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The Use of LP-RAPD for Assessing Genetic Relatedness among Selected Banana Cultivars

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ABSTRAK

Analisis LP-RAPD (Primer panjang DNA Polimorfik Gandaan Rawak) telah dijalankan ke atas lapan jenis pisang iaitu Mas (AA), Berangan (AAA), Raja (AAB), Rastali (AAB), Awak (ABB), Nipah (BBB), Kapas (AAB) dan Nangka (AAB) untuk menilai hubungan genetik antara jenis-jenis tersebut. Dua puluh lima individu daripada setiap jenis telah disampel dari negeri Perak, Selangor, Melaka dan Negeri Sembilan. Lima primer panjang terpilih iaitu PEH A3, ERICIR, PUCMI3F, BOXAIR dan PEH A6 telah digunakan untuk menggandakan genom DNA. Corak jalur DNA diperhatikan dan dianalisis. Keputusan menunjukkan fragmen terbesar ialah 2500 bp manakala fragmen yang terkecil ialah 100 bp. Primer ERICIR adalah paling polimorfik (26.5%) manakala PEH A6 merupakan primer yang paling kurang polimorfik (20.8%). Dendrogram menunjukkan terdapat tiga kumpulan utama. Kumpulan I terdiri daripada Berangan, Rastali, Mas, Nangka dan Raja. Kumpulan II merangkumi Kapas dan Awak yang mempunyai nilai jarak genetik terendah (0.3633), di mana kedua-duanya adalah jenis pisang untuk dimasak. Kumpulan I dan II mempunyai perhubungan genetik yang rapat dengan nilai jarak genetik 0.396. Pisang Nipah (BBB) jelas terasing daripada kedua-dua kumpulan tersebut dengan nilai jarak genetik 0.795. Walaupun semua jenis pisang ini berbeza antara satu sama lain secara morfologi, hasil kajian ini menunjukkan darjah keserupaan genetik antara jenis-jenis pisang tersebut adalah selari dengan kumpulan genotip, sama ada A atau B, yang dikongsi.

ABSTRACT

LP-RAPD (Long primer Random Amplified Polymorphic DNA) analysis was carried out on eight banana cultivars namely Mas (AA), Berangan (AAA), Raja (AAB), Rastali (AAB), Awak (ABB), Nipah (BBB), Kapas (AAB) and Nangka (AAB) to assess their genetic relationships. Twenty five individuals of each cultivar were collected from the Malaysian states of Perak, Selangor, Melaka and Negeri Sembilan. Five long-primers, namely PEH A3, ERICIR, PUCMI3F, BOXAIR and PEH A6 were selected to amplify the genomic DNA. The DNA banding patterns were observed and analyzed. The results showed that the largest fragment was 2500 bp and the smallest 100 bp. The ERICIR was found to be the most polymorphic primer (26.5%) whilst PEH A6 was the least polymorphic (20.8%). The dendrogram revealed three major groups. Group I consisted of Berangan, Rastali, Mas, Nangka and Raja cultivars. Group II included Kapas and Awak, which had the lowest genetic distance (0.3633) and are known as plantains by sharing the B genotype. Groups I and II were clustered closely together with a genetic distance of 0.3961 indicating a close relationship between the two groups. The Nipah (BBB) cultivar alone was distinctly separated from both Groups I and II with a genetic distance of 0.795. Although all cultivars differ morphologically, the findings agree with the degree of shared genotypes, A or B, among the cultivars.

INTRODUCTION

Cultivated bananas (including plantains) belong to the *Eumusa* section of the family Musaceae and are natural hybrid polyploids (diploid, triploid or tetraploid) of two species of Musa: *Musa acuminata* (genome A) and *Musa balbisiana* (genome B). These tallest monocotyledons (Stover and Simmonds, 1987) have become the premier fruiting plants of Southeast Asia and are considered to be of great socioeconomic importance in the countries of the region. Bananas rank second or third in importance among the industrial fruits of India, Malaysia and Taiwan, and are important export commodities for countries such as Malaysia and those in Central America (Valmayor, 1987). All cultivars are classified into various genomic groups such as AA, AAA, AB, AAB and ABB based on the morphological scoring method (Simmonds, 1987). Cultivars containing the B genotype have starchy and acidic fruits and they are usually eaten boiled, fried or roasted. Cultivars containing the A genome have sweet and fine textured fruits, and they are mainly eaten raw or as a dessert. Among the popular dessert bananas in Malaysia are Pisang Mas (AA), Berangan (AAA) and Rastali (AAB) while the popular cooking types are Pisang Raja (AAB), Nangka (AAB), Awak (ABB), Nipah (BBB) and Kapas (AAB) (Jamaluddin, 1990).

Mas has a small fruit, 8.0-12.0 cm in length and 3.0-4.0 cm in diameter. The peel is thin and golden yellow in colour when ripe. The pulp is firmly attached, yellow in colour, aromatic and very sweet. The fruit of Berangan is medium to large in size and the peel is thick, golden yellow in colour and covered with slight to heavy blemishes. The flesh of Berangan is very aromatic and sweet. Rastali has a thin peel, yellow orange in colour when ripe and covered with moderate to heavy black blemishes and slightly sour in taste. The fruit of Raja is angular and the skin is thick and coarse and develops an orange-yellow colour when ripe. The flesh of Raja is coarse in texture. The Nangka plant is short to medium and the fruit is long, pointed and angular. The peel is thick and remains green when ripe. The pulp of Nangka is creamy, starchy and slightly sour in taste. The Awak has a small to medium fruit and becomes yellow when ripe. The skin of Awak is thick and the pulp is whitish, firm and consistently sticky. The Nipah has a short, stout and angular fruit with a thick skin that turns yellow when ripe. The fruit of Kapas is shorter than the Awak and the skin turns yellow in colour when ripe. The taste is sweet with slight subacid flavour (Jamaluddin, 1990).

The classical approaches for the identification of banana cultivars are based on morphological characters. However, morphological changes caused by environmental factors are major obstacles to accurately identify the varieties (Kaemmer *et al.*, 1992). DNA markers have proven to be useful, efficient and reliable methods for genetic characterization, studying genetic diversity and relationships among populations and varieties because they are not affected by environmental conditions (William *et al.*, 1990).

Random Amplified Polymorphic DNA (RAPD) is widely and successfully used for determining genetic diversity in a number of plant species, such as plum (Ortiz *et al.*, 1997), lemon (Deng *et al.*, 1996) and grapes (Qu *et al.*, 1996). The RAPD technique is relatively quick, inexpensive and requires no prior sequence information of the target genome. Small amounts of DNA are sufficient. It requires the use of no radioactive isotopes and yet can detect a good number of polymorphisms (William *et al.*, 1990; Welsh and McClelland, 1990). However, this technique requires careful optimization that can affect the reproducibility of the results (Yang and Quiros, 1993). To overcome the reproducibility problem, the long primer Random Amplified Polymorphism DNA (LP-RAPD) technique (Gillings and Holley, 1997) is used. This technique provides a more sensitive and reproducible PCR method because longer primers can make the PCR amplification more selective and thus be able to easily distinguish closely related organisms.

TABLE 1
Primers used in the LP-RAPD method (Gillings and Holley, 1997)

Primers	Sequence (5''Æ3')
PEH A3	CAGCAGAACCCGCGCCTGATCCAG
PUCM13F	CGCCAGGGTTTTCCCAGTAGTCAC
BOXAIR	CTACGGCAAGGCGACGCTGACG
PEH A6	ATCGCACTTGATGATGCGCAGGCCGTT
ERICIR	ATGTAAGCTCCTGGGGATTCAC

The objective of this study was to assess the genetic relatedness among eight local banana cultivars by using the LP-RAPD technique.

MATERIALS AND METHODS

Plant Material

Eight local banana cultivars, namely Pisang Mas (AA), Berangan (AAA), Rastali (AAB), Raja (AAB), Nangka (AAB), Awak (ABB), Kapas (AAB) and Nipah (BBB), were used in this study. Twenty five samples of each cultivar were collected from the Malaysian states of Perak, Selangor, Melaka and Negeri Sembilan.

DNA Isolation

Young leaves from each individual sample were stored at -20°C prior to DNA extraction. Total DNA was extracted following the CTAB method of Doyle & Doyle (1990) with some modifications.

One tenth gram (0.1 g) of leaves was ground by using cold mortar and pestle in 1 ml of 65°C preheated CTAB buffer [2% (w/v) CTAB, 1.4M NaCl, 0.2% mM EDTA, 100mM Tris-HCl (pH 8.0) and 1% (v/v) PVP-40] and incubated at 67°C for 60 minutes. The lysate was extracted with 0.79 ml of chloroform/ isoamylalcohol (24:1) and centrifuged for 15 min at 1000 rpm at 4°C. In order to precipitate the DNA, the aqueous portion was mixed with an equal volume of cold isopropanol.

The DNA precipitate was washed in 1 ml of 70% ethanol with 3M ammonium acetate and air dried before being resuspended in 200ml of TE buffer [5 mM Tris, 0.1 mM EDTA, pH 7.5].

The DNA purity was determined by using a spectrophotometer. The absorbance was read at wavelengths of 260 nm and 280 nm. DNA purity is indicated by the ratio of absorbance at 260 nm and 280 nm of 1.8 to 2.0.

DNA Amplification

The amplification was performed as described by William *et al.* (1990) with some modifications. The amplification reactions consisted of 50 ng of template DNA; 1X PCR reaction buffer [10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1% Triton®X-100], 3.0 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP and dGTP; 1.5 units of Taq DNA Polymerase and 1 μM primer. The primers used in this study are shown in Table 1 (Gillings and Holley, 1997). Sterilized deionized water was added to make up 10 μl of total reaction volume. Samples were amplified in a MJ Research PTC –100 thermal cycler. The thermal cycler was programmed for predenaturation at 94°C for 5 min. Samples were processed through 39 cycles consisting of denaturation at 94°C for 30 sec, annealing at 52°C for 1 min and extension at 72°C for 2 min. For the last cycle, the extension step at 72°C was extended to 10 min. The amplified fragments were separated on 2.0 % agarose gel using 1x TBE buffer. After staining with ethidium bromide the bands were visualized and photographed using Alpha Imager Digital Imaging System (Siber Hegner Sdn. Bhd.).

TABLE 2
Size range of fragments (bp), number of amplified bands, number of monomorphic bands and percentage of polymorphic bands produced by five primers in eight banana cultivars

Primer	Fragments size (bp)	No. of amplified band	% of monomorphic band	% of polymorphic band
Peh A3	100-2500	44	74.6	25.4
ERICIR	100-2500	36	73.5	26.5
PUC M13F	150-1600	38	79.2	20.8
BOXAIR	230-2000	33	73.9	26.1
PEH A6	140-1500	41	76.9	23.1

Analysis of DNA Amplification

Clear and sharp bands of the amplified DNA using different primers were compared among banana cultivars. Each band was scored as present (1) or absent (0). Genetic distances (D) were calculated using the formula of Nei & Li (1979). A dendrogram was constructed based on the genetic distance matrix data by using the Unweighted Pair Group with Arithmatic Mean (UPGMA) method (Sneath and Sokal, 1973).

RESULTS

From the 10 long primers tested, five primers gave clear and high resolution banding patterns. A combination of five selected primers produced a total of 192 scorable bands ranging in size from 100 bp to 2500 bp. Among these, 145 were monomorphic and 47 were polymorphic in the eight banana cultivars. Each primer generated between 33 and 44 scorable bands (Table 2). The highest number of scorable bands was generated by PEH A3 while the lowest was by BOXAIR. The most informative primer was ERICIR which produced the highest percentage of polymorphic bands (26.5%) whilst the lowest percentage (20.8%) was produced by PUCM13F (Table 2).

The average number of polymorphic bands detected per primer was 29. Among the cultivars, Kapas had the highest percentage of polymorphic bands whilst Raja had the lowest. Table 3 shows that the PEH A3 primer produced 300 bp, 400 bp and 1000 bp bands as common bands in all cultivars. The Raja, Rastali, Nipah, Mas, Awak and Nangka cultivars are distinguishable among themselves by the presence of unique 1500 bp, 366 bp, 320 bp, 1250 bp, 800 bp and 550 bp bands, respectively. Rastali also can be marked at 325 bp.

The ERICIR primer produced a 750 bp band which was present in all cultivars as a common band. The Mas was clearly distinguished from the other cultivars by the presence of a 2500 bp band. The 1375 bp band only existed in the Nangka cultivar. All cultivars had 1125 bp except for Mas. The 416 bp band was only absent in the Nipah cultivar while the 375 bp band was observed in the Raja cultivar. The 400 bp, 450 bp and 500 bp bands can also be used to distinguish Nipah from the other cultivars.

The BOXAIR primer showed that all cultivars had a 250 bp band. The Nipah cultivar can be distinguished from the other cultivars by the absence of a 325 bp band which existed in all other cultivars. The 600 bp band was present in all AAB cultivars except for Kapas while Berangan, Raja and Mas cultivars shared a 1000 bp band. The Rastali cultivar can be discriminated from the other cultivars by the presence of the 1125 bp and 1375 bp bands. The PEH A6 primer revealed that all cultivars had a 217 bp band (*Plate 1*). The Nipah was clearly distinguished from the other cultivars by the absence of 225 bp band. Awak and

TABLE 3
Distribution of LP-RAPD markers within eight banana cultivars.
'+' indicates presence and '-' indicates absence of band

Primer/Locus	bp	Cultivar							
		Berangan	Nangka	Raja	Rastali	Nipah	Kapas	Awak	Mas
PEH A3	1500	-	-	+	-	-	-	-	-
	1250	-	-	-	-	-	-	-	-
	1125	+	-	+	-	-	+	-	-
	1000	+	+	+	+	+	+	+	+
	900	+	+	-	-	+	+	-	+
	800	-	-	-	-	-	-	+	-
	750	+	+	-	-	-	+	-	-
	700	+	+	+	+	+	+	+	+
	650	+	+	-	-	-	-	-	+
	550	-	+	-	-	-	-	-	-
	475	-	+	+	+	-	+	-	-
	400	+	+	+	+	+	+	+	+
	366	-	-	-	+	-	-	-	-
	320	-	-	-	-	+	-	-	-
	300	+	+	+	+	+	+	+	+
	325	-	-	-	+	-	-	-	-
ERICIR	2500	-	-	-	-	-	-	-	+
	1500	-	-	-	+	-	+	+	+
	1375	-	+	-	-	-	-	-	-
	1125	+	+	+	+	+	+	+	-
	900	-	-	-	+	-	+	+	-
	850	-	-	-	-	+	+	+	-
	800	-	-	+	-	-	-	-	-
	750	+	+	+	+	+	+	+	+
	700	-	-	-	-	+	-	-	-
	650	+	-	+	+	+	-	+	-
	600	-	-	+	-	-	-	-	-
	500	-	-	-	-	+	-	-	+
	450	-	-	-	-	+	-	-	-
	433	+	+	+	+	-	-	+	+
	416	+	+	+	+	-	+	+	+
	400	-	-	-	-	+	-	-	-
	375	-	-	+	-	-	-	-	-
	312	-	-	+	+	+	+	-	+
	300	+	-	+	-	-	-	-	-
PUCM13F	1250	-	+	-	+	-	-	-	+
	1000	-	-	-	+	+	-	+	-
	925	-	-	-	-	-	-	-	+
	900	+	+	+	-	-	-	-	-
	750	-	-	-	+	-	-	-	-
	700	+	+	+	-	-	-	+	+
	575	-	-	-	-	-	-	+	-
	550	+	+	+	+	+	+	-	-

Table 3: Continued

Primer/Locus	bp	Cultivar							
		Berangan	Nangka	Raja	Rastali	Nipah	Kapas	Awak	Mas
	525	-	-	-	-	-	-	-	+
	500	-	-	+	-	+	+	+	-
	450	-	+	-	-	-	-	-	-
	425	+	+	+	+	+	+	+	+
	375	+	+	+	-	-	-	-	-
	350	+	+	-	-	+	-	-	+
	266	+	+	+	-	+	+	-	+
	250	+	+	+	-	+	-	-	-
	216	+	+	+	-	+	+	-	-
	171	+	-	-	+	-	-	-	-
	128	-	-	-	+	-	-	-	-
BOXAIR	2000	-	-	+	+	-	-	-	+
	1500	+	+	+	-	-	-	-	-
	1375	-	-	-	+	-	-	-	-
	1250	+	-	+	-	-	+	+	-
	1125	-	-	-	+	-	-	-	-
	1000	+	-	+	-	-	-	-	+
	900	-	-	-	-	+	-	-	+
	800	+	+	-	-	-	-	+	+
	750	-	-	+	-	-	-	+	-
	700	-	-	-	-	-	+	-	-
	650	+	-	+	+	-	+	+	+
	600	-	-	-	+	-	-	-	+
	550	-	-	-	+	-	-	-	+
	500	-	+	-	-	+	-	+	+
	475	-	+	-	-	-	-	-	-
	450	+	-	-	-	+	+	+	+
	400	-	+	+	+	-	+	+	+
	350	-	-	-	-	+	-	-	+
	325	+	+	+	+	-	+	+	+
	250	+	+	+	+	+	+	+	+
PEH A6	1500	+	-	-	-	-	-	-	-
	1250	+	-	-	-	-	+	-	-
	1000	+	-	-	-	-	-	+	-
	700	+	-	-	-	-	+	+	-
	650	+	-	-	-	-	-	+	-
	600	-	-	-	-	-	-	+	-
	500	+	+	-	-	-	-	-	-
	350	-	-	-	-	-	-	+	-
	267	-	-	-	+	+	-	-	-
	225	+	+	+	+	+	+	+	-
	217	+	+	+	+	+	+	+	+
	175	+	+	+	-	+	+	-	-
	150	+	+	-	-	-	+	+	+
	125	-	+	+	+	+	+	-	+

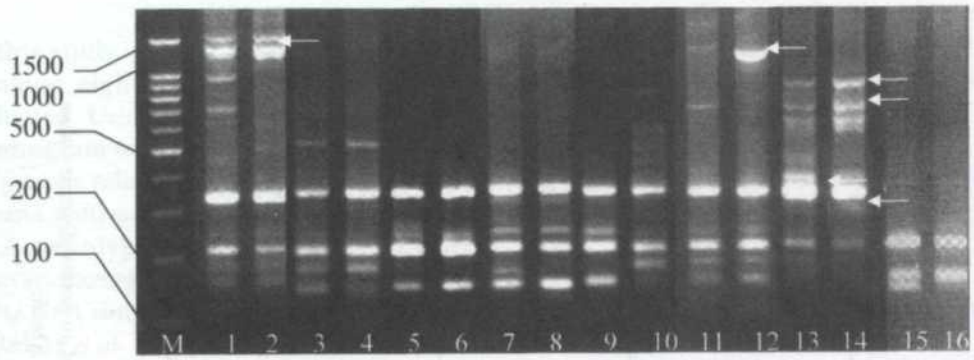

Plate 1: DNA banding patterns among eight banana cultivars obtained by using the long-primer PEH A6 (M = 100 bp ladder; lane 1 and 2 = Berangan; lane 3 and 4 = Nangka; lane 5 and 6 = Raja; lane 7 and 8 = Rastali; lane 9 and 10 = Mas; lane 11 and 12 = Kapas; lane 13 and 14 = Awak; lane 15 and 16 = Nipah

TABLE 4
Genetic distance values among eight banana cultivars

	BERANGAN	NANGKA	RAJA	RASTALI	NIPAH	KAPAS	AWAK	MAS
BERANGAN	0							
NANGKA	0.74823	0						
RAJA	0.78977	0.79455	0					
RASTALI	0.74226	0.78955	0.74786	0				
NIPAH	0.82475	0.79233	0.79806	0.78011	0			
KAPAS	0.80261	0.81485	0.82394	0.80412	0.79628	0		
AWAK	0.76815	0.76134	0.77332	0.74654	0.79111	0.72661	0	
MAS	0.7507	0.77489	0.78525	0.76092	0.79213	0.80595	0.76868	0

Berangan both shared the 1000 bp and 650 bp bands. The Awak cultivar was characterized by the presence of 350 bp and 600 bp bands while Berangan had a 1500 bp band. Kapas was distinguished from the other cultivars by the presence of a 1250 bp band.

The PUCM13F primer produced a 425 bp band in all cultivars. The 375 bp band can be used to distinguish the Raja, Berangan and Nangka cultivars from the other cultivars. The 750 bp and 128 bp bands were observed only in the Rastali cultivar while the 575 bp band was only present in the Awak cultivar. The 925 bp and 525 bp bands were only exhibited in the Mas cultivar and can be considered as marker bands.

The values of the genetic distance between pairs of banana cultivars are presented in Table 4. The dendrogram constructed by using the UPGMA method demonstrated that three major groups existed in the collection. Group I consists of Berangan (AAA), Rastali (AAB), Mas (AA), Nangka (AAB) and Raja (AAB) cultivars. Group II included Kapas (AAB) and Awak (ABB), which had the lowest genetic distance (0.3633) and are known as plantains by sharing the B genotype. Group I and II are clustered closely together with a genetic distance of 0.3961, indicating a close relationship between the two groups. The Nipah (BBB) cultivar alone was distinctly separated from both Groups 1 and II with a genetic distance of 0.795.

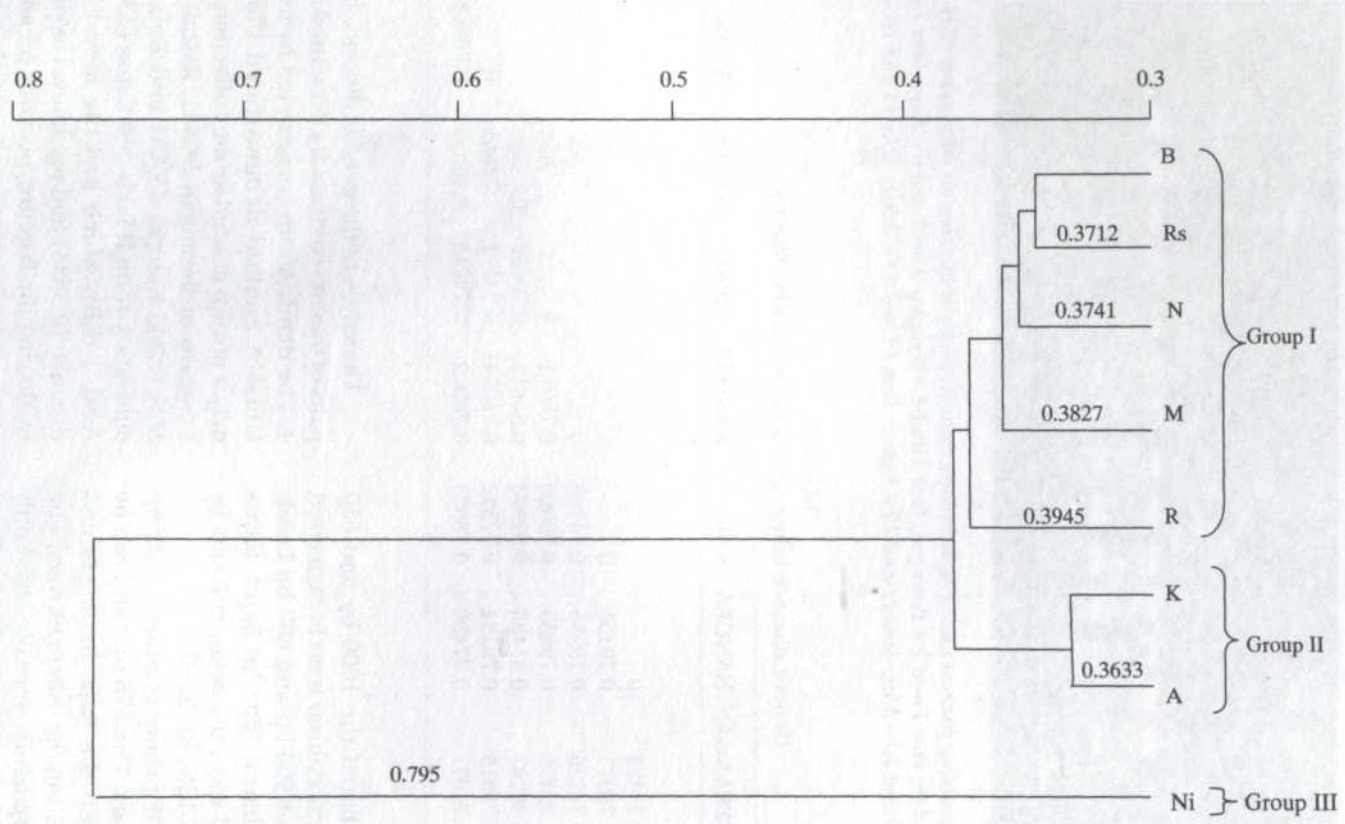

Fig. 1: Dendrogram showing the clustering of eight banana cultivars constructed by using the UPGMA method based on genetic distance values. (B = Berangan, Rs = Rastali, N = Nangka, M = Mas, R = Raja, K = Kapas, A = Awak, Ni = Nipah)

DISCUSSION

In this study, genetic distances among the common eight local banana cultivars were evaluated. Using the genetic distance data, a dendrogram was constructed to demonstrate the genetic relationships among the eight local banana cultivars. All cultivars sharing at least an A genotype were separated from the cultivars having at least a B genotype, as shown in *Fig. 1*. A similar observation was also made by Rekha *et al.* (2001). In general, low genetic distance was demonstrated between cultivars sharing the A genotype and within Group II itself. All the cultivars are different morphologically, especially the pseudostem colour, height and fruit shape as reported by Jamaluddin (1990) and also fruit size, leaf shape and flavour (Valmayor *et al.*, 2000). However, the results revealed that they are genetically closer and share at least an A genotype.

The Nipah cultivar which has a triploid BBB genotype was distantly separated from the other two groups although some of them shared at least a B or BB genotypes. This showed that the contribution of the B or BB genotypes in the triploid bananas did not significantly affect the genetic relatedness among the eight banana cultivars.

The LP-RAPD technique allowed the detection of polymorphisms by only using five long primers. The amplification products were generally reproducible and reliable. Some aspects need further investigations, as there was a confusion regarding the grouping of two popular bananas, Berangan (AAA) and Mas (AA), in the AAB group. Some variations might occur in both cultivars due to human selection or geographical factors as reported by Sagredo *et al.* (1998).

CONCLUSIONS

The results of this study have proven that LP-RAPD is an effective molecular technique to be used in assessing the genetic variation and relationships among the eight banana cultivars. Low genetic distance was demonstrated between cultivars sharing the A genotype. All cultivars sharing at least an A genotype were separated from cultivars having at least a B genotype. Among the eight cultivars, Kapas and Awak had the lowest genetic distance while Nipah was distantly separated from the other cultivars.

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