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RAPD Analysis of the Genetic Diversity Among Accessions of Micropropagation Bananas from Indonesia

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Abstract. Molecular Characterization of banana (*Musa spp*) is very important for the purpose of conservation and improvement of plants. This research was conducted to investigate phylogenetic of Musa spp cultivar namely Cavendish, Barangan, Raja Buluh and Kepok. RAPD analysis was performed on four accessions of *Musa spp*, originating from West Java, Indonesia. The samples used were taken from the plant result of micropropagation at Tissue Culture laboratory Lebak Bulus, Jakarta, Indonesia. The result of PCR-RAPD visualization produces bands whose size ranges from 200-1500 bp. The primers used were able to amplify four samples clearly. The DNA amplification results show that the four primers can produce 62 polymorphic and monomorphic bands. A total of 30 polymorphic bands is capable of producing on PCR-RAPD process with 48.29%. Primer OPB 3 produces the most polymorphic bands of 10 bands whereas OPB 4 produces only four polymorphic bands. Primer OPB 3 is capable of producing the highest percentage of polymorphic loci that is 55.56%. Dendogram results show that at the coefficient of 0.68 separation occurs in the Raja Buluh with other samples. While in the coefficient of 0.70, the varieties of Kepok shows the separation of Raja Buluh and Cavendish. At the coefficient of 0.90, the Barangan and Cavendish varieties are in the same branching which tools that the two have close phylogenetic. RAPD sequence analysis has provided more information on the inter-species phylogenetic status of these four cultivars of *Musa spp*.

1. Introduction

Bananas (*Musa spp*) are a fruit plant that could thrive in Indonesia [1]. Bananas are also an export product in many countries, with an annual production of 102 million tons worldwide [2]. Bananas appertain fruit which undergoes a rapid ripening process after harvest, leading to short shelf life [3], [4]. Different types of bananas are cultivated for the purposes of society as a nutrient intake [5], [6]. Top seeded bananas varieties in Indonesia are Kepok, Cavendish, Barangan, and Raja Buluh. Each type of *Musa spp* accession has its own uniqueness that attracts a lot of public attention [7]. Not a few also farmers in Indonesia who cultivate the banana in the garden and yard [8]. The development of potency and production of bananas as a source of germplasm conservation is indispensable through the

selection and breeding of genetically superior varieties [1], [9]. Through micropropagation techniques, various types of superior varieties of bananas are multiplied and cultivated [9], [10]. The cultivation of banana varieties is a combination of different genomes to form diploids (AA, AB), triploid (AAA, AAB, ABB) and tetraploid (AAAA, AAAB, ABBB), depending on the number of multiples of the basic chromosome [11], [12]. Heretofore, *Musa spp* tissue culture is widely used by using explant source and pathway [13]. However, genetic protocol stability testing has not been done [14]. Further research is needed to establish the genetic character of the *Musa spp* cultivation plant. Detailed molecular characteristics of accession are required for efficient management of utilization of *Musa spp* diversity [6], [7], [15].

On the grounds that each banana cultivar has different physico-chemical properties including its chemical composition, genetic analysis is required to tip the data [7], [15]. Various molecular techniques were performed to assess the genetic stability of *Musa spp* clones derived from micropropagation [5], [16]. Recently, RAPD and ISSR techniques have been used successfully in detecting genetic similarities and inequalities in *Musa spp* [17]. This technique could be applied to a number of in vivo and in vitro methods [18]. RAPD markers are able to detect diversity and group diversity based on DNA band patterns that might indicate the presence or absence of chromosomal carriers of genes or alleles which are desired [14]. One of the advantages of RAPD markers is that they are not influenced by the growing environment and plant growth phases [19]. This RAPD technique is based on the use of a mutually changing nucleotide sequence to amplify the genomic segments of DNA randomly through PCR utilization to show the degree of polymorphism [8], [16], [20]. The arbitrary short primer (a 10-base sequence sequence pairs) is used to generate some random DNA fragments in the PCR reaction with low annealing conditions [21].

Sequencing RAPD could present data with better resolution on intra-genus and above levels [17], [22]. With a single primer, RAPD can detect polymorphisms although there is no specific nucleotide sequence information [23]. Primary function of polymorphism as a genetic marker can be used to build individual genetic maps [24]. Our research will present analysis of genetic variation of four local varieties of Musa spp in Indonesia using RAPD fragments. The final result is expected to be used for the development and cultivation of better varieties of *Musa spp*.

2. Materials and Methods

2.1 DNA Extraction

Total genomic DNA was extracted from young and fresh leaves of four Musa spp. cultivars i.e. Cavendish (CV), Barangan, Raja Buluh and Kepok (K) collected from tissue culture plans at Lebak Bulus Tissue Culture Laboratory, Jakarta, Indonesia. Genomic DNA from four Musa spp. cultivars was extracted using the modified CTAB method [8]. The quality and quantity of each DNA were checked in 1% agarose gel electrophoresis by NanoDrop spectrophotometry (Thermo Fisher Scientific, USA) respectively. Isolated genomic DNA was diluted to 10 ng/L and stored at 20oC for use [22].

2.2 PCR Amplification

Amplification of the DNA segment was performed using a single primary decanucleotida. We present four primers i.e. OPB 01 (GAC GGA TCAG), OPB 1-04 (GGT ACT CCCC), OPB 03 (TGC CGG CTTG), OPB 04 (TGG ACC GGTG) in this work. 15 µl total DNA sample consisting of 10 ng DNA sample; 0,2 nM dNTPs; 1,5 ml buffer reaction; 5 pmole primer; 2mM MgCl2; and 1 unit Taq DNA polymerase (Promega) was amplified on Thermalcycler (Takara Gradient PCR) programmed for 45 cycles. The PCR is performed under conditions of pre-denaturation at 94oC for 5 minutes, followed denaturation 94oC for 1 minute, annealing 36oC for 1 minute, and extension 72oC for 2 minutes. After 45 cycles completed, then continued the extension process of 72oC DNA fragment for 4 minutes. Then the dye loading solution is added to increase the molecular weight of the DNA. The PCR amplification results are visualized using Electrophoresis 1% agarose gel in TEA (Tris-EDTA) buffer. The agarose gel is dipped in EtBr solution with a final concentration of 1ml / 100 ml for 10 minutes. The DNA fragment separation results are detected by under the ultraviolet Geldoc, then

drawn using a Polaroid camera. As a standard, DNA size is used 100 bp DNA ladder (Promega) to define the band size of DNA amplification.

2.3 Data analysis

The results of DNA bands emerging from the RAPD process showed the molecular weight of DNA. The banding pattern profile resulting from DNA analysis was detected using 254nm UV and sprayed with general spray reagent (Serium (IV) Sulfate). The parameters observed were the presence or absence of the tape on the agarose gel after UV irradiation. The amplification results are changed to a binary matrix where "1" denoted the presence and "0" denoted the absence of a band on the product. Only clear, consistent bands and polymorphic bands were used to create binary matrices for statistical analysis using NTSYS-pc. The amplification products obtained were compared to each other [7], [8]. Genetic similarity was calculated between species and grouped with UPGMA (Unweightedpair Group Method Averaging) dendogram to illustrated the genetic relationships among the analyzed *Musa spp* genotypes [25].

3. Result

The accessions of *Musa spp* (Kepok, Cavendish, Barangan, and Raja Buluh) have an important role as a source of food in all walks of life. Therefore, it is urgent to preserve the genetic resources of *Musa spp* varieties and to integrate conservation issues with needs. This can only be achieved once the genetic diversity of the existing varieties of *Musa spp* is clear. In this study, RAPD analysis was performed to examine the phylogenetic relationship between accessions of *Musa spp* of micropropagation result. DNA quality and quantity tests have been performed with NanoDrop Spectrophotometry and agarose gel electrophoresis. The amplification results of the total quality and quantity DNA genome of four Musa spp cultivars yielded the purity and concentration of DNA presented in Table 1.

Tabel 1. Total number of purity and genomic DNA concentrations of four variety Musa spp

Sample	Code	Purity (μl/ml)	Concentrations (μl/mg)
Cavendish	CV	0,8425	151,771
Barangan	BR	0,9694	113,591
Raja Buluh	RB	1,093	111,711
Kepok	K	0,5902	36,904

Table 1 shows the highest level of DNA purity found in the Raja Buluh with a purity of 1.093 µl/mg. While the highest level of DNA concentration found in Cavendish varieties with a value of 151,771 µl/mg. The lowest purity was in Kepok varieties with a purity of 0.5902 µl/mg and a concentration of 36.904 µl/mg.

Of the four primers tested, they all yield stable and readable bands for assessment. Four primers exhibit intraspecific and interspecific bands that can be continued for investigation of genetic diversity (Figure 1). All primers showed interspecific polymorphisms while some primers exhibited intraspecific variation (Table 2).

Tabel 2. The number of primers used for DNA amplification and level of polymorphism

Primer	Sequence	Total bands	Polymorphic	Monomorphic	Polymorphic Percentage
OPB 1	GAC GGA TCAG	20	8	12	40
OPB 1-04	GGT ACT CCCC	8	8	0	100
OPB 3	TGC CGG CTTG	18	10	8	55.56
OPB 4	TGG ACC GGTG	16	4	12	25
	Total	62	30	32	48.39

A total of 62 polymorphic and monomorphic bands were produced with an average number of bands per primer of 15.5. a total of four RAPD primers are capable of producing 30 polymorphic bands with a 48.29% percentage. Primary OPB 3 produces the most polymorphic bands of 10 bands whereas OPB 4 produces only four polymorphic bands. It can also be noted that, primers of OPB 1-04 do not produce monomorphic bands. Primer OPB 3 yields the highest percentage of polymorphic locus that is 55.56%. The highest polymorphic bands illustrate that the primer can show the presence of allele variation in the sample, whereas the monomorphic band exhibits the same allele properties.

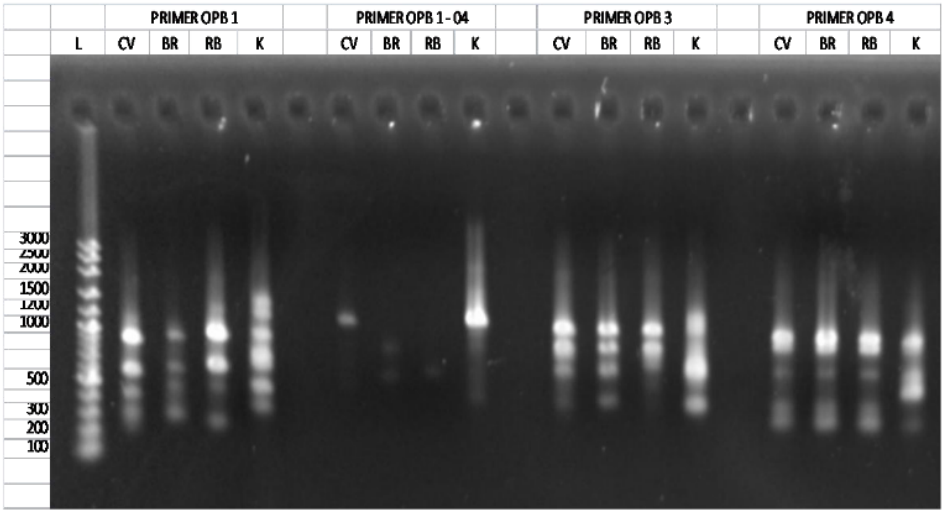

Figure 1. Electrophoretic pattern of DNA extracted with 5 RAPD markers from four *Musa spp*. Accessions.

The result of PCR-RAPD visualization produces bands whose size ranges from 200-1500 bp. Using the k-means non-hierarchical clustering, the *Musa spp* accessions could be divided into three clusters at 70% similarity coefficient and in the 67.5% coefficient similarity produces one cluster as shown in the dendrogram. The genetic similarity coefficient of four accessions ranged from 67.5% to 90%. Cluster A included two accessions namely Cavendish and Raja Buluh, Cluster B comprised of one accessions namely Kepok. Cluster C also having only one accessions namely Barangan (Figure 2). Dendrogram built on the percentage of similarity was calculated after collecting data from 62 RAPD loci from four accessions of *Musa spp*.

4. Discussion

Our results prove that the primers of OPB 01, OPB 1-04, OPB 03, and OPB 04 successfully amplify the genome of *Musa spp* well. Quality and quantity tests have been successfully tested with Spectrophotometry. The purity test results and the concentration obtained can be considered good enough in all samples. In this result, Kepok varieties do show low purity and low concentration among other species. However, that is not so good value of purity and concentration and does not guarantee that genomic DNA isolation fails completely. If there are too few DNAs, then the test results can be negative due to the minimum limitations of the means . Very few genomic DNAs make it hard to detect by the tool, but they can be used as templates in the PCR-RAPD process.

PCR-RAPD has the ability to multiply very powerful DNA fragments because this RAPD process requires only a small amount of DNA tamplate to reproduce certain DNA fragments [19]. In principle the PCR process takes place in the extension where taq polymerase begins its activity to extend the primary DNA from the 3 'end. The rate of nucleotide preparation by the enzyme at 72oC is estimated to be between 35 and 100 nucleotides per second, depending on the buffer, target DNA molecule and pH. The amplification results with four RAPD primers yielded several DNA fragments of bands,

primer OPB 01 yielding 20 fragments 8 of which are polymorphic while the other 12 are monomorphic fragments. In primers OPB 1-04 produces 8 fragments which are entirely polymorphic, while OPB 03 produces 18 fragments, 10 fragments are polymorphic and 8 fragments are monomorphic. In primary OPB 04 can produce a total of 16 fragments consisting of 4 fragments polymorphic and 12 monomorphic.

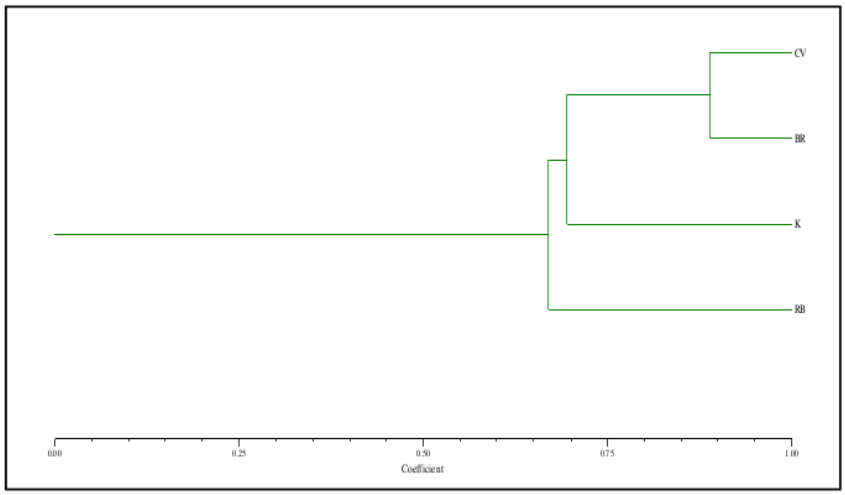

Figure 2. Dendrogram depicts the genomic relationship between the four *Musa spp*. accessions.

A good mark is a marker that has a high polymorphism value and denotes a double dominant allele. The fragment bands then become binary data and processed with NTSYS (Numerical Taxonomy and Multivariate) software to construct phylogenetic trees. From Figure 1 shows that at the coefficient of 0.68 there is separation on the varieties of Raja Buluh with others. While the coefficient of 0.70 varieties Kepok also show the separation of varieties Barangan and Cavendish. At the coefficient of 0.90, the Barangan and Cavendish varieties are in the same branch. This shows that the closest phylogenetic is in Barangan and Cavendish. In this study Musa spp used is the Eumusa group which generally has the number of chromosomes with n = 11, so that in the cultivation known musa spp diploid with the number of chromosomes 22, tripoid number of chromosomes 33, and tetraploid the number of chromosomes 44. Raja Buluh, Barangan and Cavendish cultivar have the same genetic code that is code A which has acuminata properties, while for Kepok accession has a code B has the nature of balbisiana [11].

When viewed from the morphological form, the four Musa spp cultivars have a shape that resembles, the difference can be known if the Musa spp already has fruits [4], [10]. Barangan varieties have the characteristics of skin color red yellow fruits with brown spots. Moreover, Cavendish has a yellowish white flesh, slightly sour sweet, and soft. Additionally, the skin of the fruit is a bit thick yellowish green to light yellow. The variety of Raja Buluh has a rather thick flesh, sweet taste, and strong aroma. Furthermore, at the time ripe skin color of yellow fruits mottled brown. The color of the meat shows reddish white. Kepok has characteristic of the shape of the fruit is slightly flat so sometimes called Musa spp sprinkled and when cooked full yellow fruit.

The closest phylogenetic seen on the dendogram is found in Barangan and Cavendish varieties. In Figure 1 the two samples are in the same group, ie, the AAA triploid acuminata group and when viewed from its morphology have almost the same characteristics whereas in Kepok is included in the BBB triploid balbisiana and Raja Buluh balbisiana group is still a triploid acuminate group but with AAB code which is the offspring of acuminata and balbisiana.

5. Conclusion

This study is a prerequisite for the development of suitable molecular methods in *Musa spp* genomic polymorphism with selected primers. The work is currently in the process of gaining deeper insights into the genetic diversity and molecular characteristics of Musa spp germplasm. Primary test and selection is an important stage in the analysis of genetic diversity with PCR-RAPD. Primer OPB 3 (TGC CGG CTTG) is the primary that can provide the highest percentage of polymorphic loci that is 55.56%. Our results also explain that the varieties of Cavendish, Barangan, Raja Buluh and Kepok have genetic diversity. From the results of the dendogram shows that Cavendish and Barangan have the closest genetic proximity to the coefficient of 0.90. We also provide information that RAPD analysis in general is still feasible to be used to identify phylogenetics in *Musa spp*.

Acknowledgements

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