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Full Length Research Paper

Genetic relationship among *Musa* genotypes revealed by microsatellite markers

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A banana germplasm was established containing 44 *Musa* genotypes collected from various locations in Malaysia. To detect their genetic variation and to rule out duplicates among cultivar, microsatellite markers were used in their analysis. The microsatellite profiles of 44 *Musa* genotypes of various origins were detected by 130 alleles at nine microsatellite loci. Genetic distances and relationships were determined with one locus designated as MaOCEN08 being most polymorphic. The highest percentage of similarity observed was 43%. This was between *Musa* sp. cv. Raja and *Musa* sp. cv. Mas and this indicated that these two genotypes are the closest relatives. The analysis also revealed that an unknown cultivar was 100% dissimilar from the rest of the *Musa* genotypes. It was therefore concluded that all accessions in the germplasm collection are of different genotypes and none are duplicates. Seven clusters were established to group the genotype, whereby the groupings did not imply their ploidy levels.

Key words: Banana, germplasm, microsatellites, plantain, genetic relationship.

INTRODUCTION

There are approximately 50 species of *Musa* worldwide, with 68 accessions reported in Malaysia (Pollefeys et al., 2004). Researchers believe their center of diversity to be either Malaysia or Indonesia (Daniells et al., 2001). The number of new species in Malaysia has increased recently. Two new wild banana species, *Musa bauensis* Hākkinen and Meekiong (Hakkinen, 2004) and *Musa barioensis* Hakkinen (Hakkinen, 2006) were found in Bau and Bario, two locations belonging to two different divisions in Sarawak, Bau and Bario, respectively. The latter, *M. barioensis* is abundant in the Bario Kelabit highland and is the only *Musa* species that occurs in that area. *Musa* species are grouped according to ploidy levels, the number of chromosome sets they contain and the relative proportion of *Musa acuminata* (A) and *Musa balbisiana* (B), both with 2n = 2x = 22 chromosomes, in their genomes (Simmonds and Sheperd, 1955; Lescot et al., 2008). Triploid hybrids (AAA, AAB and ABB) are the most familiar and are the seedless and cultivated varieties (cultivars) of banana. Diploids (AA, AB and BB) and tetraploids (AAAA, AAAB, AABB and ABBB) are much rare. The latter essentially being experimental hybrids. Based on their morphological characteristics and chromosome numbers, *Musa* was separated into four sections, namely, *Musa*, *Rhodochlamys, Callimusa* and *Australimusa*. Wong et al. (2002) used amplified fragment length polymorphism (ALFP) to assess the validity of these sections and suggested that the species of sect. *Rhodochlamys* should be combined into a single section with sect. *Musa* and likewise species of sect, *Australimusa* to be merged with those in sect. *Callimusa*. Considering that new wild banana species are still being discovered, it is essential to characterize them during each sample collection so that cultivars are not mistaken as species. Most collections are identified according to their common names but this can easily lead to duplication of sample collections as different regions have different common names. Morphological characterization and determination of their genomes are inadequate and thus require molecular support. Various markers have been developed for genetic relationship studies but

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microsatellites are more favorable when com-pared to random amplified polymorphic DNA (RAPD), ALFP and restriction fragment length polymorphism (RLFP). This is because microsatellites are co-dominant in nature, are highly polymorphic and are also genus specific. Microsatellite markers are small arrays of tandemly arranged bases spread throughout the genome. They are sequences made up of a single sequence motif, often more than six bases long, and can be mono-, di- or trinucleotide, tandemly repeated, and arranged head-to-tail without interruption by any other bases or motifs (Dograr et al., 2000; Hancock, 2000; Ha et al., 1988). Microsatellites are now generally considered to be the most powerful genetic markers for genetic mapping and evolutionary studies (Yue et al., 2009). During the establishment of these banana collections from Peninsular Malaysia, most of the genotypes were assigned by their local name. Two wild species identified as *M. acuminata* and *Musa vietnam* were included. Initial steps were needed to determine whether the genotypes were duplicates or of different cultivars. However, identification of genotypes is usually delayed when relying on their morphological characteristics. Therefore, it was essential to have genetic analysis performed using microsatellite markers, with the objective of estimating the genetic diversity, genetic distance and genetic relationships among the 44 *Musa* genotypes.

MATERIALS AND METHODS

A total of 44 *Musa* genotypes collected from Peninsular Malaysia were examined (Table 1). Collections were established in 2006 and maintained under field conditions at the Department of Crop Science, Faculty of Agriculture, University Putra, Malaysia. DNA was extracted from leaf samples using GeneAll® Plant DNA extraction kit and quantified in 1.4% agarose gel stained with ethidium bromide. Polymerase chain reaction (PCR), was carried out in a total volume of 20 µl consisting of 30 ng of genomic DNA, 1.5 mM $MgCl_2$, 100 µM of each dNTPs, 0.2 µM of the forward and reverse primers and 1.5U of Taq Polymerase (Creste et al., 2006). Ten primers (Table 2) previously developed from *M. acuminata* (Creste et al., 2006) and *M. balbisiana* (Buhariwalla et al., 2005) were used in this study. Amplifications were carried out in a thermalcycler programmed with a touchdown cycle, with an initial step at 94°C for 4 min, followed by 10 cycles of 40 s at 94°C, 40 s at annealing temperature decreasing from 55 to 45°C by 1°C every cycle, and 60 s at 72°C, followed by 30 identical cycles with annealing temperature at 45°C for 40 s (Creste et al., 2006). The PCR products were finally separated on 2% agarose gel stained with ethidium bromide. Allele sizes were recorded and analyzed. The genetic similarities among the 44 *Musa* genotypes were estimated using Jaccard's coefficients. Cluster analysis was performed on the similarity matrices using the unweighted pair group method with arithmetic averages (UPGMA), all utilizing the NTSYS-pc version 2.1 software (Rohlf, 2000). POPGENE program version 1.31 was used to estimate the genetic diversity parameters (Yeh et al., 1999).

RESULTS

Of the ten primers screened, only one (MaOCEN17) did not show any amplification for the 44 *Musa* genotypes. A total of 130 alleles were observed at the 9 polymorphic microsatellites loci. The number of alleles (A) (Table 3) ranged from 9 to 21 averaging to 14.44. The effective allelic number (Ae) ranged from 1.15 to 9.50 and was 5.49 on the average. The overall size of amplified products ranged from 100 to 538 bp, with locus MaOCEN08 having the highest number of alleles (21 alleles) and locus Mb1-63 having the lowest (4 alleles) (Table 3). Locus MaOCEN08 also had the highest number of effective alleles (13.08 effective alleles) and locus Mb1 - 63 also had the lowest (1.5 effective alleles). The polymorphic information content (PIC) value is a reflection of allele diversity and frequency among the cultivars. The higher the PIC value of a locus, the higher the number of alleles detected (Wong et al., 2009). The PIC value for the loci ranged from 0.1692 to 0.6229 with MaOCEN08 primer having the highest value. The expected heterozygosity (He) exhibited a range of variation from 0.13 at Mb1-63 locus to 0.93 at MaOCEN08 locus generating a mean of 0.69. The Nei's gene diversity for the 9 loci was between 0.1302 and 0.9236 and Shannon's information index ranged from 0.3238 to 2.8046.

By comparing these indices, it was observed that the most polymorphic loci was MaOCEN08, revealing a high genetic variation among the 44 M genotypes, while the least polymorphic loci was Mb1-63, revealing a low genetic variation among the 44 *Musa* genotypes. The Nei's gene diversity index also showed that occurrence of the MaOCEN08 locus was the highest among the genotypes. The observed heterozygosity (Ho) was 0 and the expected heterozygosity (He) averaged to 0.6885 and ranged from 0.1317 to 0.9342. The coefficient of genetic differentiation (Fst) value was 1.000 indicating that the genetic variation accounted was 100%.

Based on the dendrogram in Figure 1, it was clearly shown that the 44 *Musa* genotypes could be further classified into seven clusters. Cluster 1 has five genotypes, namely, Raja, Mas, Awak Tak Berbiji, Belalai Gajah and Kelat Siam; while Cluster 2 consists of Nangka Manis, Toman, Kapas, Gala, Embun, Kelat lega, Raja Udang, Rasa and Keling; Cluster 3 consists of ten members, that is, Abu Filipina, Rotan, Awak Asli, Bakar Sayur, Batu, Rastali, Nipah, Musa acuminata, Hutan Jantung Ungu and Lilin; Cluster 4 had the most members, that is, Nangka, Susu, Jari Buaya, Berangan, Abu Keling, Kelat Perlis, Abu, Buloh, Kari, Berangan Asli, Bakar Baling and Serendah; whereas Cluster 7 consists of only one member, that is, Unknown X. Cluster 5 has four members, namely, Hutan Jantung Kuning, Lang, Hutan and M. vietnam; while Cluster 6 consists of Karang, Lemak Manis and Basah.

DISCUSSION

Primers developed from locus MaOCEN08 were most

Table 1. List of *Musa* species and cultivars utilized for the genetic analysis. All genotypes were collected from Peninsular Malaysia. Collections were established in 2006 and maintained under field conditions in University Putra, Malaysia.

S/N	Genotype	Designation	Genome group	Origin
1	*Musa acuminata*	MAcum	AA	UPM Serdang, Selangor
2	Rotan	Rotan	AA	UPM Serdang, Selangor
3	Kapas	Kapas	AA	UPM Serdang, Selangor
4	Masam/Keling	Keling	AA	Petaling, Selangor
5	Jari Buaya	JBuaya	AA	Petaling, Selangor
6	Lemak Manis	LemakM	AA	Sabak Bernam, Selangor
7	Mas	Mas	AA	Sabak Bernam, Selangor
8	Hutan Jantung Kuning	HJKun	AA	UPM Serdang, Selangor
9	Hutan Jantung Ungu	HJUngu	AA	UPM, Serdang, Selangor
10	Hutan	Hutan	AA	UPM, Serdang, Selangor
11	Lilin	Lilin	AA	Muar, Johor
12	Bakar Baling	BakarB	AAA	Batu Pahat, Johor
13	Raja Udang	RajaU	AAA	UPM Serdang, Selangor
14	Toman	Toman	AAA	Batang Berjuntai, Selangor
15	Embun	Embun	AAA	UPM Serdang, Selangor
16	Buloh/Raja Setalon	Buloh	AAA	Sabak Bernam, Selangor
17	Bakar Sayur	BakarS	AAA	Segamat, Johor
18	Berangan Asli	BeraA	AAA	Malaka
19	Berangan	Berang	AAA	UPM, Serdang, Selangor
20	Serendah	Seren	AAA	UPM, Serdang, Selangor
21	Susu	Susu	AAA	Jerantut, Pahang
22	Belalai Gajah	Gajah	AAB	Kota Setia, Perak
23	Rastali	Rast	AAB	Sabak Bernam, Selangor
24	Nangka	Nangka	AAB	UPM Serdang, Selangor
25	Raja	Raja	AAB	Sabak Bernam, Selangor
26	Nangka Manis	NangM	AAB	Sabak Bernam, Selangor
27	Lang	Lang	AAB	Sabak Bernam, Selangor
28	Abu Keling	AbuK	ABB	Sabak Bernam, Selangor
29	Kari	Kari	ABB	Batang Berjuntai, Selangor
30	Awak Tak Berbiji	AwakTB	ABB	Sabak Bernam, Selangor
31	Kelat Siam	KelatS	ABB	Kuala Terengganu, Terengganu
32	Batu	Batu	ABB	Sabak Bernam, Selangor
33	Kelat Perlis	KelatP	ABB	Segamat, Johor
34	Abu	Abu	ABB	UPM Serdang, Selangor
35	Kelat Lega	KLega	ABB	Tanah Merah, Kelantan
36	Awak Asli	AwakA	ABB	Segamat, Johor
37	Nipah	Nipah	BBB	Segamat, Johor
38	Abu Filipina	AbuF	BBB	UPM Serdang, Selangor
39	Gala	Gala	BB	Tanah Merah, Kelantan
40	Rasa	Rasa	Unknown	Tanah Merah, Kelantan
41	Unknown X	X	Unknown	UPM Serdang, Selangor
42	Karang	Kara	Unknown	Tanah Merah, Kelantan
43	Basah	Basah	Unknown	Sabak Bernam, Selangor
44	*Musa vietnam*	Mviet	Unknown	Tenom Agriculture Park, Sabah

suitable for genetic diversity studies among the *Musa* genotypes because it produces more alleles. It was also the most polymorphic loci, and revealed a high genetic variation among the *Musa* genotypes. From the analysis of genetic diversity among the 44 *Musa* genotypes, it was observed that the genetic diversity was very high and Fst value of 1.000 clearly indicated that all the members of the germplasm were of different genotypes and not

Table 2. Sequences of 10 *Musa* microsatellite primers used to assess genetic diversity among the 44 *Musa* genotypes, including their predicted product and range of allele size.

S/N	Label	SSR motif	Sequence of primer 5'-3'	Predicted product size (bp)	Range of allele size (bp)
1	Mb1-63	(GA)20	F : AATGGCTGCCTGCCATGCA R: GAATCAAAGAGGCGAGAAGACGA	191	173 - 183
2	Mb1-69	(TC)15	F : CTGCCTCTCCTTCTCCTTGGAA R : TCGGTCATGGCTCTGACTCA	386	363 - 416
3	Mb1-113	(GA)18	F : AGGTGCCACACAGTTCAGACA R : CAACCCAAACCTGTTCGACCAA	399	360 - 538
4	Mb1-134	(GA)21	F : ATGCCCAAGAAGGGAAGGGAA R : TAATGCCGGAGGATCAGTGTGA	398	390 - 450
5	Mb1-139	(GA)21	F : AGGGAGGGATCAGAAGGAGCA R : CGGCTTCCTTCTGAAGGTTCCA	190	No amplification
6	MaOCEN01	(CT)17	F : TCTCAGGAAGGGCAACAATC R : GGACCAAAGGGAAAGAAACC	210-250	234 - 318
7	MaOCEN04	(AG)20	F : GCCGAGAGAGGAAATGGAA R : CTGAGAGGGTAGAGTGGATG	130-180	100 - 192
8	MaOCEN08	(GA)16	F : ATCAGCAACACAGGAAGAAAAG R : AATCTGGACGAGGGAGCA	240-270	250 - 361
9	MaOCEN13	(TC)16	F : GCTGCTATTTTGTCCTTGGTG R : CTTGATGCTGGGATTCTGG	141-200	139 - 229
10	MaOCEN17	(TC)15	F : CGCTTCTGCCTTGTTTCTGT R : TGGGTTGACTCCTTTTGTGG	150-190	155 - 278

SSR, Simple sequence repeat; bp, base pair

Table 3. Genetic variation of microsatellite loci and genotype genetic structure of the primers used.

Loci	Sample size	A	Ae	I	Ho	He	Nei	Fis	Fst	PIC
MaOCEN01	88	11.0000	1.8615	1.2006	0.0000	0.4681	0.4628	1.0000	1.0000	0.1692
MaOCEN04	88	19.0000	4.4201	2.2216	0.0000	0.7827	0.7738	1.0000	1.0000	0.2326
MaOCEN08	88	21.0000	13.0811	2.8046	0.0000	0.9342	0.9236	1.0000	1.0000	0.6229
MaOCEN13	88	14.0000	3.0730	1.7434	0.0000	0.6823	0.6746	1.0000	1.0000	0.2195
MaOCEN17	88	19.0000	9.3077	2.5881	0.0000	0.9028	0.8926	1.0000	1.0000	0.4899
Mb1-63	88	4.0000	1.1496	0.3238	0.0000	0.1317	0.1302	1.0000	1.0000	0.2874
Mb1-69	88	9.0000	2.4506	1.3599	0.0000	0.5987	0.5919	1.0000	1.0000	0.2723
Mb1-113	88	15.0000	4.5877	2.0912	0.0000	0.7910	0.7820	1.0000	1.0000	0.3058
Mb1-134	88	18.0000	9. 4902	2.5819	0.0000	0.9049	0.8946	1.0000	1.0000	0.5272
Mean	88	14.4444	5.4913	1.8795	0.0000	0.6885	0.6807	-	-	-
Standard deviation		5.5703	4.1419	0.8049	0.0000	0.2596	0.2567	-	-	-

A, Observed number of alleles; Ae, effective number of alleles; I, Shannon's diversity index (Lewontin, 1972); Ho, observed heterozygosity; He, expected heterozygosity; Nei, Nei's expected heterozygosity (1973); Fis, Wright's fixation index (1978); Fst, coefficient of genetic differentiation; PIC, polymorphism information contents.

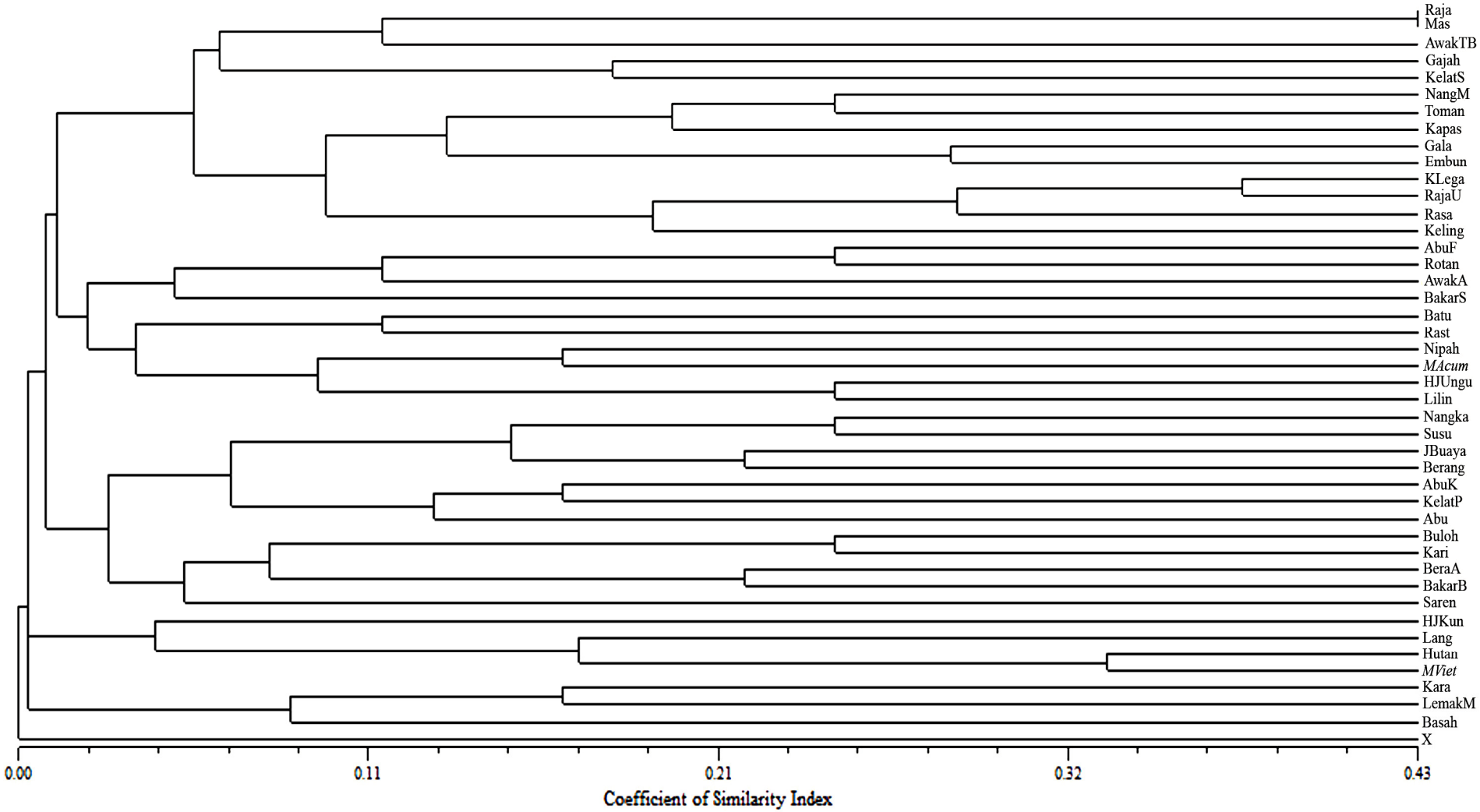

Figure 1. Dendrogram showing genetic similarities among 44 *Musa* genotypes. Dendrogram was generated by UPGMA from similarity data based on Jaccard's. This dendrogram demonstrates the genetic relationships among 44 *Musa* genotypes analysed with microsatellites.

duplicates, suggesting that there was high genetic variation among genotypes. It was logical because the genetic analysis involved populations from different *Musa* genotypes.

This study showed that the nine microsatellite markers were effective and useful for analyzing the genetic diversity of *Musa* genetic resources. This was in line with the results from some previous researches. Microsatellite DNA was

favoured over other markers because of its wide genome coverage, high polymorphism, multiallelic nature, co-dominance, high reproducibility and easy amplification using the PCR (Tautz, 1989; Ostrander et al., 1992; Karagyozov et al., 1993; Morgante et al., 2002; Creste et al., 2003, 2004; Ning et al., 2007). Microsatellite is a co-dominant marker, so it is an effective marker for determining the allele associated with specific traits of the species tested (Kaemmer et al., 1997; Creste et al., 2003; Creste et al., 2004; Ning et al., 2007).

Banana cultivars have diversified to a complex germplasm pool mostly through somatic mutation and somaclonal variations (Vuylsteke et al., 1991; Osuji et al., 1997). These variations were detected by microsatellites markers where nine primers allowed discrimination of all genotypes. Clustering, however, was not according to their ploidy levels (Figure 1). This was also reported for other *Musa* germplasm by Creste et al. (2003), Oriero et al. (2006) and Ning et al. (2007). In group I, Raja and Mas showed high similarity indicating that the two cultivars are 43% genetically close to each other, while the unknown cultivar which has zero percentage of similarity was separated from the rest of the group entirely, showing that this cultivar is distant from the rest of the cultivars. This unknown cultivar was clustered into a cluster in which it is the only member. Identifying the genetic relationships with additional information on the morphological characteristics would facilitate researchers in choosing genotypes for future hybridization programmes. High genetic variations were detected among the 44 *Musa* genotypes in the germplasm collection, and the most polymorphic loci were MaOCEN08. The highest percentage of similarity observed was 43% between Raja and Mas, indicating that these two genotypes are the closest relatives. An unknown cultivar was 100% dissimilar from the rest of the *Musa* genotypes. It was concluded that all accessions in the germplasm collection are of different genotypes and none are duplicates. Seven clusters were obtained and each member was grouped and not according to their ploidy levels.

Abbreviations

AFLP, Amplified fragment length polymorphism; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; **DNA**, deoxyribonucleic acid; **PCR**, polymerase chain reaction; **µL**, microlitre; **ng**, nanogram; **mM**, millimolar; **µM**, micromolar; **U**, unit; **$MgCl_2$**, magnesium chloride; dNTPs, deoxynucleotide triphosphates; **Taq Polymerase**, thermostable DNA polymerase; **NTSYS**, numerical taxonomy and multivariate analysis system; **POPGENE**, population genetic analysis; **UPGMA**, unweighted pair group method with arithmetic averages; **A**, observed number of alleles; **Ae**, effective number of alleles; **I**, Shannon's diversity index; **Ho**, observed heterozygosity; **He**, expected heterozygosity; **Nei**, Nei's expected heterozygosity; **Fis**, Wright's fixation index; **Fst**, coefficient of genetic differentiation; **PIC**, polymorphism information contents.

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