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DNA EXTRACTION FROM LOCAL SOUVENIR OF SUKU ANAK DALAM JAMBI WITHOUT LIQUID NITROGEN USING CTAB METHOD

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Abstract

The Anak Dalam Tribe (SAD) of Jambi Province, Indonesia, relies on forest resources for sustenance, with sebalik sumpah seed accessories representing their cultural heritage. However, modernization threatens this indigenous knowledge and the sebalik sumpah tree's existence. Genetic conservation is vital for preserving biodiversity, necessitating efficient DNA extraction methods. This study addresses challenges in DNA extraction from processed sebalik sumpah seeds. Adopting the CTAB method without liquid nitrogen, we optimized DNA precipitation incubation times to enhance yield and purity. The modified DNA extraction method from the sebalik sumpah seed coat is qualitatively assessed based on the gel photo resulting from the PCR product electrophoresis. The results show that the DNA extraction method using CTAB buffer without liquid nitrogen and with modified incubation time during precipitation can extract DNA from processed materials such as the sebalik sumpah seed coat. Substituting liquid nitrogen with silica sand and PVP and a 4-day incubation period is the most optimal modification. These findings underscore the importance of optimized extraction for processed materials, crucial for genetic conservation efforts and preserving indigenous heritage.

Keywords; *CTAB, conservation, DNA Extraction, SAD, sebalik sumpah*

Introduction

The Anak Dalam Tribe (SAD) is a traditional tribe in the province of Jambi, Indonesia, who live in the forests and rely on forest products, including non-timber forest products (NTFPs), to meet their daily needs (Mustakim et al., 2023; Susanti et al., 2020; Ulfa & Mitaria, 2019). One of the well-known local souvenirs of the SAD is accessories made from the skin of the seed of the sebalik sumpah tree. Although initially only utilized by the SAD community in Jambi, these accessories are now distinctive souvenirs from the Muaro Jambi Temple area (Susanti et al., 2020). These accessories serve as unique adornments worn in the daily lives of SAD members and depict their local wisdom and close relationship with nature. The sebalik sumpah souvenir is made from the dried skin of the seed of the sebalik sumpah tree (Mustakim et al., 2023). These seeds are produced by the sebalik sumpah tree, which bears fruit 30 years after planting with seeds. However, this local wisdom is threatened by the increasingly dominant wave of modernization, which could lead to the loss of their traditional knowledge and cultural values (Trisnawati et al., 2021). On the other hand, the sebalik sumpah tree is an essential part of the identity and natural heritage of the SAD. The sebalik sumpah tree grows

in the Bukit Duabelas National Park (TNBD), and the Bukit Tigapuluh National Park (TNBT), but its existence is currently quite challenging to find (Susanti et al., 2020). The lack of inherited local wisdom has led to few people in the surrounding forest areas recognizing and planting sebalik sumpah trees (Trisnawati et al., 2021), making conservation efforts for these trees urgent to prevent their extinction. One of the efforts to conserve these trees is through genetic conservation.

Genetic conservation is an effort to maintain genetic diversity within specific species populations, to preserve the survival of those species, and to prevent the loss of genetic variations crucial for adaptation to environmental changes, disease resistance, and population recovery after disturbances or extinction events (Romesh & Bhagirath, 2023; Turhadi & Hakim, 2023). The initial step in genetic conservation activities involves obtaining the species' genome DNA, which is done through DNA extraction. DNA extraction consists of retrieving DNA from biological cells or tissues and separating it from other components such as proteins, lipids, and polysaccharides (Emilia & Anhar, 2021). The aim is to purify the DNA for further molecular analysis (Gupta, 2019). Successfully extracted DNA can be utilized for molecular analyses such as Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), and DNA sequencing. These analyses provide deep insights into the genetic structure and functionality of the studied plant species (Nalini et al., 2003; Buchori et al., 2023). Selecting the appropriate DNA extraction method is a crucial initial step in determining subsequent molecular activities' success (Ramlah et al., 2022; Sari & Restanto, 2022). Therefore, choosing an extraction method that aligns with the characteristics of the species under study and considers the analytical needs is essential (Retnaningati, 2020).

This research addresses the challenges of DNA extraction from processed plant materials, such as the sebalik sumpah seeds, commonly used as souvenirs. Standard and straightforward DNA extraction methods for plant leaves, shoots, roots, and other living tissues have been widely used (Rachmayanti, 2009; Azyenela et al., 2023). However, DNA extraction from processed plant materials still presents significant challenges. Common issues encountered in DNA extraction from processed materials include DNA structure damage due to processing, contamination by chemical compounds or microbes, use of preservatives, physical tissue damage, as well as the presence of polysaccharides and other interfering components (Lear et al., 2018; Rohland et al., 2018; Dra´bkova, 2021). One of the main challenges in conventional extraction methods is using liquid nitrogen to disrupt plant cell walls (Sharma et al., 2010). Handling liquid nitrogen requires careful procedures and can sometimes be difficult to access, especially in remote areas. Moreover, the cost of liquid nitrogen can also be a limiting factor for large-scale DNA extraction.

Research conducted by Jankowiak et al. (2005), Andreasen et al. (2009), Höpke et al. (2018), and Marinček et al. (2022) reported successful DNA extraction from herbarium specimens using the Cetyl Trimethyl Ammonium Bromide (CTAB) method. Based on this literature, this study modified the extraction method using CTAB buffer without dependence on liquid nitrogen. We also optimized the incubation time during the DNA precipitation stage, as the appropriate incubation time can enhance the yield and purity of the extracted DNA (Langga et al., 2012). This research is expected to create a more practical, economical, and effective DNA extraction approach, particularly in processed materials.

METHOD

The research was conducted in the molecular laboratory at the Bogor Botanical Gardens (KRB) scientific conservation area from November until December 2023. The materials used in DNA extraction process were sebalik sumpah bracelet souvenirs originating from the Muaro Jambi Temple area (Figure 1), Cetyl Trimethyl Ammonium Bromide (CTAB) buffer, polyvinylpyrrolidone (PVP), chloroform, isoamyl, cold isopropanol, washing buffer (76%

ethanol, 10 mM ammonium acetate), Tris-EDTA (TE) buffer, and silica sand. The materials used in the amplification process using Polymerase Chain Reaction (PCR) were ddH2O, DNA primer ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL), My TaqTM Master Mix 2X (Bioline), and DNA sample. The visualization of PCR product used Agarose, Tris-Acetate-EDTA (TAE) buffer 1X, Gelred, DNA Ladder 100 bp.

Figure 1. sebalik sumpah bracelet souvenirs: the materials used in the study.

The equipment used included a mortar and pestle; 1000 µl and 200 µl pipette tips; 2 ml and 1.5 ml microtubes; 1000 µl and 100 µl micropipettes (PhysioCare); microtube rack; VWR vortex; Gyrozen centrifuge, VWR incubator, VWR digital heat block, EMS-Myfuge spin down, electrophoresis Mupid EXU Chamber, UV transilluminator Bio-Rad Gel DocTM EZ Imager, analytical balance Precisa, Erlenmeyer flask, plastic wrap, microwave, spatula, agarose gel mold, and Takara PCR Thermal Cycler Dice.

DNA Extraction

DNA extraction was conducted using the modified CTAB method Doyle & Doyle (1987) developed. DNA extraction involves four stages: cell lysis or disruption of cell walls and membranes, separation of DNA from other particles, precipitation or deposition of DNA, and DNA washing for purification (Dairawan & Shetty, 2020). Modifications were implemented in the physical extraction stage, and the incubation time was optimized during the DNA precipitation stage. Physical extraction of the sample (sample grinding) was performed without liquid nitrogen and with the addition of PVP. The sample used is plant material processed into bracelets made from the seed of the sebalik sumpah tree. The seed coat is reduced in size using branch scissors to facilitate crushing. The finely ground sample, 100 mg, is ground using a mortar and pestle. Liquid nitrogen, commonly used in sample grinding, is replaced with silica sand and PVP at 1 spatula. The ground sample is then transferred to a 2 ml microtube with a volume of 0.5 ml, followed by adding 1 ml CTAB extraction buffer solution. The mixture is then vortexed for 10-20 seconds to ensure no clumps are formed. Subsequently, the mixture is incubated for 20 minutes in a heat block at a temperature of 60°C with inversion every 5 minutes.

In this procedure, 500 µl of chloroform and isoamyl (in a ratio of 24:1) were added, mixed by inverting several times, and centrifuged for 15 minutes at 7,200 rpm. The supernatant was transferred to a new 1.5 ml microtube, and then 500 µL of cold isopropanol was added and inverted several times. The sample was incubated at -20°C according to the method used by Barbier et al. (2019), with modifications to the incubation time for 1, 4, 7, and 30 days. This modification aims to enhance the yield and purity of the extracted DNA. After incubation, the sample was centrifuged for 10 minutes at 12,000 rpm at 25°C. The supernatant was discarded, and the sample was dried at room temperature. After drying, 1 ml of washing buffer was added and centrifuged for 10 minutes at 12,000 rpm at 25°C. The supernatant was discarded, and the sample was dried again. Then, 100 µl of TE buffer was added, then spun down for 10-20 seconds.

PCR Amplification

The extracted DNA is then amplified via PCR to duplicate the DNA sample and produce visible DNA bands upon visualization. Since the samples are processed materials, the chance of obtaining pure DNA without amplification is meager. Therefore, visualization before amplification is not performed to conserve materials and streamline time. The primers used for amplification are rbcL primers, chosen in hopes of efficiently amplifying the DNA sample. The rbcL primers have a high success rate in the amplification stage (Basith, 2015; Syahreza et al., 2023). The reagents required for PCR are prepared as a PCR master mix, which aims to homogenize all reagents used in each sample. The total volume of PCR used to check whether the DNA sample was successfully amplified is 6 µl per tube. The composition of the ingredients includes 0.5 µl each of reverse and forward rbcL primers, 1 µl ddH20, 3 µl my taq, and 1 µl DNA template. The tubes are then centrifuged until homogenized and loaded into a Thermal Cycler machine. The amplified DNA regions are mentioned in Table 1.

Table 1. DNA region of rbcL for PCR amplification

Primer	Nucleotide
*rbcL*aF	5-ATGTCACCACAAACAGAGACTAAAGC-3'
*rbcL*aR	5-GTAAAATCAAGTCCACCRCG-3'

Kress et al., 2009

DNA Visualization

Visualization of PCR products is conducted through the process of electrophoresis on a 1% agarose gel. The gel is prepared by weighing 0.6 grams of agarose and 60 ml of 1x TAE, which are then added to an Erlenmeyer flask. The flask is covered with plastic wrap and heated for 2 minutes (with homogenization every minute). Before heating, the plastic wrap is punctured using a tip. After heating, the agarose gel solution is supplemented with 1 µl of gel red and homogenized. Subsequently, the gel solution is poured into a mold and left to solidify. Once solidified, the gel is placed into an electrophoresis apparatus containing a buffer solution. PCR DNA samples, 5 µl each, are loaded into the gel wells. As a reference, a 1 kb DNA ladder marker is loaded into the first well. Electrophoresis is run at 100 volts for 30 minutes. Visualization of the resulting bands is performed using a GelDoc UV Transilluminator.

RESULTS AND DISCUSSION

The extraction process is crucial for DNA bands' purity and successful visualization. The quality of DNA extraction is considered poor if impurities (proteins) are not fully degraded during extraction, leading to their mixture with the target DNA and the presence of protein contamination originating from non-lysed cell components or the phenol solution added during extraction (for DNA precipitation) (Bellard et al., 1973). In this study, optimization of DNA extraction from the sebalik sumpah seed coat was performed using the CTAB method. The grinding process of the sebalik sumpah seed coat sample does not involve liquid nitrogen but substitutes it with silica sand and PVP. Restu et al. (2012) stated that CTAB buffer successfully extracted DNA from plants containing high phenol and carbohydrate levels without damaging the DNA (Sari & Restanto, 2022). The sebalik sumpah seed plant is woody and rich in polysaccharides and polyphenols (Couz & Fritz, 1990). The addition of CTAB buffer helps to remove polysaccharides that can become contaminants from plant cells (Fang et al., 1992; Sari & Restanto, 2022), while PVP helps to reduce discolouration due to high phenol content (Porebski et al., 1997; Surzycki, 2000). The incubation time during the DNA precipitation step is modified to improve the success of DNA extraction from processed samples. Incubation was performed at -20°C for 1, 4, 7, and 30 days. The aim was to determine the optimal DNA sedimentation time to enhance the yield and purity of the extracted DNA.

Since the samples used have been processed, the likelihood of obtaining pure DNA without amplification is very low. Therefore, DNA amplification bands in PCR products are observed to assess the success of the DNA extraction process in this study. The amplification of the sebalik sumpah seed coat DNA extracted is done using PCR with a thermocycler machine. The basic principle of this device is to amplify target DNA fragments with the assistance of oligonucleotide primers (Zein & Prawiradilaga, 2013). The primer pair used for amplifying the extracted DNA in this study is the universal DNA Barcoding primer for plants, namely rbcL. The rbcL primer is known to have a high success rate in amplifying plant DNA (Basith, 2015; Syahreza et al., 2023). The PCR reaction conditions for the rbcL primer are presented in Table 2.

Table 2. PCR reaction conditions for the rbcL primer

Stage	Temperature (°C)	Time (second)	Cycle
Initial denaturation	94	300	1
Denaturation	94	15	
Annealing	52	15	35
Extension	72	20	
Post Extension	72	300	1

The modified DNA extraction method from the sebalik sumpah seed coat is qualitatively assessed based on the gel photo resulting from the PCR product electrophoresis. Through electrophoresis of the PCR products, it can be observed whether the DNA isolated from the sebalik sumpah seed coat sample was successfully amplified, as well as the purity level of the resulting DNA. Evaluation of DNA purity can also be performed using ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA), where DNA is considered pure if the A260/280 nm ratio ranges between 1.8 – 2.0 (Vesty et al., 2017; Yelli et al., 2022). The electrophoresis process uses 1% agarose gel due to its ease of application (Nugroho et al., 2019). Agarose gel can separate DNA fragments of various sizes, ranging from several hundred to 20,000 base pairs (Nugraha et al., 2014). Ardiana (2009) also stated that high-quality amplification results will appear as thick and clean bands. Visualization of PCR products at various incubation times is presented in Figure 2.

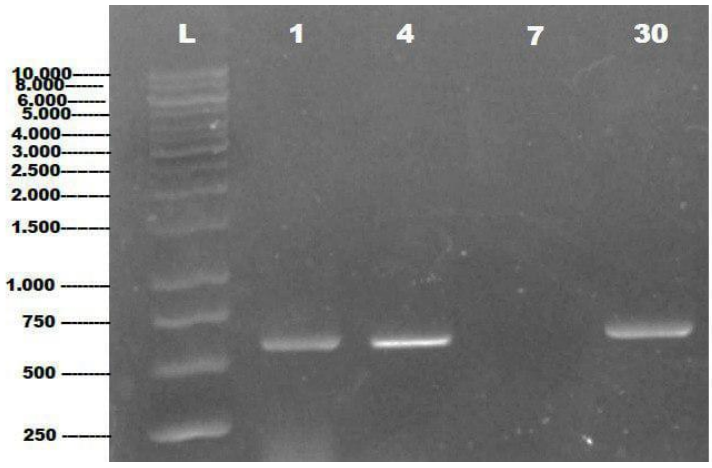

Figure 2. Visualization of sebalik sumpah PCR products at various incubation times.

Based on the visualization results of PCR products through electrophoresis (see Figure 2), it is shown that DNA bands are only observed at incubation times of 1, 4, and 30 days. In comparison, no DNA bands are produced at 7 days of incubation. The observed DNA bands appear clear and singular, indicating the absence of other compounds from the

extraction process. The observed DNA fragment length is approximately 600 bp, consistent with the range of rbcL gene fragment sizes ranging from 500-1400 bp (Sagala & Sogandi, 2022). Visually, the DNA bands at 4 days of incubation appear thicker than those at 1 and 30 days. The thickness of the PCR-generated DNA bands observed in this agarose gel electrophoresis may be influenced by the initial DNA concentration used as the DNA template (Roslim & Fitrianti, 2021).

Additionally, PCR results can also be affected by the PCR process itself. Suppose the PCR product contains much nonspecific DNA and exhibits size variations, resulting in DNA bands appearing as smears on the agarose gel. In that case, PCR disruptions may lead to DNA band formation failure (Lorenz, 2012; Ediwirman & Mansya, 2009; Sadikin et al., 2021). The success of DNA amplification indicates that the DNA extraction process has been successful. The success of the DNA extraction means that DNA extraction is highly likely to be achieved even in samples that have changed shape or in processed materials, as demonstrated by Höpke et al.'s (2019) study, which successfully extracted DNA from herbarium specimens aged 19-69 years using CTAB buffer. Additionally, Marinček et al.'s (2022) study stated that the age of the herbarium does not affect the DNA results obtained, indicating that older herbarium specimens can yield DNA with the same or better results than younger ones.

DNA samples with good quantity and quality are required for further molecular research. Therefore, the procedures and materials used for DNA isolation need to be optimized to not only produce quality products but also be more economical (Yelli et al., 2022) and in this study, the most optimal incubation time occurred during a 4-day incubation, as indicated by the high intensity of the observed DNA bands. The DNA bands generated during incubation for 1, 4, and 30 days showed no smearing, indicating that these bands have good quality. Less or no smearing can indicate better DNA quality (Qubais, 2015). Good DNA quality can be determined based on thick and well-centered DNA bands, according to Bagaskara et al. (2018). According to Roslim & Fitrianti (2021), the thickness and thinness of DNA bands indicate DNA concentration, whereas thicker DNA bands indicate higher DNA concentration, and vice versa.

In the context of this research, the success of DNA amplification is influenced by the incubation time and several other factors. For example, environmental conditions such as temperature and laboratory humidity can affect the stability of reagents and the accuracy of amplification results (Bailey et al., 2008). Furthermore, the results obtained from the extraction method without liquid nitrogen and the optimization of incubation time in this study have important implications for environmental research. By obtaining high-quality DNA samples from processed materials, such as souvenirs or herbarium specimens, biodiversity, and species conservation research can be conducted more effectively and accurately. Genetic identification of old specimens can also be a critical factor in understanding the evolution and environmental changes over time (Bieker & Martin, 2018).

Conclusions and Recommendations

The DNA extraction method using CTAB buffer without liquid nitrogen and with modified incubation time during precipitation can extract DNA from processed materials such as the sebalik sumpah seed coat. Substituting liquid nitrogen with silica sand and PVP and a 4-day incubation period is the most optimal modification. Additional validation methods, such as DNA sequencing, can be utilized. DNA sequencing confirms the accuracy of the amplified DNA fragments, thereby providing further certainty regarding genetic identification and analysis.

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