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The Efficiency of Long Primers Compared to The Short Primer for RAPD Technique in Date Palm

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ABSTRACT

Random Amplified Polymorphic DNA (RAPD) applies single arbitrary short primers (8-12 nucleotides) to produce many amplified discrete DNA. Limited reports and studies were done on the use of long primers (over 12 bases). This study was performed to investigate the potential value of long primers (15-21 bases) for generating RAPD polymorphisms. We compared both short and long primers in RAPD assays of two date palm cultivars grown in Malaysia: Ajwa and Barhi. The number of produced polymorphic fragments ranged in order from 2 and 38 bands for short and long primers in Ajwa. Meanwhile, more polymorphic fragments were generated by long primers in Barhi, which were 50 and only five bands for short primers. 18-mer GY107 and 20-mer CO4 primers yielded 100% polymorphism in Ajwa and Barhi, respectively. Moreover, long primers produced more DNA fragments and a wider range of DNA fragment sizes (from 140-1600 bp, with respect to 300-1000 bp obtained with 10-mer primers). Hence, a significant correlation was observed between primer length and the number of polymorphic fragments within the long primer group, suggesting that increasing primer length above 15 bases may demonstrate enhanced production of more polymorphism.

Keywords: *Long primer, short primer, RAPD, polymorphism, date palm*

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INTRODUCTION

The first entry of dates palm cultivation into Malaysia was reported in late 2010 [1]. Several established privately-owned farms are located in Terengganu, Kelantan, and Johor [2]. The suitability of the climate of Malaysia for date palm plantation has compelled the government to promote the potential of this plant in the agricultural sector. Despite the rapid growth of this sector, the lack of research in date palm, especially at the molecular level, could affect the sector's long-term viability. Information of the genetic variations in all plant species is the main key for improving the breeding and guiding strategies for their effective conservation [3].

The random Amplified Polymorphic DNA (RAPD) technique is widely applied as a molecular marker for the genetic diversity analysis in plants [4]. The benefits of RAPD markers are that it does not require any data concerning the DNA sequence to be amplified, are easy and quick, more cost-effective, and can differentiate taxa below the species level [5]. The related techniques are DNA Amplification Fingerprinting (DAF) [6] and Arbitrary Primed PCR (AP-PCR) [7]. They have differed from the RAPD marker in terms of the primer length, stringency conditions, and the method of separating and detecting the fragments. The consistency of RAPD analysis is well known and not affected by the plant source or age [7,8]. This method has been effectively utilized for the genotyping analysis of ornamental plants [9] and clonal variation of plant species [10]. Successful application of the RAPD technology has been reported for taxonomic studies and genetic analyses of plants [11] and in plant breeding and phylogenetic relationships studies [12].

Successful building of genetic maps in species such as Arabidopsis [13], bananas [14], and slash pine [15] using the RAPD method has been published. Many reports regarding the effectiveness of RAPD for genetic diversity studies in various endangered plant species can be found [16,17,18,19]. Several competitive parameters influenced the RAPD performance, such as low annealing temperature and short or long primer and template [20]. Hence, optimization and internal control of this method are crucial to obtain reproducible RAPD results. RAPD reaction can also be influenced by several critical methodological variations, including the DNA quality and quantity [4], presence of RNA [21], choice of DNA polymerase [22], primer concentration [4], choice of thermal cycler [23], use of ethidium bromide vs silver for detection of products [6] and Mg concentration [24].

The RAPD arbitrary sequence of short oligonucleotides is used to amplify the segments of the target genome. The high G+C content of 10-mer primers, generally 50-80%, is preferred for better stability amplification reactions. A study reported less possibility of finding perfect or near-perfect homologies between a longer primer and the target sites [25]. Thus, a decrease in genomic target sites. However, primers as short as five bases can also generate complex banding patterns [6]. There are few reports on using long primers (over 12 bases). However, no data were presented

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comparing RAPD results between 10-mer primers and longer primers except in genetic mapping and fingerprinting of grape and pear. In addition, genetic diversity assessment using RAPD markers has only been reported in date palm species, particularly those of Saudi Arabia, Tunisia, Pakistan, and Egypt. No studies have looked at the genetic variation and polymorphism of Malaysian date palm. This study successfully utilized the RAPD technique, especially the long primers (15-21 bases), to generate more polymorphism in Ajwa and Barhi date palm species.

EXPERIMENTAL

Sample Collection

Fresh young leaves (white to yellow) of the Ajwa and Barhi date palm cultivars were collected from the farms located at Johor and Perak, respectively. Each cultivar is represented by 50 young leaf samples selected from the different trees aged three years old. Hence, 100 young leaf samples were used in this study. The samples were individually placed in sealable polythene bags, transported to the laboratory, and then kept frozen at -70 °C and lastly analyzed through the RAPD method.

DNA Extraction

The standard CTAB (Cetyltrimethylammonium bromide) method extracted the total genomic DNA from 500 mg of young leaf samples [26]. The leaves were ground to a fine powder with autoclaved and pre-chilled mortar and pestle using liquid nitrogen. After grinding, the powder was transferred into a 1.5 mL microcentrifuge tube and resuspended in a pre-warmed extraction buffer (1.5 M NaCl, 100 mM Tris-HCl pH-8, 40 mM EDTA, 1 % PVP, 3 % C-Tab, and 1 % β-mercaptoethanol). The suspension was incubated at 65 °C for 1 hour. Then, an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added, mixed well by gentle inversion and centrifuged. The supernatant was mixed with a 0.6 volume of ice-cold isopropanol in a new tube to precipitate the DNA. After centrifugation, 70 % ethanol was used to wash the DNA pellet, then air-dried and dissolved in 50 µL tris-EDTA buffer (TE). The RNA was eliminated by RNase treatment. The quantity and quality of genomic DNA were assessed by spectrophotometry. Optimal values of samples between 1.8 to 2.0 were used for the RAPD reaction.

PCR Conditions and Electrophoresis

Genomic DNA was amplified using five different RAPD primers [27]. The amplification was performed in a total volume of 20 µL containing 1 x PCR buffer (Banglore Genei), 0.2 mM dNTPs,

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0.1 µM of primer, 1.5 mM $MgCl_2$, 50 ng of genomic DNA, and 1 U Taq DNA polymerase (Banglore Genei). Negative control was also included in the PCR reaction to discard any contamination. The reaction mixture was submitted to the following RAPD program: initial denaturation step at 94 °C for 5 min, followed by 40 cycles of 94 °C, 30 s; 36 °C for short primers and 44-60°C for long primers, 1 min; and 72°C, 2 min. After the final extension step at 72 °C for 8 min, the samples were kept at 4 °C. PCR products were analyzed by electrophoresis on 1.5 % (w/v) agarose gels in 0.5 x TBE Buffer at 80 V for 1 hour. The gels were visualized by ethidium bromide (0.5 µg/mL) staining using the Image LabTM Software Version 6.0 (Bio-Rad).

RESULTS AND DISCUSSION

Various sizes of 21, 20, 18, 15 and 10 nucleotides of RAPD primers were screened to amplify the DNA in this study. Table 1 shows 58 reproducible bands, which comprises 18 monomorphic bands and 40 polymorphic ones for Ajwa date palm. Meanwhile, Table 2 shows that a greater total amplified band and polymorphic fragments were generated for Barhi date palm, 71 and 55, respectively. Hence, all the primers tested produced approximately 69-77 % of polymorphisms in both cultivars. Minimum one variable locus was yielded by all the primers in both cultivars. The optimal template DNA concentration was 50 ng, and 0.1 µM of primer concentration was applied. However, no significant effect of the DNA concentration is observed in these results.

Table 1: Effect of primer length (10 to 21 bases) on number of RAPD fragments and number of polymorphic fragments on Ajwa date palm

Primer length (Bases)	Maximum amplified band	No. of monomorphic bands	No. of polymorphic bands	Polymorphism (%)	Range of DNA fragments (bp)
10	6	4	2	33	500-1000
15	8	5	3	38	350-850
18	13	0	13	100	250-1600
20	15	5	10	67	400-1300
21	16	4	12	75	150-1400
Total	58	18	40	69	

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Table 2: Effect of primer length (10 to 21 bases) on number of RAPD fragments and number of polymorphic fragments on Barhi date palm

Primer length (Bases)	Maximum amplified band	No. of monomorphic bands	No. of polymorphic bands	Polymorphism (%)	Range of DNA fragments (bp)
10	9	4	5	56	300-700
15	9	5	4	44	350-800
18	15	3	12	80	200-1300
20	20	0	20	100	140-1600
21	18	4	14	78	250-1500
Total	71	16	55	77	

Monomorphic and polymorphic bands were two types of bands detected among the amplified DNA products. All individuals with the presence of similar size bands are monomorphic. Meanwhile, those bands present in one or more but not in all individuals are polymorphic. At least one individual contains unique bands [28]. About 33-56 % of polymorphic bands were produced by the short primers (10 to 15 bases) in both cultivars. Primers 18-mer GY107 and 20-mer CO4 yielded 100% polymorphism in the Ajwa and Barhi date palm, respectively. Almost the same polymorphism, which was 75-78% detected in both cultivars revealed by the 21-mer primer. Greater polymorphisms yielded in Barhi compared to the Ajwa date palm. In conclusion, we observed that the long primers gave high reproducible RAPD results, including distinct polymorphic amplified fragments compared to the short primers. These results are consistent with the observation on grape and pear [27,29].

Figure 1 and Figure 2 show the successful amplification products with the highest polymorphisms revealed using long primers in both cultivars. Amplification of DNA fragments by the long primers, including a wider range of DNA fragment sizes (from 140 to 1600 bp, with respect to 300 to 1000 bp obtained with 10-mer primers), and a greater amount of polymorphism than short ones were observed. A similar pattern of results was obtained with the study on poplar and Egyptian date palm cultivars [30,31]. Several studies used long primers in genomic fingerprinting [32]. However, limited data comparing RAPD results between 10-mer primers and longer primers were recorded and published [33,34]. Indefinite explanation or clarification on why the long primers exposed more polymorphism. Importantly, lower G+C content (from 39 % to 55 %) of the long primers used in this study compared to the standard 10-mer primers (more than 50 %). However, more amplified bands were produced than 10-mer primers. There is no definite answer to conclude whether the primer length, lower G+C content or a combination of both increased the number of bands generated by long primers. Hence, future works are needed to study

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the correlation of primer length and G+C content on RAPD polymorphisms in various date palm cultivars.

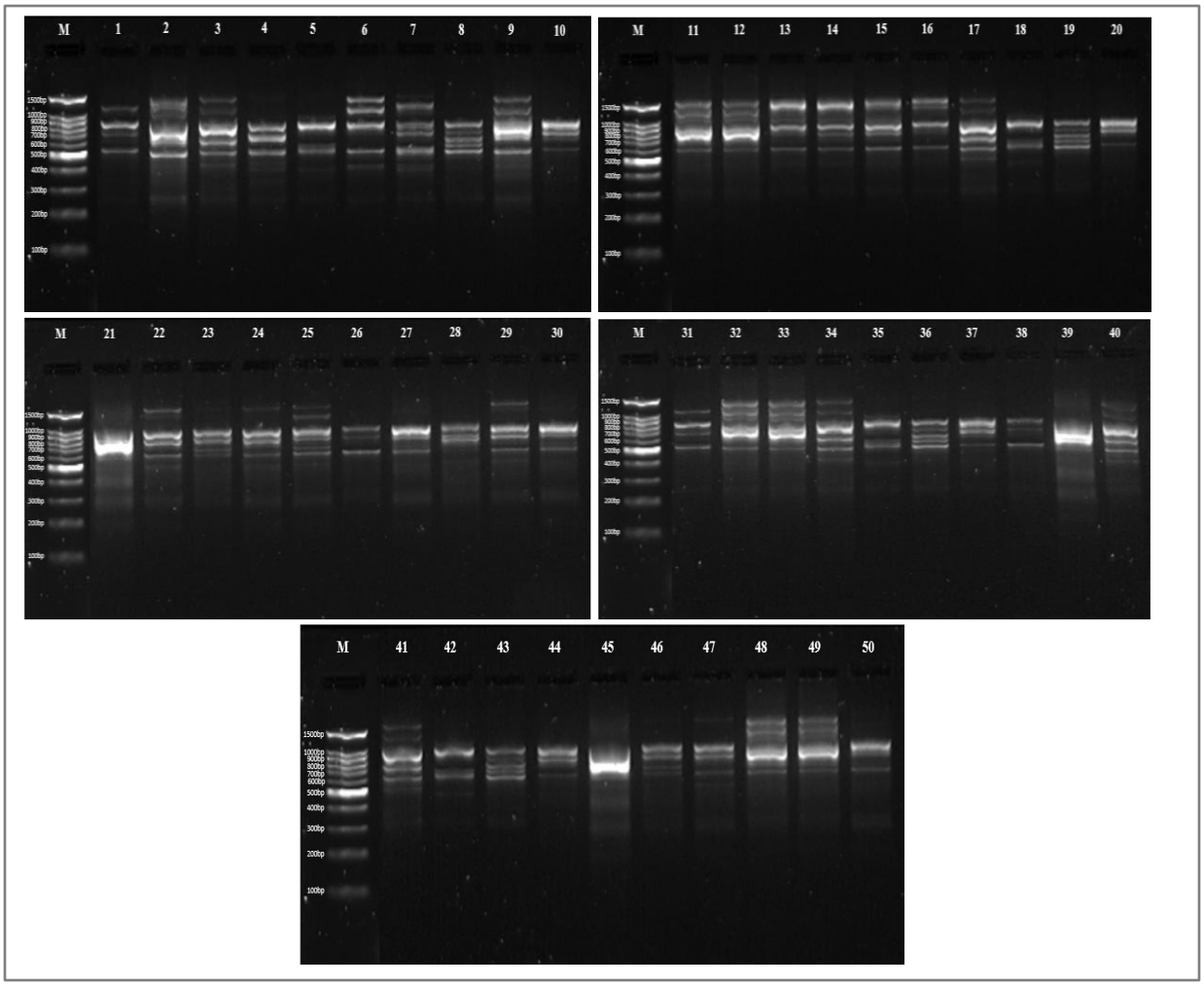

Figure 1: RAPD profiles of Ajwa date palm DNA generated with the 18-mer GY107 GTTCAGGGCTGTTTATAG. Lane 1 to 50 represented by DNA samples from the different trees and 100 bp of DNA ladder (M) was used

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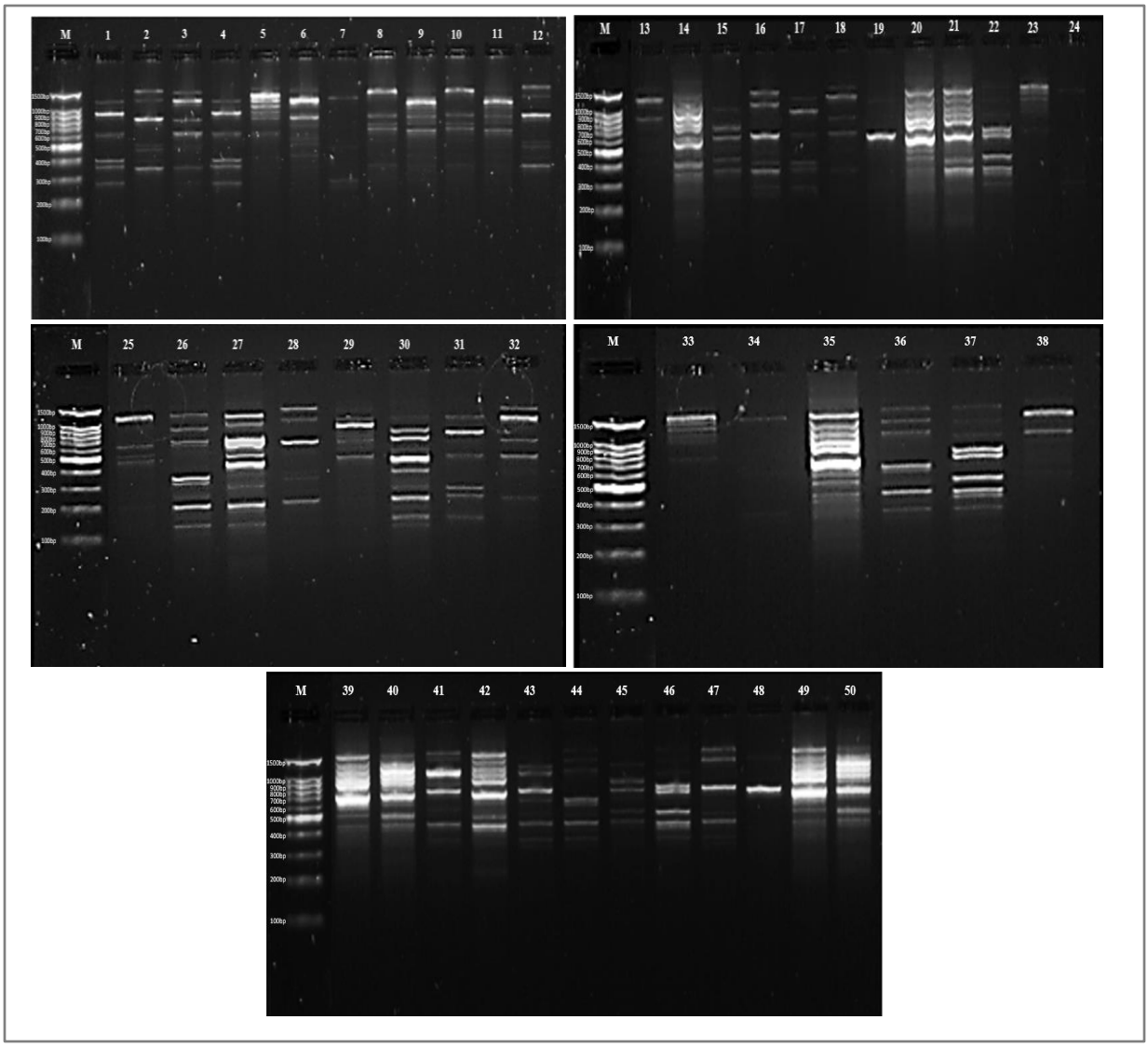

Figure 2: RAPD profiles of Barhi date palm DNA generated with the 20-mer CO4 TGCCTTCCATTCGTAGCCAA. Lane 1 to 50 represented by DNA samples from the different trees and 100 bp of DNA ladder (M) was used

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One possibility could be explained if the primer length affects the production of more fragments, in which the extra bases at the 5' end anneal to the template followed by the binding of 3' bases to the template resulting in a new template-primer complex. In another way, it may improve the existing template-primer complex. The intergenic or repetitive DNA regions are usually targeted by the primers with less than 50 % G+C content, which is proven successful compared with 10-mer primers in mapping telomere and centromere regions [27]. Two methods can reveal the identity of amplified products and the fundamental activities in amplification reaction: 1) Probing the genomic DNA with the amplified fragments; 2) Sequencing of the amplified fragments. Although more cost is needed to synthesize the long primers, high polymorphism can be exposed. Despite the limited number of cultivars and primers tested, the results of this study may be used as a reference or guideline for future diversity assessment and genetic analysis of date palms in Malaysia.

CONCLUSION

Our results indicated that the long RAPD primers have a higher potential to generate polymorphism in Ajwa and Barhi date palm species than the short ones. The 18-mer GY107 and 20-mer CO4 were the most efficient, with 100 % polymorphism detected in the Ajwa and Barhi date palm, respectively. The data from our present study will serve as baseline information for future RAPD assessments. Further study should be done by using varieties of date palm cultivar and RAPD primer to ensure more reliable clarification regarding the relationship of primer length and G+C content on RAPD polymorphisms.

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AUTHOR'S CONTRIBUTION

Aisyah Mohd Ismail carried out the research, wrote and revised the article. Farida Zuraina Mohd Yusof conceptualised the central research idea, provided the theoretical framework, designed the research, supervised research progress, anchored the review, revisions and approved the article submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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