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A new image of plantain diversity assessed by SSR, AFLP and MSAP markers

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Abstract

Using both SSR and AFLP markers, the genetic diversity of 30 plantains constituting a representative sample of the phenotypic diversity was assessed. The results confirmed a very narrow genetic base of this cultivar group. SSR and AFLP data support the hypothesis that these cultivars may have arisen from vegetative multiplication of a single seed. MSAP were used to survey cytosine methylation status at CCGG sites in order to obtain an alternative source of diversity data. A higher degree of polymorphism was revealed allowing the classification of the samples into three clusters. No correlation was observed between the phenotypic classification and methylation diversity. Implications for breeding programs are discussed.

Abbreviations: AFLP – Amplified Fragment Length Polymorphism; MSAP – Methylation Sensitive Amplification Polymorphism; SSR – Simple Sequence Repeat; RFLP – Restriction Fragment Length Polymorphism; RAPD – Random Amplified Polymorphic DNA; CARBAP – Centre Africain de Recherches sur Bananiers et Plantains; CTAB – Cetyltrimethylammoniumbromide.

Introduction

Banana is one of the most important tropical and subtropical food crops. Cultivated bananas are mostly triploid, highly sterile, parthenocarpic and originate from two wild diploid species noted the A and B genome for *Musa acuminata* Colla and *M. balbisana* Colla, respectively (Simmonds & Shepherd, 1955).

Production of bananas is divided into two major groups, sweet or dessert bananas which are produced for both local consumption and export, and cooking bananas which are mostly produced for local consumption and are of major economic importance for tropical and sub-tropical regions of Africa, South and Central America, Asia and Pacific areas. Among the varieties of cooking bananas, the plantain subgroup (AAB) is the most appreciated for organoleptic, cooking and agronomic qualities. Genetic improvement effort focuses on resistance to black sigatoka disease, to which all plantain landraces are highly susceptible. The breeding strategy is based on crosses between partially fertile triploid plantain with a wild or cultivated diploid donor of resistance. This strategy requires a better knowledge of genetic diversity to improve the choice of both diploid and plantain parents.

Genetic diversity in banana genomes have been assessed using different molecular markers. Wild diploid *M. acuminata* species are classified into four major groups i.e., *banksii*, *zebrina*, *malaccensis* and *burmannica/burmanicoides* based on microsatellite and RFLP markers (Carreel et al., 1994; Grapin et al., 1998).

Relationships among diploid and triploid cultivars with wild relative species have also been investigated using nuclear and cytoplasmic

markers leading to identification of Cavendish and Gros-Michel putative diploid parents (Lanaud, 1999) and to strong genetic relationships between wild and cultivated clones (Grapin et al., 1998).

To date, little is known regarding the genetic diversity within the plantain subgroup. The most common hypothesis is that this group is composed of very similar genotype, many of which arose as mutant 'sports'. However, important morphological variation is observed amongst plantains landraces, particularly in inflorescence characters, plant size and several other characters which are commonly used for classifying germplasm (Tezenas du Montcel, De Langhe & Swennen, 1983).

Genotypic and phenotypic comparative analysis performed using RAPD and AFLP techniques (Crouch et al., 2000; Ude et al., 2003) concluded that no strong correlation exists between genetic diversity and phenotypic characters. This highlights the fact that classification based on agro-morphological characters, if used for collection management, is not suitable for maximizing genetic diversity in collections or for choosing parents in breeding strategies.

DNA methylation plays an essential role in regulating plant development through its influence on gene transcription, and is involved in a number of specific biological processes such as gene silencing or mobile element control (Finnegan, 1996). An AFLP-based technique for surveying cytosine methylation at CCGG sites has been developed (Reyna-Lopez, Simpson & Ruiz-Herrera, 1997; Fraga and Esteller, 2002). These methylation-based molecular markers are different from others in terms of genetics, transmission of characters, and the evolution/mutation process (Xiong et al., 1999; Cervera, Ruize-García & Martinez-Zapater, 2002). These markers can target different genomic parts and help in understanding epigenetic processes which could be involved in phenotypic diversity of plantain landraces.

Objectives of the present study were:

- to assess genetic diversity inside the plantain subgroup using reliable molecular markers as SSR and AFLP
- to assess if epigenetic molecular characters could be useful for characterization of plantain germplasm.

Material and methods

Plant material

A sample of 30 of the 132 plantain landraces issued from the CARBAP collection representing the phenotypic diversity was used (Table 1). Sixteen accessions are issued from the list of 41 described in Musalogue (Daniells et al., 2001) as representatives of the diversity of plantain landraces. Accessions more recently introduced and characterized at CARBAP were chosen to enlarge the sample. A well identified mutant pair was added, Mbirinyong and Mbirinyong green mutant. Five accessions were added in order to enlarge size diversity of Giant, True Horn and French Horn types, respectively, Gui and Congo 2, Nothing but green and Nothing but red, and Penkelon Noir. Ihitisim, Daluyao, Bungaoisan, Douala, Mbotoko vert, Niangafelo and Madre del Platano were used due to their particular bunch or cooking traits. DNA was extracted using a modified CTAB method (Baurens et al., 2004).

Microsatellites

Analysing 9 SSR loci on 59 diploid *M. acuminata* genotypes, Grapin et al. (1998), revealed a number of alleles by locus, ranging from 4 to 12, with an average of 8. The structure of the genetic diversity obtained was in agreement with the organization of the subspecies previously described. These 9 microsatellite loci (Table 2) were used in the present study to assess genetic diversity in plantain. Amplification protocol was used as detailed by Lagoda et al. (1998).

MSAP

The principle of restriction activity of the two isoschizomers *Hpa*II and *Msp*I detailed elsewhere (Xiong et al., 1999; Fraga & Esteller, 2002) is based on different sensitivity to methylation of their recognition site CCGG: *Msp*I cleaves non-methylated (CCGG) and methylated ($C^{5m}CGG$) sites on the internal cytosine, whereas *Hpa*II will cleaves only the non-methylated site (CCGG). Comparison of DNA amplification patterns obtained from *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I restricted DNAs therefore provides information on the methylation status of the internal cytosine of the CCGG recognition

Table 1. List of plantain landraces used in the study showing bunch type, plant size and color of the plants

	Name	Bunch type	Plant size	Color
1	CONGO 2	French	Giant	Green/Red
2	FRENCH SOMBRE*	French	Medium	Dark green
3	FRENCH CLAIR*	French	Medium	Green
4	BUNGAOISAN	French	Medium	Green
5	FRENCH ROUGE*	French	Giant	Red/Black
6	KELONG MEKINTU*	French	Medium	Green
7	MOTO MO LIKO*	French	Medium	White chimeric
8	ELAT*	French	Medium	Green
9	MBOTOKO VERT	French	Medium	Green/Red
10	MESSIATSO*	French	Medium	Green/Red
11	MADRE DEL PLATANO	French	Medium	Green
12	GUI	French	Giant	Green
13	NJOCK KON*	French	Dwarf	Green
14	RED YADE*	French	Dwarf	Red
15	BATARD*	French horn	Medium	Green
16	3 VERT*	French horn	Medium	Green
17	BIG EBANGA*	False horn	Giant	Green
18	DOUALA	False horn	Medium	Green
19	NIANGAFELO	False horn	Medium	Green
20	MBOUROUKOU No1*	False horn	Medium	Green/yellow
21	MOTO EBANGA*	False horn	Medium	White chimeric
22	MBIRINYONG	False horn	Medium	Red
23	MBIRINYONG GREEN MUTANT	False horn	Medium	Green
24	PENKELON NOIR	French horn	Medium	Green/Red
25	IHITISIM	True horn	Giant	Green/Red
26	DALUYAO	True horn	Medium	Green
27	2 HAND PLANTY*	True horn	Medium	Green
28	IGIHOBE*	True horn	Medium	Green
29	NOTHING BUT GREEN	True horn	Giant	Green
30	NOTHING BUT RED	True horn	Giant	Green/Red

* Indicate accessions described in Musalogue.

sites. The protocol used for the methylation-sensitive amplified polymorphism (MSAP) technique was adapted from Reyna-López, Simpson & Ruiz-Herrera, (1997), and detailed by Baurens et al. (2003). Briefly, for each sample of plant material, 500 ng of DNA was cleaved with *Eco*RI, then divided into two aliquots to be treated one with either *Hpa*II or *Msp*I. The resulting DNA fragments were ligated with linkers, then PCR-amplified using primers carrying one additional 3′ nucleotide. The resulting PCR products were used as templates for a labeled selective amplification with 8 primer pairs (E-AC/HM-ACA, E-AC/HM-ACC, E-AC/HM-ACG, E-AC/HM-ACT, E-AG/HM-ACA, E-AG/HM-ACC, E-AG/HM-ACG, E-AG/HM-ACT). *HM-p*rimers were end-labeled with 50 μCi of γ^{33}P-ATP. The amplified DNA fragments were separated on polyacrylamide gels and autoradiographed for 2–4 days. This procedure was repeated and each DNA sample was analyzed twice.

Data scoring and analysis

For all types of markers, bands were scored visually on autoradiography.

Microsatellites
SSR patterns were scored according to the number of alleles at each locus.

Table 2. Microsatellite locus polymorphism in plantain

Primer Name*	Polymorphic	Number of alleles
25–26	no	3
33–34	no	3
103–104	no	2
91–92	no	2
93–94	no	3
35–36	no	2
125–126	yes	3
129–130	no	2
101–102	no	2

*Numbers in primer name column refer to primer pairs defined for microsatellite locus amplification (Lagoda et al., 1998). AGMI 25–26, 33–34, 103–104, 91–92, 93–94, 35–36, 125–126, 129–130, 101–102 refer to microsatellite locus available as sequence data in nucleotide international database (http://www.ncbi.nlm.nih.gov) through accession number X87262, X87265, X87261, X90747, X87258, X87263, Z85959, Z85971, X87260. Number of alleles corresponds to the number of microsatellite alleles detected in the whole plantain sample.

MSAP

Amplified bands in both *Hpa*II and *Msp*I lanes indicate unmethylated sites (coded as 1) while the bands found only in the *Msp*I lane reflect internally methylated C5mCGG site (coded as 0). Dissimilarity matrix were calculated with DARWin 4.2 software (Perrier, Flori & Bonnot, 2003) using the Dice index: $D_{i,j} = b + c/2a + (b + c)$ were a is the number of markers present in i and j, b is the number of markers present in i and absent in j and c is the number of markers absent in i and present in j. Distance matrix were then subjected to Neighbor Joining analysis (Saitou and Nei, 1987) to construct a dendrogram.

AFLP

As described previously, MSAP allows for assaying cytosine methylation at CCGG sites. Data from MSAP amplification can also be used as a source of AFLP markers for the *Eco*RI–*Msp*I profiles.

Results

SSR

A total of 22 alleles were scored from 9 loci, with an average number of 2.4 alleles per loci. Only one polymorphic locus was observed. Table 2 shows the number of allele at each microsatellite locus. On the 30 plantain landraces surveyed, 27 were identical for the 9 loci. Locus 125–126 shows that plantains Kelong Mekintu, Penkelon Noir and Mbouroukou No1 exhibit an additional allele 15 nucleotide larger than other bands. This result supports the hypothesis of a unique origin (i.e., a unique meiotic event) of plantain landraces and total absence of sexuality in the evolution of plantains. The additional allele observed in these samples can be assigned to mutation process only, because meiotic event involving highly heterozygous polyploid would have produced allele shuffle in the other loci. The appearance of a new allele through mitotic multiplication is very unlikely and if it had occurred, it would involve very close allele size (increase or decrease of few repetitions of the microsatellite motif). In our result an allele size difference of 15 nucleotides is too large to be explained this way. Thus, the most probable explanation is that a mutation occurred in the annealing site of SSR primers leading to null allele for the accessions which do not exhibit the allele.

Considering that plantains are AAB triploids, the majority of loci are highly heterozygous. Loci with three alleles, are representative of the diversity of *Musa* species and sub-species involved in the genomic composition of plantains and could reveal the nature of the original cross from which all plantain landraces are issued. As four microsatellite loci had three alleles, the origin of plantain can be assigned to a cross involving a highly heterozygous *M. acuminata* parent as donor of a non reduced gamete and a diploid *M. balbisiana*.

CCGG site methylation analysis

Of 633 CCGG site surveyed, 582 sites remained unmethylated, 35 sites arose from methylated sites in all studied plantain landraces (5.5%), 16 markers showed methylation polymorphism within the plantain sample (2.5%) and one was considered as methylation insensitive polymorphism as defined by Cervera, Ruiz-García and Martineze-Zapter, (2002) (Figure 1). Using data generated from these 16 markers, genetic distances were calculated (Table 3). On the NJ tree, three clusters appear despite low bootstrap values (Figure 2) with no correlation with major phenotypic traits listed in Table 1. Inside cluster III, a subcluster is

differentiated inside of which Elat and Madre del Platano cannot be distinguished. Two other groups of indistinguishable plantain appear: Igihobe – Congo2, and Penkelon noir – Kelong mekintu – Mbouroukou No1. Finally, Mbirinyong cannot be distinguished from Mbirinyong green mutant as expected for somaclonal variants issued one from the other. All other cultivars have unique methylation fingerprints.

AFLP

From 633 bands provided by 8 primer pairs, only one methylation insensitive polymorphism level appears (0.2%). This marker reflects genetic diversity. Samples No1, 12, 13, 21, 25, 26, 27, 28, 29 are different from the others but the same as each other. All plantains are grouped into two distinct fingerprints. This supports results obtained with microsatellite analysis which highlight the fact that plantain landraces genetic diversity is very narrow.

Discussion

Genetic relationships within plantain landraces have been assessed using 9 microsatellite loci showing very low polymorphism within plantain group still these loci have been previously described as highly polymorphic in *Musa* (Grapin et al., 1998). Recently, Creste et al., (2003) characterized diploid and polyploid cultivars from Brazil using the same number of microsatellite loci but the seven plantain landraces and cultivars from Brazil could not be distinguished.

If microsatellites are known to be highly polymorphic, only a small number of loci have been assessed and the genome coverage is low. AFLP analysis is a complementary approach due to the generation of a high number of band levels representing numerous loci. Eight AFLP primer pairs produced 260 polymorphic band levels in *Musa* germplasm (Ude et al., 2002) but in their study, the four representatives of the plantain group were not differentiated.

In our study, we used *Msp*I as the frequent cutter, whereas the original AFLP protocol (Vos et al., 1995) used *Mse*I. *Msp*I and *Mse*I, have different recognition site (TTAA for *Mse*I and CCGG for *Msp*I), which targets sites with different GC content (i.e., AT rich versus GC rich regions). However, CCGG and TTAA sites are both spread throughout the rice genome (Xiong et al., 1999; Ashikawa, 2001). We hypothesize, consequently, that AT rich regions versus GC rich region should be equivalent for molecular markers analysis in term of genome coverage.

Genetic diversity in our study was revealed by only a single microsatellite locus (i.e., primer pair 125/126). Our results based on SSR clearly support the hypothesis that plantain group is based on very narrow genetic basis. This is in contradiction with the results obtained with a sample of 76 plantains by Crouch et al. 2000 using RAPDs. However the reliability of this type of marker has been controversially discussed (Penner et al., 1993). Ude et al. (2003) observed genetic diversity using AFLP, with a sample of 25 plantains from both Nigeria and Cameroon but highlight some surprising results concerning large genetic distance (31.7%) between an accession and its somaclonal variant and between two accessions considered previously as being synonyms (52%).

Finally, the origin of the 30 plantain cultivars used in this study is supposed to be a single plant which has been vegetatively multiplicated. During this multiplication phase and selection, point mutations or epigenetic mutations occurred and have been stabilized by the vegetative multiplication itself to produce the phenotypic differences observed. In this study, we observed that methylation status at CCGG sites of the genome is different amongst cultivars. Cytosine methylation survey at CCGG site is a useful molecular tool for highlighting differences inside the plantain subgroup.

Polymorphism was detected with the methylation survey was almost non-existent with AFLP markers. It has been shown that methylation status of CCGG site in plants can be stable due to the active process of methylation maintenance and is strictly controlled during development (Finnegan, 1996). In our study all DNA samples were obtained from fully expanded leaves from field grown mature plants and methylation markers revealed using MSAP are stable in a particular organ even through vegetative multiplication (Peraza-Echeverria, Herrera-Valencia & James-Kay, 2001). Moreover, the methylation status of CCGG sites is stable in some rice cultivars even through sexual multiplication

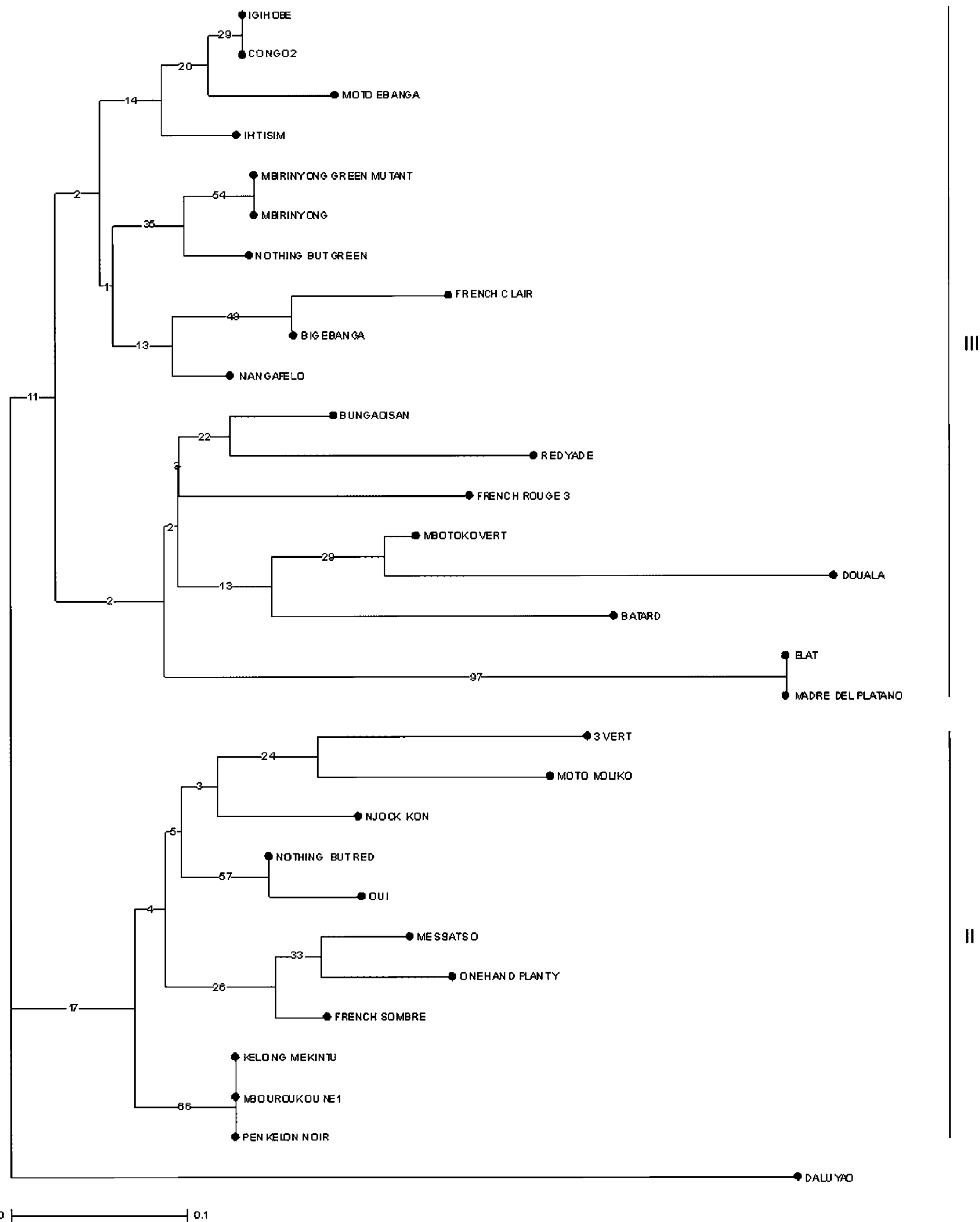

Figure 1. MSAP analysis of 8 plantains using Eco-AC/HM-ACC. Lanes 1–8 and 9–16 corresponds to the same plantain samples (No. 12–20) treated with *Hpa*II and *Msp*I respectively. Unmethylated sites corresponds to presence of bands in both *Hpa*II and *Msp*I subsets. Methylated sites are identified by the presence of band in *Msp*I subset but not in *Hpa*II subset (labelled with m). Methylation polymorphism amongst plantains landraces are indicated by a white arrow. Band level showing methylation insensitive polymorphism (common presence or absence in *Hpa*II and MspI subsets) is indicated (*).

Table 3. Dissimilarity matrix of pair wise distances based on Dice index

Ind.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
2	0.375																												
3	0.188	0.438																											
4	0.313	0.313	0.375																										
5	0.438	0.313	0.500	0.250																									
6	0.250	0.125	0.313	0.188	0.438																								
7	0.375	0.375	0.313	0.313	0.563	0.250																							
8	0.250	0.500	0.188	0.313	0.438	0.375	0.375																						
9	0.313	0.438	0.250	0.125	0.250	0.313	0.438	0.313																					
10	0.375	0.125	0.438	0.313	0.438	0.125	0.250	0.500	0.438																				
11	0.250	0.500	0.188	0.313	0.438	0.375	0.375	0.000	0.313	0.500																			
12	0.313	0.313	0.375	0.375	0.500	0.313	0.188	0.438	0.500	0.313	0.438																		
13	0.375	0.375	0.563	0.313	0.563	0.250	0.375	0.625	0.438	0.375	0.625	0.313																	
14	0.500	0.375	0.563	0.188	0.313	0.375	0.500	0.375	0.313	0.375	0.375	0.438	0.375																
15	0.313	0.563	0.375	0.375	0.500	0.438	0.688	0.313	0.250	0.563	0.313	0.625	0.438	0.313															
16	0.563	0.438	0.625	0.250	0.375	0.313	0.313	0.563	0.375	0.313	0.563	0.500	0.313	0.438	0.625														
17	0.188	0.313	0.125	0.250	0.375	0.188	0.438	0.313	0.125	0.313	0.313	0.500	0.438	0.438	0.250	0.500													
18	0.500	0.625	0.438	0.313	0.313	0.500	0.625	0.375	0.188	0.625	0.375	0.688	0.625	0.375	0.313	0.438	0.313												
19	0.125	0.250	0.188	0.188	0.313	0.125	0.375	0.250	0.188	0.250	0.250	0.438	0.375	0.375	0.313	0.438	0.063	0.375											
20	0.250	0.125	0.313	0.188	0.438	0.000	0.250	0.375	0.313	0.125	0.375	0.313	0.250	0.375	0.438	0.313	0.188	0.500	0.125										
21	0.125	0.375	0.313	0.188	0.313	0.250	0.500	0.250	0.188	0.375	0.250	0.438	0.375	0.375	0.188	0.438	0.188	0.375	0.125	0.250									
22	0.188	0.188	0.250	0.250	0.250	0.188	0.438	0.313	0.250	0.188	0.313	0.375	0.438	0.313	0.375	0.500	0.125	0.438	0.063	0.188	0.188								
23	0.188	0.188	0.250	0.250	0.250	0.188	0.438	0.313	0.250	0.188	0.313	0.375	0.438	0.313	0.375	0.500	0.125	0.438	0.063	0.188	0.188	0.000							
24	0.250	0.125	0.313	0.188	0.438	0.000	0.250	0.375	0.313	0.125	0.375	0.313	0.250	0.375	0.438	0.313	0.188	0.500	0.125	0.000	0.250	0.188	0.188						
25	0.125	0.375	0.313	0.188	0.313	0.250	0.375	0.375	0.188	0.375	0.375	0.313	0.250	0.375	0.313	0.438	0.188	0.375	0.125	0.250	0.125	0.188	0.188	0.250					
26	0.375	0.625	0.563	0.563	0.563	0.500	0.625	0.500	0.563	0.500	0.500	0.688	0.500	0.625	0.438	0.438	0.438	0.375	0.375	0.500	0.375	0.438	0.438	0.500	0.375				
27	0.375	0.125	0.563	0.438	0.438	0.250	0.375	0.625	0.563	0.125	0.625	0.313	0.375	0.500	0.563	0.438	0.438	0.750	0.375	0.250	0.375	0.313	0.313	0.250	0.375	0.500			
28	0.063	0.313	0.250	0.250	0.375	0.188	0.438	0.313	0.250	0.313	0.313	0.375	0.313	0.438	0.250	0.500	0.125	0.438	0.063	0.188	0.063	0.125	0.125	0.188	0.063	0.313	0.313		
29	0.125	0.250	0.313	0.313	0.313	0.250	0.500	0.375	0.313	0.250	0.375	0.313	0.375	0.375	0.313	0.563	0.188	0.500	0.125	0.250	0.125	0.063	0.063	0.250	0.125	0.375	0.250	0.063	
30	0.313	0.188	0.500	0.250	0.375	0.188	0.313	0.563	0.375	0.188	0.563	0.125	0.188	0.313	0.500	0.375	0.375	0.563	0.313	0.188	0.313	0.250	0.250	0.188	0.188	0.563	0.188	0.250	0.188

Distances were computed with DARWin 4 software. Sample numbers correspondences are listed in Table 1.

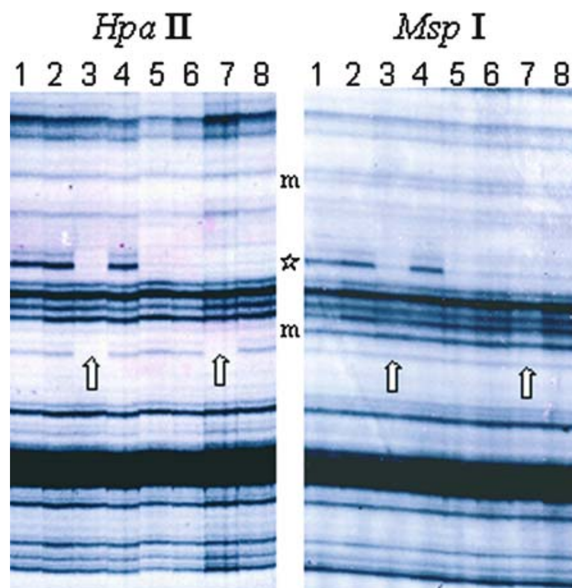

Figure 2. Dendrogram constructed with the NJ tree method (Saitou & Nei, 1987), based on a dissimilarity matrix (Table 3) calculated with the Dice index. Methylation data were used to create the data matrix. Three clusters I, II and III are identified.

(Xiong et al., 1999). Thus it is very unlikely that methylation differences observed in this study are issued from a labile process.

However, as no strong correlation exists between phenotypic major traits and genotypic (Crouch et al., 2000) and methylation diversity in plantain, the question of predictability of characters through crosses involving plantains as parents is still unresolved. Even under the hypothesis of methylation–phenotype correlation, evolution, transmission and control process of methylation is unclear and different from random mutation process (Cervera et al., 2002).

Thus, it seems that parents for plantain improvement programs should be carefully chosen, not only based on phenotypic diversity with the hope of maximizing heterozygosity in the progeny, but also on other criteria to be developed as transmission and prediction of epigenetic characters is unknown.

Future research of this study will include survey of transmission of methylation in progenies of banana involving plantain parents.

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