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Variation of *B* Genome in *Musa* Accessions and Their New Identifications

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ABSTRACT

Thirty-four accessions of *Musa* were identified using 18 primer pairs: 9 of SSH, 3 of specific polymerase chain reaction (PCR) and 6 of PCR-restriction fragment length polymorphism PCR-RFLP. These primers could generate 5 specific bands for the *A* genome and 10 specific bands for the *B* genome. The results were: 1) *M. balbisiana* accessions could be classified into two subgroups, B_N and B_E; 2) 5 out of 13 hybrid accessions, which had been previously identified morphologically as *ABB*, namely Kluai Namwa Dang, Kluai Nommi, Kluai Tip, Kluai Okinawa and Kluai Nang Phaya, were revealed by this study to be *AAB* by their markers; 3) of five *balbisiana* accessions, which had previously been known as *BBB*, four, namely Kluai Phama Haek Khuk, Kluai Hin, Kluai Saba and Kluai Thep Phanom, were identified as *ABB* and the fifth, Kluai Lep Chang Khut, was identified as *AAB*. All primers also generated data for phylogenetic analysis and construction that showed a distinction between the *A* genome, the *B* genome and a relationship among their hybrids as well. The *M. balbisiana* accessions could be classified into three subgroups that were not related to the geographical distribution. All markers used helped to create an effective classification of the *Musa* genome (especially in the *B* genome, which had not been reported as categorized into subgroups), and revealed genetic diversity and relationships among the *Musa* accessions.

Keywords: *Musa*, DNA marker, *B* genome, genetic relationship

INTRODUCTION

Musa is a genus of giant perennial herbs belonging to the Musaceae family of the order Scitamineae. Edible bananas are considered to have originated from two wild diploid species, *M. acuminata* Colla (the *A* genome) and *M. balbisiana* Colla (the *B* genome) and have been classified into different groups according to their genome composition by the system of Simmonds and Shepherd (1955), which relies primarily on 15 morphological characters. Current breeding efforts for the improvement of bananas rely on introgression of useful genes from the wild and cultivated diploid progenitors, especially in the *B* genome, which are related to resistance to pests and diseases (Sotto and Rabara, 2000) and determination of the starch type, while the *A*

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genome is related to susceptibility to diseases (Silayoi, 2002). The genome composition of the banana showed some correlation to a grouping by use, so that bananas derived from the *M. balbisiana* genome were cooking bananas and bananas derived from the *M. acuminata* genome were dessert bananas (Valmayor *et al*., 2000). Therefore, there is a need to study genetic diversity and relationships among the wild and cultivated diploid accessions and their polyploidy ones. Polymorphisms of genomic DNA are very useful for tagging genetic traits and studying biological diversity among species, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP). These methods have been used to investigate the genetic variability present in *Musa* germplasm (Ude *et al*., 2002; Phothipan *et al*., 2005). Suppression subtractive hybridization (SSH) is one of the principal methods used to generate primers for revealing the genome or transcriptome of an organism (Marenda *et al*., 2005). The screened unique genomic DNA fragments can be used as species-specific probes for discerning the species they represent from all other species (Li *et al*., 2006). The objective of this study was to find out the specific PCR-based markers for banana genome identification.

MATERIALS AND METHODS

Plant materials

A total of 34 accessions of bananas containing 2 samples of *M. acuminata* (AA group), 12 of *M. balbisiana* (BB group), 13 of triploid hybrids (ABB group), 5 of *M. balbisiana* (BBB group) and 2 of tetraploid hybrids (ABBB group) were collected from the Pak Chong Research Station, Nakhon Ratchasima and the Department of Horticulture, Kasetsart University, Bangkok, Thailand.

DNA extraction

Genomic DNA was extracted from the cigar leaves of all accessions, according to Agrawal *et al*. (1992) and quantified.

Suppression subtractive hybridization (SSH)

Genomic DNA of *M. balbisiana* (Kluai Tani Eisan), *M. balbisiana* (Kluai Tani Tai) and *M. balbisiana* (Kluai Tani Nuea) were extracted and used as the driver, and then genomic DNA of *M. balbisiana* (Kluai Tani Nakom) was used as the tester. Tester and driver DNA were firstly digested with *Mse*I. Fragments of tester DNA were ligated with an adapter and amplified by polymerase chain reaction (PCR). The driver DNA was labeled with biotin using a *random prime DNA labeling* method and hybridized onto amplified tester genomic DNA. Single strand DNA samples were separated after hybridization and amplified by PCR. The PCR products were ligated into the pGEM-T-Easy vector. The SSH library was constructed by transforming the ligation mixture into competent *E. coli* cells. The plasmids were isolated and the nucleotide sequences of inserted size were determined. Sequences were analyzed with BLASTN to determine related sequences. New pairs of primer were designed by the Fast PCR program and applied to detect variation among banana samples by PCR, using the following cycling conditions: pre-denaturing before cycle of the polymerase was accomplished by incubating the reaction mixture at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, T_{an} for 30 s and 72°C for 45 s, followed by 72°C for elongation at 5 min in the last cycle (Table 1). PCR products were separated in 1.5% agarose gel, stained with 0.3 μg/ml ethidium bromide and visualized under UV light.

Specific – PCR

Amplification using three of the specific oligonucleotide primer pairs was developed using the Sequence Characterized Amplified Region

(*SCAR*) technique by Onto (2003), i.e., marker M4(A_1-1), M5 (A_2-1) and M22 (B-1) were performed in a 15 µl reaction mixture containing 5 pmole of each primer, 2 mM of dNTPs, 50 mM $MgCl_2$, 0.5 unit *Taq* polymerase, 10x PCR buffer supplied with the polymerase and 100 ng genomic DNA. Amplifications used the following cycling conditions: activation of the polymerase was accomplished by incubating the reaction mixture at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, T_{an} for 1 min and 72°C for 2 min, followed by elongation at 72°C for 5 min in the last cycle (Table 1). PCR products were separated on 1.5% agarose gel.

PCR-RFLP of the organellar genomes

Two chloroplast and four mitochondrial primer pairs were used to amplify selected regions of the organellar genomes (Boonruangrod *et al*., 2008). PCR amplification of these chloroplast and mitochondrial regions was performed in 25 ml reaction mixture containing 0.2 µM of each primer, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.2 unit *Taq* polymerase and 1x PCR buffer supplied with the polymerase and 50 ng genomic DNA. Amplifications used the following cycling conditions: activation of the polymerase was accomplished by incubating the reaction mixture at 94°C for 15 min, followed by 35 cycles of 94 °C for 45s, T_{an} 45s and 72°C for D_{el}, followed by elongation at 72°C for 10 min in the last cycle (Table 1). Subsequently, the fragments were restriction digested with specific enzymes. The banding patterns were visualized either by polyacrylamide gel electrophoresis using 6% polyacrylamide gel in TBE and followed by silver

Table 1 18 Primer pairs.

Marker	Primer	% GC content	T_{an} (°C)
SSH	A10	50.0	68.0
	A18	47.8	69.5
	B44	47.6	67.5
	C1	50.0	70.0
	C23	45.0	67.5
	D19	47.6	67.0
	D30	50.0	65.0
	Contiq2	45.0	64.0
	Contiq3	45.0	64.0
Specific primer	M4 (A_1-1)	54.4	55.0
	M5 (A_2-1)	46.3	42.0
	M22(B-1)	55.8	53.0
PCR-RFLP	Chloroplast		
	tRNAlys (*trnK* gene)	52.5	58.0
	tRNAleu-tRNAleu		
(tRNAleu intron 1)	55.0	58.0	
	Mitochondria		
	nad1exonB/nad1exonC	52.5	57.5
	Rps14/cob	60.0	54.5
	Ccb203	65.0	56.0
	cox II (cytochrome		
	oxidase subunit II gene intron)	45.0	52.0

staining or by agarose gel electrophoresis using 1% agarose in TBE and subsequent staining of the gels with 0.3 μg/ml ethidium bromide.

Genetic relationship analysis

Selected bands from the DNA fingerprint of SSH, specific primers and PCR-RFLP techniques were changed to binary data (0 and 1) and were analyzed with the software NTSYS-pc 2.01e. Similarity coefficients were calculated using simple matching. Clustering was grouped using the unweighted pair group method with arithmetic average (UPGMA).

RESULTS

A total of 34 accessions of *Musa* were identified using 18 primer pairs of SSH, specific-PCR and PCR-RFLP (Table 4). Four primer pairs generated five specific bands for the *A* genome. Primer contiq 2 and contiq 3 gave *A* specific bands of 500 and 570 bp, respectively. Primer M4 (A_1-1) gave a 280 bp DNA fragment specific to the A_1 genome and also gave a 1,200 bp fragment specific to the A_2 genome. Primer M5 (A_2-1) showed A_2 specific fragment of 209 bp and seven primer pairs generated 10 specific bands for the *B* genome. Primer C1 and C23 showed *B* specific fragments of 187 and 420 bp, respectively. Primer D30 gave *B* specific fragments of 525 and 262 bp. Primer contiq 2 gave a 216 bp DNA fragment specific to the *B* genome. Primer contiq 3 showed *B* specific fragments of 550 and 300 bp. Primer M22 (B -1) gave a *B* specific fragment of 186 bp. The *B* genome was classified into two subgroups using primer M5 (A_2-1) that presented the 500 bp of B_N specific fragment in *M. balbisiana* (*BB* : # 2, 4, 5, 6, 7, 8, 9, 11 and 12). Numbers 1-12 were referred to by the list of *Musa* accessions in Table 2, and the 700 bp fragment that was specific to B_E in *M. balbisiana* (*BB* : # 1, 3 and 10) (Figure 1) (Table 3). Interestingly, 5 out of the 13 hybrid accessions, namely Kluai Namwa Dang, Kluai Nommi, Kluai Tip, Kluai Okinawa, Kluai Nang Phaya previously identified as *ABB*, but markers in the current study revealed them as *AAB*. Moreover, of the five *balbisiana* accessions, which had previously been known as *BBB,* four, namely Kluai Phama Haek Khuk, Kluai Hin, Kluai Saba and Kluai Thep Phanom, were identified by these primers as *ABB*, while the fifth, Kluai Lep Chang Khut, was identified as *AAB*.

For PCR-RFLP analysis, two cpDNA and four mtDNA were selected. The sizes of fragments from the total number of 73 bands were found ranging from 55 to 800 bp. Sixty-four bands were polymorphic. Fragments generated by mtDNA primers gave the same pattern or monomorphism in all BB accessions, but fragments from the cpDNA primers were polymorphic and could be constructed into a dendogram from all BB accessions (data not shown) in a phylogenetic tree pattern as shown in Figure 2.

Figure 1 M5 (A_2-1) pattern showing B_N specific fragment (500 bp) and B_E specific fragment (700 bp). Numbers on the top (1-34) refer to the list of *Musa* accessions in Table 4. Lane M is the 1 kb ladder DNA size standard.

Table 2 Genome of 34 *Musa* accessions.

No.	Name	Previous genome	Genome
1	Kluai Tani Eisan	*BB*	B_EB_E
2	Kluai Tani Tai	*BB*	B_NB_N
3	Kluai Tani N*akhon Si Thammarat*	*BB*	B_EB_E
4	Kluai Tani Dam	*BB*	B_NB_N
5	Kluai Tani Tamai	*BB*	B_NB_N
6	Kluai Tani A15, Lampang.	*BB*	B_NB_N
7	Kluai Tani 252, Buriram.	*BB*	B_NB_N
8	Kluai Tani	*BB*	B_NB_N
9	Kluai Tani Buriram	*BB*	B_NB_N
10	Kluai Tani *Tadluan*g	*BB*	B_EB_E
11	Kluai Tani Nuea	*BB*	B_NB_N
12	Kluai Tani Nakhom	*BB*	B_NB_N
13	'Kluai Namwa Maliong'	*ABB*	$A_2B_NB_N$
14	'Kluai Namwa Dang'	*ABB*	$A_1A_2B^*$
15	'Kluai Namwa Lueang'	*ABB*	$A_2B_NB_N$
16	'Kluai Namwa Khom'	*ABB*	$A_2B_NB_N$
17	'Kluai Namwa Dam'	*ABB*	$A_2B_NB_N$
18	'Kluai Namwa Ngoen'	*ABB*	$A_2B_NB_N$
19	'Kluai Hakmuk Khiao'	*ABB*	$A_1B_NB_N$
20	'Kluai Hakmuk Thong'	*ABB*	$A_1B_NB_N$
21	'Kluai Hakmuk Khao'	*ABB*	$A_1B_NB_N$
22	'Kluai Nommi'	*ABB*	AB_EB_E
23	'Kluai Tip'	*ABB*	$A_1A_2\ B_E{}^*$
24	'Kluai Okinawa'	*ABB*	$A_1A_2B^*$
25	'Kluai Nang Phaya'	*ABB*	$A_1A_2B^*$
26	'Kluai Phama Haek Khuk'	*BBB*	$A_1B_EB_E{}^*$
27	'Kluai Lep Chang Khut'	*BBB*	$A_1A_2B_N{}^*$
28	'Kluai Hin'	*BBB*	$A_1B_EB_E{}^*$
29	'Kluai Saba'	*BBB*	$A_1B_EB_E{}^*$
30	'Kluai Thep Phanom'	*BBB*	$A_1B_EB_E{}^*$
31	'Kluai Thep Pharot'	*ABBB*	$AB_NB_NB_N$
32	'Kluai Thip Pharot'	*ABBB*	$AB_NB_NB_N$
33	'Kluai Sa'	*AA*	A_1A_2
34	'Kluai Homchan'	*AA*	A_1A_2

Note: * A difference between previous and present identification.

A_1 : *A* genome of Kluai Pa Phrae (*M. acuminata*) (Auvuchanon, 2001)

A_2 : *A* genome of Kluai Pa Phatthalung ('Kluai Hom Champa') (*M. acuminata*) (Auvuchanon, 2001)

B_N : *B* genome of Kluai Tani Nuea (*M. balbisiana*)

B_E : *B* genome of Kluai Tani Eisan (*M. balbisiana*)

Phylogenetic tree of banana cultivars

The phylogenetic tree showed two main clusters, which were AA-ABB and BB, ABB, BBB, ABBB banana genome accessions. Numbers 1-34 were referred to in the list of *Musa* accessions in Table 4. The AA-AAB clusters consisted of five cultivars (# 33, 34, 14, 24 and 25) while the BB, ABB, BBB, ABBB clusters consisted of 29 cultivars. These cultivars were divided into five subgroups. The first subgroup consisted of 3 out

Table 3 Genome specific fragments.

Genome	Primer	Band size (bp)
A	contiq 2	500
	contiq 3	570
A_1	M4 (A_1-1)	280
A_2	M4 (A_1-1)	1200
	M5 (A_2-1)	209
B	C1	187
	C23	420
	D30	525
	D30	262
	contiq 2	216
	contiq 3	550
	contiq 3	300
	M22 (B -1)	186
B_N	M5 (A_2-1)	700
B_E	M5 (A_2-1)	500

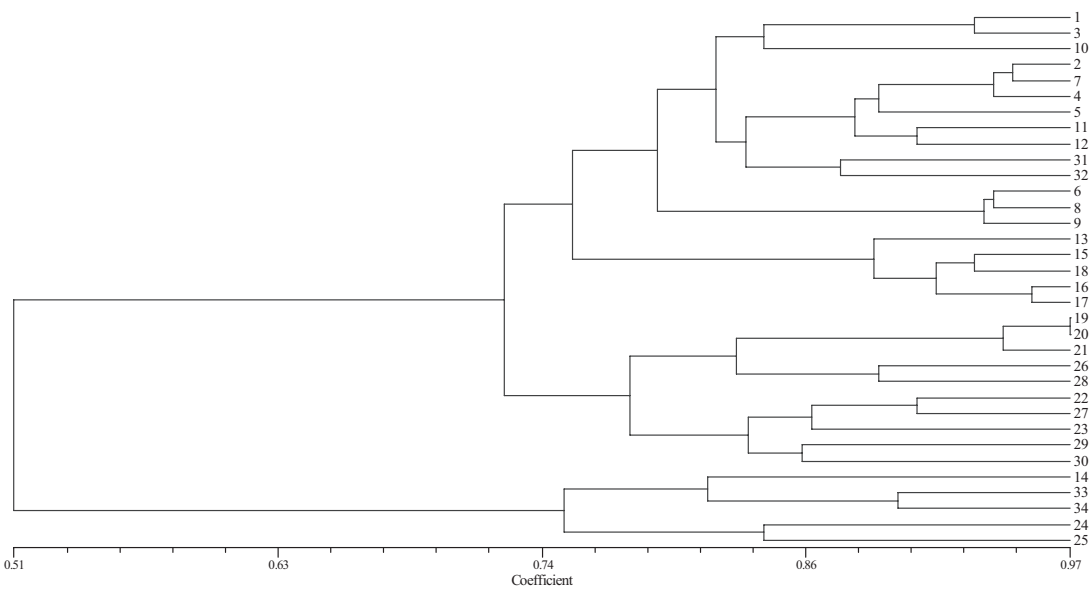

Figure 2 Phylogenetic tree of banana accessions by NTSYS-pc 2. 01e. Numbers 1-34 refer to the list of *Musa* accessions in Table 4.

of 12 BB bananas (# 1, 3 and 10). The second subgroup consisted of 6 out of 12 BB bananas (# 2, 4, 5, 7, 11 and 12) and the ABBB bananas (# 31 and 32). The third subgroup consisted of 3 out of 12 BB bananas (# 6, 8 and 9). The fourth subgroup consisted of 5 out of 13 ABB bananas (ABB: # 13, 15, 16, 17 and 18), while the last subgroup consisted of 5 out of 13 ABB bananas and all five BBB bananas (ABB: # 19, 20, 21, 22 and 23; BBB: # 26, 27, 28, 29 and 30). The phylogenetic tree showed that *M. balbisiana* accessions could be classified into three subgroups (Figure 2).

DISCUSSION

The variation of the *B* genome in bananas of the *Musa* genera has never been reported. In this study, nine useful primer pairs generated by SSH (three specific PCR and six PCR-RFLP primers) were used as PCR-based marker to identify the closely related species of the *B* genome in *Musa*. The results showed that variation of the *B* genome was lower than variation of the *A* genome, as was also reported by Phothipan *et al.* (2005). Specific PCR primers and PCR-RFLP primers revealed genetic variability within the *B* genome as well. These results agreed with Ude *et al*. (2002) and Phothipan *et al*. (2005), who reported similar variation in *M. balbisiana* and divided this accession into two groups. The current study, on the other hand, further divided *M. balbisiana* into two subgroups and clearly showed them to be in B_N and B_E. This could provide some information on the differentiation of morphological traits in *M. balbisiana* and their hybrids, such as resistance to pests and diseases and determination of starch type.

PCR-RFLP also revealed the inheritance of the chloroplast and mitochondrial genome of *Musa*, using primer designs based on their sequences, such as *tRNA* genes. Cytoplamic polymorphism was observed only in the mitochondrial genome of the *B* genome banana accessions. The variation of the cytoplasmic genome could be detected in their hybrids, ABB (cooking banana), BBB and ABBB groups that contained high starch. This was similar to the results of Boonruangrod *et al.* (2008), which stated that the cooking banana had received a mitochondrial genome from *M. balbisiana* and plantain (AAB) had received mitochondrial genome from *M. acuminata* (Carreel *et al*., 2002). The investigation of Silayoi and Babpraserth (1983), which formerly revealed that the *ABB* genome of 5 out of 13 hybrid accessions, namely Kluai Namwa Dang, Kluai Nommi, Kluai Tip, Kluai Okinawa, Kluai Nang Phaya by the scoring method of Simmonds and Shepherd (1955) were currently identified as *ABB*. In contradiction to *M. balbisiana* accessions, namely Kluai Phama Haek Khuk, Kluai Hin, Kluai Saba, Kluai Thep Phanom and Kluai Lep Chang Khut which were previously morphologically identified as BBB (Valmayor *et al*. 2000; Silayoi, 2002), the current study showed them to be *ABB*, while Kluai Lep Chang Khut was *AAB*. These results were in agreement with the reports that identified Kluai Saba ,Kluai Phama Haek Khuk and Kluai Hin as *ABB* (Silayoi, 1991; Boonruangrod, 2008). Furthermore, these results showed that morpho-taxonomy must be supplemented with techniques, such as DNA markers, in order to guarantee the level of sensitivity needed for the correct identification of the genetic diversity in the genus *Musa* (De Langhe, 1990). The information acquired on the banana genome was quite interesting and further study should be conducted to confirm these findings. The phylogenetic tree showed two main clusters, which were AA-ABB and BB, ABB, BBB, ABBB banana genome accessions and the second axis separated the BB and ABB (cooking bananas) in agreement with the previous report of Ude *et al*. (2002), which discriminated the *M. acuminata* and *M. balbisiana* accessions in the first axis and the second axis separated the BB, ABB (cooking bananas) and AAB (plantains).

CONCLUSION

Eighteen primer pairs of SSH, specific PCR and PCR-RFLP were used in *Musa* genome identification. Among 12 accessions of *Musa babisiana*, 2 *B* genomes were revealed as B_N and B_E. Hybrid accessions, namely Kluai Namwa Dang, Kluai Nommi, Kluai Tip, Kluai Okinawa, Kluai Nang Phaya previously identified as *ABB* were revealed as *AAB*. Similarly, *M. balbisiana* accessions, namely Kluai Phama Haek Khuk, Kluai Hin, Kluai Saba, Kluai Thep Phanom, formerly known as *BBB*, were identified as *ABB*. Kluai Lep Chang Khut was classified as *AAB* instead of *BBB*. The phylogenetic tree also presented the variation of *B* genomes, but it was not related to geographical distribution.

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