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Comparison Of The Effectiveness of rbcL and matK in Amplifying The Genome of Diospyros

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Abstract-
Ebenaceae is a large angiosperm family that includes many endemic species; one of the genera is Diospyros. The aim of identified chloroplast genome marker in the Diospyros family is to enable precise species identification by analyzing a unique DNA sequence of a target gene. The current study was carried out to create a marker for different species of Diospyros growing in South Sulawesi. Three putative species of Diospyros (each represented by specimens collected from two districts in South Sulawesi) were evaluated using two regions in the chloroplast genome (matK and rbcL) in order to discriminate them at the species level. Results showed that matK yielded 891 bp after alignment. However, there was no precise identification to species level. The combined rbcL gene showed 100 percent amplification in three species, but combined MatK gene just 50 percent showed amplification. Considering the overall performance of these loci, we suggest the rbcL gene for amplification and matK + rbcL combination may amplify to determine Diospyros growing in South Sulawesi to the species level as distinguished on morphological grounds. These findings show the necessity of finding other candidate genes or markers that can potentially help delineate the various species of Diospyros and other related Diospyros genera. This study is very needed, especially for endemic species.

Keywords: Chloroplast, Diospyros, Genome, matK, rbcL

I. INTRODUCTION

Diospyros celebica is one of the genera having the highest species number in Family Ebenaceae and also becomes one of the largest Genus in Angiospermae. The Genus has more than

500 species (1). Indonesia is the endemic region of Genus Diospyros. Bakh is an endemic species to Sulawesi (2). This species has been widely found in South Sulawesi, Central Sulawesi, and West Sulawesi. There are also ten other Diospyros species distributed in Central and Eastern Indonesia, i.e., Sulawesi, Bali, Nusa Tenggara, and Maluku.

The previous exploration by (3) showed eight Diospyros species found in Tangkoko nature preservation (D. *minahassae*, D. *pilosanthera*, D. *cauliflora*, D. *maritima*, D. *hebecarpa*, D. *malabarica*, D. k*orthalsiana*, and D. *ebenum*). There are also D. *buxifolia* and D. *javanica* that thrive in North Sulawesi. Information from the Natural Resource Conservation Center of South Sulawesi (Sulawesi BKSDA) through direct communication stated that exploration by the Indonesian Institute of Science (LIPI) observed two species of Diospyros in Lohe Island, D. *nigra* and D. f*oliosa* (Rich. Ex A. Gary) Bakh.

Phylogenetic and barcoding analyses of endemic Diospyros in Indonesia have never been studied before. Phylogenic analysis of species in the same Genus can be performed using a DNA barcode. The DNA barcode can provide information on nucleotide sequences from a specific region that can be used for identifying an individual (4).

DNA barcoding is an effective method for identifying and documenting the species in the taxonomy group (5). It also has been applied for analyzing Genus Diospyros in New Caledonian, which is located in Southwest Pacific (6). There are two main genes used by the DNA barcode consortium in plant identification, i.e., matK dan rbcL (7). Both genes are chosen due to their ability to distinguish individuals. They are from the chloroplast genome and located in the coding region (8).

Universal primers used in DNA barcoding (matK and rbcL) need to be screen in order to determine the suitable primers for phylogenetic analysis between species in Genus diospyros that thrive in a tropical area, such as Indonesia. The objective of the study was to evaluate and compare the success rate of matK and rbcL in amplifying DNA for barcoding and Phylogenetic analysis of Indonesia's endemic Diospyros.

II. MATERIALS AND METHODS

A. Genetic Material and DNA extraction

The samples of Genus Diospyros used in the study were collected from different areas. D. malabarica was from the orchard collection area of the Regional Tree Seed/Seedling Office (Sulawesi BPTH), D. *buxifolia* was collected from Kalaena natural preservation, *D. macrophylla* was from Ponda-Ponda natural preservation, and *D. celebica* was from Poso, Central Sulawesi. Leaf sample collection of *D. buxifolia* and *D. macrophylla* in natural preservation areas were conducted after obtaining the permit from Sulawesi BKSDA. The leaf samples were then extracted using DNeasy Plant Mini Kit DNA extraction protocol (Qiagen) (9) with little modification by adding RNase solution into the DNA at the last step of extraction.

B. Primer Selection and DNA Amplification

Two chloroplast primers used in the study were rbcL and matK (four rbcL, four primers, and seven matK primers). From these primers, we obtained four rbcL primer and 12 matK primer combinations. Sixteen of the total rbcL and matK combinations were used in the study. Primer names and sequences are presented in Table 1 based on information from (10). Primer screening was performed to obtain information on whether primer pairs can amplify the DNA samples and determine the suitable annealing temperatures (11).

The DNA was amplified using PCR (Sensoquest Thermocycler) machine. PCR amplifications were conducted using the following steps: one cycle of pre-amplification at 95°C for 3 minutes, 35 cycles of amplification steps at 95 °C for 30 seconds (template denaturation), annealing temperature for 50 seconds (primer annealing), and 72 °C for 1 minute (primer extension), and one cycle of final extension at 72 °C for 5 minutes and then stored at 4°C. During the primer screening process, gradient temperature was performed using ±5°C from the given melting temperature in order to obtain the suitable annealing temperature (Ta).

The amplification products were then separated using horizontal electrophoresis on 2% agarose and TBE (1x) at 100V for 80 minutes. Agarose dyeing was done by adding GelRed (biotium) right after agarose dissolve completely. The visualization was performed by Geldoc (Biostep Gel Documentation System). To measure the amplified products, we used a DNA ladder of 50bp (bioline).

III. RESULTS AND DISCUSSION

Three out of four rbcL primer (75%) combinations could amplify DNA of Genus Diospyros and then used in the sequencing process, whilst only three out of 12 matK primer combinations (25%) were able to generate clear bands; thus, a total number of primer pairs used in this study was six primer pairs. The primer pair that could amplify and generate clear bands are described in Table 2.

There were one species out of four evaluated Diospyros species that showed unsuccess amplification using matK4. RbcL primers were able to amplify all evaluated samples (Figure 1). It indicated that rbcL has a higher success rate of amplification on Genus Diospyros than matK primer.

This study presents similar results to previous studies that analyze plants from different tropical regions. A previous study on 2052 samples, representing 655 species, 259 genera, and 76 families from a tropical rain forest area in Xishuangbanna, China, reported that rbcL managed to amplify up to 98% of the all evaluated samples, whilst matK amplified only 90% (12). It was similar to the study by (13). They observed that the rbcL sequences only confirmed five seagrass species out of seven morphologically identified species, and the sequences generated from this study cannot discriminate Halophila ovalis and H. minor.

A study by (14) used five DNA barcodes (rbcL, matK, trnH-psbA, ITS, ITS2) were evaluated for species identification ability across 669 samples representing 314 species and 100

genera in the Arecaceae. Among the four analyses used, the barcode combination ITS2 + matK + rbcL gave the highest resolution among all single barcodes and their combinations, followed by ITS2 + matK. Among 669 palm samples analyzed, 110 samples (16.3%) were found to be misidentified. Those studies indicated that rbcL has a higher success rate than matK in amplifying the DNA. It is not only observed on Genus Diospyros in the tropical area but also on different Genus in other tropical forests and arid areas. The rbcL showed the best performances: the greatest amplification success, the best sequencing performance both in terms of the number of sequences obtained and in terms of quality of the sequences obtained (15)

The sequence of rbcL is more universal so that it is easier to amplify the evaluated DNA samples and has a higher success rate of the amplification process. Moreover, this primer is also easier to sequence yet has a lower ability to distinguish the individuals (16). On the other hand, matK has a lower success rate on amplification but a higher accuracy level in distinguishing the individuals at the species level. Study in the Genus Amentotaxus, consisting of five or six species, is confined to South China, Northeast India, Laos, and Vietnam, the species discrimination rate increased for all two-barcode combinations, except for matK+trnH-psbA (17).

DNA barcoding studies in plants use one or more plastid regions, such as rbcL and matK, as well as the non-coding spacer trnH-psbA and ITS (Internal Transcribed Spacer) nuclear ribosomal DNA (18–22). Figure 2 presents that matK4 was unable to amplify the D. *macrophylla* species. A similar result also observed in a previous study by (23) matK used in that study was failed to amplify one of the evaluated species, Viscum articulatum. The unsuccessful amplification of D. *macrophylla* samples using matK is due to the different sequences of primers with DNA templates. MatK has large sequence variation so that half of the binding site sequence from this species are different, which eventually lead to amplification failures (19).

The development of DNA barcodes is very needed, especially for endemic species. Research (24) results show for the first study to report a strategy for developing specific DNA barcodes of Orchidaceae plants, laying the foundation for the conservation, evaluation, innovative utilization, and protection of Orchidaceae germplasm resource. Their work highlighted the potential of the barcoding approach for the rapid identification of plant species in order to solve taxonomic disputes and support the commercial traceability of floreal products. In the study of rice report, The six rice-specific chloroplast barcodes revealed that 17% of the 53 seed accessions from rice seed banks or field collections were mislabeled. These findings are expected to improve rice biodiversity by clarifying the concept of rice species, assisting in the identification and use of rice germplasms, and promoting rice biodiversity(25).

IV. CONCLUSION

This study showed universal primer of rbcL and matK genes could be used for amplifying Genus Diospyros's DNA. The success rate of amplification of rbcL was higher than matK. As many as 75% of rbcL were able to amplify all DNA samples, whereas matK only amplified 25%

of the total samples. The primers that were successfully amplifying the samples showed sharp bands and suitable for gene sequencing.

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REFERENCES

[1] Turner B, Munzinger JÔ, Duangjai S, Temsch EM, Stockenhuber R, Barfuss MHJ, et al. Molecular phylogenetics of New Caledonian Diospyros (Ebenaceae) using plastid and nuclear markers. Molecular Phylogenetics and Evolution [Internet]. 2013;69(3):740–63. Available from: http://dx.doi.org/10.1016/j.ympev.2013.07.002

[2] Larekeng SH, Restu M, Susilowati A, Rachmat HH. Genetic diversity of parental and offspring population in ebony (Diospyros celebica bach) revealed by Microsatellites marker. International Journal on Emerging Technologies. 2019;10(2):178–85.

[3] Suryawan. Ady, Kinho, J mayasari, A. Potensi Permudaan Alami Jenis-Jenis Eboni (Diospyros spp.). INFO BPK MANADO, ISSN 2252-4401. 2011;1(1):21–34.

[4] Bafeel SO, Arif IA, Bakir MA, Khan HA, al Farhan AH, al Homaidan AA, et al. Comparative evaluation of PCR success with universal primers of maturase K (matK) and ribulose-1, 5-bisphosphate carboxylase oxygenase large subunit (rbcL) for barcoding of some arid plants. Plant OMICS. 2011;4(4):195–8.

[5] Danielsen F, Beukema H, Burgess ND, Parish F, BrÜhl CA, Donald PF, et al. Plantaciones de biocombustible en terrenos boscosos: Doble peligro para la biodiversidad y el clima. Conservation Biology. 2009;23(2):348–58.

[6] Duangjai S, Samuel R, Munzinger J, Forest F, Wallnöfer B, Barfuss MHJ, et al. A multi-locus plastid phylogenetic analysis of the pantropical Genus Diospyros (Ebenaceae), with an emphasis on the radiation and biogeographic origins of the New Caledonian endemic species. Molecular Phylogenetics and Evolution [Internet]. 2009;52(3):602–20. Available from: http://dx.doi.org/10.1016/j.ympev.2009.04.021

[7] Mursyidin DH, Makruf MI. KEANEKARAGAMAN DAN KEKERABATAN GENETIK ARTOCARPUS BERDASARKAN PENANDA DNA KLOROPLAS matK & rbcL: KAJIAN IN SILICO. Floribunda. 2020;6(5).

[8] Meena RK, Negi N, Uniyal N, Shamoon A, Bhandari MS, Pandey S, et al. Chloroplast-based DNA barcode analysis indicates the high discriminatory potential of matK locus in Himalayan temperate bamboos. 3 Biotech [Internet]. 2020 Dec 13;10(12):534. Available from: http://link.springer.com/10.1007/s13205-020-02508-7

[9] Restu M, Gasir G, Larekeng SH. High outcrossing rate and pollen dispersal distance of diospyros celebica Bakh. (Ebenaceae), an endemic tree species in Sulawesi Island, Indonesia. Biotropia. 2017;24(3).

[10] Duangjai S, Samuel R, Munzinger J, Forest F, Wallnöfer B, Barfuss MHJ, et al. A multi-locus plastid phylogenetic analysis of the pantropical Genus Diospyros (Ebenaceae), with an emphasis on the radiation and biogeographic origins of the New Caledonian endemic species. Molecular Phylogenetics and Evolution. 2009 Sep;52(3):602–20.

[11] Larekeng SH, Paelongan R, Cahyaningsih YF, Nurhidayatullah, Restu M. Primer screening and genetic diversity analysis of jabon Putih (Anthocephalus cadamba (roxb) Miq.) based on random amplified polymorphic DNA (rapd) markers. International Journal of Current Research and Review. 2020 Dec 1;12(24):173–8.

[12] Huang X-C, Ci X-Q, Conran JG, Li J. Application of DNA Barcodes in Asian Tropical Trees-A Case Study from Xishuangbanna Nature Reserve, Southwest China. 2015;

[13] Stevanus, Pharmawati M. Biodiversity and phylogenetic analyses using DNA barcoding rbcl gene of seagrass from Sekotong, West Lombok, Indonesia. Biodiversitas. 2021;22(1).

[14] Le DT, Zhang YQ, Xu Y, Guo LX, Ruan ZP, Burgess KS, et al. The utility of DNA barcodes to confirm the identification of palm collections in botanical gardens. PLoS ONE. 2020;15(7 July).

[15] Giovino A, Martinelli F, Perrone A. The technique of plant DNA barcoding: Potential application in floriculture. Caryologia. 2020;73(2).

[16] Arif MF, Aristya GR, Subositi D, Sari AN, Kasiamdari RS. Short communication: Rbcl and matk chloroplast DNA composition of green chireta (Andrographis paniculata) from Indonesia. Biodiversitas. 2019;20(12).

[17] Ming Gao L, Li Y, Pahn LK, Jun Yan L, Thomas P, Phan KL, et al. DNA barcoding of East Asian Amentotaxus (Taxaceae): Potential new species and implications for conservation. Journal of Systematics and Evolution. 2016;9999(9999):1–9.

[18] Weihong B, Li D, Li X. DNA barcoding of Actinidia (Actinidiaceae) using internal transcribed spacer, matK, rbcL and trnH-psbA, and its taxonomic implication. New Zealand Journal of Botany. 2018;56(4).

[19] Maulidya NN, Rohimah S, Ramadany Z, Ratnasari T, Su'udi M. Assessment of the DNA Barcodes Characteristic of Phalaenopsis deliciosa based on matK, rbcL, and ITS. Biogenesis: Jurnal Ilmiah Biologi. 2020;8(2).

[20] Umer S, Safdar N, Muhammad K. The molecular identification of Zanthoxylum armatum DC of Pakistan based on DNA barcoding. Bangladesh Journal of Plant Taxonomy. 2020;27(2).

[21] Jiang KW, Zhang R, Zhang ZF, Pan B, Tian B. DNA barcoding and molecular phylogeny of Dumasia (Fabaceae: Phaseoleae) reveals a cryptic lineage. Plant Diversity. 2020;42(5).

[22] Nithaniyal S, Majumder S, Umapathy S, Parani M. Forensic application of DNA barcoding in the identification of commonly occurring poisonous plants. Journal of Forensic and Legal Medicine. 2021;78.

[23] Muttaqin Z, Budi RSW, Wasis B, Corryanti, Siregar IZ. DNA barcode characterization of mistletoe infestation in teak Clonal Seed Orchard (CSO) in Padangan, East Java Province, Indonesia. Biotropia. 2017;24(2).

[24] Li H, Xiao W, Tong T, Li Y, Zhang M, Lin X, et al. The specific DNA barcodes based on chloroplast genes for species identification of Orchidaceae plants. Scientific Reports, www.nature.com/scientific reports. 2021;11(1424):1–15.

[25] Zhang W, Sun Y, Liu J, Xu C, Zou X, Chen X, et al. DNA barcoding of Oryza: conventional, specific, and super barcodes. Plant Molecular Biology. 2021;105(3).

TABLES

Table 1. rbcL and matK primers name and sequence for primer selection of Diospyros

Gene	Primer name	Sequence
rbcL	rbcL724R	5'-TCG CAT GTA CCT GCA GTA GC-3'
	rbcL1F	5'-ATG TCA CCA CAA ACA GAA AC-3'
	rbcL1460R	5'-TCC TTT TAG TAA AAG ATT GGG CCG AG-3'
	rbcL636Fn	5'-TAT GCG TTG GAG AGA CCG TTT C-3'
matK	matK1300R	5'-CGA AGT ATA TAY TTY ATT CGA TAC A-3'
	matK800F	5'-CAT GCA TTA TGT TAG GTA TCA AGG-3'
	matK1710R	5'-GCT TGC ATT TTT CAT TGC ACA CG-3'
	matK1070F	5'-CCA TAG TTC CAA TTA TTC CTC TG-3'
	matK1900R	5'-ATT CGA GTA ATT AAA CGT TTT ACA A-3'
	matK55F	5'-CCC CCA YAT ATT TGA TAC CTT CTC-3'
	matK880R	5'-CCA GAA ATT GAC AAG GTA ATA TTT CC-3'

Source : Duangjai et al. 2009

Table 2. Primer and band quality generated by each selected primer

No	Primer Combination	Ta (°C)	Band
	Primer rbcL		

1	R1 rbcL724R rbcL1F	55,9	Clear
2	R3 rbcL1460R rbcL1F	60	Clear
3	R4 rbcL1460R rbcL636Fn	55,9	Clear
	Primer matK		
4	M1 matK300R matK800F	55	Clear
	M4	55,9	Clear
5	matK1710R matK800F		
	M5	54,2	Clear
6	matK1710R matK1070F		

FIGURES

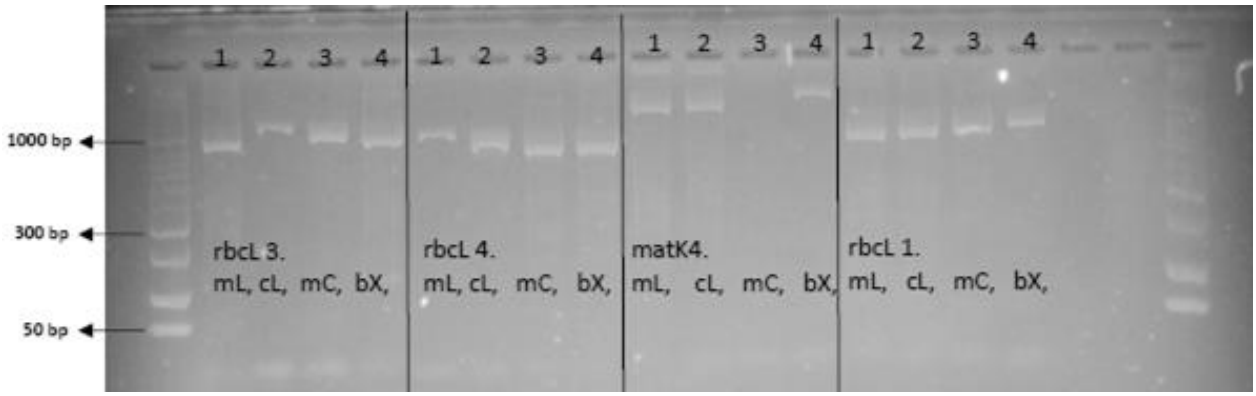

Figure 1. Presents that Genom chloroplast markers amplify the Diospyros genus.
Notes : (1) mL : *Diospyros malabarica*
(2) cL : *Diospyros celebica*
(3) mC : *Diospyros macrophilla*
(4) bX : *Diospyros buxifolia*