DNA isolation and amplification of raru leaf (*Cotylelobium melanoxylon* Pierre and *Cotylelobium lanceolatum* Craib)

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**Abstract.** *Cotylelobium lanceolatum* Craib and *Cotylelobium melanoxylon* Pierre or locally known as raru are trees used as ingredients for traditional Batak drinks called “tuak”. Destructive and uncontrolled harvesting of raru leads to a decline in its population and genetic diversity. Morphological distinctions for *C. melanoxylon* and *C. lanceolatum* have been conducted even though there are some weaknesses of this identification, thus molecular based marker are tools which is preferred for solved the problem. However, an appropriate DNA isolation method is basic and important step for molecular studies to obtain high-quality genomic DNA so that further analysis can proceed. Therefore, our study focused to obtain the successful DNA isolation method using the modified CTAB (*Cetyl Trimethyl Ammonium Bromide*) method and its amplification on three candidate of DNA barcodes i.e. *trnL-trnF*, *trnH-psbA*, ITS1-ITS4. In total six genomic DNA were isolated from the silica-gel dried leaf of the two raru species and amplified with *trnL-trnF*, *trnH-psbA*, and ITS1-ITS4 primer-pairs. The results showed that the modified CTAB method proved to be successful and applicable for the extraction of DNA of *C. melanoxylon* Pierre and *C. lanceolatum* Craib. Furthermore, the *trnL-trnF* primer-pair was successfully amplified compared with *trnH-psbA* and ITS1-ITS4 indicating that the primer-pair can be used as a DNA barcode candidate for further DNA sequencing and analysis.

1. Introduction

*Cotylelobium melanoxylon* Pierre and *Cotylelobium lanceolatum* Craib (raru) are tropical trees that belong to the member of the Dipterocarpaceae family [1,2] and grows scattered on Sumatera and Borneo Island [3]. In North Sumatera, the raru bark is commonly used by Bataknese as an ingredient in their traditional alcoholic drinks called “tuak” to increase the rate of fermentation and to improve tuak flavour[4,5]. Moreover, raru leaves and bark are also used by local people for traditional medicine to cure various diseases such as diabetes, diarrhea, malaria, and fever. According to [6] and [7], raru bark contain isoampelopsin F, ε-viniferin, vaticanol A, E, G, ampelopsin F and lyoniresinol,
which prospective for anti-diabetic drugs. In addition, wood from these trees is widely used for building materials, ships, bridges, and railroads.

Destructive harvesting of raru in wild forests and natural regeneration problems sharply reduce the population of these species [8], which then causes the C. melanoxylon to be categorized as the Least Concern (LC) species [2] and C. lanceolatum to be Vulnerable (VU) species [1] according to IUCN Red List of Threatened Species. In addition, disturbed raru habitat and populations will increase selfing rates and genetic drift in all individuals [9]. Therefore, conservation efforts of these species, such as conservation through molecular approach, need to be carried out to avoid the species from extinction. Information on DNA isolation methods, the development of DNA markers, and population genetics analysis in raru species is still very limited, thus, the information was needed for further molecular analysis [10].

DNA isolation on herbaceous and tree species are rather difficult due to the presence of several impurities compound [10] such as phenols, tannins, and polysaccharides [11], which inhibit DNA polymerase activities during the PCR process. Therefore, these compounds should be eliminated in the process of isolation. The DNA yield from the isolation process also varies among species of the same genera and among different tissue of the same tree [12]. Generally, isolation of DNA from microbes, plants, and animals still uses the CTAB (Cetyl Trimethyl Ammonium Bromide) method even though several other isolation methods have also been developed by many researchers. The success of DNA isolation using the CTAB method for higher plants has been reported by [13–15], but there are no reports for raru. Therefore, this present study focused on genomics DNA produced from the DNA isolation process using CTAB method, and amplification patterns using three candidate of DNA barcodes i.e. trnL-trnF, trnH-psbA, and ITS1-ITS4.

2. Methods

2.1. Plant materials

Fresh leaves of Cotylelobium lanceolatum were collected from 3 seedlings that grew naturally on Natuna Island, Riau Archipelago, Indonesia and fresh leaves of Cotylelobium melanoxylon seedlings were collected from 3 seedlings that grew naturally on Lingga Island, Riau Archipelago, Indonesia. A total of sixth-leaf samples from the two species were stored in ziplock plastic bags containing silica gel. The samples then kept at room temperature until DNA extraction was carried out.

2.2. DNA isolation

Genomic DNA from the dried leaf samples of Cotylelobium lanceolatum and C. melanoxylon were isolated using the modified CTAB method adopted from [16] and [17]. The 4-10 pieces of 1 cm x 1 cm dried leaf from each individual were grinded into a fine powder using mortar and pestle, after that the powder transfered into 1.5 ml microcentrifuge tube. In microcentrifuge tube, the 700 µl of 2% CTAB extraction buffer and 100 µl of polyvinyl-pyrrolidone (PVP) were added and then mixed using vortex for 1-2 min a while until well mixed. The tube, then incubated in 65 °C water bath for 45 minutes. Mix the samples every 15 min gently by inverting the tube, then cool down to the room temperature for 15 minutes. After incubation process the chloroform:isoamyl alcohol in equal volume of (24:1 v/v) and 10 µl of phenol were added an then mix by slight inversion for 5 m. The tube then treated for 10 min in Centrifuge at 13,000 rpm in room temperature (RT). After that, transfer the upper aqueous phase contains the DNA carefully to a new 1.5 ml microcentrifuge tube using a wide bore pipet and added NaCl 500 µl and cold isopropanol alcohol 300 µl then invert gently to precipitate DNA until the formation of DNA threads. Samples then incubated for 1 hour in temperature -20 °C. After that, the samples then centrifuged for 10 min at room temperature (RT) using 13,000 rpm, then discard supernatant carefully.
The DNA pellet then washed with 300 μl of 70% ethanol to increase DNA purity. The pellet then centrifuged for 10 min at room temperature (RT) at 13,000 rpm. The 70% alcohol was discharged from tubes and the tube then inverted for 15 min on desiccator. DNA pellet then re-suspend in 50 μl of TE buffer. The pellet was centrifuged for 2 min at 13,000 rpm in room temperature (RT), pellet then stored till further use at -20 °C.

2.3. Evaluation of DNA isolation
The DNA quantification was performed by applying a mix of 3 μL of DNA and 1 μL of DNA loading dye per sample in each well of 1.5% agarose gel. The agarose was stained with GelRed® Nucleic Acid Stain 10000X Water (Biotium). Horizontal electrophoresis was run for 30 minutes at 100 V using 1X TAE Buffer. Gel visualization was observed on a UV Transilluminator and photographed using Canon EOS 1000D camera.

2.4. DNA amplification
DNA was amplified with two cpDNA universal primer-pairs i.e. trnL-trnF, and trnH-psbA (trnHf05 and psbA3f primers), and one nuclear DNA universal primer pair i.e ITS1-ITS4 (Table 1) by Polymerase Chain Reaction (PCR). Amplification was processed in PCR machine by using 20 μl solution containing 10 ng genomic DNA, each forward and reverse primers 5 pmol, and Go Taq 10 μl of ® Hot Start Colourless Master Mix (Promega, Wisconsin, USA) according to instructions of the manufacturer’s. The Bio-Rad MJ Research PTC-100 Thermal Cycler was used for the PCR amplification under the following conditions: 95 °C for 2 min of initial denaturation, followed by denaturation at 95 °C for 1 min using 30-35 cycles, annealing for 30 s and extension at 72 °C for 2 min, and at 72 °C for 7 min in final extension. Annealing temperature for each primer-pair presented in table 1. Amplified products were electrophoresed and then visualized in a 2.0% agarose gel stained with GelRed® Nucleic Acid Stain 10000X Water (Biotium) using UV Transilluminator.

Table 1. The Sequence and annealing information of 2 cpDNA and 1 nuclear universal primer-pair combinations used in Cotylelobium lanceolatum Craib and Cotylelobium melanoxylon Pierre

| Region | Primer Sequance (5’-3’) | Size (bp) | Annealing (°C) | Reference |
|--------|-------------------------|-----------|----------------|-----------|
| trnL-trnF | F: ATTTGAACCTGAGACGACGAG | 550 | 50 | [18] |
| R: GAAATCGGTAGACGCTACG | 550 | 50 | [18] |
| trnH-psbA | F: CGCGCATGGTGGATTCACAATCC | 450 | 54 | [19] |
| R: GTTATGCATGAACGTAATGC | 450 | 54 | [20] |
| ITS1-ITS4 | F: TCCGTAGGGAACCTCGCG | 290 | 52 | [21] |
| R: TCCTCCGCTTATTGATATGC | 290 | 52 | [21] |

F: Forward, R: Reverse

3. Results and Discussion

3.1. DNA isolation
The perennial plants genomic DNA isolation is more difficult due to the presence of polysaccharides and polyphenols [12]. According to [3], Cotylelobium lanceolatum and C. melanoxylon leaves contain some secondary metabolites such as flavonoids, tannins, saponins, triterpenoids, and hydroquinone. The secondary metabolites contained in DNA isolation samples will interfere with some biological enzyme activities such as a polymerase, ligase, and endonuclease restriction [11]. During isolation process, the polysaccharides and nucleic acids are found to form a gelatinous mass, thereby become physically inhibiting for DNA action for modifying enzymes e.g. DNA polymerase, restriction enzymes, ligase, etc. [22]. Besides disturbing some enzyme activities, the content of secondary metabolites also causes a brownish color to DNA [23].
The addition of antioxidants such as polyvinyl-pyrrolidone (PVP) and β-mercaptoethanol into the extraction buffer in CTAB buffer can help to eliminate phenolics in plant DNA[10]. Apart from these difficulties, in general, the isolation of DNA using the modified CTAB method in the present study was considered successful with sufficient amounts of DNA indicated by a clear band even though the visualization of electrophoresis results in agarose gels were not apparent (figure 1). This result indicated that CTAB method was applicable for the extraction of DNA with a good yield from C. lanceolatum and C. melanoxylon leaves.

![Image](image_url)

**Figure 1.** Quality of DNA isolated from Cotylelobium lanceolatum Craib (1-3) and C. melanoxylon Pierre (4-6) dried leaf using a modified CTAB method.

### 3.2. PCR amplification

The success of PCR amplification can be shown from a clear single band resulted from the electrophoresis process when visualized in agarose gel because good quality DNA is characterized by high molecular weight without being contaminated with inhibitors such as proteins, polysaccharides, phenolics, or other secondary metabolites. According to [24], also reported that thick and non-multi DNA bands see in agarose gel show high DNA concentrations and the total DNA extracted in intact condition. In this study, of the three primer pairs (trnL-trnF, trnH-psbA, ITS1-ITS4) used to amplify DNA Cotylelobium lanceolatum and C. melanoxylon, only one primer (trnL-trnF) was successfully amplified indicated by a single band when the PCR product was visualized in agarose gel (figure 2) suggested that the primer-pair can be used as DNA barcode candidate for further DNA sequencing. The successful of trnL-trnF primer amplification has also been reported for several tree species such as Cinnamomum verum [25], Rutaceae family [26], Moraceae [27], Styrax sumatrana, and Dryobalanops sumatrensis [10].

Meanwhile, the primer-pair of trnH-psbA and ITS1-ITS4 failed to amplify the DNA of C. melanoxylon and C. lanceolatum, which indicated by the appearance of non-single band and the band position was below 50 bp, as [30] reported that the failure of PCR amplification can lead to many non-specific DNA products of varying sizes that appear as a smear of bands or no products form at all on agarose gels. This result suggested that these two primer-pairs are not recommended for use in further analysis. The failure to PCR amplification can be caused by several factors such as inhibitor substances found in the sample, primer length, annealing temperature, genome size, polysaccharides, and other substances in DNA that become inhibitors in the PCR process [10].
In the case of *C. melanoxylon* and *C. lanceolatum*, the primer-pair of *trn*H-*psb*A and ITS1-ITS4 failed to amplified perhaps because of (i) the presence of polyphenols content in the leaf samples that inhibited amplification during the PCR process, as [29] reported that polyphenol compounds can interfere with the work of several enzymes such as the enzyme ligase, polymerase, and endonuclease, and (ii) the annealing temperatures used in this study were not suitable for *trn*H-*psb*A and ITS1-ITS4. The too-high annealing temperature will cause the primer cannot bind properly to the template DNA and the too low annealing temperature will result in more bindings, more bands, and non-specific amplification during the PCR reaction. Therefore, annealing temperature optimization is necessary for further studies. In addition, ITS primers used in this study are indeed more often used in the fungal group because ITS is more selective in producing good DNA products in the fungal groups [28].

**Figure 2.** Electrophoresis results and UV transilluminator visualization of PCR product amplified by *trn*L-*trn*F, *trn*H-*psb*A, and ITS1-ITS4. PCR amplification of *trn*L-*trn*F in *Cotylelobium lanceolatum* (1-3) and *Cotylelobium melanoxylon* (4-6), *trn*H-*psb*A in *Cotylelobium lanceolatum* (7-9) and *Cotylelobium melanoxylon* (10-12), and ITS1-ITS4 in *Cotylelobium lanceolatum* (13-15) and *Cotylelobium melanoxylon* (16-18).

### 4. Conclusions

DNA isolation using the modified CTAB method was considered successful with sufficient amounts of DNA suggesting that the method was applicable for DNA extraction with a good yield from *Cotylelobium lanceolatum* Craib and *C. melanoxylon* Pierre silica-gel dried leaves. Furthermore, PCR amplification using two cpDNA universal primer-pairs (*trn*L-*trn*F and *trn*H-*psb*A) and one nuclear DNA universal primer pair (ITS1-ITS4) indicated that only *trn*L-*trn*F primer-pair was successfully amplified compared with *trn*H-*psb*A and ITS1-ITS4 suggesting that the primer-pair can be used as a DNA barcode candidate for further DNA sequencing and analysis.

### References

[1] Ly V, Nanthavong K, Pooma R, Luu HT, Khou E, Newman MF 2017 *Cotylelobium lanceolatum*. The IUCN Red List of Threatened Species: e.T33069A2832191
https://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T33069A2832191.en

[2] Barstow M 2019 *Cotylelobium melanoxylon* The IUCN Red List of Threatened Species 2019:e.T33070A68069829 DOI:10.2305/IUCN.UK.2019-1.RLTS.T33070A68069829.en.

[3] Pasaribu G, Bonifasius S, Gustan P 2007 *Jurnal Penelitian Hasil Hutan* 25 (4): 327-333.

[4] Heyne K. 1987. *Tumbuhan berguna Indonesia* Jakarta: Badan Litbang Kehutanan, Departemen Kehutanan. Jakarta [Indonesian]
[5] Susilowati A, Iswanto AH, Kusuma YS, Rachmat HH, Elfiati D, Larekeng SH, Ginting IM, Rangkuti AB 2020 Proc. Int. Conf on Agriculture, Environment and Food Security (Medan) vol 454 (IOP Conf. Series: Earth and Environmental Science) p 1-8

[6] Soerianegara, Lemmens RHMJ 1994 Plant Resources of South-East Asia 5 Timber Trees Major Commercial Timbers Bogor: Prosea

[7] Matsuda H, Asao Y, Nakamura S, Hamao M, Sugimoto S, Hongo M, Yoshikawa M 2009 Chem Pharm Bull 57 (5): 487-494.

[8] Susilowati A, Hartini KS, Elfiati D, Rachmat HH, Kusuma YS, Sinaga MZE, Suhartati T 2020 Biodiversitas 21(2): 724-730

[9] Susilowati A, Rachmat H Henti, Elfiati D, Kholibrina R Cut, Kusuma S Yosie and Siregar H 2019 Biodiversitas 20: 1681-87.

[10] Susilowati A, Rachmat HH, Rangkuti AB, Elfiati D, Ginting IM 2018 Proc. The 8th International Symposium for Sustainable Humanosphere (Medan) vol 374 (IOP Conf. Series: Earth and Environmental Science ) p1-6

[11] Moyo M, Amoo SO, Bairu MW, Finnie JF, Van Staden J 2008 South African Journal of Botany 74:771–775.

[12] Cheng FS, Brown SK, Weeden NF 1997 Hort Science 32: 921–922.

[13] Porebski S, Bailey L, Baum B 1997 Plant Mol. Biol. Rep 15(1):8-15

[14] Mace ES, Buhariwalla HK, Crouch JH 2003 Plant Mol. Biol. Rep 21(4):459-460

[15] An Z, Huang H 2005 A Plant Physiol. Commun 41(4):513-515

[16] Weising K, Nybon H, Wolff K, Kahl G. 2005. DNA Fingerprinting in Plants: Principles, Methods, and Applications. 2nd ed Florida: Taylor & Francis

[17] Aritonang KV, Siregar IZ, Yunanto T. 2007. Manual Analisis Genetik Tanaman Hutan di Laboratorium Silvikultur Bogor

[18] Taberlet P, Gielly L, Pautou G, Bouvet J. 1991 Plant Molecular Biology 17:1105-1109.

[19] Sang T Crawford DJ Stuessy TF 1997 American Journal of Botany. 84: 1120–1136.

[20] Tate JA, Simpson BB 2003 Systematic Botany 28: 723–737

[21] White TJ, Bruns TD, Lee SB, Taylor JW 1990 Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics In. PCR Protocol: A Guide Methods and Application California: Academic Press Inc

[22] Sharma AD, Kaur P, Prabhjeet S 2002 Plant molecular biology reporter 20 (4) 415-415

[23] Khanuja SPS, Shasany AK, Darokar MP and Kumar S 1999 Plant Molecular Biology Reporter 17(1) p. 1-7.

[24] Irmawati. 2003. Perubahan Keragaman Genetik Ikan Kerapu Tikus Generasi Pertama Pada Stok Hatchery Thesis Bogor: Institut Pertanian Bogor [Indonesian].

[25] Abeyesinghe PD, Wijesinghe KGG, Tachida H, dan Yoshida T 2009 Research Journal of Agriculture and Biological Sciences 5(6):1079-1088.

[26] Morton CM 2009 Organisms Diversity and Evolution 9:52–68.

[27] Nepal MP and Ferguson CJ. 2012. Systematic Botany 37(2): 442–450.

[28] Suparman 2011 Analisis filogenetik Genus Mangifera Menggunakan Gen rbcL DNA Kloroplas Tesis Bandung: SITH-ITB

[29] Arif IA, Bakir MA, Khan HA, Ahmed A, Al Farhan AH, Al Homaidan AA, Al Sadoon M, Bahkali AH, Shobrak M 2010. Int J Mol Sci. 11: 3149-3157

[30] Lorenz TC 2012 Journal of Visualized Experiments 63 3998