random amplified polymorphic DNA marker (RAPD) has been gainfully employed to investigate genetic variability (Brown *et al*, 1993). Hence, the present study is an attempt to characterize some of the commercially important bananas and their land races grown in Kerala using molecular markers and D^2 analysis.

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MATERIAL AND METHODS

PLANTING MATERIAL

This study was conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. Ecotypes of banana used in the study are presented in Table 1.

Banana clones belonging to six genomic groups were used in the study. Five suckers (replication) of each ecotype were maintained at the Instructional Farm, College of Agriculture, Vellayani. Suckers of almost uniform size were collected from different parts of Kerala, planted and maintained here. Spacing adopted was 2.0m x 2.0m. The experiment was laid out in Completely Randomized Block (CRD) design with five replications, as per the Panse and Sukhatme (1967). Observations were recorded on plant height, pseudostem girth, number of suckers per plant, leaves per plant, leaf length, leaf width, crop duration, number of fingers per hand, fingers per bunch, bunch weight, hand weight, number of hands per bunch, length of bunch, weight; length, girth and volume of finger, pulp/peel ratio, TSS, acidity, total sugars, sugar/acid ratio and shelf life of the fruit. Data were analyzed statistically.

Table 1. Cultivars banana types ploidy and genomic composition of banana ecotypes

Sl. No.	Clone	Type	Ploidy level	Genomic composition
1.	Red banana	Dessert	3x	AAA
2.	Vellakappa	Dessert	3x	AAA
3.	Robusta	Dessert	3x	AAA
4.	Vellayani Nendran	Dessert	3x	AAB
5.	Padalamurian	Dessert / cooking	3x	AAB
6.	Myndoli	Dessert / cooking	3x	AAB
7.	Chengazhikodan	Dessert / cooking	3x	AAB
8.	Attu Nendran	Dessert / cooking	3x	AAB
9.	Kaliethan	Dessert / cooking	3x	AAB
10.	Koonoor Ethan	Dessert / cooking	3x	AAB
11.	Mysore Ethan	Dessert / cooking	3x	AAB
12.	Zanzibar	Dessert / cooking	3x	AAB
13.	Quintal banana	Dessert / cooking	3x	AAB
14.	Changanasseri Nendran	Dessert / cooking	3x	AAB
15.	Manjeri Nendran	Dessert / cooking	3x	AAB
16.	Palode Palayankodan	Dessert	3x	AAB
17.	PKNNR	Dessert	3x	AAB
18.	Chandra Bale	Dessert	3x	AAB
19.	Pisang Ceylon	Dessert	3x	AAB
20.	Mottapoovan	Dessert	3x	AAB
21.	Vellapalayankodan	Dessert	3x	AAB
22.	Monthan	Cooking	3x	ABB
23.	Peyan	Cooking	3x	ABB
24.	Kadali	Dessert	2x	AA
25.	Pisang Lilin	Dessert	2x	AA
26.	Njalipoovan	Dessert	2x	AB
27.	Kunnan	Dessert	2x	AB
28	Ilavazha	Leaf purpose	2x	BB

RAPD analysis

DNA extraction

Emergent young leaves before they fully unfurled were used for DNA extraction in all the ecotypes of banana following the procedure of (modified) Walbot (1998). Leaves were collected in the morning hours and washed under running tap water, and then in distilled water, two to three times after chopping the leaves coarsely. After wiping off the water using tissue paper, the chopped leaves were placed in a cool, dry porcelain mortar and ground well to a fine powder in liquid nitrogen. About 1 g of emerging leaves is used for DNA extraction

Powdered leaf samples were transferred to the extraction buffer (168g urea, 28ml 5M NaCl, 20ml 1M Tris HCL at, pH 8, 16ml 0.5M EDTA and 20ml phenol, made upto 400 ml with sterile water) and placed in a water-bath at a temperature of 55^{o}C. A volume of 2.5ml of 20% SDS (5g SDS, in 25ml sterile distilled water) and a pinch of polyvinyl pyrollidone (PVP) were added and mixed gently. Then, 25ml of phenol:chloroform:isoamyl alcohol (25:24:1) solution was added and this was centrifuged at 10000rpm for 10 min at 4^{o}C. The supernatant was mixed with equal volume of phenol:chloroform:isoamyl alcohol and centrifugated at 10000rpm for 10 min. The above steps were repeated 2-3 times until the interphase disappeared. To the upper phase so collected, 1/10th volume of 3.0M sodium acetate and double the volume of 70% cold, absolute ethanol were added. The pellet was dried and dissolved in 100µl to 200µl of TE buffer and stored at 4^{o}C. DNA quantification was done using UV-Vis spectrophotometer.

DNA amplification reactions were performed in 25µl medium containing 20ng of genomic DNA, 2.5µl 1x buffer, 5pM primer, 200µM each of dNTPs (each of dATP, dTTP, dCTP and dGTP) and 0.6 units of Taq DNA polymerase. PCR was carried out with initial denaturation at 95^{o}C for

3.0 min, followed by 45 cycles of denaturation at 95°C for 1.0 min, annealing at 36°C for 1.5 min and extended at 72°C for 2.0 minutes. The synthesis step of the final cycle was extended further by 6.0 min. Finally, products of amplification were cooled to 4°C. Forty one decamer primers were screened for efficiency using DNA isolated from 'Attu Nendran' as a representative sample. Of the 41 decamer primers used, 34 yielded amplification products. These primers produced 123 bands, of which 116 bands (94.31%) were polymorphic and seven bands (5.61%) were monomorphic. Twenty five primers showed a high level of polymorphism. Finally, six most-polymorphic primers were used for RAPD analysis of all the 28 banana clones. PCR amplification was made by six different random primers (OPA-01, OPA-03, OPA-13, OPB-04, OPB-10 and OPB-12 (Operon Technologies, USA) and these were selected for further amplification. Amplification products were mixed with loading buffer containing bromophenol blue, separated electrophoretically on 1.4% agarose gel containing 0.5µg/ml ethidium bromide. For data analysis, only amplification products reproducible over two amplifications were included. PCR products were Difenoconazoled as discrete variable: a Difenoconazole '+' for presence and '-'for absence of a homologous band. A genetic Similarity Matrix was constructed using the Jaccard's Co-efficient method (Jaccard, 1908). Based on similarity coefficient, the distance between clones was computed with the help of a software package NTSYS (version 2.02i). A dendrogram was constructed by UPGMA method and, association between various genotypes was estimated from the dendrogram (Fig. 1).

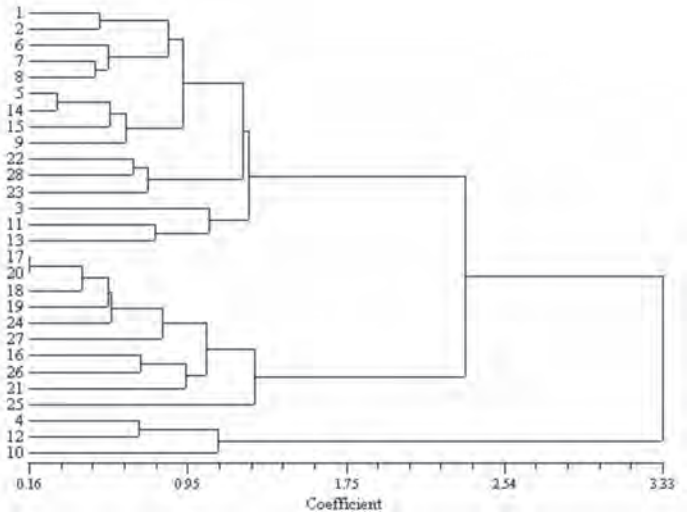

Fig 1. Dendrogram of 28 banana ecotypes of Kerala using RAPD marker (for clone number, refer Table 1)

D^2 analysis

Morphological observations were recorded from 28 banana ecotypes based on the method of Stover and Simmonds (1987). Data on plant morphology, quantitative yield, fruit and quality parameters were recorded. D^2 statistics, a measure of distance, based on multiple characters proposed by Mahalanobis (1936) was used. Grouping of varieties was done by Tocher's method (Rao, 1952). Relative contribution of characters to divergence at the cluster as well as genotype level was assessed on the basis of coefficient of variation of individual traits. Average intracluster distance was calculated using the following formula.

D^2 is defined as:

$$D^2 = \sum_{i=1}^{k} d_i^2 = (Yi^1 - Yi^m)^2, (1 \pm m)$$

where Yi^1 and Yi^m are uncorrelated means of the 1^{th} and m^{th} clones for the i^{th} character. Average intercluster distance was calculated by taking each cluster and its distance from another cluster. Cluster diagram was drawn with the help of square root of D^2 values showing relationships within and between clusters (Fig. 2).

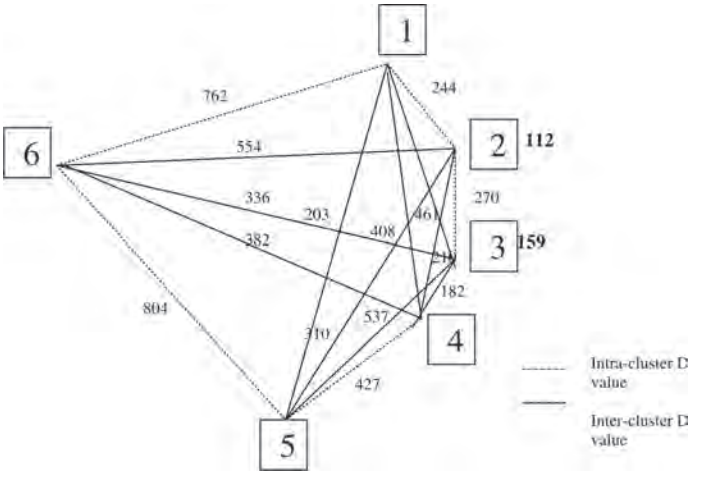

Fig 2. Cluster diagram showing relationship between different clusters

RESULTS AND DISCUSSION

D^2 analysis

Analysis of Variance showed highly significant differences between genotypes for each of the twenty three characters studied. From the present study, all the 28 genotypes can be grouped into six clusters (Table 2).

Maximum number of genotypes are included in Cluster II (14 genotypes), viz., Red banana, Vellakappa, Robusta, Padalamurian, Chengazhikodan, Kaliethan, Vellayani Nendran, Attu Nendran, Mysore Ethan, Manjeri Nendran, Changanasseri Nendran, Monthan, Peyan and Ilavazha. This is followed by Cluster I (9 genotypes), namely, Palode Palayankodan, PKNNR, Chandra Bale, Pisang Ceylon, Mottapoovan, Kadali, Pisang Lilin, Njalipoovan and Kunnan. Cluster IV (Quintal banana), Cluster V (Vellapalayankodan) and Cluster VI (Koonoor Ethan) formed individual clusters. Cluster III contained only two genotypes, namely, Myndoli and Zanzibar. Ecotypes occurring in Cluster I were Palode Palayankodan, PKNNR, Mottapoovan, Chandra Bale and Pisang Ceylon. Another member of Palayankodan, Vellapalayankodan, fell under Cluster V.

Table 2. Group constellations in twenty eight ecotypes of banana

Cluster	No. of ecotypes	Ecotype/s
C_1	9	Palode Palayankodan, PKNNR, Chandra Bale, Pisang Ceylon, Mottapoovan, Kadali, Pisang Lilin, Njalipoovan, Kunnan
C_2	14	Red banana, Vellakappa, Robusta, Padalamurian, Chengazhikodan, Kaliethan, Myndoli, Attu Nendran, Mysore ethan, Manjeri Nendran, Changanasseri Nendran, Monthan, Peyan, Ilavazha
C_3	2	Vellayani Nendran, Zanzibar
C_4	1	Quintal banana
C_5	1	Vellapalayankodan
C_6	1	Koonoor Ethan

Inter and intra cluster distance among the six clusters was variable (Table 3). Intercluster D values express diversification among groups of genotypes resembling each other, based on 23 characters under the study, while intra-cluster D values express the magnitude of divergence between clones within a cluster. Intra-cluster genetic distance, D values, ranged from 96 (Cluster I) to 159 (Cluster III) indicating wide divergence. Maximum inter-cluster distance was observed between Cluster VI and Cluster V (803), followed by Cluster VI and Cluster I (762), while, minimum inter-cluster distance D (182) was between Cluster IV and Cluster III. Intercluster distances and their mutual relationship are depicted in Fig. 2. Intercluster distance is higher than intracluster distance in all the cases indicating greater divergence of genotypes between clusters. Similar relationships were also observed by Valsalakumari *et al* (1985) in banana and Balasubramanyan *et al* (2009) in mango.

Table 3. Estimation of average intra and inter cluster D for six clusters constructed from 28 ecotypes of banana

	Cluster Number					
	C_1	C_2	C_3	C_4	C_5	C_6
C_1	**96**	244	460	408	202	762
C_2		**112**	269	210	310	553
C_3			**159**	182	536	336
C_4				**0**	427	382
C_5					**0**	803
C_6						**0**

Bold figures in diagonals are intra-cluster distances

Contribution of individual character towards divergence (Table 4) revealed that maximum contribution to total differences was made by number of fingers per hand (15.48%), shelf life of fruit (14.88%), number of fingers per bunch (13.69%) and finger girth (8.93%). Lowest contribution of frequency was recorded in leaf width, TSS, pseudostem girth, number of leaves per plant, number of suckers per plant, hand weight, pulp/peel ratio, fruit volume and level of acidity.

Table 4. Contribution of various characters to divergence

S. No.	Character	Frequency	% Contribution
1.	Plant height (cm)	5	2.98
2.	Psendostem girth (cm)	2	1.19
3.	No. of leaves	2	1.19
4.	Leaf length (cm)	1	0.60
5.	Leaf width (cm)	4	2.38
6.	No. of suckers	2	1.19
7.	Crop duration (days)	3	1.79
8.	Bunch weight (kg)	11	6.55
9.	No. of hands per bunch	13	7.74
10.	No. of fingers per bunch	23	13.69
11.	No. of fingers per hand	26	15.48
12.	Finger length (cm)	3	1.79
13.	Finger girth (cm)	15	8.93
14.	Finger weight (g)	5	2.98
15.	Bunch length (cm)	5	2.98
16.	Hand weight (kg)	2	1.19
17.	Pulp-peel ratio	2	1.19
18.	Fruit volume (cc)	2	1.19
19.	TSS (°Brix)	1	0.60
20.	Acidity (%)	2	1.19
21.	Total sugars (%)	7	4.17
22.	Sugar /acid ratio	7	4.17
23.	Shelf life (days)	25	14.88

RAPD analysis

Six random primers that were used for amplification (OPA-01, OPA-03, OPA-13, OPB-04, OPB-10 and OPB-12) gave scorable polymorphic bands. Number of

polymorphic and monomorphic bands obtained from these primers are presented in Table 5. Number of bands produced by each primer varied with genotype. The primers yielded 46 scorable bands with an average of 7.66 bands per primer. Nucleotide sequence of the above - mentioned primers is presented in Table 6. A total of 46 bands were analyzed and a dendrogram was constructed (Fig. 1).

From the dendrogram, at a distance of 0.20 it is seen that diploid groups were different from triploid groups. Among the diploids, AB genomic clones, namely, Kunnan and Njalipoovan were found to be grouped together. AB genotypes are different from the BB genotype. Kadali and Pisang Lilin (belonging to AA group) Ilavazha (of BB group) formed three separate groups, at a distance of 0.20. Ecotypes belonging to AA genotype formed a single cluster at a distance of 0.25. In D^2 analysis too, ecotypes belonging to AA group fell under the same cluster. Ilavazha (BB) was different from both AA and AB genotypes with respect to molecular profile. Ilavazha (BB group) formed a single cluster and also had maximum genetic divergence among diploid clones. Ilavazha is different in geographical origin and is morphologically too different from other diploid clones. In D^2 analysis of diploid clones, Kadali, Pisang Lilin, Njalipoovan and Kunnan formed a cluster, while, Ilavazha formed a separate cluster.

Among triploids, clones belonging to AAA group were different from AAB or ABB as seen in the dendrogram.

Table 5. Number of polymorphic and monomorphic bands obtained with different primers

Primer	Number of polymorphic bands	Number of monomorphic bands
OPA-01	9	-
OPA-03	8	-
OPA-13	5	1
OPB-04	9	-
OPB-10	9	1
OPB-12	6	1
Total	46	3

Table 6. Nucleotide sequence of promising primers and number of informative RAPD markers

Primers	Sequence	Number of informative RAPD markers
OPA-01	5'CAGGCCCTTC3'	9
OPA-03	5'AGTCAGCCAC3'	8
OPA-13	5'CAGCACCCAC3'	5
OPB-04	5'GGACTGGAGT3'	9
OPB-10	5'CTGCTGGGAC3'	9
OPB-12	5'CCTTGACGCA3'	6

Vellakappa and Red banana, both belonging to the AAA group, grouped together. In D^2 analysis also, these two clones clustered together. Shanmugavelu *et al* (1992) reported that Vellakappa was probably a mutant of Red banana, as these two naturally resembled each other in several aspects. Robusta, falling under AAA group formed a separate cluster in the dendrogram. This might be due to the dwarf stature of Robusta, which is a distinct character of the Cavendish group. However, in D^2 analysis, all three clones belonging to the AAA genome (Red banana, Vellakappa and Robusta) fell under a single cluster. Rekha *et al* (2001) also observed that Robusta did not group together in the dendrogram with other clones belonging to AAA genome, namely, Red banana, Grand Naine and Dwarf Cavendish.

In the present study, eight out of 12 Nendran ecotypes belonging to AAB genome grouped together with respect to molecular profile. Ecotypes like Chengazhikodan, Myndoli, Kaliethan, Vellayani Nendran, Zanzibar Mysore Ethan, Changanasseri Nendran and Manjeri Nendran formed a single group D^2. Koonoor Ethan and Attu Nendran formed independent clusters. The same trend was seen in RAPD profiles too. Simi (2001) reported Manjeri Nendran, Myndoli, Chengazhikodan, Attu Nendran, Changanasseri Nendran and Mysore Ethan as falling under the same cluster. Koonoor Ethan morphologically varied from the rest of Nendran ecotypes and was also of a geographically different origin. Fruit characters like length, girth and weight of finger, shelf life and TSS in this clone were higher, compared to other Nendran ecotypes. Padalamurian and Quintal banana grouped together at a distance of 0.25. Quintal banana, considered Giant Plantain, recorded higher plant height, pseudostem girth, days from flowering to harvest, crop duration and bunch weight. Hence, in morphological clustering, it is under a different cluster from Padalamurian, although the two fell under the same cluster as per RAPD analysis. From RAPD profiles, among the six Palayankodan ecotypes, five clones (Mottapoovan, Pisang Ceylon, PKNNR, Chandra Bale and Palode Palayankodan) are grouped together, the exception being Vellapalayankodan. In D^2 analysis too, Palode Palayankodan, Mottapoovan, PKNNR, Pisang Ceylon and Chandra Bale formed a single cluster while Vellapalayankodan formed a separate cluster. Vellapalayankodan belonging to this particular clone is unique, characterized by robust growth characters, with higher value for crop duration, pseudostem girth, plant height, number of fingers per bunch, bunch length, number of

suckers per plant, number of hands per bunch, finger length, sugar/acid ratio and shelf life of fruit. It has an ashy-white fruit skin which is uncommon in Palayankodan ecotypes. This result indicated that including cultivars with desirable characters, and traits such as disease resistance and high inter-cluster distance, would result in a highly segregating generation in breeding programmes. In selecting cultivars for hybridization, considerable care should be taken to select specific clusters and specific cultivars from selected clusters. Our study showed that cultivars belonging to the same genomic group was highly variable, as, these belonged to different clusters.

In D^2 analysis, Monthan and Peyan came under a single cluster and were similar in 21 characters out of 31. Clones belonging to ABB genomic group (Monthan and Peyan) at a distance of 0.20 formed an independent cluster and were different from other triploid genomic clones (AAA or AAB). In clustering based on D^2 analysis, Monthan and Peyan were found to belong to a single group. However, as per Rekha *et al* (2001) found that all the cultivars of ABB group (Monthan, Cuba, Muthia, Karpooravally and Klue Teparod) came under a single cluster.

The above molecular characterization tallied with association of genotypes based on genomic classification of Simmonds. Some aspects needed further investigation as there was confusion regarding grouping of cultivars such as Quintal banana, Vellapalayankodan, Myndoli, Attu Nendran, Koonoor Ethan, Robusta and Ilavazha. These clones formed separate clusters which might be due to less number of decamer primers used in this study.

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