DNA isolation and amplification of *Dryobalanops oblongifolia* DREY and *Dryobalanops lanceolata* BURCK

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Abstract. Dryobalanops belong to a member of Dipterocarpaceae, which has a high economic value. Due to illegal harvesting for its wood, the population tends to decrease every year, so rapid conservation effort was needed. *D. oblongifolia* and *D. lanceolata* were important members of dryobalanops, which have listed as endangered (EN), according to IUCN. This study aims to provide information on the DNA isolation and amplification process. This data was needed as basic information for further research, such as genetic diversity, population genetics, phylogeny studies, or biogeography aspects. DNA isolation was performed using the CTAB method (*Cetyl Trimethyl Ammonium Bromide*). The amplification process was performed with three barcode markers those were *trnL-trnF*, *rbcL*, and *matK*. The results of DNA isolation showed quite good DNA yield. Amplified DNA using all primers showed successfully with an annealing temperature of 49°C for *matK*, 50°C for *trnL-trnF*, and 55°C for *rbcL*.

1. Introduction

Dryobalanops is one of the tree genera that produce non-timber products [1], which has high economic value. There are several species found in Indonesia: *Dryobalanops sumatrensis* and *Dryobalanops oblongifolia* in Sumatra Island, while *Dryobalanops lanceolata* and *Dryobalanops oblongifolia* found in Borneo Island. Currently, the presence of Dryobalanops continues to decrease in its habitat. The International Union for Conservation of Nature (IUCN) listed *D. oblongifolia* as the least concern conservation status [2], *D. lanceolata* is in the endangered category [3], and *D. sumatrensis* is in the vulnerable category [4]. This is due to several causes, including land conversion, illegal logging, and mass fruiting flowering character of Dryobalanops, which occurred every five years.

One of the non-wood products from Dryobalanops is an essential oil known as Borneol (C10H18O). In the industrial sector, pure borneol and isoborneol are used as raw material for making perfumes and esters. Borneol, in China, is also known as Bing Pian. One of its uses is as an additive to sanitary napkins (bio-panty), which is useful for reducing pain and pressure during menstruation, reducing muscle and joint pain, helping to clear blood clots, and preventing germs from multiplying [5, 6].

With such great benefits, it is necessary to make conservation efforts to maintain the existence of Dryobalanops. One of the conservation efforts that can be done is by using molecular markers. Genetic diversity research needs to be done. This is a fundamental requirement for the development of...
an appropriate conservation strategy and sustainable forest management. Research on DNA (Deoxyribonucleic Acid) isolation and molecular markers are needed for basic knowledge for proper conservation efforts.

A common method for DNA isolation is using CTAB (Cetyl Trimethyl Ammonium Bromide) buffer because it has the advantage that it can be used to isolate plant DNA which contains high polysaccharides and polyphenols, where polysaccharides and polyphenols in plant DNA are known as inhibitors or inhibitors in the PCR process [7]. The successful use of the CTAB method for tree species has been reported in *Eurycoma longifolia* Jack [5], *Styrax sumatrana* [8], *Taxus sumatrana* [9], *Shorea javanica* [10], and *D.aromatica* [11]. In the amplification process using PCR, a specific primer is needed to produce the targeted region's appropriate band. In this study, thenon-coding region*trnL-trnF* Intergenic Spacer, Ribulose Bisphosphate Carboxylase (*rbcL*) gene, and *Maturase K* (*matK*), which is the standard marker for barcoding, were used. This research aims to fill information gaps regarding the success of optimal DNA isolation using the CTAB method and reference markers for further molecular research.

2. Materials and methods

2.1. Plant material

The leaves *D. oblongifolia* obtained from Bukit Tiga Puluh National Park, and *D.lanceolata* was obtained from Kalimantan. For each population, three individuals were collected. So that the total of all samples for DNA isolation is six samples.

2.2. DNA isolation and DNA quality test

DNA isolation was carried out on all dried leaf samples previously prepared in silica gel plastic clip bags. The isolation method was carried out by the CTAB method, with several minor modification steps that have been carried out by [12].

The quality test of isolation DNA was carried out using agarose media and an electrophoresis machine. Making agarose uses 1% agarose, which is put into an Erlenmeyer tube, then heated in the microwave for 2.5 minutes until the solution is clear. After that, added Gel Red® or 0.5 µl of agarose dye. Agarose is printed in an electrophoresis mold container and cooled until it freezes. The components needed for DNA electrophoresis include *D. oblongifolia* and *D. lanceolata* DNA, DNA leader, and loading dye (Blue Juice). Then the loading dye (Blue Juice) is mixed and placed as a DNA dye on 1 µl of parafilm, and added Dryobalanops DNA as much as 3 µl. Then it is mixed and placed in an agarose palette (well). The DNA leader is placed in the agarose palette at the left end, provided that the location of the mold well is on the negative axis so that energy flows. Electrophoresis was carried out for 30 minutes with 100 Volts, and after that, agarose was visualized with a UV transilluminator machine to see the electrophoretic DNA results.

2.3. PCR amplification

PCR amplification was performed with a final volume of 16 µl, then the amplicon was seen in 2% agarose. Making agarose for PCR is the same as making agarose in DNA quality testing. The PCR composition of the product in one micro tube included 2 µl of DNA, 1 µl of forward primers, 1 µl of reverse primers, 45 µl of NFW, and 8 µl of Green GoTaq®. PCR products in one microtube are spin down until they are evenly mixed.

PCR was performed using a PCR PTC-100 Programmable Thermal Cycler machine. The samples were put into the PCR block and arranged in a balanced manner. The melting stage (denaturation) with a temperature of 94 °C for 30 seconds, the attachment stage (annealing) with a temperature of 55 °C for 30 seconds, and the elongation stage (elongation) with a temperature of 72 °C for 1 minute. DNA visualization is carried out by means of a UV transilluminator machine in the same way as the DNA quality test.
Table 1. Primers used to amplify the DNA of *D. oblongifolia* and *D. lanceolata*.

| Primary code   | Primary base arrangement       | expected sequence length | Reference |
|----------------|--------------------------------|--------------------------|-----------|
| *trn* L-c (forward) | TATTTGAACTGGTGACAGG            | 550 bp                   | [13]      |
| *trn* L-f (reverse)   | CGAAATCGGTAGCGCTAGG            | 550 bp                   | [13]      |
| *rbcLa*-F             | ATGTCACCACAAACAGAG             | 554 bp                   | [14]      |
| *rbcLa*-R             | GTAAAATCAAGTCCACCRCG           | 554 bp                   | [15]      |
| *MatK*-1RKIM-f        | ACCCAGTCCATCTGGGAAT            | 850 bp                   | [16]      |
| *MatK*-3FKIM-r        | CGTACAGTACTTTTGTGTTC           | 850 bp                   | [16]      |

3. Results and discussion

3.1. Isolation genomic DNA

The choice of DNA isolation method must be done quickly, efficiently, and minimize contamination potential. In this study, the CTAB method was used. This method was chosen because it has the advantage that it can be used in all types of plants and types of plants containing lots of chemical compounds such as polyphenols.

DNA isolation is carried out in 3 main steps, namely, destruction of cell walls (lysis), separation of DNA from solid materials such as cellulose and protein, and DNA purification [17]. Destruction of the cell wall (lysis) by grinding using an extraction buffer. The crushing process is carried out to damage plant cells or tissue and is followed by incubation at a temperature of 65-70 °C. Then, DNA is separated from other cellular components such as protein, RNA (*Ribonucleic Acid*), and fat. High-quality DNA is characterized by high molecular weight DNA, without contaminating substances, such as proteins, polysaccharides, phenolics, or other secondary metabolites. The results of DNA isolation using the CTAB method showed that this method gave better DNA results in terms of quality and quantity.
CTAB buffer as a substitute for liquid nitrogen for extraction can produce quality DNA products (lines 1, 4, 5, 6), as shown by the genomic DNA bands (Figure 1). Thus, the CTAB buffer can be used to isolate DNA in *D. oblongifolia* and *D. lanceolata* plants. A good quality DNA extraction product was indicated by a thick and clean DNA band when visualized using gel electrophoresis. However, there are several bands of thin electrophoresis results (lines 2, 3). Very thin bands or bands that look taily during visualization can be caused by the presence of impurities in the DNA. Chemical compounds such as phenols, tannins, and alkaloids are found in the leaves [18]. According to [19], Dryobalanops leaves contain several secondary metabolites: 35% terpenoid compounds, 10% alcohol, 20% sesquiterpenes, and 35% resin. These secondary metabolite compounds inhibit the process of DNA isolation and PCR reactions in several types of trees. The presence of substances in the leaves can also inhibit the process of the rate of chemical reactions or the presence of secondary metabolites that are not completely washed, resulting in contaminants.

3.2. Amplification of polymerase chain reaction
In this study, the isolation DNA was amplified by PCR using a specific primer pair from the non-coding region *trnL-trnF*. The *rbcL* gene, or *matK* as reported [20]. DNA quality of the samples tested (Figure 1) quite good, *trnL-trnF* primer with an annealing temperature of 50°C (line 3) amplified DNA bands at its territory, but the occurrence of a double band (line 1, 2, 4, 5, 6) amplified but not on its area, so it needs to be donemodification of the annealing temperature. The *rbcL* primer (line 12) showed quite good DNA bands but very thin (lines 8, 9, 10) DNA bands amplified on the region but occurred multi-band (lines 7 and 11) are amplified but not in the area. *MatK* primers with an annealing temperature of 49°C (lines 13, 14, 16, 17, 18) were amplified but not in the region (line 15). The DNA bands were amplified in the area but double band occurred. The DNA sample is declared successfully amplified if the electrophoresis analysis results show the presence of a single DNA band with a size according to a previously known marker.

A single DNA band will easily amplify. The DNA needed in the PCR process must have a high level of purity. However, in *trnL-trnF* primers the DNA band was thinner. From previous studies, *trnL-trnF* has successfully amplified the *Cinnamomum verum* species with an annealing temperature of 50°C [21], family Rutaceae [22], and the Moraceae family [23]. As with statements [24], DNA purity and concentration affect the amplified DNA band's intensity in each primer. The thin band resulting from DNA amplification is caused by compounds such as phenolics and polysaccharides in the DNA template. Another cause of visually invisible bands or very thin bands is that the DNA concentration is too low [25].

![Figure 2](image.png)

*Figure 2.* The results of electrophoresis by visualization of uv transiluminator from DNA optimization of *Dryobalanops oblongifolia* and *Dryobalanops Lanceolata.*
The rbcL primer with a temperature of 55°C and 56°C produced a double band amplified band (lines 8, 9, 10) (Figure 2). This can happen because the DNA concentration is less than optimal, and DNA is not clean. According to [26] also states that the number of bands produced by each primer is different. Meanwhile, according to [27] high DNA concentrations may still contain contaminants such as phenols and other secondary metabolites, which can interfere with the process of primary attachment to DNA. The process of annealing to complementary DNA becomes imperfect so that no DNA doubling is formed.

This research has been carried out in several temperatures, but the most optimal temperature is 55°C and 56°C. The temperature setting of the annealing phase in the PCR process greatly affects the primary adhesion process so that a one degree temperature change will cause the primer to fail to adhere [28]. According to [29-31], the orientation and number of complementary sequences in the plant genome to primers are directly related to the number of PCR amplification products.

4. Conclusions
The isolation of samples from D. oblongifolia and D. lanceolata using the CTAB method showed promising results. PCR applications using primers trnL-trnF, rbcL, matK for both species showed a slightly low result, so it is necessary to optimize the annealing temperature by modifying the material components for the PCR process.

References
[1] Purwaningsih 2004 Review: Ecological Distribution of Dipterocarpaceae Species in Indonesia. Biodiversity 5 2 pp 89-95.
[2] Barstow M 2018 Dryobalanops oblongifolia The IUCN Red List of Threatened Species.
[3] Ashton P 1998 Dryobalanops lanceolata The IUCN Red List of Threatened Species
[4] Barstow M, Randi 2018 Dryobalanops aromatica The IUCN Red List of Threatened Species
[5] Susilowati A, Rachmat HH, Rangkuti AB, Elfiati D and Ambarwati A 2018 Optimizing Genomic DNA Isolation and PCR Amplification For Pasak Bumi (Eurycoma longifolia) Proceeding International Conference on Basic Sciences and Its Application KnE Engineering pp 30–9
[6] Duke S 2005 Plants Containing Borneol Phytochemical and Ethnobotanical Databases (Portland: Institute for Traditional Medicine)
[7] uraki, Ahmad B, Magaji, UF, Abdulrazak UL, Yusuf BA and Hamza AB 2017 Optimized Cetyltrimethylammonium Bromide (CTAB) DNA Extraction Method of Plant Leaf With High Polysaccharide and Polyphenolic Compounds for Downstream Reliable Molecular Analysis Academic Journal 16 24 pp 1354-65
[8] Rachmat HH, Susilowati A, Elfiati D, Hartini KS and Faradillah WN 2017 Short Communication: Strong Genetic Differentiation of The Endemic Rosin-Producing Tree Styrax sumatrana (Styracaceae) in North Sumatra, Indonesia Biodiversity 18 4 1331-5
[9] Rachmat HH, Subiakto A and Kamiya K 2016 Short Communication: Genetic Diversity And Conservation Strategy Considerations For Highly Valuable Medicinal Tree of Taxus sumatrana in Indonesia Biodiversity 17 2 pp 487-91
[10] Rachmat HH, Kamiya K and Harada K 2012 Contrasting cpDNA Variation in Two Indonesian Endemic Lowland Dipterocarp Species and Amplification For Their Conservation Faculty of Agriculture, Ehime University 3-5-7 Tarumi, Matsuyama-shi 790-8566 Japan 15 16 pp 783-8
[11] Ginting IM 2018 Screening of Amplified DNA Regions for the Identification of Camphor (Dryobalanops Aromatica) (Indonesia: The University of Northern Sumatra Field)
[12] Aritonang KV, Siregar IZ and Yunanto T 2007 Manual of Forest Plant Genetic Analysis at the Silviculture Laboratory of the Faculty of Forestry (Bogor: Bogor Agricultural University)
[13] Hao DC, Huang BL, Chen SL and Mu J 2009 Evolution of The Chloroplast trnL-trnF Region in the Gymnosperm Lineages Taxaceae and Cephalotaxaceae Biochem Genet 47 pp 351-69
[14] Levin RA, Wagner WL and Hoch PC 2003 Family-level relationships of Onagraceae Based on Chloroplast rbcL and ndhF Data American Journal of Botany 90 pp 107-15 (modified from Soltis, P et al, 1992) Proceedings of the National Academy of Sciences USA 89 pp 449-51

[15] Kress WJ, Erickson DL, Jones FA, Swenson NG and Perez R 2009 Plant DNA Barcodes and a Community Phylogeny of a Tropical Forest Dynamics Plot in Panama Proceedings of The National Academy of Sciences 106 pp 18621–6

[16] Kuzmina ML, Johnson KL, Barron HR and Hebert PD 2012 Identification of Vascular Plants of Churchill, Manitoba, Using a DNA Barcode Library BMC ecology 12 25

[17] Nicholl DST 1993 An Introduction to Genetic Engineering Department of Biological Science (Paisley: The University of Paisley)

[18] Weising K, Nybom H, Wolff K and Kahl G 2005 DNA Fingerprinting in Plants: Principles, Methods, and Applications Second Edition (London: CRC Press)

[19] Burkill IH 1996A Dictionary of The Economic Products of Malay Peninsula (Kuala Lumpur: Ministry of Agriculture and Co-operatives)

[20] Kress WJ, Wurdack KJ and Zimmer EA 2005 Use of DNA Barcodes to Identify Flowering Plants PNAS 102 23 pp 8369-74

[21] Abeyesinghe PD, Wijesinghe KGG, Tachida H and Yoshda T 2009 Molecular Characterization of Cinnamon (Cinnamomum verum Presl) Accessions and Evaluation of Genetic Relatedness of Cinnamon Species in Sri Lanka Based on trnL Intron Region, Intergenic Spacers Between trnT-trnL, trnL -trnF, trnH -psbA and Nuclear ITS Research Journal of Agriculture and Biological Sciences 5 6 pp 1079-88

[22] Morton CM 2009 Phylogenetic Relationships of The Aurantioidae (Rutaceae) Based on The Nuclear Ribosomal DNA ITS Region and Three Noncoding Chloroplast DNA Regions, atpB-rbcL Spacer, rps16, and trnL-trnF Organisms Diversity and Evolution 9 pp 52–68

[23] Nepal1 MP and Ferguson CJ 2012 Phylogenetics of Morus (Moraceae) Inferred from ITS and trnL-trnF Sequence Data Systematic Botany 37 2 pp 442–50

[24] Purba YS and Martanti D 2008 Genetic Diversity Based on Random Amplified Polymorphic DNA Markers on Amorphophallus Mueller Blume in Java Biodiversity 9 4 pp 245-9

[25] Haris N, Aswidinoor H, Mathius NT and Purwantara A 2003 Genetic Similarities of Rubber Clones (Hevea brasiliensis Muell Arg) Based on Amplified Fragment Length Polymorphisms (AFLP)Method Plantation Tower 71 1 pp 1-15

[26] Hartati D 2006 Genetic Diversity of Sengon (Albizia falcataaria L Fosberg) Through DNA Markers at the Center for Plantation Forest Research and Development (P3HT) (Bogor: Essay Bogor Agricultural Institute)

[27] Joko T, Nanda K and Sedyo H 2011 Optimization of the PCR Method for the Detection of Pectobacterium caroto Vorum, the cause of orchid soft rot Indonesian Plant Protection 17 2 pp 54-9

[28] Aris M 2011 Identification, the pathogenicity of bacteria, and the 16srna gene's utilization for the detection of ice-ice in seaweed (Kappaphycus alvarezzii) cultivation (Bogor: Essay Bogor Agricultural Institute)

[29] Azrai M 2005 Utilization of Molecular Markers in the Selection Process of Plant Breeding AgroBiogen 1 1 pp 26-37