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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Received: March 17, 2020; Revised: September 13, 2020; Accepted: September 19, 2020

**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 Pst1 (P11-700) and Mse1 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, *Pst1*, *Mse1*, genetic diversity.

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**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 20<sup>th</sup> 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-Malong, Garut; Majalengka (Setiawan, 2017), Brastagi; Karo-Sumatra Utara (Hanaflah *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 overripen to have the astringency removed, while the non astringent persimmon can be eaten immediately after the harvest and does not require to overripen. 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. Accords to Delfianti *et al.*, (2019) and Ridwan & Iskandar (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 breeding programs 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).
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° 29’ 05.641”S - 110° 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 1,310 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 quantified 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 Psrl and Mse1 restriction enzymes (Suryati et al., 2013). The reaction mixture included 5 μl of DNA (100 ng/μl); 0.25 μl restriction enzymes Psrl and Mse1; 0.5 μl Psrl adapter and Mse1 adapter; 0.5 μl ATP 10 mM; 2.5 μl NEB buffer 10 X; 0.2 μl T4 ligase; and 15, 8 μl dH2O. 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 Psrl (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.

  Selective Amplification: Selective amplification used seven primer combinations (Mulvli et al., 1999), the Psrl primer 5’ GACTCGTGATCGACAGA3’ was labeled with 1 IRDye 700 (P11-700) ; while Mse1 primer sequences are reported in Table 2.

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 Quality Test

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Concentration (ng/μl)</th>
<th>λ 260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Jrakah</td>
<td>80.5</td>
<td>1.94</td>
</tr>
<tr>
<td>2.</td>
<td>Gebyok</td>
<td>85.2</td>
<td>1.91</td>
</tr>
<tr>
<td>3.</td>
<td>Dampit</td>
<td>90.3</td>
<td>1.97</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Amplicons (n)</th>
<th>Polymorphic bands (n)</th>
<th>Polymorphism (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P11-M48</td>
<td>GATGAGTCCTGAGTAAAC</td>
<td>64</td>
<td>49</td>
<td>76</td>
</tr>
<tr>
<td>2.</td>
<td>P11-M49</td>
<td>GATGAGTCCTGAGTAAACG</td>
<td>50</td>
<td>35</td>
<td>70</td>
</tr>
<tr>
<td>3.</td>
<td>P11-M50</td>
<td>GATGAGTCCTGAGTAAACAT</td>
<td>55</td>
<td>43</td>
<td>78</td>
</tr>
<tr>
<td>4.</td>
<td>P11-M51</td>
<td>GATGAGTCCTGAGTAAACCA</td>
<td>73</td>
<td>51</td>
<td>69</td>
</tr>
<tr>
<td>5.</td>
<td>P11-M53</td>
<td>GATGAGTCCTGAGTAAACCG</td>
<td>45</td>
<td>19</td>
<td>42</td>
</tr>
<tr>
<td>6.</td>
<td>P11-M55</td>
<td>GATGAGTCCTGAGTAAACGA</td>
<td>84</td>
<td>70</td>
<td>83</td>
</tr>
<tr>
<td>7.</td>
<td>P11-M58</td>
<td>GATGAGTCCTGAGTAAACGT</td>
<td>70</td>
<td>57</td>
<td>81</td>
</tr>
</tbody>
</table>

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 primer 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 persimmon (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 dendrogram. Persimmon samples formed two groups namely IA and IB. IA group consists of Jarakah and Gebyok accessions that had a similarity coefficient of around 0.675. The IB subcluster 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 polymorphisms generated by the AFLP markers. Of the seven primers combinations used, the three most suitable ones for the analysis of persimmon genetic diversity were P11-M48; P11-M49; and P11-M51. The clustering procedure generated three main branches that clearly separated the genotypes Jarakah (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.

Acknowledgement

Author declare thanks to the University of Sebelas Maret-Indonesia for funding this research from PUT-UNS (PNBP).

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