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DETECTING DNA POLYMORPHISM ON MULBERRY (*Morus Sp.*) USING RAPD AND ISSR MARKERS

MUHAMMAD BIMA AKZAD, SITTI NURAENI AND SITI HALIMAH LAREKENG*
Faculty of Forestry, Universitas Hasanuddin, Makassar, South Sulawesi, Indonesia
[MBA, SN, SHL].
[*For Correspondence: E-mail: sitih5h.82@gmail.com].

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ABSTRACT

Mulberry molecular analysis in Indonesia has not been widely reported, so research towards molecular genetics needs to be done. *Random Amplified Polymorphic* DNA (RAPD) and *Inter Simple Sequence Repeats* (ISSR) markers are reliable methods for identifying varieties by DNA polymorphism analysis to reveal genetic diversity of three provenances in South Sulawesi. This study aims to see the opportunities for polymorphisms using RAPD and ISSR markers in mulberry plants so that it is considered very important to be used as basic information in mulberry plant breeding and conservation programs in the future. The results of primary selected using RAPD markers on mulberry. Successfully amplified 8 out of 10 primers with clear polymorphic bands, namely primers OPK-20, OPA-15, OPA-05, OPAD-11, OPG-19, OPAE- 11, OPP-08, and OPD-20. Whereas, the primary ISSR 10 markers, namely UBC 810, UBC 813, UBC 814, UBC 820, UBC 822, UBC 823, UBC 824, UBC 827, UBC 830, and UBC 868 were successfully amplified with polymorphic and bright bands so that they can be used for diversity analysis for mulberry.

Keywords: Mulberry; primer selections; amplification; RAPD; ISSR.

INTRODUCTION

The molecular markers that have been successfully developed have primarily overcome the issues associated with the phenotype-based classification. Well-known molecular markers for diversity analysis are Restriction Fragment Length Polymorphism (RFLP), Cleaved Amplified Polymorphic Sequence (CAPS), Sequence-Tagged Site (STS), Simple Sequence Repeat (SSR), Single Nucleotide Polymorphism (SNP), Random Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Region (SCAR), Amplified Fragment Length Polymorphism (AFLP), and Inter Simple Sequence Repeats (ISSR) markers [1].

RAPD marker can be relied on to detect polymorphic nucleotide sequences with PCR (Polymerase Chain Reaction) procedure, and a

single RAPD primer is useful for generating molecular markers [2]. This technique has several advantages over RFLP (Restriction Fragment Length Polymorphism) technique because of its simplicity, fast, only requires a small amount of DNA as a template, and does not require initial information on the target genome. Several studies assessing genetic diversity and taxonomic relationships of various plants have been conducted based on this technique. However, RAPD amplification profile is influenced by the parameters that affect the PCR reaction. The probability of reproducing the same RAPD band pattern is low, and consequently, the application of RAPD has been limited [3].

ISSR marker is a multi-locus marker based on the amplification (duplication) of DNA fragment, which is flanked by simple nucleotide sequence repeat in a reverse orientation. These sequences are scattered throughout the genome. The role of repetitive fragments in chromosomes can be a high probability area of crossing over during chromosome reduction (meiosis). Amplification with ISSR primers reveals several loci per primer than RAPD analysis [4]. This primer does not require a specific locus because it will search for any site in the genome that contains a microsatellite motif [5]. Analysis using ISSR markers has been carried out on many plant species, with various purposes, such as the genetic diversity and relationships of mulberry in India [6] and the study of *Morus Alba* genetic stability via tissue culture in China [7].

Mulberry (*Morus sp*) is a native plant from China widespread throughout tropical and sub-tropical climates. It is a long-lived plant and can adapt well to several types of soil. It has a vital role in the silk business because its leaves are the forage for silkworms (*Bombyx mori*) [8].

One of the first steps that can be taken to protect against mulberry pests and diseases is through a molecular approach. This approach using DNA markers has succeeded in forming molecular markers capable of detecting specific genes and traits, especially traits associated with the desired character.

Molecular analysis on mulberry in Indonesia has not been reported. Thus research towards molecular genetics needs to be conducted. This study aimed to analyze DNA polymorphisms by using RAPD and ISSR markers on mulberry plants. It will provide basic genetic information regarding this species for supporting future mulberry plant breeding and conservation programs.

MATERIALS AND METHODS

Plant Materials

The samples were collected from several plantations in Soppeng, Enrekang, and Wajodistricts. Laboratory activities were carried out at Laboratory of Biotechnology and Tree Breeding, Faculty of Forestry, Universitas Hasanuddin, Makassar.

DNA Isolation

The DNA isolation process was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method with modifications [9]. Young leaf samples without leaf venations were weighed as much as 200 mg and then placed into a mortar. 500 µl of CTAB buffer was added, the leaves were crushed into powder, poured into a 2 ml tube, and vortexed for 15 seconds. The tubes containing the solution were incubated in a water bath at 65°C for 90 minutes; the solution was reversed every 30 minutes. A 100 ml of isoamyl alcohol chloroform solution was added to the incubated samples. The tube containing the solution was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a new tube then poured with 800 ml isopropanol solution. The supernatant was centrifuged at 1,000 rpm for 5 minutes. The supernatant solution was removed; the tube was then dried overnight.

The process of separating DNA from other components was carried out by adding 500 µl of TE buffer into a dry tube then centrifuged at 10,000 rpm for 10 minutes. The solution was transferred to a new tube, 100 µl of chloroform was added and centrifuged at a speed of 10,000 rpm for 10 minutes. The solution was transferred to a new tube, and 100 µl of sodium acetate and 800 µl of isopropanol were added. The tubes were centrifuged at 10,000 rpm for 10 minutes. The solution was discarded, the precipitate was

collected and dried overnight. A total of 100 µl ddH_2O was added to the dry tube and centrifuged at 10,000 rpm for 1 minute. The extracted DNA solution was added with 4 µl of RNAs and centrifuged at a speed of 10,000 rpm for 1 minute. Lastly, the DNA solution was stored in a freezer at -20°C.

Primer Selection

Primer selection is conducted to determine RAPD and ISSR primers that produce clear DNA bands that representing loci. This phase was performed by amplifying the same DNA samples using different primers under the same condition. Thus, the optimum conditions and the band variation level produced by each primer can be obtained. Four samples used in the selection were taken from each provenance. The total number of samples tested was 12 samples. The selection process was done to select polymorphic primers, which can amplify the DNAs and determine the right annealing temperature.

RESULTS AND DISCUSSION

Primers selection on 10 RAPD primers had selected eight polymorphic primers, OPK-20, OPA-15, OPA-05, OPAD-11, OPG-19, OPAE-11, OPP-08, and OPD-20. Two out of 10 did not produce any bands, namely OPO-14, and OPC-11. Meanwhile, all ISSR primers successfully amplified the 12 samples tested. The primers used for further analysis were primers that produced clear, bright, and polymorphic bands [10]. Visualizations of DNA bands using RAPD and ISSR primers are presented in Figs. 1 and 2.

The primers successfully amplified DNA bands with different sizes, numbers, and intensities. The number and intensity of DNA bands produced by PCR amplification are highly dependent on how the primer recognizes its complementary DNA sequence in the DNA template used [11].

The primer selections using OPA-15 and OPAD-11 (Fig. 1) showed polymorphic bands; hence these primes can distinguish the individuals by performing different allele sizes of the bands. The OPA-15 amplified 11 samples, OPAD-11 only amplified 4 samples. In comparison, OPAC-12 and OPC-11 showed no DNA bands were generated (data not shown in the electropherogram). OPA-15 primers showed successful amplification in cocoa [12], bamboo [13], and Korean mulberry [14].

Table 1. Mulberry amplification results using RAPD and ISSR primers

No.	Primer Name	Annealing temperature (°C)	Number of samples amplified	Notes
RAPD (Random Amplified Polymorphic DNA)				
1	OPK -20	42,2	8	Polymorphic
2	OPA-15	37	11	Polymorphic
3	OPA-05	32,6	7	Polymorphic
4	OPAD-11	36,6	7	Polymorphic
5	OPG-19	37,5	4	Polymorphic
6	OPAE-11	40,5	4	Polymorphic
7	OPAC-12	-	-	No band
8	OPC-11	-	-	No band
9	OPP-08	39,3	7	Polymorphic
10	OPD-20	42,8	11	Polymorphic
ISSR (Inter-Simple Sequence Repeats)				
1	UBC 810	49,1	11	Polymorphic
2	UBC 813	45,1	11	Polymorphic
3	UBC 814	48,4	11	Polymorphic,
4	UBC 820	49,3	11	Polymorphic
5	UBC 822	45,3	12	Polymorphic
6	UBC 823	49,8	12	Polymorphic
7	UBC 824	47,9	10	Polymorphic
8	UBC 827	50,2	12	Polymorphic
9	UBC 830	52,1	11	Polymorphic
10	UBC 868	47,7	12	Polymorphic

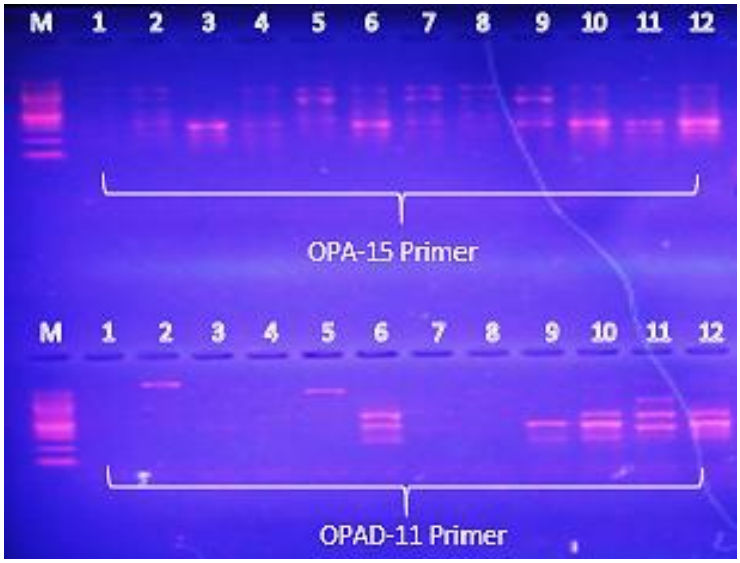

Fig. 1. Electropherogram of PCR amplification products using RAPD primers OPA-15and OPAD-11

(Notes: M = 100 bp Marker and 1-12 = mulberry)

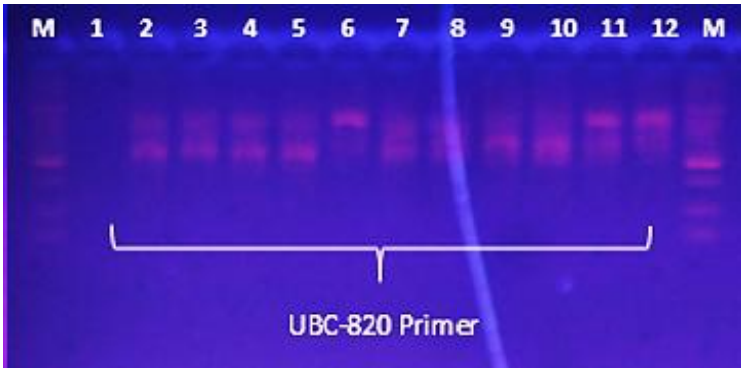

Fig. 2. Electropherogram of PCR amplification products using ISSR primer UBC-820

(Notes: M = Marker and 1-12 = mulberry)

The low consistency of PCR bands using RAPD could be due to imperfect annealing primer to the DNA template as the result of inaccurate PCR reaction concentration and DNA template quality [15,16]. Ali et al. [17] stated that DNA template is closely related to the primer concentration. It is necessary to optimize the ratio between the concentration of DNA template and primer. An incompatible ratio between primer and DNA template causes the RAPD band product to be inconsistent.

The ISSR primer amplification using UBC 820 (Fig. 2) produced bright and polymorphic bands. However, some primers showed smeary and thick bands. A smeary band can be a residue of the solution carried during the isolation process, or it could also be DNA that was degraded in the isolation process [18]. Also, the location of the DNA band far above the DNA marker band indicates that the size of the isolated DNA is very large. The higher the DNA size, the slower the band migrates in agarose during the

electrophoresis process. Primers with smear bands cannot be used for genetic diversity analysis because they may lead to data misinterpretation [10].

Selection of annealing temperature is a step that affects the success of amplification because primer attachment to an already open DNA strand requires an optimal temperature. Too high temperature causes amplification failure because there is no primer adhesion; otherwise, if the temperature is too low, it causes the primer to stick to another side, resulting in low specificity of the DNA formed. The annealing temperature selection is based on the primer used. The conventional breeding process has been undertaken to breed mulberry cultivar during the past few decades in order to develop the mulberry varieties with high yield crops. Thus, the evaluation of the genetic relationship of Morus genotypes is important for measuring the genetic divergence among different mulberry cultivars, predicting progeny performance, and selecting parents for hybridization [19].

CONCLUSION

Primers selection using RAPD markers on mulberry (*Morus sp*.) had successfully amplified 8 out of 10 primers with clear polymorphic bands, namely OPK-20, OPA-15, OPA-05, OPAD-11, OPG-19, OPAE-11, OPP-08, and OPD-20 primers. Whereas, the 10 primers ISSR markers that successfully amplified polymorphic and bright bands were UBC 810, UBC 813, UBC 814,UBC 820, UBC 822, UBC 823, UBC 824, UBC 827, UBC 830, and UBC 868. Thus, these primers will be used in the genetic diversity analysis of mulberry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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