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Original Research

## Optimization of DNA Isolation Dried Leaf Samples of Endangered Plants *Dipterocarpus cinereus*


Ruri Fitriyani[1], Afifatul Achyar[1]*, Iyan Robiansyah[2]

[1]Department of Biology, Faculty of Science and Mathematics, Universitas Negeri Padang, West Sumatera.
[2]Research Center for Plant Conservation and Botanical Gardens, National Research and Innovation Agency, Bogor, West Java 16911
*__Corresponding author:__ e-mail address: afifatul.achyar@fmipa.unp.ac.id.



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**Abstract**

Dipterocarpus cinereus (Lagan bras) is an endemic tree of Mursala, a small island in Central Tapanuli, North Sumatra. Lagan bras is a tree that has good quality for shipbuilding or construction materials. This plant has a critical status by the IUCN Red List 2020 so that conservation efforts need to be carried out. Conservation efforts are still limited to the status of conservation, ecology and distribution as well as vegetative propagation while molecular studies have never been carried out. Therefore, it is necessary to know the optimum DNA isolation method because DNA quality is very important as a determinant of the success of molecular analyses such as PCR. Various techniques can be used to isolate DNA, depending on the type of plant used. Each type of plant contains different secondary metabolite compounds that require an optimum isolation method. This study aims to obtain the optimum DNA isolation method and produce good quality genomic DNA from the dried plant Dipterocarpus cinereus from the LIPI Plant Conservation Research Centre and Botanical Gardens collection. This research was conducted at the Genetics and Biotechnology Laboratory, State University of Padang in October 2021. The DNA isolation method used was the modified CTAB method with variations in leaf size, the use of PVP, and grinding with and without liquid nitrogen as well as the Promega and Qiagen isolation kit methods. The results showed that a large quantity of DNA and high quality DNA could be produced in plant samples by modifying DNA isolation using the Cetyl Trimethyl Ammonium Bromide (CTAB) method as well as the addition of polyvinylpolypyrrolidone (PVP) antioxidants and the use of liquid nitrogen with a leaf size of 2x2 cm.



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### Introduction

Dipterocarpus cinereus or Lagan Bras is a member of the genus Dipterocarpus which has a diameter of 40 cm. This tree is an endemic tree species on Mursala Island, North Sumatra whose wood is targeted for ship construction. Was said to be extinct in 1998, Lagan bras was rediscovered as many as 30 adult individuals in 2018 and was included in the category of critically endangered (Critical Endangered) by the IUCN Red List (Robiansyah, 2020). Therefore, it is necessary to conduct molecular studies to determine the genetic variation in this plant. To obtain genetic information of this endemic plant, an optimal isolation method is needed.

DNA isolation is the process of separating DNA from other cell components such as proteins, carbohydrates, fats and others. Good quality DNA is used for activities such as molecular markers, genome library creation and sequencing. DNA isolation consists of three main stages, namely cell wall

destruction (lysis), separation of DNA from other components and DNA purification (Corkill, 2008). Good quality DNA obtained from the extraction is an initial requirement that must be met in molecular studies (Syafaruddin et al., 2011). In general, a good DNA extraction procedure includes three important things, namely it must be able to produce DNA with high purity, DNA must be intact, and high concentration (Romlah, 2018).

The main problem that often arises in the process of isolating plant DNA is the presence of polysaccharide compounds, proteins, polyphenols, RNA and other secondary metabolites found in plants that inhibit the DNA isolation stage (Varma et al., 2007). The amount of these components varies according to plant species, plant parts used, environmental conditions, and environmental conditions. According to Heikrujam (2020), generally fresh leaves aged 15-20 days are easier to isolate. The plants isolated in this study were leaves that had been air-dried using silica gel which caused the leaves not to be fresh anymore, so it was necessary to optimize DNA isolation in order to get the best results. The main objective of the optimization of the isolation method is to obtain a relatively fast, inexpensive and consistent method for extracting high-quality DNA and better yields (Heikrujam, 2020).

Cetyl trimethyl ammonium bromide (CTAB) method is a method commonly used in extracting plant genomic DNA which contains a lot of polysaccharides and polyphenolic compounds. CTAB is a type of cationic surfactant used for the lysis of plant cell walls. The CTAB method does not require more expensive costs than using a kit (Ardiana, 2009). Optimization of the isolation method can be carried out on the composition of the lysis buffer solution or physical handling techniques in separating genomic DNA from other compounds. In principle, the optimization of the isolation method aims to protect genomic DNA from degradation due to secondary compounds released when cells are destroyed or damage due to physical handling (Restu et al., 2012).

## Method

### Materials

The main ingredients used in this study were samples of dried leaves of D. cinereus which had been dried using silica gel. This sample is a collection of the Indonesian Endangered Plant Conservation Research Group, the Research Center for Plant Conservation and the LIPI Botanical Gardens. Other materials used for DNA isolation were cetyl trimethyl ammonium bromide (CTAB), sodium dodecyl sulfate (SDS), polyvinyl pyrrolidone (PVP), liquid nitrogen, trisbase, HCl, sodium chloride (NaCl), ethylene diamine tetra-acetic acid (EDTA), Promega isolation kit, DNA Mini Kit Qiagen, Plant Mini Kit Qiagen, ultra-pure distilled water (ddH2O), chloroform, isopropanol alcohol, isoamyl, 70% ethanol and buffer TE 1X pH 8. The tools used are ice box, scissors , marker pen, zip plastic, micropipette, micropestle, microtube rack microtube, spatula, bath, centrifuge, vortex, spin downtube falcon , and tweezers.

### Method

This research was carried out at the Genetics and Biotechnology Laboratory, Faculty of Mathematics and Natural Sciences, Padang State University in October-December 2021. Optimization of DNA isolation that has been carried out is using the modified CTAB-based Doyle and Doyle method, Promega Isolation Kit method, QIAamp DNA method Mini Kit and DNeasy Plant Mini Kit Qiagen method. The research activities included the following stages:

**Modified method of Doyle & Doyle (1990) based on CTAB.**

In this method, various treatments were carried out, namely variations in the size of isolated plant leaves, grinding using liquid nitrogen and without using liquid nitrogen as well as with and without the addition of PVP. First of all, name the tube with a marker pen. Leaf samples were cut and then crushed with liquid nitrogen (depending on the treatment) and put into sterile microtubes. A total of 670 L of extraction buffer was added then the sample was ground with the help of a micropestle until homogeneous. Then 50 L SDS 20% was added, then the reaction was vortexed until

homogeneous and incubated in a water bath at 65℃ for 15 minutes. Then as much as 100 L of 5 M NaCl was inserted into the tube, then the tube was inverted slowly. A total of 100 L CTAB 1X was added to the sample tube and then vortexed and added PVP (depending on treatment) at the end of the spatula in the specified tube. Samples were incubated at 65℃ for 15 minutes in a water bath and then centrifuged at 12,000 rpm for 15 minutes.

The supernatant was transferred to a new microtube and added 900 L of chloroform:isoamyl alcohol (24:1) and then vortexed. The sample was centrifuged at a speed of 12,000 rpm for 15 minutes. The supernatant was again transferred to a new microtube and 600 L of cold isopropanol was added and the tube was slowly inverted. At this stage the DNA will look like strands of white thread. Then the sample was centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was washed with 200 L of 70% ethanol. Samples were centrifuged at 12,000 rpm for 5 minutes. The supernatant (ethanol) was discarded and the pellet was dried on a clean dry tissue, then the pellet was dissolved in TE 1X pH 8. DNA samples could be stored in a -20℃ refrigerator or the concentration and purity could be measured.

**Promega Kit**

Method The isolation method is in accordance with the Promega Kit protocol. Leaves measuring 2x2 cm were put into a microtube and then frozen using liquid nitrogen and mashed with the help of a micropestle. Then 600 L of nucleid acid solution was added and then vortexed. Samples were incubated at 65℃ for 15 minutes. Then the sample was added with 200 L of protein precipitation solution and vortexed at high speed and then centrifuged at 13,000 rpm for 3 minutes so that the protein would precipitate. Next is the DNA precipitation step, the supernatant is transferred into a new tube which already contains 600 L of isopropanol and the tube is slowly inverted until the DNA threads are visible. Centrifugation at 13,000 rpm for 1 minute. The next step was washing and rehydrating DNA with the addition of 600 L of 70% ethanol and then slowly inverting the tube. Then it was centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the microtube was dried in an inverted position on a tissue for 10-15 minutes. A total of 50 L DNA rehydration solution was added and incubated for 1 hour at 65℃ or 24 hours incubation at 4℃. The concentration and purity of DNA can be measured with a nanophotometer.

**QIAamp DNA Mini Kit**

Method The isolation method complies with the QIAamp DNA Mini Kit Kit protocol. Leaves measuring 2x2 cm were cut and sterilized with 70% alcohol and then put into microtube and crushed with liquid nitrogen with the help micropestle . A total of 180 L of buffer ATLThe mixture was vortexed for 5 seconds and incubated in a heatblock at 65℃ for 1 hour. A total of 200 L buffer AL and vortexed for 15 seconds. The samples were incubated in a heat block at 70℃ for 10 minutes. Microtube spindown to ensure there is no liquid in the tube. A total of 200 L absolute ethanol (96-100%) was added and vortexed for 15 seconds. The lysate was pipetted into a QIAamp Mini spin column (in a 2 ml collection tube). Centrifugation for 5 minutes at 8,000 rpm. The remaining leaves that stick to the tube wall are removed with the help of tips. Flow-through and collection tubes are discarded. The QIAamp Mini spin column was placed in a new collection tube, 500 L buffer AW1 was then centrifuged at 8,000 rpm for 5 minutes. Flow-through and collection tubes are discarded. The QIAamp Mini spin column was placed in a new collection tube, 500 L buffer AW2 was then centrifuged at 13,000 rpm for 5 minutes. The flow-through was removed and a QIAamp Mini spin column was placed in the tube and centrifuged at 13,000 rpm for 2 minutes (dry centrifugation). The QIAamp Mini spin column was placed in a 1.5 mL sterile microtube and 50 L of buffer AE and incubated at room temperature for 1 minute. Centrifugation at 13,000 rpm for 1 minute for elution 1. Repeat previous steps for elution 2.

**DNeasy Plant Mini Kit**

Method The isolation method was in accordance with the DNeasy Plant Mini Kit protocol. A weight of 100 mg was cut and put into a sterile microtube and 400 L of buffer AP1 and 4 L of RNase A were added, then vortexed and incubated for 10 minutes at 65℃ in a heatblock. Then 130 L of buffer

P3 was added. Vortex and incubate in refrigerator -20℃. Samples were centrifuged for 5 minutes at 13,500 rpm. The lysate was pipetted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at 13,500 rpm. The flow-through was transferred into a sterile microtube without disturbing the pellet formed. A total of 1.5 volumes of AW1 buffer were added and mixed using a micropipette. A total of 650 L of the mixture was transferred to a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifugation for 1 minute at 8,000 rpm. Flow-through discarded. The spin column was transferred to a new collection tube and 500 L of buffer AW2 was added and centrifuged for 1 minute at 8,000 rpm. The flow-through was removed and 500 L of buffer AW2 was added. Centrifugation for 2 minutes at 13,500 rpm. The spin column was transferred to a sterile 1.5 ml microtube and 100 L of buffer AE for elution and then incubated for 5 minutes at room temperature. Centrifugation for 1 minute at 8,000 rpm. Repeat the previous step for elution 2.

**Results and Discussion**

**Table 1**. Nanophotometer results from DNA isolation

| Method | concentration (ng/ L) | DNA purity (A260/A280) |
|---|---|---|
| CTAB-Leaf size 1x1 cm | 55.30 1.301 | CTAB |
| -Leaf size 2x2 cm | 127.50 | 1.326 |
| CTAB-Leaf size 3x3 cm | 135..50 | 1.313 |
| CTAB-Scrubbing with liquid nitrogen | 65.00 | 1,427 |
| CTAB-Crushing without liquid nitrogen | 117.10 | 1,262 |
| with liquid nitrogen and adding PVP | 55.20 | 2,029* |
| without liquid nitrogen and adding PVP | 117.10 | 1,667 |
| Promega Kit | 80.85 | 1,367 |
| QIAamp DNA Mini Kit Qiagen | 123.60 | 1,680 |
| DNeasy Plant Mini Kit Qiagen | 335.35 | 0.971 |

Specification: The * mark indicates the optimum DNA isolation method.

Based on Table 1. The optimal leaf size used for isolation was 2x2 cm with a concentration of 127.50 ng/µL and purity at wavelength A260/280 was 1.326. Grinding the sample using liquid nitrogen gave DNA extraction results with a purity of 1,427 and a DNA concentration of 65.00 ng/µL. The purity and quality of DNA greatly affect the results that will be obtained in the next stage. A DNA molecule is said to be pure if its absorbance ratio is between 1.8-2.0 (Darmo, 2011).

The methods used in DNA isolation are very diverse and depend on the type of plant or tissue to be used (Syamsurizal et al., 2021). But basically there are three factors that determine the success of DNA isolation, namely 1). Plant tissue homogenization, 2). The composition of the addition of buffer solution at the time of grinding plant leaves and 3). Removal of plant polysaccharides. The CTAB method is commonly used for the isolation of plant DNA. This method uses liquid nitrogen in the early stages of extraction to produce the best DNA quality (Pharmawati, 2009). CTAB is a kind of detergent that can degrade cell walls, denature proteins, and separate carbohydrates (Suprapto, 2003). This method does not require more costs than using a kit (Ardiana, 2009).

The DNA extraction process encountered several difficulties due to the large number of phenolic compounds which were very disturbing during the DNA isolation process. Polyphenolic compounds are irritating contaminants and often cause failure of DNA isolation and purification. Things like this are often found in other plants, including gambier (Fauza et al., 2007) and sunan candlenut (Syafaruddin & Santoso, 2011). Polysaccharides interfere with the DNA isolation process because polysaccharides will interact with DNA to form a very viscous solution. Contaminants mixed in DNA can inhibit the amplification reaction, so that the quantity and quality of DNA produced is very low.

These problems can be overcome by adding PVP (polyvinylpyrrolidone) and purification repeatedly, so that DNA amplification can be carried out and can be used for further analysis.

**Conclusion**

The most optimal DNA isolation method used for endangered plants Dipterocarpus cinereus is the CTAB-based Doyle & Doyle (1990) method modified with 2x2 cm leaf size, grinding using liquid nitrogen and the addition of PVP. The PCR results showed that the results of the isolation using the CTAB method with grinding using liquid nitrogen and the addition of PVP could be amplified so that genetic variations could be observed. The CTAB method is also cost-effective than other methods.

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