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Molecular Verification and Diversity Analysis of Indonesian BB, AAB and ABB Banana Cultivars

Amin Retnoningsih[1,2] • Rita Megia [1*] • Alex Hartana[1]

[1] Bogor Agricultural University, Faculty of Mathematics and Life Sciences, Department of Biology, Dramaga Campus, Bogor 16680, Indonesia
[2] Present address: Semarang State University, Department of Biology, Sekaran Campus, Gunungpati, Semarang 50229, Indonesia

Corresponding author: * **ritamegia@cbn.net.id**

ABSTRACT

Genetic diversity of bananas in Indonesia is considered to be high. Phenotypic identification of banana accessions is sometimes incorrect due to instability of morphological characters. The aims of this study were to verify Indonesian bananas accessions containing the B genome previously identified based on morphological characters, to construct a molecular determination key for the genomic groups, and to reveal genetic relationships among the accessions based on microsatellite markers. The DNA of 92 banana accessions classified as containing the B genome was analyzed using 8 pairs of microsatellite primers. The results showed that 9 accessions were incorrectly classified as containing the B genome. Of the remaining 83 accessions, 76 were accurately classified as AAB, ABB, or BB; and 7 accessions needed to be reassigned to a different subset of the B-genome containing accessions. Alleles MaCIR108 size longer than 270 bp were considered as the diagnostic characters for the B genome, while one allele Ma-3-90 size of 152 bp could be used to distinguish AAB from ABB genomic groups. The molecular determination key constructed classified the 83 accessions into 3 genomic groups, consisted of 8 accessions of BB, 33 accessions of AAB, and 42 accessions of ABB. Genetic relationship analyses of the 83 accessions based on the 8 primers detected only 67 genotypes due to the presence of 8 identical genotypes consisting of 24 accessions. The accessions clustered according to their genomic groups. ABB formed fewer clusters than the AAB genomic groups indicating less diversity in the ABB group.

Keywords: allele, genomic group, genetic relationships, molecular determination key
Abbreviations: CIMMYT, International Maize and Wheat Improvement Center; **INIBAP**, International Network for the Improvement of Banana and Plantain; **NTSYS**, Numerical Taxonomy and Multivariate Analyses System; **SHAN**, Sequential, Agglomerative, Hierarchical and Nested; **SIMQUAL**, Similarity of Qualitative Data; **UPGMA**, Unweighted Pair-Grouping Method with Arithmatic Average

INTRODUCTION

For over 50 years, genomic constitution in banana has traditionally been determined based on morphological characters following the scoring system described by Simmonds and Shepherd (1955). The modified version proposed by Silayoi and Chomchalow (1987) suggested that 15 morphological characters be used for identifying genomic group of AA/AAA, AAB, AB/AABB, ABB, ABBB, and BB/BBB. The phenotypic identification of banana accessions are sometimes incorrect due to instability of morphological characters (de Vicente *et al*. 2005), mainly in identifying accessions which have intermediate characters between *M. acuminata* and *M. balbisiana*. Incorrect identification tended to increase among closely related cultivars. Therefore it was not surprising that Indonesian banana germplasm were classified differently by different researchers (Pudjoarinto *et al.* 1994; Hadisunarso *et al*. 1995; Jumari 2000).

Genomic groups have an important role in banana classification. Evolutionary relationships among banana accessions were hardly interpreted due to the occurrences of hybridization and polyploidy (Pillay *et al*. 2004). Several banana genomic groups were also questionable due to the restricted variability on the morphological characters, so validation the groups using more accurate techniques is necessary. Characteristics of a cultivar could be easily detected through nucleotide distinction of the DNA. Resolution of molecular markers are commonly higher than that of morphological appearance (Ford-Lloyd *et al*. 1997), which make DNA nucleotides better than morphological markers for identifying cultivars (de Vicente *et al*. 2005).

Microsatellites have been employed to investigate the genetic diversity of *Musa* species (Creste *et al*. 2003, 2004). They were powerful for estimating an intra-specific genetic distance and effective for analyzing polyploidy (Bruvo *et al*. 2004). Microsatellites are chosen as markers in genetic analyses since they produce high polymorphic alleles even in closely related lines (Semagn *et al*. 2006). These markers had also been used to characterize and identify fruit crops cultivars, such as grape (Arnold *et al*. 2002), strawberry (Ashley *et al*. 2003), sweet orange (Novelli *et al*. 2005), apple (Galli *et al*. 2005) rasberry and blackberry (Stafne *et al.* 2005), and mango (Ukoskit 2007).

A previous study in Indonesian pure *acuminata* cultivars (Retnoningsih *et al*. *in press*) showed that microsatellites could be used for clarifying the taxonomic status of banana containing the pure A genome based on the absence of diagnostic character of B genome in the accessions. As these markers were able to be used for genomic group fingerprint (Kaemmer *et al*. 1997; Creste *et al*. 2003) and the fact that Indonesian banana germplasm are considered to be high (Daniells *et al*. 2001) support the opportunity for constructing a determination key using molecular data such the key created by Guzow-Krzeminska *et al*. (2001). The key is applied to determine genomic groups of the accessions of either inter- or intra-specific of *M. acuminata* and *M. balbisiana*.

The aims of the study were to verify cultivars of banana containing B genome previously identified based on morphological characters, to construct a molecular determination key for banana genomic groups and to investigate the genetic relationships among the accessions using microsatellite markers.

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MATERIALS AND METHODS

Plant materials

Cigar youngest leaf of 92 banana accessions morphologically identified and categorized into BB, BB/BBB, AAB, and ABB genomic groups (Jumari 2000; Jumari and Pudjoarinto 2000; INIBAP 2002a; Siddiqah 2002; Edison *et al*. 2004) were used. The accessions were collected from: field at Jasinga, Bogor; Banana Germplasm Center belongs to Agricultural Service (Diperta) Yogyakarta Municipality; and RIF (Research Institute for Fruit) Collection Center at Solok, West Sumatera (**Table 1**).

Microsatellite analyses

Eight microsatellite primers were used in this study (**Table 2**). One of them, MaCIR108 is known as a fingerprint for distinguishing banana cultivar containing the B genome from the pure A genome (Kaemmers *et al*. 1997; Creste *et al*. 2003). A genomic DNA was extracted following Dixit (1998) procedure with modification. Leaves were crushed in liquid nitrogen and homogenized in the extraction buffer containing 100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl, 10 mM β-mercaptoethanol, and 20% SDS. After purification using 10 mg/ml of RNAse free at 37°C for 1 hr, precipitation of DNA was carried out without PEG solution treatment. DNA concentration was measured by spectrophotometer and then the DNA was diluted in TE to a suitable concentration.

Microsatellites were amplified using PCR in a 10 µl reaction solution containing 15-20 ng of genomic DNA, 1 µl 10 x PCR buffer with 20 mM $MgCl_2$, 0.2 µl 10 mM dNTPs, 0.2 µl 10 µM of each primer, and 0.06 µl 5u μl^{-1} *Taq* DNA Polymerase Native (GenScript Corp.). The amplification was performed for 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 50, 53, 55 and 56°C (depending on the primers) for 30 s, and extention at 72°C for 30 s. These cycles were preceded by a 4 min denaturation at 95°C, and ended by a 10 min final extension at 72°C (Kaemmer *et al*. 1997). PCR reactions were carried out in a Perkin Elmer 2400 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR products were then separated on a gel containing 6% (w/v) polyacrylamide and 7 M urea (Sigma-Aldrich Chemie, Germany) at 45-60 W constant power for 2 to 3 hrs. Then, the gel was stained using a modified silver staining method described by Creste *et al*. (2001). All solutions were prepared using distilled water.

Data analyses

Each variant fragment of microsatellite was assumed as an allele. For each allele was scored as present (1) or absent (0). The size of each allele was estimated using 100 bp DNA ladder (Invitrogen). Designation of allele submitted to the relative size of allele followed CIMMYT (2004). Eight loci of microsatellites are named as a for the largest fragment to h for the smallest fragment. Any band below the fragment is named according to the letter designation followed by number, for instance a_1 for the largest of allele a, etc.

A similarity matrix was calculated based on Jaccard similarity index using Similarity of Qualitative Data (SIMQUAL) procedures. The matrix was used for cluster analyses to examine genetic relationships among the accessions. Cluster analyses was performed using Sequential, Agglomerative, Hierarchical and Nested (SAHN) clustering of Unweighted Pair-grouping Method with Arithmatic Average (UPGMA) by the Numerical Taxonomy and Multivariate Analyses System (NTSYS) pc version 2.02 (Rohlf 1998).

RESULTS AND DISCUSSION

Verification of banana accessions containing the B genome

All primers produced well-defined alleles exhibiting a polymorphic banding pattern. A total of 75 alleles with size ranged from 110 to 436 bp were obtained in this study (**Table 3**). The highest degree of polymorphism was detected in MaCIR108 and the lowest was observed at MaCIR327b.

The primer MaCIR108 which was known as a fingerprint for distinguishing banana cultivar containing the B genome from the A genome (Kaemmer *et al*. 1997; Creste *et al*. 2003) produced 13 alleles which 4 alleles (d_1-d_4) size longer than 270 bp, while 9 alleles (d_5-d_{13}) size shorter than 270 bp. A previous study on 59 accessions of pure. *Acuminata* (Retnoningsih *et al.* 2010) revealed that the alleles MaCIR108 shorter than 270 bp were considered as characters of the A genome while those longer than 270 bp were considered as characters of the B genome. This result was in agreement with Kaemmer *et al*. (1997) who mentioned that the different allele size in the MaCIR108 was due to the flanking region which is 49 bp longer in the B genomic allele than in the A genomic allele.

Based on the allele size, 9 of the 92 accessions did not have the MaCIR108 allele longer than 270 bp. It means that those 9 accessions had only the A genome. Therefore, they have incorrectly been classified into the group of banana containing the B genome. The absence of the MaCIR108 allele longer than 270 bp indicated that the cultivars belong to pure *M. acuminata*. Since the number of the alleles MaCIR108 shorter than 270 bp found were three, they should have been included in the triploid AAA (**Table 4**).

The 9 cultivars above were most probability mislabeled in the field because the name 'Kepok Klutuk' and 'Kepok Ungu' are known specific for Indonesian banana containing the B genome. Mislabeled was a common phenomenon in the *ex situ* germplasm collections because morphological appearances for characterization and identification were sometimes difficult to be interpreted due to the influence of environmental and cultivation factors (Hernandez *et al*. 2001). The occurrence of mislabeled in collections of olive germplasm were reported by Lopes and Mendonça (2004), in many barley collections by Hamza *et al*. (2004), and in banana collections by Karamura *et al*. (2000). This misidentification could have great influence on breeding efficiency (Lopes and Mendonça 2004).

Diagnostic characters and a molecular key for determination banana genomic groups

The 92 accessions studies consisted of cultivars morphotaxonomically identified as 8 accessions of BB, 1 accessions of BB/BBB, 37 accessions of AAB and 46 accessions of ABB (**Table 4**). Four alleles MaCIR108 size of longer than 270 bp were recognized as diagnostic characters for the B genome, and 9 alleles MaCIR108 size of 270 bp or less for the A genome. The four alleles MaCIR108 were designated as d_1, d_2, d_3, and d_4, with size estimation 295, 289, 287, and 275 bp, respectively. They were consistently present or absent in certain genomic groups. In all BB cultivars only allele d_1 was observed and no allele MaCIR108 equal or shorter than 270 bp was found. On the other hand on BB/BBB cultivar, besides d_1 and d_3, one allele MaCIR108 shorter than 270 bp was obtained.

The study exhibited that 4 out of the 37 AAB accessions only produced the alleles MaCIR108 with size of 270 bp or less. In the morphotaxonomically AAB genomic group, 23 accesions produced allele d_3 with 2 alelles of the MaCIR108 shorter than 270 bp; 7 accessions had allele d_4 also with 2 alleles shorter than 270bp – all indicating that they were correctly identified as AAB. One accession had alleles d_1 and d_2 with one allele of the MaCIR108 shorter than 270 bp –so it should be molecularly considered as ABB. From the remaining AAB genomic group, 2 accessions produced only allele d_1 with one allele of the MaCIR108 shorter than 270 bp (**Table 5**).

For the 46 accessions morphologically identified as ABB, 5 accessions had only alleles of the MaCIR108 shorter than 270 bp. Together with the 4 accessions had only alleles of the MaCIR108 shorter than 270 bp from the AAB group, they were considered as the mislabelled cultivars. Thirteen accessions produced alleles d_1, d_2 and one allele of the MaCIR108 shorter than 270 bp, while 5 accessions had

Table 1 List of 92 accessions used in study.

Accession	Subgroup[a]	Genomic group	Region	Source[b]
Batu	Klutuk	BB	Medan	Diperta
Batu Krawang	Klutuk	BB	Unknown	Diperta
Klutuk Awu	-	BB	Uknown	RIF
Klutuk Batu	-	BB	Uknown	RIF
Klutuk Hitam	-	BB	Jasinga Bogor	Bogor
Klutuk Warangan	Klutuk	BB	Kulon Progo	Diperta
Klutuk Watu	Klutuk	BB	Uknown	Diperta
Klutuk Wulung	Klutuk	BB	Cilongok Banyumas	Diperta
Boi		BB/BBB	Genyem Jayapura	RIF
Angleng	-	AAB	Jasinga Bogor	Bogor
Austroli	Plantain	AAB	Dongkelan Yogyakarta	Diperta
Bangkahulu	-	AAB	Unknown	Diperta
Beleum	-	AAB	Jasinga Bogor	Bogor
Burlangge	-	AAB	Sentani Jayapura	RIF
Haseum	-	AAB	Jasinga Bogor	Bogor
Jambe Saat	Triolin	AAB	Cipaku Bogor	Diperta
Kapas	-	AAB	Jasinga Bogor	Bogor
Ketan	-	AAB	East Java	Diperta
Ketip Gunungsari	-	AAB	Cipaku Bogor	Diperta
Koja Susu	-	AAB	Unknown	Diperta
Longong	-	AAB	Cipaku Bogor	Diperta
Nangka	-	AAB	Jasinga Bogor	Bogor
Nangka	-	AAB	Unknown	RIF
Pisang Seribu	Pisang Seribu	AAB	Unknown	Diperta
Pulut	Pulut	AAB	Sukoharjo	Diperta
Raja	-	AAB	Uknown	RIF
Raja Bagus	Raja	AAB	Sleman	Diperta
Raja Bandung	-	AAB	Bantul	Diperta
Raja Bulu	-	AAB	Jasinga Bogor	Bogor
Raja Kasman	Plantain	AAB	Kulon Progo	Diperta
Raja Lini	Raja	AAB	Guning Kidul	Diperta
Raja Marto	Plantain	AAB	Tembarak Temanggung	Diperta
Raja Nangka	Plantain	AAB	Giwangan Yogyakarta	Diperta
Raja Puser	Plantain	AAB	Gunung Kidul	Diperta
Raja Sabrang	-	AAB	Kulon Progo	Diperta
Raja Sereh	-	AAB	Jasinga Bogor	Bogor
Raja Sereh	-	AAB	Uknown	RIF
Raja Sereh	-	AAB	Purworejo	Diperta
Raja Talun	-	AAB	Kotagede Yogyakarta	Diperta
Roid	-	AAB	Cipaku Bogor	Diperta
Rojomolo	Plantain	AAB	Malang	Diperta
Susu	-	AAB	Jasinga Bogor	Bogor
Tanduk	-	AAB	Jasinga Bogor	Bogor
Tanduk ex Kedondong	-	AAB	Cipaku Bogor	Diperta
Tanduk Hijau	Plantain	AAB	Surakarta	Diperta
Triolin	Triolin	AAB	Bantul	Diperta
Abu Awak	Awak	ABB	Unknown	Diperta
Apu	-	ABB	Jasinga Bogor	Bogor
Awak	Awak	ABB	Samarinda	Diperta
Awak Rawa	Awak	ABB	Unknown	Diperta
Bawean	Kepok	ABB	Banyumas	Diperta
Brentel	Kepok	ABB	Tlekung Malang	Diperta
Byar	-	ABB	Purworejo	Diperta
Comot	Awak	ABB	Gunung Kidul	Diperta
Gablok	Sobo	ABB	Unknown	Diperta
Gandul	Sobo	ABB	Giri Banyuwangi	Diperta
Gedah	Awak	ABB	Cipaku Bogor	Diperta
Kapas	-	ABB	Tirto Pekalongan	Diperta
Kaso	Awak	ABB	Cipaku Bogor	Diperta
Kates	Kepok-	ABB	Giri Banyuwangi	Diperta
Kepok	-	ABB	Jasinga Bogor	Bogor
Kepok Asam	-	ABB	Bantul	Diperta
Kepok Awu	-	ABB	Gunung Kidul	Diperta
Kepok Brot	Kepok	ABB	Purworejo	Diperta
Kepok Byar	Kepok	ABB	Unknown	Diperta
Kepok Gandul	Kepok	ABB	Gunung Kidul	Diperta
Kepok Gajih	Kepok	ABB	Unknown	Diperta
Kepok Klutuk	-	ABB	Bantul	Diperta
Kepok Kuning	Kepok	ABB	Dongkelan Yogyakarta	Diperta
Kepok Kuning	-	ABB	Unknown	RIF
Kepok Kuningan	Kepok	ABB	Tegalsari Girimulyo	Diperta
Kepok Ladrang	Kepok	ABB	Unknown	Diperta

Table 1 (Cont.)

Accession	Subgroup[a]	Genomic group	Region	Source[b]
Kepok Lumut	-	ABB	Bantul	Diperta
Kepok Ungu	Sobo	ABB	Pendowoharjo Bantul	Diperta
Kepok Urang	Kepok	ABB	Gunung Kidul	Diperta
Klutuk Susu	Awak	ABB	Umbulharjo Yogyakarta	Diperta
Lempeneng	Sobo	ABB	Cilongok Banyumas	Diperta
Raja Bali	Awak	ABB	Bantul	Diperta
Raja Entog	Awak	ABB	Purworejo	Diperta
Raja Kul	Kepok	ABB	Malang	Diperta
Raja Pendopo	Sobo	ABB	Purworejo	Diperta
Raja Siem	-	ABB	Unknown	RIF
Raja Utri	Awak	ABB	Gunung Kidul	Diperta
Raja Wesi	Awak	ABB	Tawangsari Sukoharjo	Diperta
Selayar	-	ABB	Sentani Jayapura	RIF
Siem	-	ABB	Jasinga Bogor	Bogor
Sobo Kapuk	Sobo	ABB	Giri Banyuwangi	Diperta
Sobo Kepok	-	ABB	Unknown	Diperta
Sobo Kerik	-	ABB	Tlekung Malang	Diperta
Sobo Londoijo	Sobo	ABB	Unknown	Diperta
Sobo Londoputih	Sobo	ABB	Sukoharjo	Diperta
Sobo Madura	Sobo	ABB	Giri Banyuwangi	Diperta

[a] following the subgroup determination keys constructed by Jumari (2000) and Jumari and Pudjoarinto (2000)
[b1] Diperta, (Agricultural Service), Yogyakarta
[b2] RIF, Research Institute of Fruits, Solok West Sumatera

Table 2 Primers used in this study.

Primers	Forward primer sequence Reverse primer sequence, 5′-3′	Optimized annealing temperature (°C)
MaCIR108[a]	F: TAAAGGTGGGTTAGCATTAGG	55
	R: TTTGATGTCACAATGGTGTTCC	
MaCIR327b[a]	F: AAGTTAGTCAAGATAGTGGGATTT	50
	R: CTTTTGCACCAGTTGTTAGGG	
MaCIR332a[a]	F: TCCCAACCCCTGCAACCACT	53
	R: ATGACCTGTCGAACATCCTTT	
Ma-1-17[a]	F: AGGCGGGGAATCGGTAGA	56
	R: GGCGGGAGACAGATGGAGT	
Ma-1-27[a]	F: TGAATCCCAAGTTTGGTCAAG	56
	R: CAAAACACTGTCCCCATCTC	
Ma-1-132[a]	F: GGAAAACGCGAATGTGTG	53
	R: AGCCATATACCGAGCACTTG	
Ma-3-90[b]	F: GCACGAAGAGGCATCAC	56
	R: GGCCAAATTTGATGGACT	
Ma-3-139[b]	F: ACTGCTGCTCTCCACCTCAAC	56
	R: GTCCCCCAAGAACCATATGATT	

[a] Primers designed by Kaemmer *et al.* 1997
[b] Primers designed by Crouch *et al.* 1998

Table 3 Observed microsatellite alleles sizes.

Primer	Allele size range (bp)	№ of alleles	Allele of each primer
MaCIR327b	388-436	5	a_1,a_2,a_3,a_4,a_5
Ma-1-132	330-378	8	$b_1,b_2,b_3,b_4,b_5,b_6,b_7,b_8$
MaCIR332a	260-296	10	$c_1,c_2,c_3,c_4,c_5,c_6,c_7,c_8,c_9,c_{10}$
MaCIR108	220-295	13	$d_1,d_2,d_3,d_4,d_5,d_6,d_7,d_8,d_9,d_{10},d_{11},d_{12},d_{13}$
Ma-3-139	132-177	11	$e_1,e_2,e_3,e_4,e_5,e_6,e_7,e_8,e_9,e_{10},e_{11}$
Ma-3-90	132-172	12	$f_1,f_2,f_3,f_4,f_5,f_6,f_7,f_8,f_9,f_{10},f_{11},f_{12}$
Ma-1-27	122-142	6	g_1,g_2,g_3,g_4,g_5,g_6
Ma-1-17	110-154	10	$h_1,h_2,h_3,h_4,h_5,h_6,h_7,h_8,h_9,h_{10}$
Total		75	

combination of alleles d_1, d_3 and one allele of the MaCIR108 shorter than 270 bp. All together these 18 accessions were classified in ABB group. Sixteen accessions of the ABB had only allele d_1 and one allele of the MaCIR108 shorter than 270 bp. Since morphologically these cultivars shown strong ABB characters, it was most probably that allele d_1 was actually duplex.

Table 5 showed from 46 accessions of the ABB group, 3 cultivars namely 'Byar', 'Brentel', and 'Kates' from Diperta collection should be included into the AAB genomic group due to the existence of 2 alleles of the MaCIR108 size shorter than 270 bp and one allele d_3. Five accessions had allele d_3 and 1 allele of the MaCIR108 size shorter than 270 bp. Determination of these accessions could be done by the absence/presence of f_7 locus Ma-3-90 size 152 bp. The allele f_7 consistently present in all BB and ABB cultivars but always absent in AAB and AAA genomic groups.

Table 4 List of 9 accessions in the collection incorrectly classified as containing the B genome.

Accession	Genomic group based on morphology	Genomic group based on microsatellites
Angleng	AAB	AAA
Bawean	ABB	AAA
Beleum	AAB	AAA
Kapas	ABB	AAA
Kepok Klutuk	ABB	AAA
Kepok Ungu	ABB	AAA
Ketip Gunungsari	AAB	AAA
Roid	AAB	AAA
Sobo Madura	ABB	AAA

Based on all alleles of the MaCIR108 loci and one allele of the Ma-3-90 locus, a molecular key for determination of banana genomic group was constructed.

1a. Alleles MaCIR108 size equal or shorter than 270 bp ... 2

2a. The number of alleles, one to two .....
AA/AAA genomic group

2b. The number of alleles, three .....
AAA genomic group

1b. Alleles MaCIR108 size longer than 270 bp …..…… 3

3a. Allele size 295 bp only .....……..... **BB genomic group**

3b. Combination of allele 295 bp; or 295 bp and 289 bp; or 295 bp and 287 bp; with one allele size equal or shorter than 270 bp ……...…… **ABB genomic group**

3c. Combination of allele 287 bp; with one or two alleles size equal or shorter than 270 bp 4

4a. Combination of allele size 287 bp; with 2 alleles size

Table 5 Classification of banana accessions into genomic groups based on primers MaCIR108 and Ma-3-90.

Genomic group based on morphology	Alleles						Genomic group based on microsatellites
	MaCIR108					Ma-3-90	
	d_1	d_2	d_3	d_4	d_5-d_{13}	f_7	
	295 bp	289 bp	287 bp	275 bp	≤270 bp	152 bp	
8 BB	+	-	-	-	-	+	8 BB
1 BB or BBB	+	-	+	-	+	+	1 ABB
37 AAB	-	-	-	-	+++	-	4 AAA
	-	-	+	-	+/++	-	23 AAB
	-	-	-	+	+/++	-	7 AAB
	+	-	-	-	+	+	2 ABB
	+	+	-	-	+	+	1 ABB
46 ABB	-	-	-	-	+++	-	5 AAA
	-	-	+	-	++	-	3 AAB
	+	-	+	-	+	+	5 ABB
	-	-	+	-	+	+	4 ABB
	+	-	-	-	+	+	16 ABB
	+	+	-	-	+	+	13 ABB

+ = allele present
- = allele absent
+/++/+++ = 1, 2, or 3 alleles

equal or shorter than 270 bp ..... **AAB genomic group**

4b. Combination of allele size 287 bp; with 1 allele size equal or shorter than 270 bp ..…..... 5

5a. Allele Ma-3-90 size 152 bp, present ……..……...… **ABB genomic group**

5b. Allele Ma-3-90 size 152 bp, absent ...……...… **AAB genomic group**

3d. Combination of allele size 275 bp; with one to two alleles size equal or shorter than 270 bp ..... **AAB genomic group**

A fascinating characteristic of the B genome markers was that the allele MaCIR108 295 bp and the allele Ma-3-90 152 bp were always present in accessions with two B genomes (BB and ABB), and never detected in banana with one B genome (AAB). These results confirm that genomic groups of banana accessions particularly derived from hybrid between *M. acuminata* and *M. balbisiana* could be easily and rapidly determined by microsatellite markers.

The result of determination genomic group in the present study is summarized in **Table 6**. The key demonstrated that the genomic group determination of banana based on morphological characters is not always accurate. Some AAB and ABB hybrids; and one BB/BBB accession have been incorrectly identified. The existence of pure natural BBB accession in Indonesia remains questionable (Pillay *et al.* 2004). All accessions of BB were correctly classified into its genomic group, but only 81% accessions of AAB and 83% accessions of ABB which have correctly been classified.

The key in present study should be applied for banana accessions derived from genus *Musa* section *Eumusa* only. This section is the most geographically widespread, with species ranging from India to Pacific (INIBAP 2004) contains at least 11 species (The Australian Government Office of the Gene Technology Regulator 2008), but only two wild species, *Musa acuminata* and *M. balbisiana*, involving in development of most edible bananas (Cheesman 1948; Simmonds and Shepherd 1955; Sharrock 1998). A number of distinct cultivars of major group are derived from *M. acuminata* and *M. balbisiana* either alone or in various hybrid combinations.

Molecular as well as morphological determination keys may also have limited application. The key in the study could not be used for identifying edible bananas originated from the other section, such as sect. *Callimusa*. Wong *et al*. (2002) reported that the two sections: *Callimusa* and *Eumusa* were genetically distinct. Based on AFLP analyses,

Table 6 Number of accessions classified into the genomic groups based on microsatellite markers.

Genomic group based on morphology	№ of accessions	Genomic group based on a molecular key				
		AAA	BB	BBB	AAB	ABB
BB	8	0	8	0	0	0
BB/BBB	1	0	0	0	0	1
AAB	37	4	0	0	30	3
ABB	46	5	0	0	3	38
Total	92	9	8	0	33	42

the basic number of chromosome of n = x = 10- and n = x =11- grouping are robust and justified to separate *Musa* species into different sections. The similarity between conserved regions of the two organisms from different taxonomical groups would be less significant (Guzow-Krzeminska *et al*. 2001). Therefore, different primers should be designed for construction determination keys of different taxonomical groups. Besides MaCIR108 and Ma-3-90 loci, banana genomic groups could be identified by other microsatellite primers, such as MaOCEN03, MaOCE12, MaOCE13 and MaOCE14 (Creste *et al*. 2006).

In order to avoid incorrect identification, it is envisaged to identifying banana cultivars which were difficult to be classified using morphological characters using molecular marker before they were commercially cultivated. According to Guzow-Krzeminska *et al*. (2001), a molecular determination key has potentially applied in identifying a small fragment of organisms or organisms which are difficult to be identified using traditionally keys. In addition, the determination keys provide suitable tools for researchers who are familiar with molecular techniques.

Analyses of relationships among banana accessions containing the B genome

Microsatellite has provided important informations concerning the genetic relationships among accessions of banana containing the B genome. Clustering analyses (**Fig. 1**) only detected 67 genotypes because 24 out of 83 accessions containing the B genome were genetically identical and clustered within 8 groups.

At coefficient similarity 0.22, the dendrogram showed 2 main clusters. In the first main cluster (**I**) sub cluster **Ia**, 8 accessions of BB were united with 42 accessions of ABB and separated from 26 accessions of AAB. In the second sub main cluster (**Ib**) 11 accessions of AAB were observed. The second main cluster (**II**) consisted of 7 accessions of AAB. The accessions of AAB in this cluster separated from those in the first main cluster due to the presence of allele

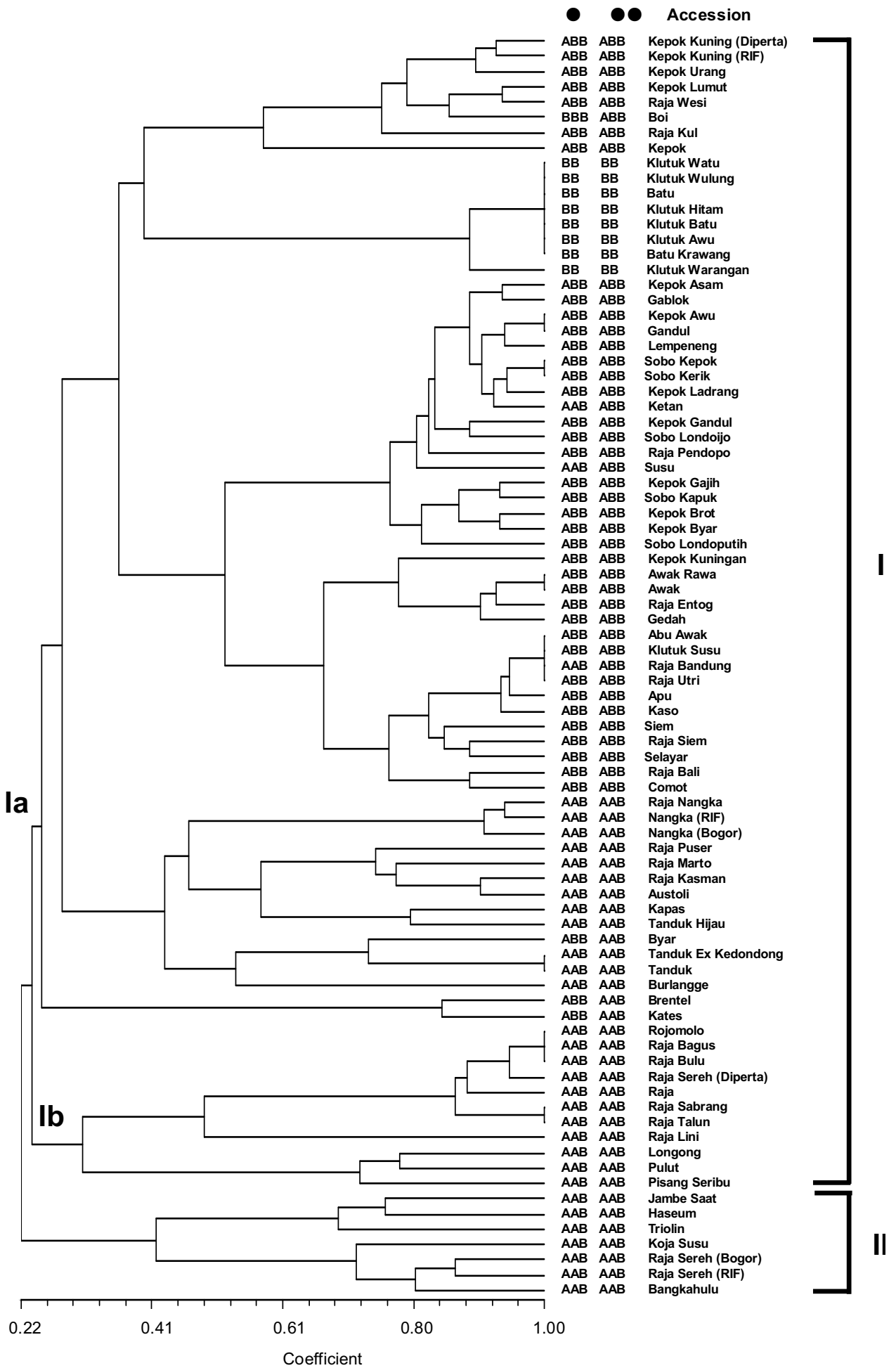

Fig. 1 Dendrogram analysis of 83 banana accessions containing B genome based on 75 alleles from 8 microsatellite primers. ● based on morphological characters and ●● based on MaCIR180 and Ma-3-90 loci.

d_4 of the MaCIR108. The results suggested that the AAB genomic group in the second main-cluster was showed to be the most distance from the other genomic groups.

The AAB accessions in the first main-cluster were separated from the BB and ABB. The accessions of BB were genetically closely related to ABB due to the existence of allele d_1 of MaCIR108 in both BB and ABB accessions. It supported the previous results using the AFLP technique (Ude *et al*. 2002). In the present study most AAB accessions were pooled in the first main-cluster due to allele d_3 of the MaCIR108 while differences among the accessions were determined by the other microsatellite primers. Molecular markers were needed to identify the presence of multiple genotypes with a single name or a single genotype with multiple names (de Vicente *et al*. 2005; Semagn *et al*. 2006). This information is important to provide accurate identification for conservation activities. In the clonally propagated crops such as banana, the conservation activities demand a complex and expensive procedures. Molecular data could be used for verifying the collections efficiently and ensured the minimum number of duplication. The number of accessions could be reduced significantly without reducing genetic variations within germplasm collections (de Vicente *et al*. 2005).

The accessions that morphologically identified as ABB namely 'Kates' and 'Brentel' in the first sub main cluster (**Ia**) were classified into AAB genomic group based on the MaCIR108 and the Ma-3-90 microsatellite loci. But they were genetically distinct from the others AAB. Both 'Kates' and 'Brentel' had 1 unique allele in each MaCIR332a and Ma-3-90 loci which were not found in others AAB. This result supported the study of Valmayor *et al*. (2000) which reported that 'Kates' was recognized as a unique cultivar originating from Indonesia. Morphologically, 'Kates' produced large, solitary fruits per hand and the fingers that were similar to small papaya.

Based on morphological characters, 2 of the 7 accessions in cluster **II**, Triolin' and 'Jambe Saat' belong to the AB genomic group (Jumari and Pudjoarinto 2000). The characteristics of these accessions tended to indicate the transition between cultivar groups of the AA, AAA and AAB to cultivar groups of the BB, and ABB. Naturally the banana accessions of AB genomic group are rare (Valmayor *et al*. 2000) so that the existence of AB hybrid especially in Indonesia still questionable. The microsatellite study demonstrated that 1 allele of the B genome and 2 alleles of the A genome were detected in accessions 'Triolin' and 'Jambe Saat'. Therefore, based on microsatellite analyses they should be placed into the AAB genomic group. In this case, variations of morphological characters were not sufficient to precisely determine genomic groups.

Based on 8 microsatellite markers, 7 of the 8 accessions of the BB were known to be identical. There were no different genotypes except for 'Klutuk Warangan'. Based on their phenotypic features, the BB cultivars were not morphologically distinct to the wild type of *M. balbisiana.* Seedless and parthenocarpy were considered as characteristics of the cultivated bananas (Stover and Simmods 1987). Within the diploid of BB accessions, the occurrence of parthenocarpy was not detected (Heslop-Harisson and Schwarzacher 2007), therefore it has pulp full with seeds. Mutations within the cultivated bananas correspond with human intervention (Carreel *et al*. 2002). Besides the BB genomic group which is less variable than the A genome diploid (Karamura 1998), the selection pressure within BB genomic group was also considered smaller than the other genomic.

Allelic composition of microsatellites in general was relatively stable (Kaemmer *et al*. 1997). It is because banana genotypes arising from cross breeding that occurred thousands years ago were maintained through vegetative propagation (Carreel *et al*. 2002). Actually, cultivated bananas were rather complex hybrids because they have been modified by various mutations and exploited by human for food for long time (Stover and Simmonds 1987).

In the present study most accessions collected from Diperta tend to be clustered according to the subgroups arranged by Jumari (2000) and Jumari and Pudjoarinto (2000) as presented in **Table 3**. Pure BB cultivars belong to subgroup Klutuk meaning seedy in Javaness. The AAB genomic groups consisted of 5 subgroups namely Raja, Plantain, Pulut, Pisang Seribu and Triolin, while the ABB genomic groups were divided into 3 subgroups, Kepok ,Sobo and Awak. Within **Ia** cultivars' 'Gablok', 'Gandul', 'Lempeneng', Sobo Kepok', 'Kepok Ladrang', and 'Raja Pendopo' clustered in subgroup Sobo; and cultivar 'Awak Rawa', 'Awak', 'Gedah', 'Abu Awak', 'Klutuk Susu', 'Raja Utri', 'Raja Entog', 'Raja Bali', 'Comot', and 'Kaso' clustered in subgrup Awak.

The cultivars of the subgroup Plantain, such as 'Raja Nangka', 'Raja Puser', 'Raja Marto', 'Raja Kasman', 'Austroli', and 'Tanduk Hijau' within cluster **Ia** was more closely related to the ABB than the AAB genomic groups. Those accessions were involved in subgroup Plantain by having an allele d_3 of the MaCIR108 size 287 bp. The subgroup Raja consisting of the cultivars'Raja Bagus' and 'Raja Lini' formed one cluster with 'Raja Sabrang', 'Longong' and 'Pulut' from the subgroup Pulut. Both subgroups clustering within **Ib** were usually used as dessert bananas. Based on morphological character, the subgroup Raja could be separated from Pulut by condition of bracts. The bract of Raja is usually indehiscent while Pulut is dehiscent (Jumari 2000). The results indicated that classification of cultivated bananas containing the B genome based on microsatellite commonly was in agreement with classification of subgroup based on morphological appearances.

Genetic relationship analyses among 83 banana accessions containing the B genome showed that the accessions clustered according to their genomic group. Similarity coefficient at 0.61 produced 11 sub-clusters AAB, 4 sub-clusters ABB, and 1 sub-cluster BB – indicating that the contribution of B genome is less diversifier than that of A genome. This result supported the study of cytometry analyses which revealed that the B genome was less diverse than of the A genome (Lysak *et al*. 1999; Kamate *et al*. 2001; Dolezel *et al*. 2004). The ABB accessions appear closer to the BB than the AAB genomic groups. It suggested that the AAB genomic group was more divergence from the original ancestors than the ABB genomic group (Pillay *et al*. 2004).

CONCLUSION

Alleles of the MaCIR108 are convincingly confirmed as diagnostic characters for distinguishing banana containing the B genome from banana containing the A genome alone. Nine accessions have incorrectly been classified into banana accessions containing the B genome. Those accessions should be placed into *M. acuminata* (AA/AAA genomic groups) due to the absence of the MaCIR108 allele size longer than 270 bp. The key of molecular determination for genomic groups is constructed based on 75 alleles obtained from analyses 92 accessions using 8 primers. Four out of the 13 MaCIR108 alleles are thought as the diagnostic characters for the B genome, whereas the remaining alleles are considered as the diagnostic character of the A genome. One of the Ma-3-90 microsatellite allele can be used to distinguish AAB from ABB genomic groups. Identification of 83 accessions containing the B genome based on molecular determination key observed 3 genomic groups consisted of 8 accessions of BB, 33 accessions of AAB, and 42 accessions of ABB genomic group. Analyses of these accessions based on 8 primers detected only 67 genotypes due to the existence of 8 genetically identical groups of 24 accessions. The dendrogram of genetic relationships showed that the accessions of bananas clustered according to their genomic groups. Similarity coefficient at 0.61 produced 11 sub-clusters AAB, 4 sub-clusters ABB, and 1 sub-cluster BB.

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