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AFLP Primer Selection for the Analysis of Genetic Diversity in Persimmon (*Diospyros kaki* L.) Originated From Central and East Java, Indonesia

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Abstract

Persimmon (*Diospyros kaki* L.) belongs to the family Ebenaceae known as the Japanese persimmon kaki. This crop was introduced in the Highlands in Central and East Java-Indonesia. A genetic analysis of a small sample of accessions was conducted using the *Amplified Fragment Length Polymorphism* (AFLP) method with IRDye700 labelled *Pst*1 (P11-700) and *Mse*1 restriction enzymes and seven primers combinations (M48, M49, M50, M51, M53, M55, and M58). The analysis resulted in a set of 441 bands, of which 117 were monomorphic and 324 polymorphic. The average percentage of polymorphic bands was 73.4%. The four persimmon accessions were genetically distinguished into three groups, with a high genetic diversity among them, while accessions B (Batu) and D (Dampit) show little differences in their profiles.

Keywords: *Diospyros kaki* L., AFLP markers, *Pst*1, *Mse*1, genetic diversity.

1. Introduction

Persimmon (*Diospyros kaki* L.) belongs to the family of Ebenaceae and is known as Japanese foot Persimmon. This plant is native to Central China and has been introduced in Korea, Japan as well as other subtropical countries (Ikegami *et al.*, 2009). In the early 20th century, this crop began to enter Southeast Asia including Indonesia (Java and Sumatra), Malaysia and Thailand (Butt *et al.*, 2015).

In Indonesia, this plant is widely grown in highlands such as Selo-Boyolali (Delfianti *et al.*, 2019), Magetan (Wardani *et al.*, 2019b), Junggo-Batu (Baswarsiati *et al.*, 2006), Dampit-Malang, Garut; Majalengka (Setiawan, 2017), Brastagi; Karo-Sumatera Utara (Hanafiah *et al.*, 2018). Persimmon can grow well at an altitude of 1,000 - 1,500 m above sea level according to Delfianti *et al.*, (2019) where plants require a mild and humid climate for survival.

Persimmon is classified in astringent and non astringent types. According to Butt *et al.*, (2015); Drahansky *et al.*, (2016); Min *et al.*, (2012) astringent persimmon tastes bitter. It is a fruit intended for cooking and requires to overripe to have the astringency removed , while the non astringent persimmon can be eaten immediately after the harvest and does not require to overripe. Persimmon cultivated in Indonesia is an astringent type and is harvested by farmers although they prefer to grow more profitable horticultural crops such as vegetables and citrus. Accordings to Delfianti *et al.*, (2019) and Ridwan & Iskandar Ishaq (2005) in Indonesia persimmon is propagated by rooted cuttings although the percentage of plants obtained is relatively small. To increase the interest of farmers in cultivating persimmons breedingprograms might be developed to produce new varieties of good quality and quantity.

There are two basic methods to study the genetic diversity: the phenotypic and the genotypic ones. According to Hanafiah *et al.* (2018), the phenotypic method uses morphological characters, but is often influenced by environmental factors so that differences between genotypes are difficult to analyze especially if they do not have a simple genetic control system. The genotypic methods are supported by molecular analysis (Syam *et al.*, 2012).

According to Jones *et al.* (1997), there are several kinds of DNA markers, namely *Random Amplified Polymorphic DNA* (RAPD), *Restriction Fragment Length Polymorphism* (RFLP), *Amplified Fragment Length Polymorphism* (AFLP), *Simple Sequence Repeat* (SSR) or DNA microsatellites.

Amplified Fragment Length Polymorphism (AFLP) is a study technique of genetic diversity based on DNA fragments obtained by restriction enzymes and selective amplification of these fragments (Makful et al., 2010; Vos et al., 1995). The basic principle of AFLP technique is to detect the difference in fragment length polymorphism among compared samples (Saunders et al., 2001).

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2. Materials and Methods

2.1. Sample collection

Persimmon (*D. kaki* L.) leaves were collected from a single tree in the following locations:

- Central Java Province, which consisted of two villages: Jrakah (coded J), located at 07^o 29' 05.641"S - 110^o 25' 27.815"E dan 1,400 m above sea level and Gebyok (coded G), located at 7°29’57.4”S - 110°28’16.1”E dan 1,499 m above sea level
- East Java Province, which consisted of two villages: Batu (coded B), located at 07°80’18.370”S - 112°52’47.787”E dan 1,318 m above sea level and Dampit (D), located at 08°14’92.001”S - 112°85’90.381”E dan 1,130 m above sea level

The distance between the two villages in Central Java province is 6.8 km from each other. The distance between villages of East Java province is 53.4 km.

2.2. DNA Isolation and quantification

DNA of leaves taken from the field was isolated using a genomic DNA Mini Kit (Plant) following the manufacturer’s instructions.

DNA was quantifyed using a spectrophotometry and concentration, and purity of DNA at λ260 nm and λ280 nm (Sambrook *et al.*, 1989; Witkowski, 1995) was recorded.

2.3. AFLP Analysis

AFLP analysis used the method of Vos *et al.*, (1995) modified on primer labeling with IRD 700. The steps were:

Restriction and ligation: The DNA was treated with the *Pst*1 and *Mse*1 restriction enzymes (Suryati et al., 2013). The reaction mixture included 5 µl of DNA (100 ng/µl); 0.25 µl restriction enzymes *Pst*1 and *Mse*1; 0.5 µl *Pst*1 adapter and *Mse*1 adapter; 0.5 µl ATP 10 mM; 2.5 µl NEB buffer 10 X; 0.2 µl T4 ligase; and 15, 8 µl dH_2O. The mixture was incubated for 24 hours at 37°C.

Pre-amplification: Pre-amplification process required 10 µl of RL plus 1.2 µl primer Pst1 (P00), 1.2 primer µl Mse1 (Mo2), 0.8 µl 10 mM dNtp, 0.4 Taq Polimerase 5 U/µl. Pre-Amplification was carried out with the following PCR profile: denaturation at 94 °C for 30 sec, 56 ° C for 30 seconds and extension of 72 °C for 60 seconds for 24 cycles. Pre-amplification product was diluted 10x and 10 µl as a DNA template was used for further selective amplifications. AFLP primer for the pre-amplification were *Pst*1 (5' GACTGCGTACATGCAG3') and *Mse*1 (5'GATGAGTCCTGAGTAAC3').

Selective Amplification: Selective amplification used seven primer combinations (Muluvi et al., 1999), the *Pst*1 primer 5' GACTGCGTACATGCAGAA3' was labeled with I IRDye 700 (P11-700) ; while *Mse*1 primer sequences are reported in Table 2.

Fragment separation and visualization: The electrophoresis of the products of selective amplification was carried out using the LI-COR 4300 DNA analyzer equipment and the acrylamide gel at 6.5%. The gel for electrophoresis was made by mixing 20 ml of KB plus 6.5% gel matrix; 12.5 µl Tetramethilenediamine (TEMED) and 150 µl ammonium Persulfat (APS) 10% (b/V). The mixture was then inserted into the glass plates and allowed to solidify. The gel was run with the TBE buffer 1X. Pre-electrophoresis was performed for 20 minutes with 20 watt to raise the temperature up to 50ºC. As much as 10 µl of DNA sample, coupled with 10 µl of loading buffer, formamid 98% (b/V), EDTA 10 mM, blue bromophenol 0.1% (b/V) with the same volume (10 µl) so that the mixture becomes 20 µl. All the samples were denatured at 94 °C for 10 minutes and then moved into the ice for approx. 5 minutes. The electrophoresis was run for a. 3 hours with 40 watt and 1500 voltage.

2.4. Data Analysis.

The DNA bands were scored and converted into binary data (1 = presence, 0 = absence). The differences between samples were analysed using a similarity matrix from which a UPGMA (unweighted pair group method with arithmetic mean) dendrogram was constructed (Rohlf 1988).

3. Result and Discussion

3.1. DNA Isolation and Quantity and QualityTest

Data on concentration and purity of persimmon DNA are reported in Table 1.

Table 1. Result of Persimmon DNA Quantification

No.	Sample	Concentration (ng/ µl)	λ 260/280
1.	Jrakah	80.5	1.94
2.	Gebyok	85.2	1.91
3.	Batu	90.3	1.95
4.	Dampit	82.4	1.97

DNA quality of the four samples of persimmon ranged between 1.91 and 1.97. According to Sambrook *et al.*, (1989); Sundari (2018); Wardani *et al.*, (2019) the absorbancy ratio of a DNA of good quality ranges from 1.8 to 2.0.

The DNA concentration was in the range of 80.5 – 90.3 ng/µl.

3.2. The AFLP analysis

The study used 7 primer combinations, including P11-M48; P11-M49; P11-M50; P11-M51; P11-M53; P11-M55; and P11-M58. The primers combination produced the number of bands reported in table 2.

Table 2. Primers combinations and number of AFLP bands produced in four persimmon accessions

No.	Primer	Sequence (5'-3')	Amplicons (n)	Polymorphic bands (n)	Polymorphism (%)
1.	P11-M48	GATGAGTCCTGAGTAA**CAC**	64	49	76
2.	P11-M49	GATGAGTCCTGAGTAA**CAG**	50	35	70
3.	P11-M50	GATGAGTCCTGAGTAA**CAT**	55	43	78
4.	P11-M51	GATGAGTCCTGAGTAA**CCA**	73	51	69
5.	P11-M53	GATGAGTCCTGAGTAA**CCG**	45	19	42
6.	P11-M55	GATGAGTCCTGAGTAA**CGA**	84	70	83
7.	P11-M58	GATGAGTCCTGAGTAA**CGT**	70	57	81
Total or average			441	324	73.4

The amount of bands obtained from the seven primer combinations and four samples were as many as 441. The largest number of bands was produced by the P11-M55 primers pair with as much as 84 bands, while the lowest number of bands was produced by the primers pair P11-M53 with only 45 bands.

The size of the AFLP bands obtained ranged from 50 to 700 bp (Figure 1).

Based on the pattern of the AFLP bands, it can be concluded that there is polymorphism on the four persimmon samples in the seven primer combinations used. Of the total of 441 bands, 117 were monomorphic and present in all samples and 324 were polymorphic. The polymorphic bands ranged from 42 to 83% according to the primers combination.

The sample clustering produced the dendrogram of Figure 2.

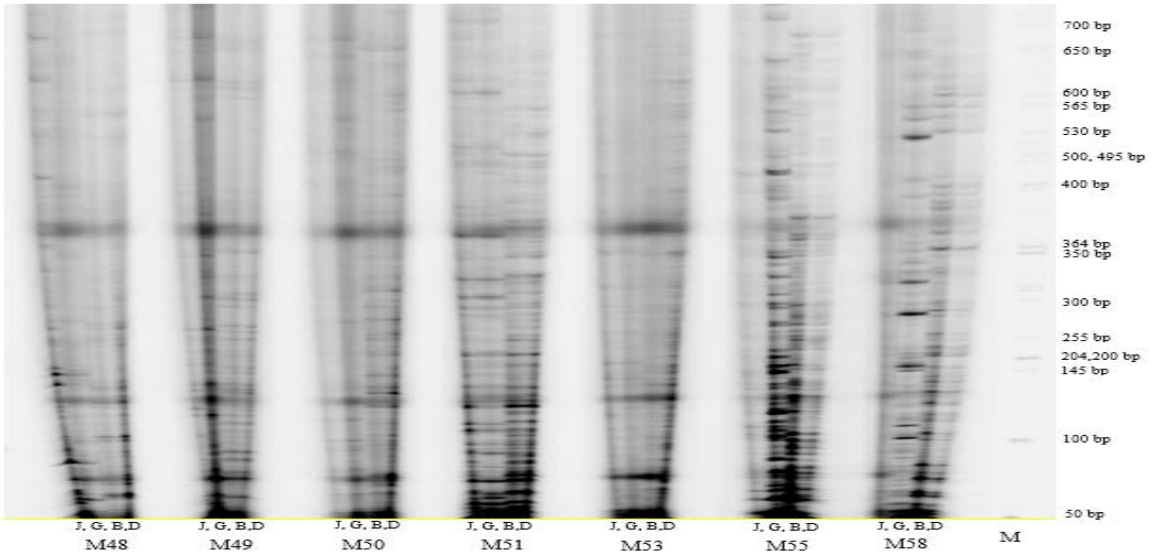

Figure 1. AFLP fragment profile of four persimon (*Diospyros kaki* L.) genotypes amplified using 7 primer combinations (P11-M48; P11-M49; P11-M50; P11-M51; P11-M53; P11-M55; P11-M58). On the right the standard size marker 50 – 700 bp.

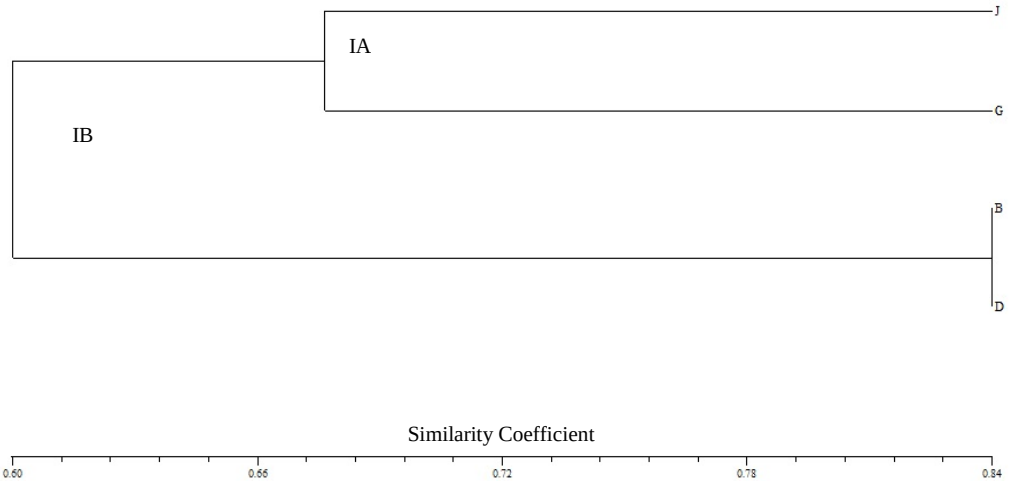

Figure 2. UPGMA cluster analysis of four persimmon samples with seven AFLP primers pairs.

Genetic relationships between persimmon individuals are grouped based on the value of the similarity coefficient on the dendogram. Persimmon samples formed two groups namely IA and IB. IA group consists of Jrakah and Gebyok accessions that had a similarity coefficient of around 0.675. The IB subclaster shows that Batu accession is genetically very close to Dampit accession with a similarity coefficient of 0.84. IA and IB groups are clustered at a similarity coefficient around 0.60.

4. Conclusion

The AFLP markers were successfully used to analyze the genetic diversity of four persimmon samples scattered in Central and East Java, Indonesia based on the number of polymorfisms generated by the AFLP markers. Of the seven primers combinations used, the three most suitable ones for the analyzsis of persimmon genetic diversity were P11-M48; P11-M49; and P11-M51. The clustering procedure generated three main branches that clearly separated the genotypes Jrakah (J) and Gebyok (G) and these two from the third group that included the genotypes Batu (B) and Dampit (D), which resulted being very similar to each other at the AFLP markers profile.

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