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Identification of dCAPS Markers that Discriminate A and B Cytoplasms in Banana (*Musa* spp.)

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Abstract

A single nucleotide polymorphic sequence (SNP) in the *trnL-F* intergenic spacer region of chloroplast DNA that discriminate *Musa acuminata* (AA) cytoplasm from *Musa balbisiana* (BB) cytoplasm has been found. This mutation was initially amplified using a pair of universal primers and converted into dCAPS (derived cleaved amplified polymorphic sequence) markers. Using these markers in combination with flow cytometric analysis, 'Pisang Klutuk' (syn. 'Pisang Awak') was found to be a triploid ABB cultivar (not a wild BB accession as previously classified). A 15-bp long deletion was also found within the *trnL-F* spacer sequence of *M. acuminata* subspecies *banksii*. This mutation will be useful as a specific marker for the cytoplasm derived from this subspecies. These maternally inheritable chloroplast DNA mutations discovered in this study are vital for better understanding the origins of banana cytoplasm to clarify the lineage of banana cultivars and the contribution of wild progenitors to cultivated types.

Accession numbers: ITC-0249, ITC-0399, ITC-0565, ITC-0617, ITC-0626, ITC-0660, ITC-0806, ITC-1016, ITC-1077, ITC-1120, ITC-1177, ITC-1209.

Key words: cpDNA, dCAPS, *Musa* spp., RFLP, SNP, *trnL-F* intergenic spacer.

Abbreviations

cpDNA, chloroplast DNA; CTAB, cetyltrimethylammonium bromide; dCAPS, derived cleaved amplified polymorphic sequence; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

Modern bananas and plantains originated from intra and inter-specific hybridization between two wild diploid species; *Musa acuminata* Colla. and *M. balbisiana* Colla. that contributed the A and B genomes, respectively (Pillay *et al.*, 2000). It was postulated that the evolution of edibility in *Musa* cultivars most probably started with wild *M. acuminata* subspecies (Simmonds, 1962) and its subsequent hybridization with a more drought-tolerant *M. balbisiana* (Jones, 1999). Due to their hybrid nature, initial attempts at taxonomic classification of individual clones were unclear and often ambiguous (Gawel and Jarret, 1991). Difficulty in classifying these clones are thought to be due to high incidence of somatic mutation (Vuylsteke *et al.*, 1988) or to the confounding influence of cytoplasmic effects on plant phenotype (Jarret, 1986). Characterization and classification of *Musa* germplasm is of paramount importance not only to taxonomists but also to plant breeders. Current breeding programs for the improvement of bananas are geared towards introgression of useful genes from both wild and cultivated diploid progenitors (Ortiz and Vuylsteke, 1994). Thus, it is necessary for breeders to eliminate large portions of the donor parent genome (Crouch *et al.*, 1998). Genes for hardiness, drought tolerance, improved nutritional value, disease resistance and starchiness for example, are believed to be contributed by *M. balbisiana* (Robinson, 1996). Vakili (1965), on the other hand, noted that *M. acuminata* ssp. *banksii/errans* has considerably more disease problems than other *Musa* species and subspecies of *M. acuminata*. *M. acuminata* cultivars, however, are sweeter and more suited to dessert use (Robinson, 1996). With knowledge of their genetic background, it will probably be easier to explain the reaction of landraces and bred

hybrids to diseases (Jones, 1999). Therefore, efforts to efficiently conserve *Musa* germplasm and their eventual utilization for breeding purposes depend upon an accurate characterization of the clones and their wild progenitors.

In this paper, we described the development of dCAPS markers based on single nucleotide polymorphic sequence (SNP) of the chloroplast *trnL-F* intergenic spacer sequence to discriminate between A and B cytoplasms in wild and cultivated *Musa* species and subspecies.

A total of 25 accessions (**Table 1**) representing wild *M. balbisiana*, *M. acuminata* including genotypes belonging to AAA, AAB and ABB groups were used to survey *Musa* A and B genomes for sequence polymorphism in the chloroplast *trnL-F* intergenic spacer region. *In-vitro* materials of wild accessions were made available from the International Network for Improvement of Bananas and Plantains (INIBAP) Transit Center, Leuven, Belgium. Some materials were also supplied as fresh cigar-leaf tissues from germplasm bank of Stanfilco-Division of Dole Philippines, Inc., Davao City, Philippines. Total genomic DNA was isolated using CTAB (Cetyltrimethylammonium bromide) method from 100 mg of frozen banana leaf tissues following the procedure of Dellaporta *et al.* (1983).

The *trnL-F* intergenic region of the chloroplast DNA was amplified by PCR (polymerase chain reaction) using two universal primers e and f as described by Taberlet *et al.* (1991). The PCR reaction mixture of 25 μl contained 1x *Taq* DNA polymerase buffer, 0.2 mM dNTPs, 10 ng of primers, 1U of Ex *Taq* DNA polymerase (Takara Biomedicals, Shiga, Japan) and 10-20 ng of total genomic DNA as template. Amplification was carried out with 35 cycles of the following thermal conditions: 1 min at 94 ℃, 1 min at 50 ℃, and 2 min at 72 ℃ using a DNA Engine thermal cycler (PT-200, MJ Research Inc., Watertown, MA). Amplified fragments were purified using Qiaquick columns (Qiagen,Chatsworth, CA) and were directly se-

Table 1 List of wild and cultivated accessions used to identify single-nucleotide polymorphic sequence (SNP) to discriminate A and B cytoplasms in *Musa* spp.

Species	Sub-group	Genome[1)]	Cytoplasm	Accession No.	Source[2)]
M.acuminata 'Hawaiin 3'	*banksii*	AA*w*	A	ITC-0617	INIBAP
M.acuminata 'Hawaiin 2'	*banksii*	AA*w*	A	ITC-1209	INIBAP
M.acuminata 'Gud Mun'	*banksii*	AA*w*	A	ITC-0806	INIBAP
M.acuminata	*malaccensis*	AA*w*	A	ITC-0399	INIBAP
M.acuminata	*zebrina*	AA*w*	A	ITC-1177	INIBAP
M.acuminata 'Khae (P) X'	*siamea*	AA*w*	A	ITC-0660	INIBAP
M.acuminata 'Calcutta 4'	*burmannicoides*	AA*w*	A	ITC-0249	INIBAP
M.balbisiana 'Tani'		BB*w*	B	ITC-1120	INIBAP
M.balbisiana		BB*w*	B	ITC-0626	INIBAP
M.balbisiana		BB*w*	B	ITC-1016	INIBAP
M.balbisiana 'Butuhan'		BB*w*	B	ITC-0565	INIBAP
'Pisang Lilin'		AA	A	-	Philippines
'Alaswe'		AA	A	-	Philippines
'Inarnibal'		AA	A	-	Philippines
'Umalag'		AAA	A	-	Philippines
'G. Cavendish'		AAA	A	-	Philippines
'Williams'		AAA	A	-	Philippines
'Laknau'		AAB	A	-	Philippines
'Bungaoisan'		AAB	A	-	Phlippines
'Pisang Seribu'		AAB	A	-	Philippines
'Pelipia'		ABB	A	-	Philippines
'Saba'		ABB	A	-	Philippines
'Cardaba'		ABB	B	-	Philippines
'Gubao'		ABB	A	-	Philippines
'Pisang Klutuk'		ABB	A	ITC-1077	INIBAP

[1)] AA*w* and BB*w* = wild *M. acuminata* and *M. balbisiana* accessions, respectively.

[2)] INIBAP = International Network for the Improvement of Bananas and Plantains, Leuven, Belgium.

quenced from both strands using e or f primer with a PRISM dye terminator cycle sequencing kit using 373A sequencer (Applied Biosystems, Foster City, CA).

Intergenic *trnL-F* sequences were initially grouped under wild *M. acuminata* and *M. balbisiana* accessions in order to identify single-base polymorphic sequences specific to A and B cytoplasms. This cpDNA region containing SNP was inputted in dCAPS Finder 2.0 software available at http://helix.wustl.edu/dcaps/dcaps.html (Neff *et al.*, 1998) to generate specific dCAPS primers, while the corresponding opposite primers were computer generated using Primer3 program developed by Rozen and Skaletsky (1998).

Regions containing SNP were amplified using combinations of dCAPS primers (**Table 2**) based on the methods as described by Michaels and Amasino (1998), with modifications. The PCR reaction mixture of 25 μl contained 1x *Taq* DNA polymerase buffer, 0.2 mM dNTPs, 10 ng of primers, 1U of Ex *Taq* DNA polymerase (Takara Biomedicals, Shiga, Japan) and 10-20 ng of total genomic DNA as template. Amplification was carried out with 35 cycles of the following thermal conditions: 5 min at 95 ℃, 15 s at appropriate annealing temperature (T*a*), and 30 s at 72 ℃. PCR products were purified using Qiaquick columns (Qiagen,Chatsworth, CA) and digested with appropriate restriction enzymes by adding 4 μl of PCR product to 6 μl of 1x restriction buffer containing 2 units of restriction enzyme. The samples were incubated for 1 h at the temperature as recommended by the manufacturer. After digestion, samples were separated by gel electrophoresis using 12% acrylamide gels in 0.5x TBE on a vertical gel chamber, stained with ethidium bromide and photographed under UV light.

Nuclear samples for flow cytometric analysis were prepared from approximately 50 mg of fresh leaf midrib from *in-vitro* plantlets. In order to release plant nuclei, tissue was chopped using a sharp razor blade in a plastic Petri dish containing 0.2 ml of commercial buffer solution (solution A of plant high resolution DNA kit type P, Partec, Münster, Germany). DNA staining was carried out by adding to the crude sample, 1 ml of staining solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30 (Wako Chemicals, Japan), 0.1% (v/v) Triton X-100, 2 mg l^{-1} DAPI (4',6-diamidino-2-phenylindole), pH 7.5. After staining for 5 min at room temperature, the nuclear suspension was filtered in 50-μm nylon mesh, and fluorescence was analyzed using Partec PA flow cytometer (Partec GmbH, Germany). The gain of the instrument was set at channel 50 using *M. acuminata* ssp. *zebrina* (accession no. ITC-1177), as diploid standard.

Chloroplast DNA (cpDNA) sequence variations are in recent years become the focus of studies to investigate intra and inter-specific relationships among wide array of plant species. In particular, non-coding regions in cpDNA are thought to display complex pattern (Clegg *et al.*, 1994) and the most frequent mutations (Palmer *et al.*, 1988; Clegg

Table 2 Restriction enzymes used in screening dCAPS markers.

Enzyme	Recognition site	Primer sequence (5' → 3')[1]	Mismatch position[2]	T*m*/ ± T*a* (℃)[3]	Expected Products (bp)[4]
*Acc*I	GTMKAC	CTTTAATTAACATAGACACAAGTA*c*TC	3/F	50/53	82/25
*Bsl*I	CC(N)$_7$GG	ATTTCCCGTGCATC*c*TCCTAGT	8/R	52/53	187/19
*Bst*XI	CCA(N)$_6$TGG	ATTTCCCGTGCA*c*CATCCTAGT	10/R	52/53	187/19
*Hpa*II	CCGG	ATTTCCCGTGCATCATCCTA*cc*	1,2/R	50/ ± 50	185/21
*Apa*LI	GTGCAC	CTTTAATTAACATAGACACAAGTA*g*Tg	1,3/F	48/ ± 50	78/29
*Xho*I	CTCGAG	ATTTCCCGTGCATCATCCT*c*G*a*	1,3/R	52/ ± 50	123/83
*Bse*RI	GAGGAG	ATTTCCCGTGCATCATCC*g*AG*g*	1,4/R	52/ ± 50	173/33
*Eco*NI	CCT(N)$_5$AGG	ATTTCCCGTGCATC*c*TCCTAG*a*	1,8/R	52/ ± 50	186/20
*Hae*III	GGCC	CTTTAATTAACATAGACACAAGTAg*g*C	2,3/F	49/ ± 50	81/26

[1] *Italicized* letters in *boldface* are mismatched bases.

[2] Numbers correspond to position of mismatched bases relative to the 3' end of dCAPS primers; F = forward primer; R = reverse primer. Opposite primers for F:5'GAAACTGGTGACACGAGGATTTT3'; R:5'ACGGATCCGAACTAAAATCCTTG3', T*m* = 53 ℃.

[3] Melting temperature (T*m*) of primers excluding mismatched bases; Variable annealing temperature (± T*a*) used in PCR reaction.

[4] Size of expected digestion products.

Table 3 Alignment of cpDNA *trnL-F* intergenic spacer sequences showing exact locations of length deletion and single-base substitution between wild *M.balbisiana* and *M. acuminata* accessions.

Group	*trnL-F* intergenic spacer sequence[1)]
M. balbisiana	41 TCTTTTTTTTCATCAGTGGT 60
M. acuminata spp. *banksii*	41 TC----------GGT 60
M. acuminata	41 TCTTTTTTTTCATCAGTGGT 60
	* * * * *
M. balbisiana	281 GACACAAGTACTCCACTGGA 300
M. acuminata spp. *banksii*	281 GACACAAGTACTCTACTGGA 300
M. acuminata	281 GACACAAGTACTCTACTGGA 300
	********** ******

[1)] Length deletion, TTTTTTTCATCAGT was detected only in *M. acuminata* ssp. *banksii*. *Italicized* letters in ***boldface*** indicate single-base difference between *M. balbisiana* and *M. acuminata trnL-F* intergenic spacer sequences.

et al., 1991). Thus, by amplification and direct sequencing of these non-coding regions, the resolution of cpDNA can be increased both for evolutionary studies, and for identifying intra-specific genetic markers (Taberlet *et al.*, 1991). In the present study, sequencing and alignment of *trnL-F* intergenic spacer showed that the size of the entire spacer sequence was in the range of 343-358 bp in *Musa* species under section *Eumusa*. The observed variation in size was mainly due to length deletion of about 15-bp (TTTTTTTTCATCAGT) found only in *M. acuminata* ssp. *banksii* (**Table 3**). Non-coding regions of cp DNA diverge through insertion/deletion changes that are sometimes site dependent (Clegg *et al.*, 1994). At least in banana (this study) and pine (Chen *et al.*, 2002), these deletions were located in the T-rich region close to the 5' end of the intergenic sequence. We believe that this deletion was caused by mechanism other than intra-molecular recombination due to the absence of short-direct repeat sequences like those found in rice (Kanno *et al.*, 1993) and wheat (Ogihara *et al.*, 1988). Further sequence analysis also revealed a single-base (T ⇔ C) substitution between *M. acuminata* (T) and *M. balbisiana* (C) wild accessions. The process of nucleotide substitution is generally considered biased toward transition, i.e. substitutions between purines (G⇔ A) or pyrimidines (T⇔ C) and a possible explanation for the apparent avoidance of A ⇔ T and G ⇔ C transversions might come from mechanisms ensuring fidelity in DNA replication (Bakker *et al.*, 2000).

Considering the diversed morphological and geographical origins of wild *M. acuminata* and *M. balbisiana* species, it is surprising to note that this region has showed very limited polymorphism. Coincidentally, plastid subtype identity (PS-ID) sequence, which is also non-coding region between *rpl16* and *rpl14* of plastid DNA in *Musa*, displayed relatively the same type and level of base substitution and deletion (Umali *et al.*, 2002). Intra-specific chloroplast DNA polymorphism could result either from normal intra-specific variation, which has not been studied for non-coding regions, or from inter-specific chloroplast DNA transfer (Taberlet *et al.*, 1991). Thus, our data indicate that intra-specific variation and inter-specific cpDNA transfer is absent, or cannot be effectively resolved using *trnL-F* intergenic spacer sequences in banana. At least for *Acanthaceae*, this locus is useful in addressing questions of phylogenetic relationships among but not within genera (McDade and Moody, 1999).

A total of 10 dCAPS primers were later generated in order to exploit this single-base mutation and develop into robust DNA markers to discriminate A from B cytoplasm in banana. Using these primers, PCR amplification showed non-specific bands from *Hae*III, *Eco*NI and *Hpa*II primers. These products tend to persist even after varying the annealing temperature and number of PCR cycles. We suspect that this result was sequence-dependent, particularly on the location and type of mismatched bases in the primers. Also, *Apa*LI and *Hae*III failed to cut the products to completion and could be an indication of mis-incorporation of target recognition sites during early PCR cycles. On the other hand, in spite of producing sharp single bands, *Xba*I, *Bse*RI, and *Xho*I did not cut their respective amplification products at all. Only *Acc*I with single mismatch at position 3 was able to reproducibly discriminate A cytoplasm while *Bsl*I

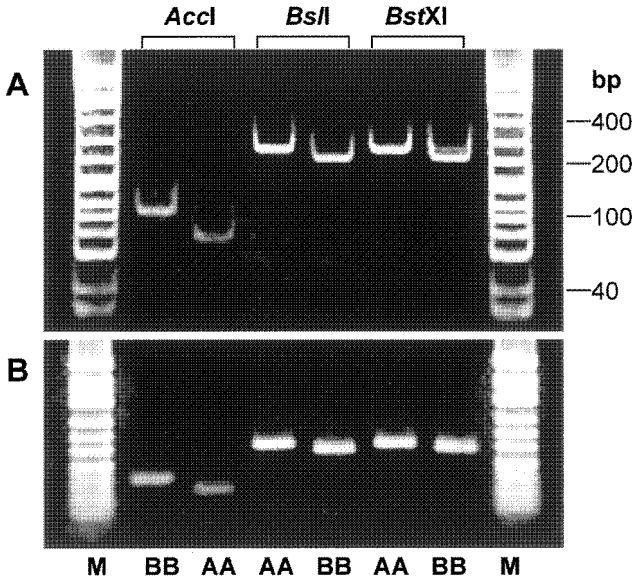

Fig. 1 dCAPS markers from *M. acuminata* (AA) and *M. balbisiana* (BB) cpDNA resolved in 12% acrylamide (A) and 3% agarose gels (B). M: Φ x174/*Hin*fI marker

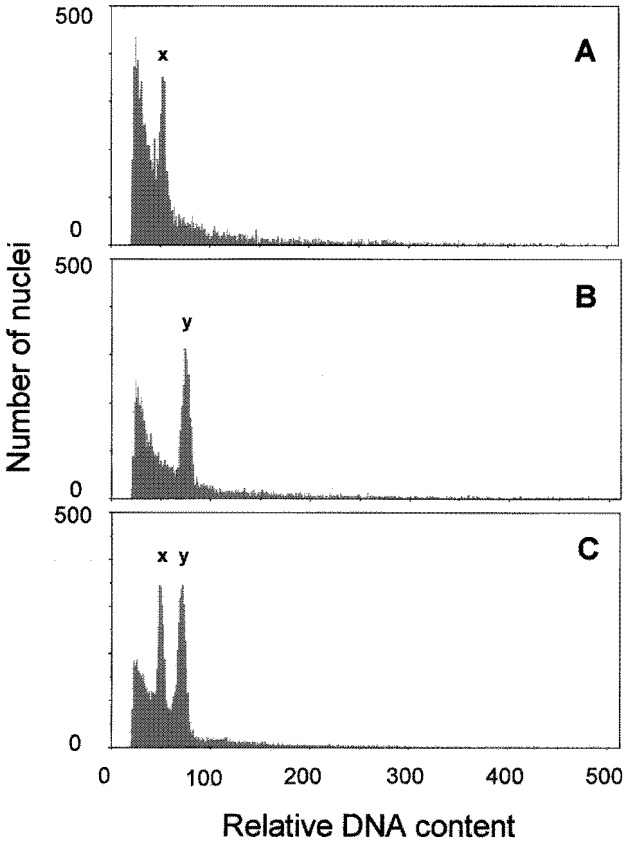

Fig. 2 Histogram of relative nuclear DNA content of nuclei isolated from diploid standard *M.acuminata* ssp. *zebrina*, AA (accession: ITC-1177) (A), 'Pisang Klutuk', ABB (accession: ITC-1077) (B), and from both the standard (x) and 'Pisang Klutuk' (y) combined (C).

and *Bst*XI with single mismatches at positions 8 and 10, respectively were able to differentiate B from A cytoplasm. Digested products were clearly visible in 12% acrylamide gel and can also be accurately scored in 3% agarose gel for faster and more economical dCAPS analysis (**Fig. 1**).

Generally, dCAPS primers that failed either in PCR reproducibility or restriction enzyme digestion are those having one or two mismatches at the end and close to the 3' end of the primer sequence. In *Arabidopsis*, Michaels and Amasino (1998) reported that primers with mismatches at position 1,2 or 2,3 did not amplify reproducibly while mismatches at position 3 or 3,4 generated digestion resistant product. One reason is that *Taq* polymerase errors during early cycles could cause amplified mistakes, yielding pools of uncuttable DNA which are not representative of the genomic template (Neff *et al.*, 1998). Furthermore, digestion-resistant fragments, could be generated by imperfect match of the primer to the template at the 3' end of the primer, that eliminate the restriction site which in turn could be due to one or more incorrect bases incorporated near the 3' end of the primer (Michaels and Amasino, 1998).

Surprisingly, survey using dCAPS markers showed 'Pisang Klutuk' (listed as wild *M. balbisiana*, BB with accession no. ITC-1077) having dCAPS profile of A cytoplasm. Incidentally, Robinson (1996) classified the same cultivar (syn. 'Pisang Awak') under the triploid ABB group. Verification using flow cytometry however confirmed that 'Pisang Klutuk' had triploid genome, apparently of ABB constitution (**Fig. 2**). With this finding, we propose to adopt Robinson's classification and place 'Pisang Klutuk' under ABB group. In addition, using RFLP chloroplast probe from lettuce (*Lactuca sativa*), 'Cardaba' and 'Saba' which are both triploid ABB, were previously grouped with hybrids having A and B cytoplasms, respectively (Gawel and Jarret, 1991). Using dCAPS markers however, 'Cardaba' revealed having the opposite B while 'Saba' showed having the opposite A-type cytoplasm (**Table 1**). While restriction fragment length polymorphism (RFLP) can also detect SNPs, this method however, is limited to the detection of SNPs that alter a recognition site for an available restriction enzyme (Neff *et al.*, 1998). Hence, when these single-base changes do not generate restriction site, critical information from SNPs resolution may be overlooked. Similarly, incidence of parallel mutations at labile sites like in the case of non-coding region upstream of *rbcL* can be positively misleading for phylogenetic studies using restriction fragment length polymorphism (RFLP) data (Clegg *et al.*, 1994).

In conclusion, we have shown that the *trnL-F* intergenic region of cpDNA in *Musa* spp. was highly conserved except for a length deletion in *M. acuminata* ssp. *banksii* and a single base substitution that was later developed into dCAPS markers. Using 'Pisang Klutuk', 'Saba' and 'Cardaba' as examples, these markers were shown to be an effective tool in validating genomic constitution in different *Musa* genotypes. Finally, our identification of a maternally inherited cpDNA mutation also provides an opportunity to better understand the origins of banana cytoplasm. Together, the lineage of banana cultivars and the contribution of wild progenitors to cultivated types may also be clarified using these markers.

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