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ORIGINAL PAPER

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PCR-RFLP of the ribosomal DNA internal transcribed spacers (ITS) provides markers for the A and B genomes in *Musa* L.

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Abstract *Musa acuminata* Colla (AA genomes) and *Musa balbisiana* Colla (BB genomes) are the diploid ancestors of modern bananas that are mostly diploid or triploid cultivars with various combinations of the A and B genomes, including AA, AAA, BB, AAB and ABB. The objective of this study was to identify molecular markers that will facilitate discrimination of the A and B genomes, based on restriction-site variations in the internal transcribed spacers (ITS) of the nuclear ribosomal RNA genes. The ITS regions of seven *M. acuminata* and five *M. balbisiana* accessions were each amplified by PCR using specific primers. All accessions produced a 700-bp fragment that is equivalent in size to the ITS of most plants. This fragment was then digested with ten restriction enzymes (*Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Hpa*II, *Msp*I, *Rsa*I, *Sau*3AI and *Taq*I) and fractionated in 2% agarose gels, stained with ethidium bromide and visualized under UV light. The *Rsa*I digest revealed a single 530-bp fragment unique to the A genome and two fragments of 350-bp and 180-bp that were specific to the B genome. A further 56 accessions representing AA, AAA, AAB, AB and ABB cultivars, and synthetic hybrids, were amplified and screened with *Rsa*I. All accessions with an exclusively A genome showed only the 530-bp fragment, while accessions having only the B-genome lacked the 530-bp fragment but had the 350-bp and 180-bp fragments. Interspecific cultivars possessed all three fragments. The staining intensity of the B-genome markers increased with the number of B-genome complements. These markers can be used to determine the genome constitution of *Musa* accessions and hybrids at the nursery stage, and, therefore, greatly facilitate genome classification in *Musa* breeding.

Keywords *Musa* · Genome markers · Internal transcribed spacers (ITS) · PCR-RFLP

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Introduction

The classification of banana genotypes is genome dependent and is based on their morphological similarity to *Musa acuminate* Colla and *Musa balbisiana* Colla (Cheesman 1947; Simmonds and Shepherd 1955). The Simmonds-Shepherd classification scheme uses the letter 'A' to designate the haploid chromosome set of *M. acuminata* and the letter 'B' to represent that of *M. balbisiana*. Thus, the export dessert bananas and east African highland bananas are classified as AAA, while the plantains and cooking bananas are AAB and ABB, respectively. Other genome combinations such as AB and AAAB also exist among cultivated bananas.

The Simmonds-Shepherd (1955) system for determining the genome composition of *Musa* cultivars relies primarily on morphological characters. However, a plant's morphology can be affected by the environment, and may require evaluation in different environments over many cropping cycles for a consistent classification of

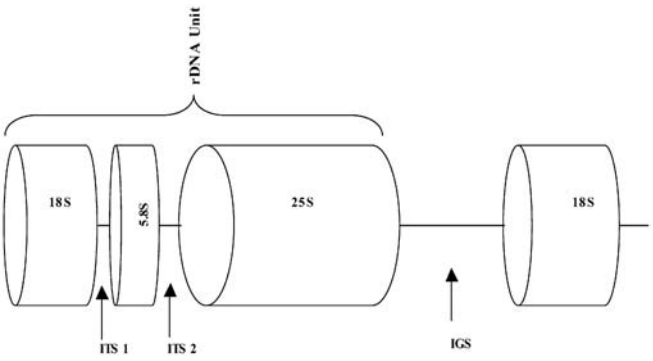

Fig. 1 Generalized ribosomal DNA unit structure. The internal transcribed spacers are denoted as ITS 1 and ITS 2. The intergenic spacer is marked IGS. The 18S, 5.8S and 25S ribosomal RNA genes are indicated

hybrids. In addition, if some characters cannot be scored, the determination cannot be complete (Perrier and du Montcel 1990). Molecular markers that can be used to determine the genome composition of bananas would be advantageous over a system relying only on morphology. Various methods to identify genomes in *Musa* have been reported. Genomic in situ hybridisation (GISH) has been used to identify the genome composition of *Musa* cultivars and hybrid progenies (Osuji et al. 1997; D' Hont et al. 2000). However the GISH technique is time-consuming, complicated and requires a high level of skill, and may not be suitable for high-throughput screening of large breeding populations. Howell et al. (1994) used RAPD markers to classify nine *Musa* genotypes into four groups representing the AA, AAA, AAB and BB genomes. However, some AAB clones clustered with the AAA group suggesting that these markers did not have enough discriminatory power. Recently, Pillay et al. (2000) identified RAPD markers linked to 'A' and 'B' genome-sequences in *Musa.* These markers are useful for determining the genome composition of initial selections from *Musa* breeding populations, but RAPD fragments represent anonymous sequences that may display segregation distortions in mapping experiments (Faure et al. 1993).

The nuclear ribosomal RNA genes (rDNA) which encode the 18S, 5.8S and 26S rRNA subunits (Fig. 1) are organized into multigene families that occur as clusters of tandemly repeated units of 250 to over 20,000 copies per genome (Rogers and Bendich 1987). The rDNA is transcribed as a single unit along with the two spacers, the internal transcribed spacers (ITS) that separate the 5.8S subunit from the 18S and the 26S subunits. Subsequently, the ITS regions are edited from the mature RNA transcript. Sequence variation in the ITS region is quite high and occurs even among closely related clones or species. Therefore, analysis of the ITS for DNA sequence variation has become a powerful method for assessing phylogenetic relationships at the lower taxonomic levels, and have been applied in a large number of plant groups including grasses (Hsiao et al. 1994), cotton (Pillay and Meyers 1999), walnut (Stanford et al. 2000) and sunflower (Clevinger and Panero 2000).

In this study, the domain comprising both ITS regions and the intervening 5.8S rDNA was amplified in polymerase chain reactions (PCR) and analysed for restriction-site differences, with the objective of identifying rDNA markers that are specific for the A and B genomes of *Musa.*

Materials and methods

Plant materials

Sixty eight accessions (Table 1) representing wild diploid *M. acuminata* Colla and *M. balbisiana* Colla species, landraces, and synthetic hybrids were used. Leaf tissue for DNA extraction was collected from plants in the field gene-bank of the International Institute of Tropical Agriculture (IITA) at Onne (4°43' N, 7°01' E) in Southeast Nigeria.

DNA extraction

DNA was extracted according to a modified protocol of Gawel and Jarret (1991). About 5 g of tissue-sample from young unfurled leaves were collected, immediately submerged in liquid nitrogen, and subsequently ground in liquid nitrogen with a mortar and pestle. The ground tissue was added to 15 ml of pre-heated extraction buffer [4% CTAB (hexadecyltrimethylammonium bromide), 100 mM of Tris-HCL (pH 8.0), 1.4 M of NaCl, 20 mM of EDTA and 4 μl/ml of mercaptoethanol] in an oak ridge tube and incubated at 65°C for 30 min. Samples were extracted with 15 ml of chloroform: isoamyl alcohol (24:1, v/v) and centrifuged at 6,000 rpm for 5 min. The upper aqueous phase was transferred to a new tube and extracted as before with chloroform: isoamyl alcohol. The DNA was precipitated by adding a two-thirds vol of ice-cold isopropanol and followed by centrifugation at 6,000 rpm for 5 min. The recovered DNA pellet was dissolved in 600 μl of TE buffer (10 mM of Tris, 1.0 mM of EDTA, pH 8.0) and RNA contamination removed by digestion with 10 μg/ml of RNAse for 30 min at room temperature. The purified DNA was precipitated by adding one-tenth volume of 3 M sodium acetate (NaOAc, pH 6.8), followed by 2 vol of cold ethanol and collected by centrifugation at 6,000 rpm for 5 min. The DNA pellet was washed with 70% ethanol, air-dried briefly and resuspended in 200 μl of TE buffer. DNA concentrations were quantified by measuring absorbance at 260-nm wavelengths using a Pharmacia Gene Quant II spectrophotometer (Pharmacia Biotech, Cambridge, U.K.). An aliquot of the isolated DNA for each sample was diluted to a final concentration of 40 ng/μl in TE buffer for use in the subsequent assays.

PCR-amplification

Two primers, ITS L (Hsiao et al. 1994) and ITS 4 (White et al. 1990), were used for PCR amplification of the ITS region. Primer ITS L (*5'-TCG TAA CAA GGT TTC CGT AGG TG-3'*) is complimentary to the 18S rDNA close to the ITS 1 border, and primer ITS 4 (*5'-TCC TCC GCT TAT TGA TAT GC-3'*) anneals to 26S rDNA near the ITS 2 border. These two primers flank the entire ITS region, which includes the intervening 5.8S subunit.

The amplification reaction was done in a 50-μl vol consisting of 100 ng of sample DNA, 0.5 μM of each forward and reverse primer, 1.5 mM of $MgCl_2$, 200 μM each of dTTP, dCTP, dGTP, dATP, 1 unit of *Taq* polymerase (Advanced Biotechnologies, Surrey, U.K.) in a reaction buffer containing 75 mM of Tris-HCl (pH 8.9) and 20 mM of $(NH_4)_2SO_4$. The thermal cycling protocol consisted of a

Table 1 List of *Musa* accessions, their genome composition, chromosome numbers and origin of plants used in this study

Serial no.	Species/cultivar/hybrid	Genome	Chromosome number	Source/origin
	M. balbisiana clones	BB_w	22	IITA[a], Onne, Nigeria
1	Los Banos	BB_W	22	IITA, Onne, Nigeria
2	Singapuri	BB_W	22	IITA, Onne, Nigeria
3	Tani	BB_w	22	IITA, Onne, Nigeria
4	1–63	BB_W	22	ITC[a], Belgium
5	Cameroon			
	M. acuminata subspecies			
6	Malaccensis	AA_w	22	IITA, Onne, Nigeria
7	Zebrina	AA_w	22	IITA, Onne, Nigeria
8	Banksii	AA_w	22	IITA, Onne, Nigeria
9	Microcarpa	AA_w	22	IITA, Onne, Nigeria
10	Truncata	AA_w	22	IITA, Onne, Nigeria
11	Burmannica	AA_w	22	IITA, Onne, Nigeria
12	Burmannicoides	AA_w	22	IITA, Onne, Nigeria
	AA cultivars			
13	Pisang tongat	AA	22	IITA, Onne, Nigeria
14	Pisang madu	AA	22	IITA, Onne, Nigeria
15	Pisang lilin	AA	22	IITA, Onne, Nigeria
16	Tjau lagada	AA	22	IITA, Onne, Nigeria
17	Heva	AA	22	IITA, Onne, Nigeria
	AAA dessert bananas			
18	Gros Michel	AAA	33	IITA, Onne, Nigeria
19	Highgate	AAA	33	IITA, Onne, Nigeria
20	Giant Cavendish	AAA	33	IITA, Onne, Nigeria
21	Dwarf Cavendish	AAA	33	IITA, Onne, Nigeria
22	Lacatan	AAA	33	IITA, Onne, Nigeria
23	Yagambi Km5	AAA	33	IITA, Onne, Nigeria
24	Red/Green	AAA	33	IITA, Onne, Nigeria
	AAA Highland Bananas			
25	Ingagara	AAA	33	IITA, Onne, Nigeria
26	Mbwazirume	AAA	33	IITA, Onne, Nigeria
27	Makara	AAA	33	IITA, Onne, Nigeria
28	Intokatoke	AAA	33	IITA, Onne, Nigeria
	AAB plantains			
29	Baka	AAB	33	*IITA, Onne, Nigeria
30	Ihitism	AAB	33	IITA, Onne, Nigeria
31	3-Hand Planty	AAB	33	IITA, Onne, Nigeria
32	Agbagba	AAB	33	IITA, Onne, Nigeria
33	Essang	AAB	33	IITA, Onne, Nigeria
34	Abomienu	AAB	33	IITA, Onne, Nigeria
35	Ukom	AAB	33	IITA, Onne, Nigeria
36	Osoaboaso	AAB	33	IITA, Onne, Nigeria
37	Batard	AAB	33	IITA, Onne, Nigeria
38	Nadzia	AAB	33	IITA, Onne, Nigeria
39	Bobby Tanap	AAB	33	IITA, Onne, Nigeria
40	Obino l'Ewai	AAB	33	IITA, Onne, Nigeria
41	Bungaoisan	AAB	33	IITA, Onne, Nigeria
	AAB dessert bananas			
42	Silk	AAB	33	IITA, Onne, Nigeria
43	Pome	AAB	33	IITA, Onne, Nigeria
	AB Ney Poovan type			
44	Kisubi	AB	22	IITA, Onne, Nigeria
45	Kamaramasenge	AB	22	IITA, Onne, Nigeria
	AAAB bananas			
46	Oura da mata	AAAB	44	IITA, Onne, Nigeria
47	Ngem	AAAB	44	IITA, Onne, Nigeria
	ABB cooking bananas			
48	Bluggoe	ABB	33	IITA, Onne, Nigeria
49	Cardaba	ABB	33	IITA, Onne, Nigeria
50	Sabra	ABB	33	IITA, Onne, Nigeria
51	Fougamou	ABB	33	IITA, Onne, Nigeria
52	Pelipita	ABB	33	IITA, Onne, Nigeria
53	Lep Chang Kut	ABB	33	IITA, Onne, Nigeria

Table 1 (continued)

Serial no.	Species/cultivar/hybrid	Genome	Chromosome number	Source/origin
	Synthetic hybrids			
54	1549–7	*OL×*C4	22	IITA, Onne, Nigeria
55	4400–8	*BT×C4	22	IITA, Onne, Nigeria
56	5105–1	*PL×C4	22	IITA, Onne, Nigeria
57	9128–3	*TL×PL	22	IITA, Onne, Nigeria
58	SH3362		22	FHIA[a], Honduras
59	15108–1	4479–1×SH3362	33	
60	14604–35	6930–1×1549–7	33	IITA, Onne, Nigeria
61	6930–1	OL×C4	44	IITA, Onne, Nigeria
62	4479–1	BT×C4	44	IITA, Onne, Nigeria
63	612–74	*BL[a]×C4	44	IITA, Onne, Nigeria
64	FHIA 1		44	FHIA, Honduras
65	FHIA 2		44	FHIA, Honduras
66	FHIA 3		44	FHIA, Honduras
67	FHIA 21		44	FHIA, Honduras
68	IC 2	AAAA	44	IITA, Onne, Nigeria

[a] IITA, International Institute of Tropical Agriculture; IITC, International Network for the Improvement of Banana and Plantain (INIBAP) Transit Centre; FHIA, Fundacion Hondurena de Investigacion Agricola; C4, Calcutta 4; BT, 'Bobby Tanap'; PL, 'Pisang Lilin'; BL, 'Bluggoe'; TL, 'Tjau Lagada'; OL, 'Obino l'Ewai'

single denaturation step of 4 min at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 50°C, 1-min extension at 72°C, with a final 7-min incubation at 72°C. To confirm successful amplification and to determine the size of the amplified fragment, 2 μl of the PCR products were separated by electrophoresis in a 1% agarose gel, stained in 1 μg/ml of ethidium-bromide solution and visualised under UV light. The sizes of the amplified DNA fragments were estimated by comparison to the 1-kb ladder (Life Technologies, Paisley, U.K.) which was loaded in each gel as molecular-weight standards.

Restriction fragment-length polymorphism

Initially the amplified fragments of the *M. acuminata* and *M. balbisiana* accessions (Table 1) were each digested sequentially with the following restriction endonucleases: *Sau*3AI, *Taq*I, *Hae*III, *Hinf*I, *Dde*I, *Rsa*I, *Alu*I, *Msp*I, *Cfo*I and *Hpa*II. Subsequently the amplified fragment of the full sample set (Table 1) was digested only with *Rsa*I. The digested DNA fragments were separated by electrophoresis on 3% agarose gels in TBE buffer (90 mm Tris, 90 mM boric acid, 2.5 μM EDTA, pH 8.3 at 5 V/cm for 1–2 h). The gels were stained and photographed as described above. Fragment sizes were estimated by comparison with a 100 base-pair ladder (Life Technologies, Paisley, UK).

Ploidy determination

The ploidy levels of synthetic hybrids were determined using a Partec Ploidy Analyzer (Partec GmbH, Germany) as described by Pillay et al. (2000).

Results and Discussion

Amplification of the ITS region produced a 700-bp fragment (Fig. 2, top, ITS band) in all the *Musa* accessions used in this study. This size is similar to that reported for other monocotyledonous plants, e.g. 591 bp for rice (Takaiwo et al. 1985), 588 bp for sorghum and 603 bp for *Crithodium monococcum* (Hsiao et al. 1994). Of the ten restriction endonucleases that were used, only *Rsa*I produced a consistent polymorphic banding pattern (Fig. 2, bottom) between *M. acuminata* and *M. balbisiana*. The *Rsa*I digest produced three fragments of 530 bp, 120 bp and 50 bp in all the *M. acuminata* accessions, while four fragments of 350 bp, 180 bp, 120 bp and 50 bp were identified in the *M. balbisiana* accessions. The 530-bp fragment (Fig. 2 fragment A) was present in all cultivars with the 'A' genome and lacking in those with exclusively 'B' genomes. The 350-bp and 180-bp fragments (Fig. 2 fragments B_1 and B_2) were present in all interspecific hybrid cultivars containing the 'B' genome, and lacking in those with exclusively 'A' genomes (Table 2). Hence, the unique 530-bp fragment was diagnostic for the presence of the 'A' genome while the 350- and 180-bp fragments were diagnostic for the presence of the 'B' genome.

The expression of these markers appeared to be quantitative, in that all cultivars possessing two sets of the 'B' genome showed stronger band intensity than did those with a single 'B' genome (Fig. 2, bottom). This may reflect differences in the copy number of different rDNA variants that are present in the cultivars. The integrity of these markers to identify the A and B genomes in a number of accessions including synthetic hybrids of known pedigree was carried out. It was possible, in conjunction with flow cytometry, to deduce the genome composition of all the plants (Table 3).

Banana improvement relies on crossing wild and cultivated diploid clones with triploid landraces to generate tetraploid hybrids having the disease resistance attributes and wider genetic diversity of the diploid parent, but retaining the superior agronomic qualities of the landrace cultivar (Rowe 1981, 1984; Vuylsteke et al. 1993). Since the tetraploid hybrids are capable of setting seeds, they are crossed with improved diploids to produce secondary triploid hybrids (Ortiz et al. 1995). Determination of the genome composition of the hybrids at an

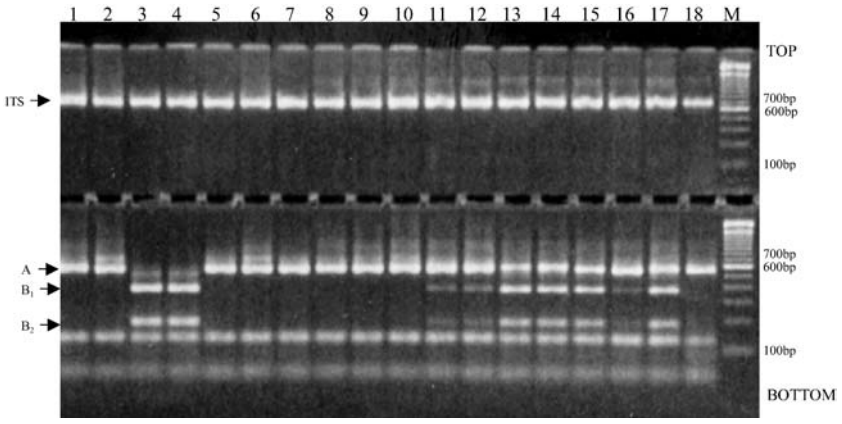

Fig. 2 TOP: fragment size of the ITS region in 18 accessions of *Musa.* BOTTOM: *Rsa*I restriction endonuclease fragment patterns of the internal transcribed spacer (ITS) sequence in *Musa* accessions. Band *A, B_1 and B_2* are diagnostic genome fragments. Accessions and lanes are *M. acuminata* ssp. malaccenssis and ssp. zebrina (AA, *1,2*); *M. balbisiana* 'Singapuri' and 'Los Banos' (BB, *3,4*); 'Pisang lilin', 'Tjau lagada' (AAcv, *5,6*); 'Lacatan', 'Gros Michel', 'Dwarf Cavendish', 'Yagambi KM5', (AAA, *7,8,9,10*); 'Baka', 'Agbagba' (AAB, *11,12*); 'Bluggoe', 'Cardaba', 'Lep Chang Kut' (ABB, *13,14,15*); 6939-1 (AAAB, *16*); 612-74 (AABB, *17*); 'Mbwazirume' (AAA, *18*). *M* indicates molecular-weight-marker lane

Table 2 Internal transcribed spacers PCR-RFLP phenotype of a representative sample of different *Musa* genotypes included in the study

Genotype	Genome group	Ploidy	Diagnostic ITS/*Rsa*I fragments[a]		
			530 bp 'A'	350 bp 'B'	180 bp 'B'
M. balbisiana	BB	2n=22	–	xx	xx
M. acuminata	AA	2n=22	xx	–	–
'Pisang lilin'	AA	2n=22	xx	–	–
'Kisubi'	AB	2n=22	xx	x	x
'Gros Michel'	AAA	2n=33	xx	–	–
'Baka'	AAB	2n=33	xx	x	x
'Bluggoe'	ABB	2n=33	xx	xx	xx

[a] xx indicates presence of marker band at full intensity, x indicates presence of marker band at faint intensity, – indicates absence of marker band.

Table 3 Genome composition of *Musa* hybrids deduced from internal transcribed spacers, PCR-RFLP phenotype and flow cytometry. Note: OL, 'Obino l'Ewai; C4, 'Calcutta 4'; PL, 'Pisang lilin'; TL, 'Tjau lagada'; BT, 'Bobby tanap'; BL, 'Bluggoe'

Acc. no.	Origin of hybrid	Ploidy	Diagnostic ITS fragments			
			530 bp	350 bp	180 bp	Genome
1549-7	OL (AAB)×C4 (AA)	2n=22	xx	x	x	AB
5105-1	PL (AA)×C4 (AA)	2n=22	xx	–	–	AA
9128-3	TL (AA)×PL (AA)	2n=22	xx	–	–	AA
SH3362	–	2n=22	xx	–	–	AA
4400-8	BT (AAB)×C4 (AA)	2n=22	xx	–	–	AA
4479-1	BT×C4	2n=44	xx	x	x	AAAB
15108-1	4479-1×SH3362	2n=33	xx	x	x	AAB
6930-1	OL (AAB)×C4 (AA)	2n=44	xx	x	x	AAAB
14604-35	6930-1×1549-7	2n=33	xx	x	x	AAB
612-74	BL (ABB)×C4 (AA)	2n=44	xx	xx	xx	AABB
FHIA 1	–	2n=44	xx	x	x	AAAB
FHIA 2	–	2n=44	xx	x	x	AAAB
FHIA 3	–	2n=44	xx	xx	xx	AABB
FHIA 21	–	2n=44	xx	x	x	AAAB
IC2	–	2n=44	xx	–	–	AAAA

early stage is useful to predict their bunch characteristics. For example, the *M. balbisiana* 'B' genome contributes to starchiness of pulp (Robinson 1996), a desirable quality in the plantains and other cooking bananas, but not in the dessert bananas. DNA markers for the A and B genomes could assist breeders to determine the genome composition of hybrid progenies and thus infer probable pulp characteristics of the resulting hybrids. It is also speculated that banana leaf streak virus (BSV) sequences appear to be linked to the B genome (Geering et al. 2000). The number of B genomes present in hybrids may become a useful indicator of the potential BSV integrant concentration when breeding for BSV resistance in *Musa* (Tenkouano et al. 2001). The DNA markers identified

in this study should be useful in banana breeding programs for identifying, at the nursery stage, those hybrids that preferentially inherited A or B genomes.

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