

Comparison of two DNA extraction methods for dry leaf Dipterocarpaceae

NITA TAUHIDA¹, ESSY HARNELLY^{1*}, ISKANDAR ZULKARNAEN SIREGAR²

¹Department of Biology, Faculty of Mathematics and Science, University of Syiah Kuala, Banda Aceh, Indonesia

²Department of Silviculture, Faculty of Forestry and Environment, IPB University, Bogor, Indonesia

Abstract. DNA extraction is the first step in obtaining high-concentration and high-purity DNA. It is considered a problem on dry leaves of Dipterocarpaceae because the leaf structure is challenging and contains several secondary metabolites that can affect results. Therefore, this research aims to obtain a suitable method for extracting DNA from preserved Dipterocarpaceae leaves. In silica gel, these leaves will dry up and become tough to destroy. DNA extraction is carried out using CTAB buffers or extraction kits. The results showed extraction with modified CTAB buffer in DNA with higher concentration and purity values than those extracted with the kit. Furthermore, DNA isolated from the modified CTAB can be used as a template in the PCR process to amplify the *matK* gene.

Keywords: CTAB, DNeasy, ekstraksi DNA, *matK*, PCR

INTRODUCTION

The Dipterocarpaceae family dominates southern Asia forests, such as in Indonesia and Malaysia [1]. Several species are endemic to Indonesian forests and provide numerous ecological and commercial benefits such as carbon stock storage and high-quality wood [2]. Dipterocarpaceae have a high economic value in wood, dominating the national and international timber trade [3]. The family offers other benefits, such as the ability to produce oil, resin, and camphor [4]. Due to the various benefits of the plants, they are in high demand and on the verge of extinction in nature. Conservation efforts are needed to ensure that the plants can continue to exist in nature [5].

Morphological or molecular identification of species is vital for conservation efforts in forest plants [5]. Furthermore, morphological identification is very challenging since the Dipterocarpaceae family members have a morphology that is harder to identify at the seedling stage [6;7]. Molecular identification, such as DNA barcoding, can confirm morphological results [7]. The extraction from

plant tissues such as leaves is the initial step in molecular identification. This process obtains a pure DNA concentration that is clear of metabolite chemicals prevalent in plants. It can isolate in various ways, including using a CTAB buffer or an extraction kit. This buffer is universally used for extracting DNA from specific materials, including plants, animals, and fungi. Several components in the buffer can assist with cell wall lysis, precipitation, and DNA washing [8]. Generally, extraction kits are used for specific types of samples, such as plants, animals, bacteria, or fungi. Various specialized extraction kits are available, such as DNeasy Plant Mini Kit, the most regularly used for plant DNA isolation [9].

The concepts of DNA extraction using CTAB buffers and plant-specific extraction kits are similar, but the processing procedures differ slightly. Therefore, this research aims to determine a suitable and optimal extraction method for DNA from Dipterocarpaceae plant leaf samples. It causes the leaves to become dry and difficult to break. The isolation of pure DNA with a high concentration requires a proper extraction procedure.

METHODOLOGY

Leaf samples from Dipterocarpaceae trees were collected in Ketambe Village, Southeast Aceh. The research site obtained three leaf samples, namely *Parashorea lucida*, *Shorea parvifolia*,

*Corresponding Author:
essy.harnelly@unsyiah.ac.id

Received: June 2022 | Revised: September 2022 | Accepted: October 2022

and *Shorea hopeifolia*. The species were taken to Laboratory for further DNA extraction using modified methods of CTAB buffer or the DNeasy Plant Mini Kit (Qiagen).

CTAB Buffer modification

Dipterocarpaceae leaf samples were isolated at the Laboratory of Genetics and Molecular Biology, Department of Biology, FMIPA, USK. Samples were collected and preserved in plastic clips at -20°C. Subsequently, they were ground with a mortar and pestle, which were cleaned after each use to avoid cross-contamination between samples. A modified CTAB isolation method obtained DNA from leaf samples [10]. A sample of finely ground leaves measuring up to 2 mg was then placed in a 1.5-2 mL tube. Each sample tube contained 700 µL of the prepared CTAB isolation buffer (10% CTAB, EDTA 0.5 M, Tri-HCl 8 pH, NaCl 5 M, 1% Mercaptoethanol, 3% PVP, and aquadest), which was evenly homogenized. The sample was incubated at 65°C for one hour, with vortexes every 10 minutes to ensure that the buffer was incorporated entirely.

Furthermore, it was centrifuged at a speed of 12,000 relative centrifugal force for 10 minutes (rcf). The resulting supernatant was transferred to a new, labelled tube. After adding 700 µL of chloroform:isoamyl alcohol (CIA) in a 24:1 ratio, it was centrifuged for 10 minutes at 12,000 rcf. This stage was repeated two times, and the supernatant was transferred to a fresh tube while cold isopropanol was added in an amount equal to the volume obtained. The sample was incubated and centrifuged at -20°C and 12,000 rcf for 30 and 10 minutes. The supernatant was removed, and 600 µL of 70 % ethanol was added to the particle at the bottom of the tube. This stage was repeated three times and centrifuged for another ten minutes at 12,000 rcf before discarding the supernatant. The pellet at the bottom of the tube was allowed to dry, and 50 µL of TE buffer was added.

DNeasy Plant Mini Kit

The crushed leaf sample was added at 20 mg and placed in a 2 ml tube with 400 µL of AP1 and 4 µL of RNase, followed by vortex homogenization. For 10 minutes, the samples were incubated in a water bath at 65°C. Furthermore, the incubated sample was homogenized in 130 µL buffer P3, and this mixture was centrifuged for 5 minutes at 14,000 rpm. The supernatant was placed in the Qiashredder Mini Spin Column, and the tube containing the sample was centrifuged for 2 minutes at 14,000 rpm. The 240 µL filtrate was transferred to a separate tube, where 1.5 times the volume of AW1 was added, homogenized, and transferred to a Qiashredder. Subsequently,

the Qiashredder tube was centrifuged for 1 minute at 8000 rpm. The filtrate was removed, and 500 µL AW2 was added to the filter, which was centrifuged at 14,000 rpm for 2 minutes and repeated up to two times. It was then transferred to a fresh 1.5 µL tube with 50 µL of AE buffer. After allowing the tube and filter to rest for 5 minutes, centrifugation was conducted for 1 minute at 8,000 rpm.

DNA Amplification

Primers used for DNA amplification of the *matK* gene were *matK*-390f (5'-CGATCTATTCA TTCAATATTTTC-3') and *matK*-136R (5'TCT AGCACACGAAAGTCAAGT-3') (11).

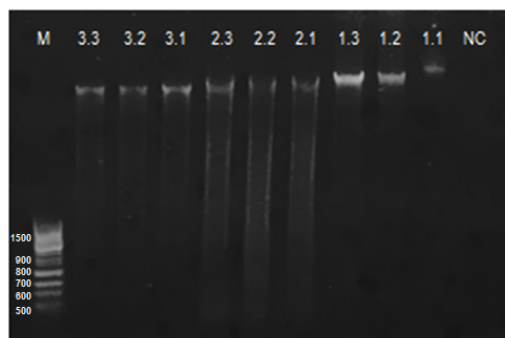
DNA was amplified using MyTaq HS Red Mix (Bioline), and the total volume for amplification was 50 µL consisting of 2 µL DNA samples and 0.5 µL of each primer (10 µM). The amplification was carried out in PCR (Sensoquest) as follows: pre-denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 40 sec, annealing at 42.6°C for 1 min, elongation at 72°C for 1 min, and one cycle of final extension at 72°C for 10 min. Furthermore, the PCR products were separated by 2.0% agarose gel electrophoresis.

RESULTS AND DISCUSSION

CTAB Buffer modification

DNA extraction using the modified CTAB buffer method produced better results than commercial kits. During the sample lysis process, 1% mercaptoethanol and 3% PVP were added to the solution. The addition of mercaptoethanol and PVP reduces contaminant chemicals from the storage of dried Dipterocarpaceae leaves. Mercaptoethanol functions as an antioxidant, reducing the brown hue in the solution during the DNA extraction procedure [12]. It can also be used to break down the protein contained in the sample [13]. Adding 3% PVP to the extraction process can assist in DNA purification. This is because PVP can attach to polyphenols and inhibit them from binding to DNA [12].

The CTAB method was modified by washing the DNA pellet three times with 70% ethanol after centrifugation, which was expected to break down contaminants in the extraction results. Since ethanol 70% helps in the precipitation process, washing the pellet after centrifugation three times is considered to produce DNA free of contaminating chemicals. Even though ethanol washing can aid precipitation, it is not recommended to use large quantities because the optimal concentration required is 70% [14].



1.1; 1.2; 1.3: *Parashorea lucida*,
2.1; 2.2; 2.3: *Shorea parvifolia*,
3.1; 3.2; 3.3: *Shorea hopeifolia*,
M: Ladder, NC: Negative Control

Figure 1. The results of electrophoresis visualization of extracted DNA using the modified CTAB method.

The DNA quality was evaluated using a 2% agarose gel electrophoresis at 75 volts for 35 minutes. The extraction using a modified CTAB buffer produced thicker bands than the kit (DNeasy Plant Mini Kit), according to electrophoresis visualization results (Figure 1). The CTAB buffer DNA has a high and pure concentration of contaminating chemicals. Even though there was a smear on samples 2.1, 2.2, and 2.3, indicating the presence of fragments or contaminating compounds, DNA bands were still visible.

The extraction results were also evaluated using a nanophotometer to measure the concentration and DNA purity. The concentration using modified CTAB ranged from 187 ng/μl to 659 ng/μl (Table 1.), according to the test. This result indicates that DNA extracted with the modified CTAB method can be used as a PCR template.

Table 1. Concentration and quantity of DNA purity extracted using modified CTAB

Name	Conc. (ng/ul)	A260/A280	A260/A230
Blank	0,0000	0,000	0,000
1.1	218,95	1,768	1,988
1.2	659,00	1,166	1,605
1.3	379,85	1,630	1,877
2.1	316,45	1,818	2,223
2.2	288,30	1,752	2,076
2.3	312,00	1,697	2,027
3.1	193,55	1,961	2,328
3.2	187,60	1,950	2,505
3.3	284,10	1,995	2,091

1.1; 1.2; 1.3: *Parashorea lucida*,
2.1; 2.2; 2.3: *Shorea parvifolia*,
3.1; 3.2; 3.3: *Shorea hopeifolia*

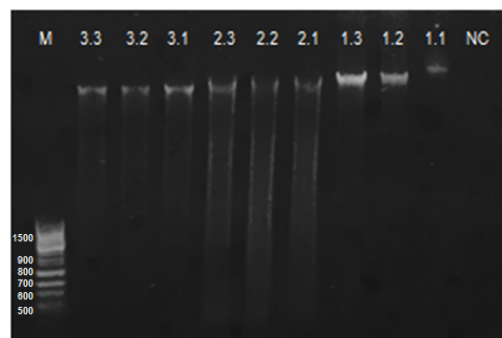
A nanophotometer was used to perform analysis and check the purity of DNA. The ratio of absorbance at wavelengths of 260 nm and 280

nm was used to determine the purity of phenol in the sample. According to the data, the A260/A280 ratio in DNA isolated from modified CTAB ranged from 1.16 nm to 2.22 nm. This value indicates that the purity of phenol is close to the recommended value of 1.80-2.00 nm [15]. The ratio of absorbance values at 260 nm to 230 nm (A260/A230) assessed the purity of DNA on carbohydrates or other organic compounds. According to the data, the A260/A230 ratio ranged from 1.60 nm to 2.50 nm. This value suggests that the extracted DNA purity for the carbohydrate is close to the suggested limit of 2.00-2.20 nm [15].

DNeasy Plant Mini Kit

DNA extracted with the DNeasy Plant Mini kit did not exhibit any bands at a visualized 2% agarose (Figure 2.). The apparent bands are very thin and fuzzy, suggesting that the kit's extraction only produces DNA with a poor concentration. This is probably caused by the buffer's inability to disrupt the cell walls in the dry leaf samples. The lysis process can be helped by increasing the incubation time to maximize the function of the buffer.

The pigmentation in the sample solution was not reduced using a lysis buffer and a spin column. Therefore, the extracted sample remains brown, indicating that contaminants are present in the recovered DNA.



1.1; 1.2; 1.3: *Parashorea lucida*,
2.1; 2.2; 2.3: *Shorea parvifolia*,
3.1; 3.2; 3.3: *Shorea hopeifolia*,
M: Ladder, NC: Negative Control

Figure 2. The results of electrophoresis visualization of extracted DNA using the Dneasy Plant Mini Kit.

The DNA concentration was deficient, ranging from 1,850 ng/μl to 12,800 ng/μl (Table 2.), according to the quantity test results. Therefore, this sample cannot be used as a template for amplification, and the needed concentration is usually 200-2000 ng/μl [15]. A260/A280 values in DNA extracted using kits also ranged

from 1.07 nm to 1.70 nm. The average A260/A280 value is very low compared to the recommended (1.80-2.00 nm).

Table 2. Concentration and quantity of DNA purity extracted using Dneasy Plant Mini Kit

Name	Conc. (ng/ul)	A260/A280	A260/A230
Blank	0,0000	0,000	0,000
1.1	11,500	1,544	2,584
1.1	12,800	1,620	3,012
1.2	10,850	1,456	4,019
1.3	11,950	1,707	3,025
2.1	9,6500	1,369	2,797
2.2	5,0500	1,074	-1,942
2.3	11,850	1,445	0,988
3.1	4,1000	1,708	-6,833
3.2	3,8500	1,604	-2,265
3.3	1,8500	1,423	-1,682

1.1; 1.2; 1.3: *Parashorea lucida*,
2.1; 2.2; 2.3: *Shorea parvifolia*,
3.1; 3.2; 3.3: *Shorea hopeifolia*,

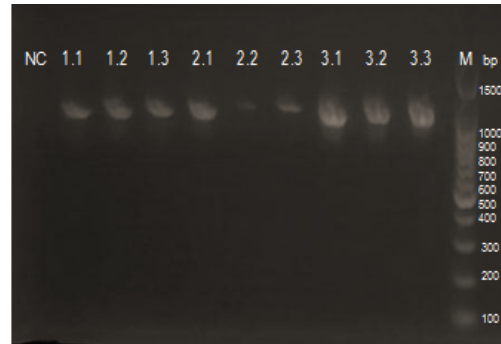
The A260/A230 ratio of DNA extracted using the kit varied from -6.833 to 4.019 nm. This demonstrates the level of contamination due to organic chemicals. Secondary metabolites are commonly found in high concentrations in mature leaves, and these molecules can contaminate extracted DNA [16;17]. The average A260/A280 value obtained using the kit is significantly below the required value (2.00-2.200 nm). This result also implies insufficient DNA to proceed to the amplification process.

Amplification

The *matK* gene was amplified using the DNA template extracted with the modified CTAB method. The BOLD system recommends *matK* as one of the genes for DNA barcodes [18, 19, 20]. The molecular identification approach to corroborate the results of morphological identification is the DNA barcode [21;22].

The *matK* gene is recommended by BOLD because it is conservative, meaning that the alterations can distinguish across species but not between individuals of the same species [23]. However, PCR amplification is considered to be problematic.

The size of the *matK* gene is 1,000 kb, according to the visualization results using a 2% agarose gel electrophoresis (Figure 3.). This is consistent with the findings of Shaw et al., where the *matK* gene is typically between 900 and 1,000 kb in size [10]. Therefore, DNA results from the modified CTAB extraction method were successfully used as a template for the gene amplification process, particularly for the *matK* gene.



1.1; 1.2; 1.3: *Parashorea lucida*,
2.1; 2.2; 2.3: *Shorea parvifolia*,
3.1; 3.2; 3.3: *Shorea hopeifolia*,
M: Ladder, NC: Negative Control

Figure 3. Electrophoresis visualization results of *matK* gene amplification in extraction samples using modified CTAB

CONCLUSION

DNA extraction on dry leaves of the Dipterocarpaceae family using CTAB buffer with the addition of 1% mercaptoethanol and 3% PVP resulted in better DNA quality and purity than a kit (DNeasy Plant Mini Kit). The modified CTAB extraction was analyzed for DNA quality using agarose gel electrophoresis and showed thick bands without degradation (smear). In addition, the DNA quantity analysis with a Nanophotometer showed promising results. The bands were successfully extracted in three samples of Dipterocarpaceae leaves using a modified CTAB method as a template for the *matK* gene amplification process.

ACKNOWLEDGMENT

The author is grateful to Syiah Kuala and IPB Universities for providing financial support for this project, the Biology Department, the Silvikultur Department, and all parties who participated and helped implement this research.

REFERENCE

- [1] Brearley, F.Q.; Banin, L.F.; Saner, P. 2017. The ecology of the Asian dipterocarps. *Plant Ecol. Divers.* **9** 429-436.
- [2] Usmani, D.; Wahyuni, S.; Melani, K. 2018. Dipterocarpaceae potential as CO₂ absorber and carbon storage in Bogor Botanical Gardens. *Prosiding Konservasi Tumbuhan Tropika: Kondisi Terkini dan Tantangan ke Depan*. Cibodas: UPT Balai Konservasi Tumbuhan Cibodas; p. 45-49
- [3] Ashton, P.S. 1989. Dipterocarp reproductive biology. In: Lieth H. Werger

- MJA (eds). *Tropical forest ecosystems: biogeographical and ecological studies. Ecosystems of the World Series, 14B*. (Amsterdam: Elsevier Science Publishers).
- [4] Purwaningsih. 2004. Sebaran ekologi dipterocarpaceae di Indonesia. *Biodivers J.* **5** 89-95.
- [5] Guan, S.L.; Yen, S.Y. 2000. Conservation of dipterocarpaceae in Peninsular Malaysia. *J. Trop. For. Sci.* **12** 593-615.
- [6] Rosdayanti, H.; Siregar, U.J.; Siregar, I. 2019. Karakter penciri morfologi daun meranti (*Shorea* spp) pada area budidaya *ex-situ* KHDTK Haurbentes. *Media Konservasi.* **24** 207-215.
- [7] Hebert, P.D.; Cywinska, A.; Ball, S.L.; deWaard, J.R. 2003. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* **270** 313-321.
- [8] Aboul-Maaty, N.A.F.; Oraby, H.A.S. 2019. Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bull. Natl. Res. Cent.* **43** 25-35.
- [9] Abdel-Latif, A.; Osman, G. 2017. Comparison of three genomic DNA extraction methods to obtain high DNA quality from maize. *Plant Methods.* **13** 1-9.
- [10] Shaw, J.; Lickey, E.B.; Schilling, E.E.; Small, R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *Am. J. Bot.* **94** 275-288.
- [11] Heckenhauer, J., Barfuss, M.H., Samuel, R. 2016. Universal multiplexable *matK* primers for DNA barcoding of angiosperms. *Appl. Plant Sci.* **4**(6), apps.1500137.
- [12] Varma, A.; Padh, H.; Shrivastava, N. 2007. Plant genomic DNA isolation: an art or a science. *Biotechnol. J.* **2** 386-392.
- [13] Mornkham, T.; Wangsomnuk, P. P.; Wangsomnuk, P.; Jogloy, S.; Pattanothai, A.; Fu, Y.B. 2012. Comparison of five DNA extraction methods for molecular analysis of Jerusalem artichoke (*Helianthus tuberosus*). *Genet. Mole. Res.* **11** 572-581.
- [14] Oda, Y.; Sadakane, K.; Yoshikawa, Y.; Imanaka, T.; Takiguchi, K.; Hayashi, M.; Kenmotsu, T.; Yoshikawa, K. 2016. Highly concentrated ethanol solutions: good solvents for DNA as revealed by single-molecule observation. *Chem. Phys. Chem.* **17** 471-473.
- [15] Čermáková, E.; Zdeňková, K.; Demnerová, K.; Ovesná, J. 2021. Comparison of methods to extract PCR-amplifiable DNA from fruit, herbal and black teas. *Czech J. Food Sci.* **39** 410-417.
- [16] Azmat, M.A.; Khan, I.A.; Cheema, H.M.; Rajwana, I.A.; Khan, A.S.; Khan, A.A. 2012. Extraction of DNA suitable for PCR applications from mature leaves of *Mangifera indica* L. *J. Zhejiang Univ. Sci. B.* **13**(4), 239-243.
- [17] Chase, M.W.; Knapp, S.; Cox, A.V.; Clarkson, J.J.; Butsko, I.Y.; Joseph, J.; Savolainen, V.; Parokonny, A.S. 2005. Land plants and DNA barcodes: short-term and long-term goals. *Proc. Biol. Sci.* **360** 1889-1895.
- [18] Fazekas, A.J.; Kesanakurti, P.R.; Burgess, K.S.; Percy, D.M.; Graham, S.W.; Barrett, S.C.H.; Newmaster, S.G.; Hajibabaei, M.; Husband, B.C. 2009. Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? *Mol. Ecol. Resour.* **9** 130-139.
- [19] Hebert, P.D.N.; Gregory, T.R. 2005. The promise of DNA barcoding for taxonomy. *Sys. Biol.* **54** 852-859.
- [20] Small, R.L.; Cronn, R.C.; Wendel, J.F. 2004. Use of nuclear genes for phylogeny reconstruction in the plant. *Aust. Syst. Bot.* **17** 145-170.
- [21] Yu, J. Xue, J.H.; Zhou, L. 2011. New universal *matK* primer for DNA barcoding angiosperms. *J. Sys. Evol.* **49** 176-181.
- [22] Chatrath, P.; Choudhary, M.; Tarafdar, A. 2013. An efficient protocol for genomic DNA isolation from field-grown mature leaves of *Pennisetum glaucum*. *J. Biotech.* **8** 30-34.
- [23] Moura, C.C. de M.; Brambach, F.; Bado, K.J.H.; Krutovsky, K.V.; Kreft, H.; Tjitrosoedirdjo, S.S.; Siregar, I.Z.; Gailing, O. 2019. Integrating DNA barcoding and traditional taxonomy for the identification of dipterocarps in remnant lowland forests of Sumatra. *Plants. J.* **8** 461-475.