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COMPARATIVE OF PCR SUCCESS WITH CHLOROPLAST MARKERS FOR BARCODING IN SELECTED FORESTRY SPECIES

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ABSTRACT

Chloroplast markers study is an approach applied for identifying molecular markers via DNA barcode. The chloroplast marker, rbcL, and matK are utilized in DNA barcoding and phylogenetic analysis. This study utilized chloroplast markers to identify some forestry species. The species evaluated in this study were *Aleurites moluccana* (Euphorbiaceae), *Arenga pinnata* (Arecaceae), *Bambusa* sp. (Poaceae), *Neolamarckia macrophylla* (Rubiaceae), and *Vatica* sp. (Dipterocarpaceae).

The amplification success rate of rbcL markers was higher than the matK marker. The chloroplast markers are matK (matK 4, matK 5) and rbcL (rbcL1, rbcL 2, rbcL 3, and rbcL 4), which have been successfully amplified in this study, can be recommended as chloroplast markers in the following studies and used to identify species. This study indicates that the chloroplast markers, as universal primers, identified the five evaluated plant families. Therefore, it is necessary to carry out sequencing analysis using other matK and rbcL primers, which have been already reported, can amplify other plant families for strengthening and supporting the genetic information and species morphological identification.

Keywords: Chloroplast markers; DNA barcode; matK; rbcL.

INTRODUCTION

Indonesia is a country known as the world's mega biodiversity. The number of flora species scattered throughout Indonesia is the reason for this designation. Various flora in Indonesia has different benefits. The abundance of flora is widely used by the people, such as candlenut as a material for making household furniture [1,2], sugar palm can produce sap [3,4], bamboo as a raw material for making houses [5,6], jabon Merah as a surface layer for plywood [7,8], and suitable for particleboard [9], and vatica as a building construction material. The distributions of these plants are numerous, and some have broad distribution impacts act their productivity. However, the classification based on morphology characters shows the difficulty for identification. Although morphological classification is easy to perform and to carry out on the field with low cost, this method shows several limitations such as limited number, complex inheritance pattern, vulnerability able to changes in the environment [10].

Complete genetic information is needed in order to support plant breeding and conservation, including information related to genetic diversity, which is still not widely available [11]. Analysis of genetic diversity requires primers that are able to detect the presence of alleles in a genotype [12-14], such as chloroplast genome primers. The rbcL and matK chloroplast genome markers are commonly recommended markers for genetic analysis in plants [15].

A previous study by [16] compared the amplification success rates of several families, *Apocynaceaenacea*, *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Convolvulaceae*, *Euphorbiaceae*, *Geraniaceae*, *Malvaceae*, *Papiliomaceae*, *Poaceae, Resedaceae, Solanaceae,* and *Zygophyllaceae* from Saudi Arabia using two chloroplast genome primers. Information about the types of molecular markers as well as the evaluation using other families for increasing the accuracy of a primer is still limited. Thus, polymorphisms of the chloroplast genome in several families for this identification need to be analyzed. This study was carried out using *Euphorbiace, Arecaceae, Poaceae, Rubiaceae*, and *Dipterocarpaceae* families with consideration to facilitate further identification.

MATERIALS AND METHODS

Sample collection was done at the 2nd Regional Seed/Seedling Forest Office, South Sulawesi. The materials were 12 DNA samples (two from the *Dipterocarpaceae* family, two from the *Euphorbiaceae* family, four from the *Poaceae* family, two from the *Araceae* family, and two from the *Rubiaceae* family). The leaves collected were wrapped according to their family in a transparent plastic wrap with detailed family identification on it and then temporarily stored in the cool box. They were transferred to the laboratory and kept at -20°C in the freezer until extraction. DNA and molecular analysis were carried out in the Laboratory of Biotechnology and Tree Breeding Laboratory, Department of Forestry, Faculty of Forestry, Hasanuddin University, Makassar, South Sulawesi, Indonesia.

DNA Isolation and Amplification

DNA was extracted from 100 mg of leaf tissue. Extraction steps were carried out using the Genomic DNA Mini Kit (Plant) protocol (Geneaid, Taiwan). The quality of extracted DNA was then assessed on 0,8% of agarose. PCR reaction mixture contained a total volume of 12.5 µL following 2 µl of DNA; 0.625 µl of primers, 6.25µl PCR mix (KAPA Biosystem), and 3µl ddH20.

The amplification process was performed using the following steps: one a cycle of pre-amplification at 95°C for 3 min., 35 cycles of amplification steps at 95°C for 30 seconds, primer annealing for 50 seconds at 49.65°C to 57.2°C (specific temperature of each primer), and 72°C for 1 min. (primer extension), and one cycle of final extension at 72°C for 5 min. All PCR conditions were the same for all primer pairs except for annealing temperature (Tm) (Table 1). A Labcycler® Thermocycler (Sensoquest, Göttingen, Germany) was used for performing amplification with PCR protocols following the KAPA Biosystem kit.

Electrophoresis and Visualization

The electrophoresis stage was done by preparing 1% agarose by heating the solution contained 3.6 g of agarose powder and 180 mL of 1X TAE (Tris-acetate-EDTA) buffer solution for ± 6 min. The fluorescent stain GelRed was added once agarose dissolved. The electrophoresis process was then conducted by inserting PCR samples into

Table 1. Primers used for amplification of matK and rbcL markers

No.	Primer Set	Primer Name	Binding	Primer Sequence (5'-3')	Temp (°C)
1	rbcL 1	rbcL 724 R	Reverse	TCG CAT GTA CCT GCA GTA GC	54,2
		rbcL 1 F	Forward	ATG TCA CCA CAA ACA GAA AC	
2	rbcL 2	rbcL 724 R	Reverse	TCG CAT GTA CCT GCA GTA GC	57,2
		rbcL 636 F	Forward	TAT GCG TTG GAG AGA CCG TTT C	
3	rbcL 3	rbcL 1460 R	Reverse	TCC TTT TAG TAA AAG ATT GGG CCG AG	54,05
		rbcL 1 F	Forward	ATG TCA CCA CAA ACA GAA AC	
4	rbcL 4	rbcL 1460 R	Reverse	TCC TTT TAG TAA AAG ATT GGG CCG AG	54,05
		rbcL 636 Fn	Forward	TAT GCG TTG GAG AGA CCG TTT C	
5	matK 1	matK 300 R	Reverse	CGA AGT ATA TAY TTY ATT CGA TAC A	50,05
		matK 899 F	Forward	CAT GCA TTA TGT TAG GTA TCA AGG	
6	matK 2	matK 300 R	Reverse	CGA AGT ATA TAY TTY ATT CGA TAC A	49,65
		matK 1070 F	Forward	CCA TAG TTC CAA TTA TTC CTC TG	
7	matK 3	matK 300 R	Reverse	CGA AGT ATA TAY TTY ATT CGA TAC A	51,1
		matK 55 F	Forward	CCC CCA YAT ATT TGA TAC CTT CTC	
8	matK 4	matK 1710 R	Reverse	GCT TGC ATT TTT CAT TGC ACA CG	54,1
		matK 800 F	Forward	CAT GCA TTA TGT TAG GTA TCA AGG	
9	matK 5	matK 1710 R	Reverse	GCT TGC ATT TTT CAT TGC ACA CG	53,7
		matK 1070 F	Forward	CCA TAG TTC CAA TTA TTC CTC TG	
10	matK 6	matK 1710 R	Reverse	GCT TGC ATT TTT CAT TGC ACA CG	55,1
		matK 55 F	Forward	CCC CCA YAT ATT TGA TAC CTT CTC	
11	matK 7	matK 190 R	Reverse	ATT CGA GTA ATT AAA CGT TTT ACA A	50,65
		matK 800 F	Forward	CAT GCA TTA TGT TAG GTA TCA AGG	
12	matK 8	matK 190 R	Reverse	ATT CGA GTA ATT AAA CGT TTT ACA A	50,25
		matK 1070 F	Forward	CCA TAG TTC CAA TTA TTC CTC TG	
13	matK 9	matK 190 R	Reverse	ATT CGA GTA ATT AAA CGT TTT ACA A	51,7
		matK 55 F	Forward	CCC CCA YAT ATT TGA TAC CTT CTC	
14	matK 10	matK 880 R	Reverse	CCA GAA ATT GAC AAG GTA ATA TTT CC	52,1
		matK 800 F	Forward	CAT GCA TTA TGT TAG GTA TCA AGG	
15	matK 11	matK 880 R	Reverse	CCA GAA ATT GAC AAG GTA ATA TTT CC	51,7
		matK 1070 F	Forward	CCA TAG TTC CAA TTA TTC CTC TG	
16	matK 12	matK 880 R	Reverse	CCA GAA ATT GAC AAG GTA ATA TTT CC	53,15
		matK 55 F	Forward	CCC CCA YAT ATT TGA TAC CTT CTC	

the wells in agarose. Wells #1 and #7 were for the Dipterocarpaceae samples (*Vatica* sp.), wells #2 and #8 were for the Euphorbiaceae samples (*Aleurites moluccana*), wells #3, #4, #9, and #10 were for the Poaceae samples (*Bambusa* sp.), wells #5 and #11 were for Araceae samples (*Arenga pinnata*), and wells #6 and #12 were for Rubiaceae samples (*Neolamarckia macrophylla*). The first and last wells (M) contained the ladder. Electrophoresis was run for 90 min at 100 V. The electropherograms were visualized and documented using gel doc (Biostep). The recorded pictures were used to determine the genotype of the evaluated samples.

RESULTS AND DISCUSSION

The results showed 6 out of 16 primers could amplify the evaluated DNA samples (Table 2).

Table 2. Primer screening of matK and rbcL markers

Primer Set	Amplification Product
matK 1	-
matK 2	-
matK 3	-
matK 4	+
matK 5	+
matK 6	-
matK 7	-
matK 8	-
matK 9	-
matK 10	-
matK 11	-
matK 12	-
rbcL 1	+
rbcL 2	+
rbcL 3	+
rbcL 4	+

Primer matK 1, matK 2, matK3, matK6, matK 7, matK 8, matK 9, matK 10, matK11, and matK 12 primers could not amplify any DNA sample. Only two matK primers could amplify the DNAs, namely matK 4 with 90% amplified band and matK 5 with 100% amplified bands. All rbcL primers amplified the DNAs. rbcL 1 and rbcL 3 had 80% and 65% of amplified band percentages, respectively. Meanwhile, rbcL 2 amplified monomorphic bands (5%). The percentage of amplification using rbcL 4 was 25%. The generated polymorphic bands are displayed in Figs. 1 and 2.

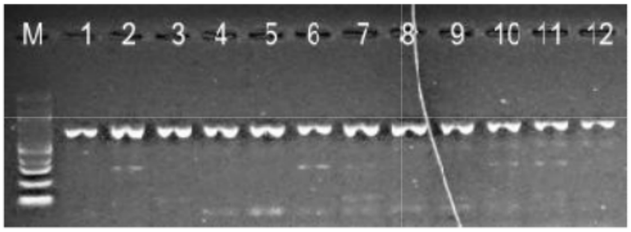

Fig. 1. Electropherogram of DNA amplification using matK 5 primers (1 and 7: *Vatica* sp. , 2 and 8: *Aleurites moluccana*, 3,4,9,10: *Bambusa* sp., 5 and 11: *Arenga pinnata*, 6 and 12: *Neolamarckia macrophylla*, M: 100 bp marker)

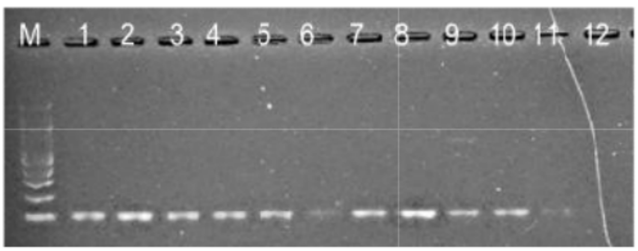

Fig. 2. Electropherogram of DNA amplification using rbcl 2 primers (1 and 7: *Vatica* sp. , 2 and 8: *Aleurites moluccana*, 3,4,9,10: *Bambusa* sp., 5 and 11: *Arenga pinnata*, 6 and 12: *Neolamarckia macrophylla*, M: 100 bp marker)

Table 3. Primer screening using chloroplast primers in five species families

Primer Set	Amplification Information				
	Dipterocarpaceae	Euphorbiaceae	Poaceae	Araceae	Rubiaceae
matK 1	-	-	-	-	-
matK 2	-	-	-	-	-
matK 3	-	-	-	-	-
matK 4	2/2	2/2	4/4	2/2	2/2
matK 5	2/2	2/2	4/4	2/2	2/2
matK 6	-	-	-	-	-
matK 7	-	-	-	-	-
matK 8	-	-	-	-	-
matK 9	-	-	-	-	-
matK 10	-	-	-	-	-
matK 11	-	-	-	-	-
matK 12	-	-	-	-	-
rbcL 1	2/2	2/2	4/4	1/2	1/2
rbcL 2	-	-	1/4	-	-
rbcL 3	2/2	2/2	3/4	1/2	-
rbcL 4	1/2	1/2	1/4	-	-

Legend: Numerator is amplified sample information
The denominator is total samples per family

The success amplification rate of rbcL primer was higher than matK using 12 DNA samples representing five families. Four (25%) samples were successfully amplified using rbcL primer, and these primers were effective in amplifying. Only two (12.5%) samples could be amplified by the matK. This finding is in line with a previous study conducted by [16], which evaluated the success rate of universal primers using matK and rbcL primers in 26 different plant species (covering 14 families) from Saudi Arabia. The amplification success rate was higher for rbcL (88%) than matK (69%).

Table 3 depicts that six primers can amplify adequately. The amplification of these primers produced DNA bands with different numbers, sizes, and intensities. They were matK 4, matK5, rbcL 1, rbcL 2, rbcL 3, and rbcL 4. The amplification rate using matK 4 was 90% because 1 out of 2 samples of the Rubiaceae family could not be amplified using this primer. MatK 5 primer produced bright and clear bands, and all samples were amplified. Thus, the presentation of successful amplification using this primer was 100%. The rbcL 1 successfully amplified DNA samples by 80% because this primer could not amplify 1 out of 2 DNA samples from Araceae and Rubiaceae. The lowest amplification rate was 5%, shown by rbcL 2. This primer could only amplify 1 out of 4 DNA samples from the Poaceae family and could not amplify other DNA samples.

The success of amplification by rbcL 3 on Dipterocarpaceae and Euphorbiaceae families was 3 out of 4 DNA samples from the Poaceae family and 1 out of 2 DNA samples from the Araceae family. Whereas rbcL 3 primer did not amplify the Rubiaceae family, thus the percentage of its amplification rate was 65%. rbcL 4 had a 25% of successful amplification rate because it was only able to amplify 3 out of 5 DNA of the evaluated families, 1 out of 2 DNA samples from the Dipterocarpaceae, and Euphorbiaceae families, and 1 out of 4 DNA samples from the Poaceae family. Meanwhile, matK 1, matK 2, matK 3, matK 6, matK 7, matK 8, matK 9, matK 10, matK 11, and matK 12 did not amplify any DNA sample. The number of amplified DNA determined the high percentage of amplification success rate. Table 3 informs that the Dipterocarpaceae and Euphorbiaceae families had 28.13% of the amplification rate using 16 primers.

Fig. 1 show that these two families had 100% amplified bands using matK 5. Whereas rbcL 2 generated monomorphic bands and rbcL 4 produced only 50% amplified bands in the Dipterocarpaceae and Euphorbiaceae families (Figure 2). All samples from the Poaceae family could only be amplified 26.56% using all primers, which were matK 4, matK 5, and rbcL 1 amplified all samples, rbcL 2 and rbcL 4 amplified only 25% samples, and rbcL 3 amplified 75% samples. The Araceae family had an 18.75% successful amplification rate using all primers (100% using matK 4 and matK 5, monomorphic bands using rbcL 2, 50% using rbcL 1 and rbcL3, and no amplified bands using rbcL 4). The Rubiaceae family had only 12.5% of the amplified samples using all primers, which were 50% using matK 4 and rbcL 1, 100% using matK 5, monomorphic bands using rbcL 2, and no band using rbcL 3 and rbcL 4. High-quality sequences could be obtained easily for *rbcL*, *matK*, and *trnH-psbA*, and *matK* performed best at resolving species among these three markers in *Actinidia* [17], red wood [18], *Artemisia vulgaris* [20], and *Atriplex pratovii* Sukhor [20]. In some cases, MatK + rbcL is not strong enough to determine Styrax growing in North Sumatra to the species level as distinguished on morphological grounds [21].

A factor that affects the amplification process is the DNA template, where the primer will be attached. Unsuitable between the DNA template and the primer sequences will not produce any amplification band [1,22]. Moreover, the low purity of the evaluated DNA during the DNA extraction process produces unclear amplification bands. The inappropriate dilution process and composition of the PCR reaction will also cause the unattached primer to the DNA target site [23]. Other factors are purity and concentration of the DNA template, which contains other compounds, such as polysaccharides and phenolic compounds. The chloroplast genome matK and rbcL markers, which have been successfully amplified in this study, can be recommended as chloroplast genome primers in the following studies and used to identify species. Identification by molecular markers via DNA barcode provides an accurate, fast, and precise alternative [24]. DNA barcoding is known to be useful to manage plant diversity inventories on a large scale and to develop conservation strategies [25]. Molecular analysis is needed to strengthen and support species morphological identification because the molecular character is more stable to environmental effects [26,27]. Identification using DNA barcode sequences has increased rapidly and has been carried out at all taxon levels (family, genus, and species) [28,29]. The most critical point of using Barcode DNA in taxonomy is the character of nucleotide bases that are "unique" or automorphic (nucleotide bases that are only owned by certain species), which differentiate them from other species. This variation of nucleotide bases will naturally differentiate the species into its group [30].

CONCLUSION

Screening chloroplast primers on Euphorbiace, Arecaceae, Poaceae, Rubiaceae, and Dipterocarpaceae families showed that the amplification success rate of the chloroplast genome rbcL marker was higher than matK marker. The chloroplast markers are matK (matK 4, matK 5) and rbcL (rbcL1, rbcL 2, rbcL 3, and rbcL 4), which have been successfully amplified in this study, can be recommended as chloroplast markers in the following studies and used to identify species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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