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A modified non-liquid nitrogen protocol for extraction of high-quality genomic DNA from the inner bark tissues of *Dalbergia cochinchinensis* (Fabaceae)

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ABSTRACT. *Dalbergia cochinchinensis* (Fabaceae) is known as Thai rosewood. It is a Thai native and widespread throughout Thailand. Due to it being a hardwood tree, it became a valuable hardwood tree species for its commercial value for luxury furniture and as a first-class prime timber, which has made it a potentially endangered species. For genetic studies of this tree, we tested four protocols of genomic DNA extraction from the inner bark based on the sodium dodecyl sulfate method and two protocols of the cetyltrimethyl ammonium bromide method. We evaluated the quantity, purity, and integrity of the extracted genomic DNA from 15 genotypes of *D. cochinchinensis* using PCR amplification and restriction enzyme digestion to develop a protocol for this species. We found that optimal concentrations of lithium chloride and polyvinylpyrrolidone could improve the quantity and quality of DNA in the extraction buffer without using liquid nitrogen. The highest concentration of high-quality DNA was obtained with protocol M5 (392 ng/μL DNA and a purity ratio of A_{260}/A_{280} equal to 1.96). In contrast, the commercial Nucleospin Plant II Mini Kit provided the lowest yield of 13.94 ng/μL DNA, with a low purity ratio of A_{260}/A_{280} (1.58). Start codon targeted and sequence-related amplified polymorphism fingerprints further demonstrated that protocol M5 developed for inner

bark tissue samples gives good DNA quality and quantity for genetic studies of *D. cochinchinensis*.

Key words: *Dalbergia cochinchinensis*; Endemic species; Genomic DNA extraction; Inner bark

INTRODUCTION

The conservation of vulnerable species is an essential strategy for preserving biodiversity levels in the world, especially in tropical regions with high biodiversity. However, illegal logging and unbridled deforestation can dramatically destroy such biodiversity, causing a breakdown in the balance of forest renewal (Amaral et al., 2004). Hence, conservation efforts around the world have focused on enhancing biodiversity. It is therefore first of all necessary to accurately identify plant species in order to facilitate their conservation and management. In general, the morphological characters of plant species are the preferred way to conduct the identification of plant species. Also, the identification of plant species requires an expert botanist who is good in genus recognition based on morphological characteristics such as flowers, leaves, and fruits. However, plant species identification remains limited by the plant growth cycle and field investigation, although the morphological characters are a suitable way to discriminate. In some species-rich or taxonomically complex groups, accurate identification is often quite difficult for endemic plant species, especially in cases where other species exist that are very similar but do not face any threat, or in cases where only certain components of the plant can be considered threatened (Webb et al., 2010; Hartvig et al., 2015). Accordingly, it is important to produce novel tools that are capable of providing an accurate means of plant species identification.

The genus *Dalbergia* encompasses approximately 250 species ranging from shrubs to trees, (Lock et al., 1994; Niyomdham et al., 1997; Lewis et al., 2005). The plants in this genus usually possess very high-quality timber that can be used for house construction, luxury furniture and high-quality music instruments (Soonhuae, 1993). This makes it a highly endangered genus. The 17th Convention on International Trade in Endangered Species of Wild Fauna and Flora (Sheikh and Corn, 2016) provides a list detailing all of the *Dalbergia* spp. species. Among them, a species known as rosewood, called *D. cochinchinensis,* is naturally distributed in tropical regions throughout Thailand, Vietnam, Laos and Cambodia (Liangsiri et al., 1993; Niyomdham, 2002). Overexploitation and illegal logging may further dramatically aggravate the decrease of this species in the field. The bark of this species forms a remarkable traded Dalbergia, for its extremely slow growth, which has caused it to become a species facing near-extinction (Liangsiri et al., 1993). In general, trees of the genus *Dalbergia* are very tall and reach 20 to 30 meters. Further, the leaves are also usually infected by fungus and very hard to analyze. It is quite difficult to get enough young healthy leaves to isolate the DNA to qualify for molecular analysis. Thus, the inner fresh bark of genus *Dalbergia* is a viable and reliable resource of genomic DNA isolation throughout the year. Furthermore, a new way to ensure the species identification of genus *Dalbergia* must be developed without leaves to fight the illegal logging of *Dalbergia* spp. However, bark tissue typically comprises from 10-20% of woody vascular plant varieties by weight, and its content includes tannins, lignin, suberin and suberin, along with several biopolymers and polysaccharides. These chemicals would normally inhibit the

enzymatic reaction and lead to unsuccessful molecular analysis. Furthermore, lignin comprises around 40% of the bark tissue, and serves to support the overall structure through crosslinks which connect the various polysaccharides, including cellulose (Vane et al., 2006). Polysaccharides can influence PCR amplification by inhibiting *Taq* DNA polymerase (Demeke and Adams, 1992; Schrader et al., 2012). It is difficult to get rid of these impurities during the process of isolation. Once oxidized, the polyphenols subsequently bind to the DNA, which limits the potential for the DNA to undergo downstream processing such as the restriction of digestion or the amplification of PCR (Sahu et al., 2012).

Molecular markers have proven to be an efficient method for identifying plants and typically show enough variation to allow different species to be distinguished (Hebert et al., 2003). The principal benefit of applying a method based on DNA is that samples can be identified without the need for expertise in taxonomy, while the tissue samples used can be very small and may be obtained from any part of the organism in question. The most important stage in any technique based on DNA is that of isolating and then amplifying the genomic DNA which is obtained from the plant tissues. Genomic DNA isolation protocols have been reported by numerous publications inferred from different plant species. However, it is still necessary to develop protocols for the extraction of DNA from certain problematic species (Cingilli and Akcin, 2005). Within the plant tissue, many compounds of plant cell wall will strongly inhibit DNA extraction and cause the failure of following molecular analysis, such as polysaccharides, proteins, and phenolic compounds (Finkeldey et al., 2010).

In this study, we develop a modified SDS-based method for isolation genomic DNA of the dried inner bark of *D. cochinchinensis* collected from Thailand without liquid nitrogen. The inner bark sample collection, pretreatment, storage and the DNA isolation protocol are also described and 15 genotypes of *D. cochinchinensis* from Kohn Kaen province were employed to test the efficiency of the protocol. The performance of the protocol was compared to commercial DNA isolation kits and two CTAB-based DNA isolation methods with leaves and dried inner bark. We hoped to provide an alternative and effective protocol for harvesting high-quality genomic DNA from dried inner bark for molecular studies of trees of the family Fabaceae.

MATERIAL AND METHODS

Sample collection

Fresh inner bark samples from 15 genotypes (KK1 to KK15) of *D. cochinchinensis* in Khon Kaen Province, Thailand were collected using rubber tapping scissors. All samples were air-dried at room temperature overnight and grounded by using a Philips juice dispenser HR2115 (with four-way blades, impact crusher, and 600-watt motor power) until fine tissue was obtained. This finely grounded bark was stored at -20ºC until use.

SDS-based extraction protocol

The SDS-based extraction protocol described below was developed for genomic DNA extraction from the inner bark powder of *D. cochinchinensis*is. Different

concentrations of LiCl and PVP were added to the developed extraction buffers to obtain the high-quality genomic DNA (Table 1). To qualify the developed SDS-based extraction protocol, two fundamental CTAB-based protocols modified from Porebski et al. (1997) and Novaes et al. (2009) were also employed to test the applicability of the developed protocols (Wangsomnuk et al., 2014), whereas the lysis agent was replaced by CTAB to isolate the samples. DNA samples were generated by the developed extraction protocols and comparisons from inner bark of *D. cochinchinensis* were further identified by molecular analysis.

Table 1. Details for the components of extraction buffers in the extraction protocols.

Protocols	Components
M1	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M2	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 50 mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M3	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 100 mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M4	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 150 mM LiCl, 1.5% SDS,2%PVP, 1.2% β-mercaptoethanol
M5	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 300 mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M6	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 1 mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M7	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 300 mM LiCl, 1.5% SDS, 0.1%PVP, 1.2% β-mercaptoethanol
M8	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 300 mM LiCl, 1.5% SDS, 0.5%PVP, 1.2% β-mercaptoethanol
M9	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 300 mM LiCl, 1.5% SDS, 1%PVP, 1.2% β-mercaptoethanol
M10	10 mM Tris-HCl pH 8.0, 50 mM EDTA, 300 mM LiCl, 1.5% SDS, 1.5%PVP, 1.2% β-mercaptoethanol
M11	300mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M12	50 mM EDTA, 300 mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol
M13	10 mM Tris-HCL pH 8.0, 300 mM LiCl, 1.5% SDS, 2%PVP, 1.2% β-mercaptoethanol

Abbreviations: SDS, sodium dodecyl sulfate; LiCl, lithium chloride; PVP, polyvinylpyrrolidone; EDTA, ethylene diamine tetra acetic acid.

Procedures:

1. 0.05 g of the finely-ground inner bark powder sample was added to 750 μL of extraction buffer (Table 1) by using SDS as lysis agent.

2. Repeated blending of the mixture was performed prior to incubation for 1 h at a temperature of 65ºC, with inversion on occasion.

3. The addition of 500 μL of chloroform: isoamyl alcohol (24:1, v/v) to the extracted system was followed by gentle mixing.

4. Then the tubes were placed in the centrifuge for 5 min at room temperature and 10,000 rpm, whereupon the supernatant was moved to a different tube, followed by steps 3 and 4 being carried out once again.

5. Once the steps for purification had been carried out repeatedly, the aqueous phase was then moved into a different tube. An equal quantity of isopropanol and 200 μL of 1.2 M NaCl were then introduced to the supernatant before the DNA was precipitated via mixing through an inversion process.

6. The tubes were then placed in the centrifuge once again for 10 min at room temperature and 10,000 rpm. The supernatant was then removed.

7. Rinsing of the pellet was performed on two occasions using 250 μL 70% ethanol before further centrifuging at 10,000 rpm at room temperature for a period of 5 minutes.

8. After the removal of the supernatant, air-drying of the pellet was completed.

9. The pellet was subsequently dissolved using 100-µL sterile deionized water. The RNA was digested for 30 min at a temperature of 37°C with RNase A (Sigma).

10. The DNA which had been extracted was placed in storage at -20°C until required.

Critical details: 1. Add β-mercaptoethanol to the extraction buffer immediately before use to decrease the possibility of oxidation.

2. Use only freshly prepared extraction buffer.

Commercial DNA extraction protocols

For comparison with the developed protocols, three commercial genomic DNA extraction kits were employed to extract the same samples to qualify the new protocols. The various commercially available extraction techniques for genomic DNA were performed in line with the instruction guidelines for the DNeasy Plant Mini Kit (Qiagen), E.Z.N.A ®Plant DNA Kit (Omega Bio-tek, USA), and NucleoSpin Plant II Mini Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). However, when using the E.Z.N.A ®Plant DNA Kit (Omega Bio-tek, USA), and NucleoSpin Plant II Mini Kit (MACHEREY-NAGEL GmbH& Co. KG, Germany), isopropanol was used instead of ethanol. The genomic DNA extracted by three commercial protocols was then evaluated and qualified, as detailed in the following description.

DNA extraction from the leaves of *D. cochinchinensis*

For further estimation of the developed protocols, the young leaves of *D. cochinchinensis* were also subjected to the best protocol that identified the inner bark materials. Here, we used 0.05 g of fresh leaves of *D. cochinchinensis* to extract the genomic DNA under the best protocol (protocol M5, see Table 1). The extraction procedure was the same as a description of the SDS-based extraction protocol (previous SDS-based procedure description).

DNA quantification and qualification

The genomic DNA quantities were assessed using a NanoDrop spectrophotometer (Thermo Scientific Co. Ltd). This approach relies upon taking a measurement of the absorbance at 260 nm. The genomic DNA quality was then evaluated through the measurement of the A_{260}/A_{280} ratios, while isolated DNA integrity underwent detection through 1% agarose gel electrophoresis in 1X Tris borate-EDTA (TBE) buffer and staining with ethidium bromide prior to examination when exposed to ultraviolet light.

SCoT marker fingerprinting

Thirty-six primers (Collard and Mackill, 2009) were synthesized and screened with genomic DNA from the bark of selected *D. cochinchinensis* genotypes to test their performance. After that, four primers (Table 2) were chosen to perform amplification on KK1 to KK15 DNA samples of *D. cochinchinensis* population. A template based on 100 ng of genomic DNA was applied in the context of a 10 µL PCR reaction system. The PCR

reaction mixture of 10 μL comprised 1x buffer (160 mM $(NH_4)_2SO_4$, 500 mM Tris-HCl, pH 9.1, 17.5 mM $MgCl_2$ and 0.1% Triton X-100; Vivantis), 0.2 mM dNTP mix, 0.2 μM of each of the primers, and 0.4 unit/10 μL of *Taq* DNA polymerase (Vivantis). The Agilent 8800 (Germany) was used for the PCR amplification stage, whereby the amplification program involved 3 min of pre-denaturalization at 95°C, followed by 40 cycles consist of denaturalization for 1 min at 95°C, before annealing for a period of 1 min at 50°C, and then extension for 2 min at 72°C. This was followed by a final single extension cycle for 5 min at 72°C. Agarose gel electrophoresis was employed for the analysis of the PCR products, while the images were obtained and visualized via the Gel Documentation Essential System (Uvitec, Cambridge).

SRAP marker fingerprinting

The amplification reaction was performed with a few modifications to the method of Li and Quiros (2001). Five primer pairs (Table 2) were chosen from 34 designed primers (Li and Quiros, 2001; Sun et al., 2007) that were pre-screened to test the efficiency of amplification of selected DNA samples. Those chosen primer pairs were then applied to perform the following amplification on entire DNA samples from both the bark and leaves of 15 accessions. An amplification template based on 100 ng of genomic DNA drawn from each of the individuals was employed in a 10 μL PCR reaction system. The PCR reaction mixture comprised 1x buffer (160 mM $(NH_4)_2SO_4$, 500 mM Tris-HCl, pH 9.1, 17.5 mM $MgCl_2$ and 0.1% Triton X-100; Vivantis), 0.5 μM of each of the primers, 0.2 mM dNTP mix, and 0.4 unit/10 μL of *Taq* DNA polymerase (Vivantis). The Agilent 8800 (Germany) was used to perform PCR amplification, while the amplification program opened with a 3-minute pre-denaturalization phase at 95°C followed by 5 cycles consist of denaturalization in duration of 1 min at 95°C, before 1 minute of annealing at 35°C followed by 2 min at 72°C for extension. Next came 35 cycles of denaturalization for 1 min at 95 °C, before 1 min of annealing at 50°C, 2 min of extension at 72°C. This was followed by one final extension cycle of 5 min at 72°C. Agarose gel electrophoresis was employed for the analysis of the PCR products, while the images were obtained and visualized via the Gel Documentation Essential System (Uvitec, Cambridge).

Table 2. Parameters of SRAP and SCoT primers used to qualify the extracted genomic DNA of 15 genotypes of *Dalbergia cochinchinensis*.

Primer name	Sequences (5'-->3')	T_m (^{O}C)	CG(%)
SRAP primers			
BG23	ATTCAAGGAGAGTGCGTGG	60.0	53
BG56	GAGAAAGGTATGAGTTGAAC	56.3	40
FC1	TCAAGGGCAGGTAAGAACAA	58.4	45
SA7	CGCAAGACCCACCACAA	54.6	59
ODD4	AGGGTAGCG TCTGAGGA	58.4	59
SCoT primers			
SCoT20	ACCATGGCTACCACCGCG	54.9	66
SCoT24	CACCATGGCTACCACCAT	50.3	55
SCoT25	ACCATGGCTACCACCGGG	54.9	66
SCoT26	ACCATGGCTACCACCGTC	52.6	61

Abbreviation: SRAP, sequence-related amplified polymorphism; SCoT, start codon targeted.

Restriction digest estimation

For further estimation of the quality of extracted genomic DNA by developed protocols, the restriction enzyme digest was applied for extended application of the genomic DNAs. Two restriction enzymes, *Pst*I-HF and *Msp*I were employed to perform the digest verification. The reaction was carried in a 40 µL reaction system consisting of 5 µg of each genomic DNA sample from the selected protocols with 2 µL of 20X *Pst*I-HF or *Msp*I enzyme (NEB), 4 µL of 10X NEB Cutsmart buffer and sterile deionized H_2O in a 40 µL final volume. The reaction was performed at 37°C overnight. Subsequently, agarose gel electrophoresis was performed to analyze the product from the reaction, making use of 0.7 % agarose gel (Agarose Molecular Biology Grade Vivantis Technology Sdn. Bhd., Malaysia). Electrophoresis was carried out with 1× TBE buffer which contained 1 µg/mL of ethidium bromide (EtBr) while the voltage was held constant for 4 h at 50 V. This allowed visualization of the digested DNA, where upon the Gel Documentation Essential System (Uvitec, Cambridge) was used to produce the images.

Data analysis

Each DNA sample from different developed protocols was tested in five replicates. One-way ANOVA analysis was conducted by using Statistic 8 software program Analytical Software (2003), while Least Significant Difference was employed to determine mean separation in the case of the F-test scores proving significant at the level of 0.05.

RESULTS AND DISCUSSION

Protocol design and DNA yields

In this study, we describe a newly developed protocol that shows high performance for extracting genomic DNA from the inner bark of *D. cochinchinensis*. The developed protocol can remove inhibiting compounds like polyphenol, tannin, secondary metabolite during the DNA extraction process. Fragment analysis revealed that the DNA quality was sufficient to perform the SCoT and SRAP DNA fingerprint analysis by using the dried inner bark samples from 15 genotypes of *D. cochinchinensis* (Figures 1, 2 and 3). Normally, researchers employ commercial kits such as DNeasy Plant Pro Kit and DNeasy Plant Mini Kit (Qiagen) to fast extraction of the genomic DNA from different forms of plant matter in a short time (high phenolic compound leaves or fresh and dried wood). However, such kits could be effective for purity, but would result in low DNA yield (Jiao et al., 2012; Yu et al., 2017; Pipan et al., 2018). It is reported that Yu et al. (2017) succeeded in obtaining genomic DNA by using the DNeasy Plant Mini Kit (Qiagen) with average DNA quantities of 18.9, 10.6 and 28.8 ng/mg from sapwood, heartwood, and twigs, respectively. However, when the DNeasy Plant Mini Kit (Qiagen) was applied to extract the genomic DNA from the inner bark of *D. cochinchinensis*, it only harvested half the DNA amount (169.92 ng/µL DNA) of that of our developed protocol M5 (Table 3). Various commercially available kits for the isolation of DNA required an additional buffer that contained PVP, β-mercaptoethanol, and EDTA at very low temperatures in order to be effective (Kalinowska et al., 2012; Broberg and McDonald, 2019). To estimate the efficiency of the developed methods for the inner

bark of *D. cochinchinensis*, we employed three commercial kits for genomic DNA extraction from the samples provided in line with the guidelines of the equipment provider: NucleoSpin Plant II Mini Kit (Macherey-Nagel), E.Z.N.A.® Plant DNA Kit (Omega Bio-tek), and DNeasy Plant Mini Kit (Qiagen). We also investigated the lysis chemical components that contributed to the yield of genomic DNA, such as SDS and CTAB based methods.

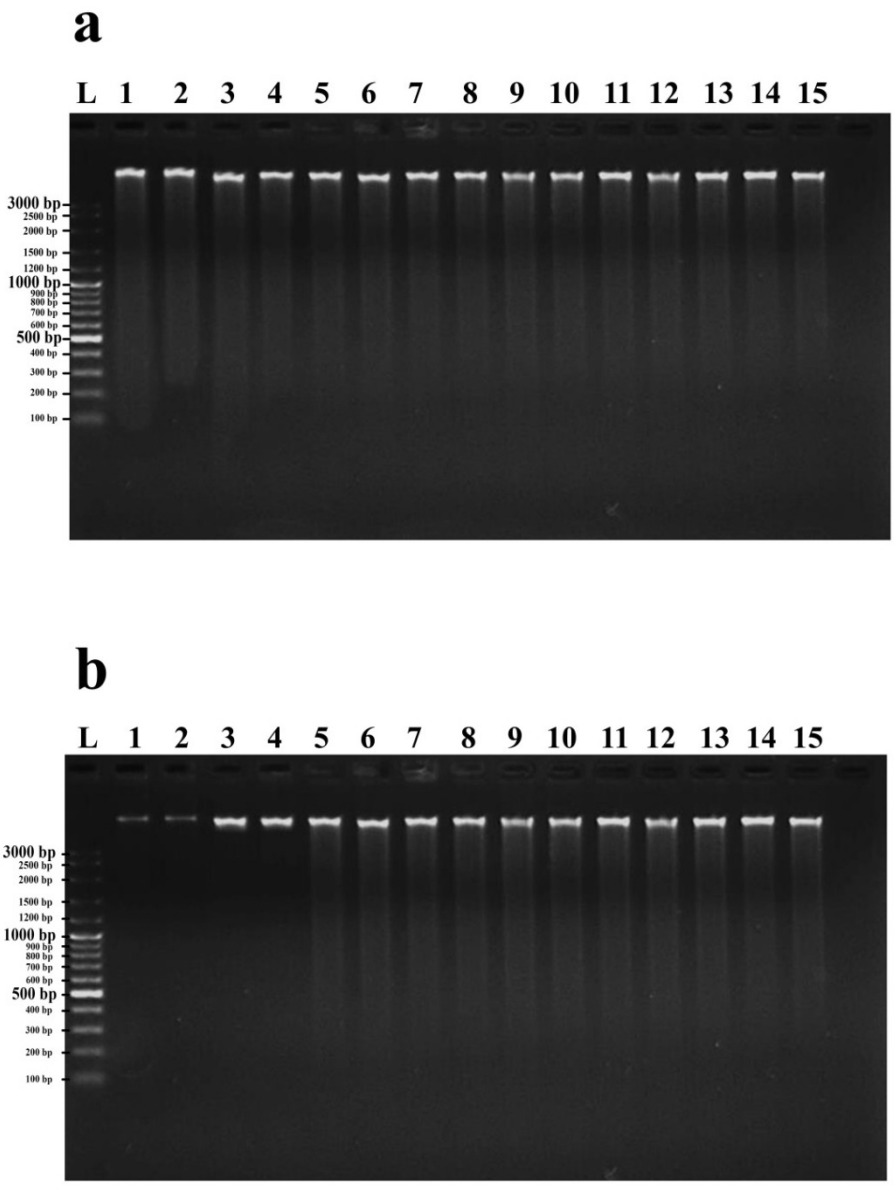

Figure 1. Agarose gel electrophoresis visualization of extracted genomic DNA of 15 genotypes of *Dalbergia cochinchinensis*. a, dried bark; b, fresh leaves.

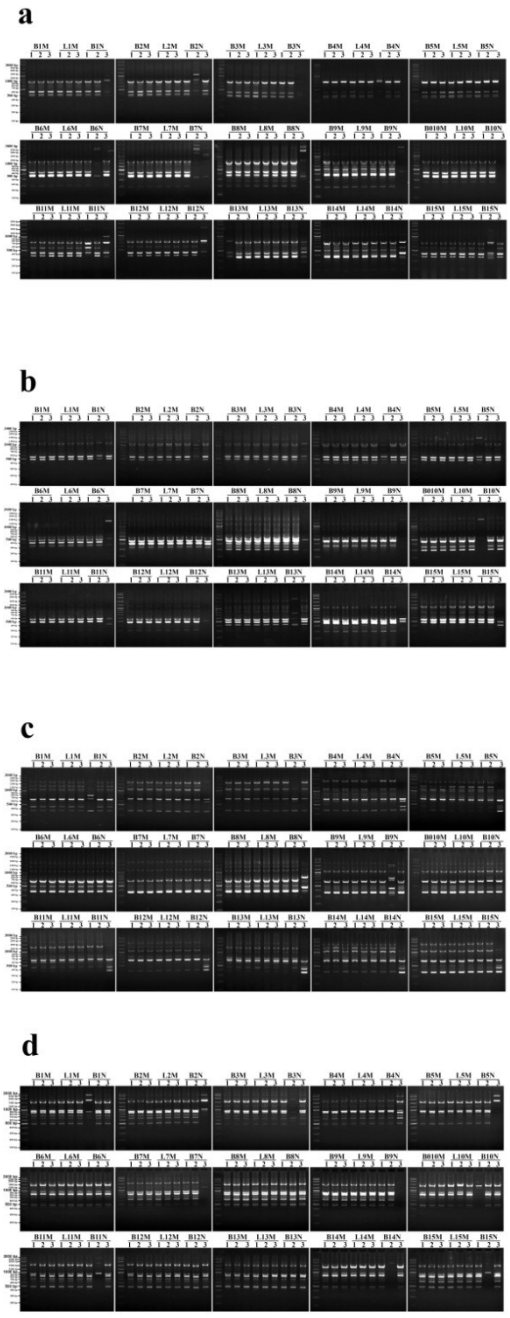

Figure 2. SCoT-DNA fingerprints amplified by SCoT20, SCoT24, SCoT25 and SCoT26 primers of 15 individuals in Figures 2a, 2b, 2c and 2d, respectively. Letters above the Figures are abbreviations showing the DNA extraction methods from bark (B) or leaf (L) tissue using the developed protocol (M5) and modified Novase et al. (2009) (N). Three replicates from each DNA sample were amplified. Numbers on the left side of the Figure are the sizes (bp) of 100 bp DNA ladder Plus (Vivantis). Abbreviation: SCoT, start codon targeted.

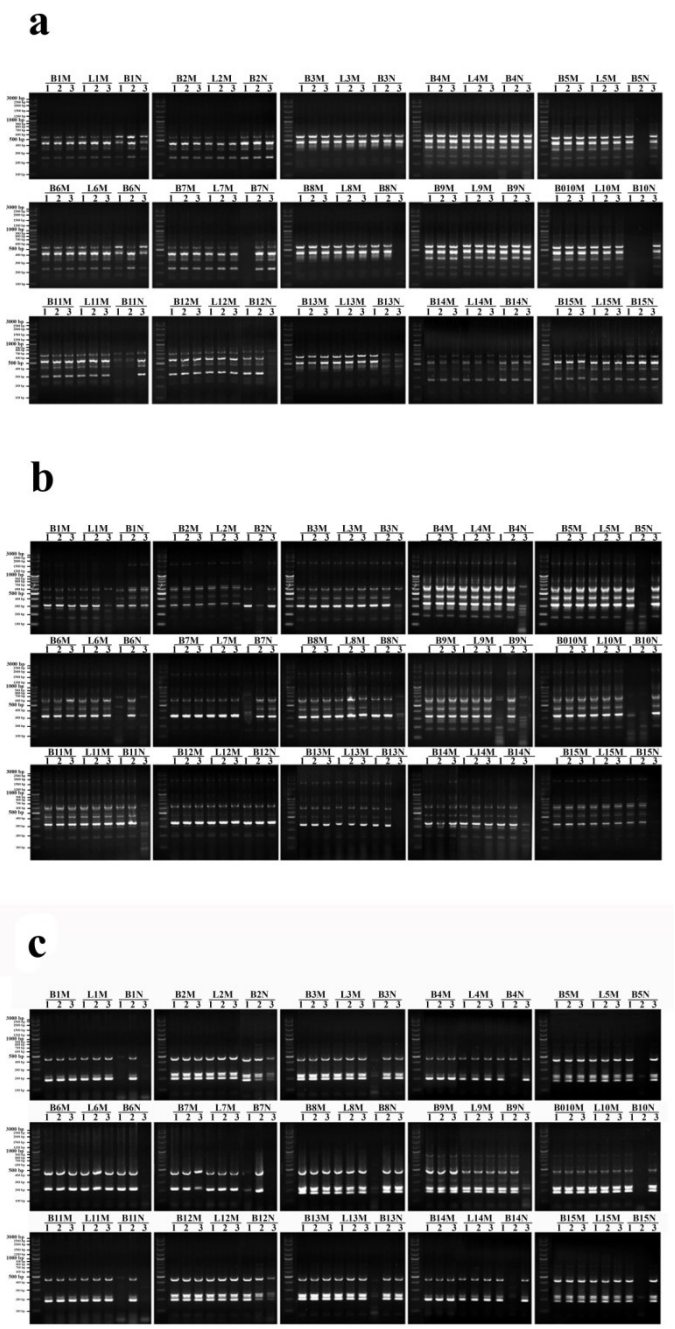

Figure 3. SRAP-DNA fingerprints amplified by FC1-BG23, SA7-BG56 and BG56-ODD04 combinations of 15 samples in Figures 3a, 3b and 3c, respectively. Letters above the Figure are abbreviations showing the DNA extraction methods from bark (B) or leaf (L) tissue using the developed protocol (M5) and modified Novase et al. (2009) (N). Three replicates from each DNA sample were amplified. The values to the Figure's left indicate the sizes (bp) for the 100 bp DNA ladder Plus (Vivantis). Abbreviation: SRAP, sequence-related amplified polymorphism.

It is usually the case that the samples of plant matter, such as bark or leaves, will undergo pre-treatment, often involving grinding to a powder, which allows the release of plant cell DNA through the use of liquid nitrogen (Cota-Sánchez et al., 2006; Ali et al., 2019), though some reports do not (Swetha et al., 2014). Here, to low down the cost of extraction and easy to access, we preferred the extracted protocol without liquid nitrogen. A Philips juice dispenser HR2115 was employed to get the fine powder of dried bark and young leaves. To get efficient DNA to perform the molecular studies verification, we also compared the initial amount of grinding bark samples during the DNA extraction process (data not shown). Results showed that 0.05 g of dried bark in 750 µl extraction buffer by the developed protocols could obtain a sufficient yield and quality of genomic DNA, broadly in line with the results from other protocols (Jiao et al., 2012; Swetha et al., 2014; Fatima et al., 2018), which suggested that the developed protocol might help a researcher save more money and easy to get sufficient DNA in a very short period.

Quantitative evaluation

To obtain the high-quality genomic DNA, the additive amount of some reagents was also investigated within the developed protocols such as LiCl, β-mercaptoethanol and PVP. The results indicated that the optimum concentration of LiCl in SDS-based extraction buffer was 300 mM, whereas a lower or higher concentration of LiCl would lead to lower DNA yields. Although adding optimum LiCl could improve the DNA yield of dried bark, there were also some contaminants like phenols to inhibit the downstream analysis of crude genomic DNA; they could not be removed during the chloroform purification process. β-mercaptoethanol and PVP are able to assist in eliminating the tannins and polyphenols which can be found in the crude extract and purify the DNA (Horne et al., 2004). Thus, the concentration of these two reagents were also investigated within the extraction buffer systems.

According to the description of Heikrujam et al. (2020), 1.2% (v/v) of the β-mercaptoethanol was used and varied concentrations of PVP were added to the extraction buffer system in different protocols to get the optimum concentration to balance the yield and quality of the DNA (Table 1). Results showed that various amounts of PVP could affect the DNA yield. A lower concentration of PVP would lead to lower DNA yield and 2% concentration of PVP was proven to be optimal. The extracted DNA ratios for A_{260}/A_{280} proved to be suitable for each of the protocols for the entire inner bark samples of *D. cochinchinensis*. When the ratio of A_{260}/A_{280} was in the range of 1.93-1.99, the extracted DNAs showed high-quality from the individuals. Normally, Tris buffer is used to steady the solution pH value, allowing the stabilization of the DNA molecules after the breaking of the membranes and cell walls as the tissue is ground. Then, as compartmentalization comes to an end, the release of the cytoplasmic material will release (Heikrujam et al., 2020). Also, beside steadying the pH value, the Tris buffer system in the developed protocols was also employed to estimate the DNA yield and quality. When protocol M11 and M12 were omitted to add 10 mM Tris-HCl (pH 8.0) in the extraction buffer, they gave lower DNA yields where protocol M13 could generate nearly two fold more with the same quality (Table 1) (Sambrook and Russel, 2001). Hence, Tris buffer not only steadied the pH value to stabilize the DNA molecules, it also could influence the DNA yields of the extraction process. Thus, the protocol M1 to M10 added 10 mM Tris-HCl (pH 8.0) to the DNA

extraction buffer system, which showed good performance during the DNA extraction process. Also, the NanoDrop instrument and agarose gel electrophoresis were employed to qualify and verify the DNA quality from different developed protocols (Bessetti, 2007).

Previous studies suggested the DNAs obtained from bark tissue were in the range of 50 ng/µL DNA, according to Asif and Cannon (2005) to 5-8.1 µg DNA/g dried tissue according to Swetha et al. (2014). Here, we developed 13 protocols to extract the DNA from the dried bark tissue of *D. cochinchinensis* (Table 1), where two modified protocols of Novaes et al., (2009), Porebski et al., (1997), along with three commercially available kits were employed to draw comparisons concerning the quantity and quality of the target DNA. DNA yields were found to fall within the range of 13.94–392.00 ng DNA per 50 mg of homogenized dry bark matters (Table 3).

Table 3. Qualification and purity of genomic DNA generated by different extraction protocols by using 50 mg inner bark tissue samples of *Dalbergia cochinchinensis*.

Protocols	Quantification (ng/µL)	Ratios (A_{260}/A_{280})
M1	167.65^{f}	1.98^{b}
M2	271.80^{cd}	1.99^{b}
M3	284.07^{c}	1.95^{bcd}
M4	359.10^{b}	1.95^{bcd}
M5	392.00^{a}	1.96^{bcd}
M6	205.80^{e}	1.94^{cd}
M7	65.85^{h}	1.95^{bcd}
M8	77.08^{h}	1.93^{cd}
M9	112.12^{g}	1.94^{cd}
M10	263.53^{d}	1.94^{cd}
M11	63.07^{h}	1.94^{cd}
M12	76.67^{h}	1.94^{cd}
M13	103.88^{g}	1.94^{cd}
Modified Novaes et al. (2009)	276.68^{cd}	1.91^{d}
Poreski et al. (1997)	112.92^{g}	1.96^{bc}
Nucleospin Plant II Mini Kit	13.94^{i}	1.58^{f}
E.Z.N.A.® Plant DNA Kit	97.50^{g}	1.75^{e}
DNeasy Plant Mini Kit	169.92^{f}	2.09^{a}
LSD (1)	.000*	.000*

Values which are significantly different according to LSD are indicated by differing letters in each column ($p \leq 0.05$) (1) *Indicates significance at the $P \leq 0.05$ level.

Results showed that protocol M5 gave the highest DNA yield of 392.00 ng/µL, which was more than 2 folds of that from QIAGEN DNeasy Plant Mini Kit (169.92 ng/µL DNA in 50 mg dried inner bark samples), while NucleoSpin Plant II Mini Kit (13.94 ng/µL DNA in 50 mg dried inner bark samples) gave the lowest DNA yield. Further examination of the extracted DNA also proved the developed protocol M5 showed high performance in the DNA extraction from the inner bark of *D. cochinchinensis*, where the commercial kits failed to get both high-quantity and high-quality DNA (Table 3). Accordingly, the protocol M5 was chosen for the genomic DNA extraction from both of bark and leaf samples of 15 *D. cochinchinensis* genotypes. According to the previously described DNA extraction *procedure* in protocol M5, the extracted DNA from the bark was harvested in ranges from 276.37 to 421.10 ng/µL DNA and 317.07 to 1367.10 ng/µL DNA from leaves, respectively. The ratios of A_{260}/A_{280} from the bark and leaves are shown in Table 4. Results indicated the success of the extraction of high-quality genomic DNA from the bark and leaves using the

protocol M5. Subsequently, the integrity of DNAs was evaluated through agarose gel electrophoresis analysis, as shown in Figure 1, which proved that the polysaccharides could be eliminated during the extraction procedure by protocol M5 (Sablok et al., 2009). Some studies reported that it is impossible to observe the extracted DNA from the bark of certain plants such as *Cunninghamia lanceolate* and Dipterocapaceae on agarose gel as a consequence of the partial degradation occurs (Rachmayanti et al., 2006; Tang et al., 2009; Jiao et al., 2012).

Table 4. Summary of results of the DNA obtained from 15 genotypes of *Dalbergia cochinchinensis*.

Genotypes Bark	DNA Conc. (ng/µL)	DNA quality (A_{260}/A_{280} ratio) Leaf	Sample	DNA Conc. (ng/µL)	DNA quality (A_{260}/A_{280} ratio)
KKU01	353.37	1.95	KKU01	676.37	2.09
KKU02	370.13	1.94	KKU02	317.07	2.08
KKU03	368.03	1.93	KKU03	467.10	2.09
KKU04	367.43	1.93	KKU04	841.53	2.09
KKU05	364.60	1.93	KKU05	467.23	2.08
KKU06	276.37	2.09	KKU06	676.37	2.14
KKU07	397.07	2.08	KKU07	497.07	2.14
KKU08	367.10	2.09	KKU08	1367.10	2.16
KKU09	374.87	2.09	KKU09	574.87	2.12
KKU10	313.90	2.08	KKU10	727.23	2.08
KKU11	352.70	2.19	KKU11	576.37	2.09
KKU12	397.07	2.08	KKU12	497.07	2.08
KKU13	421.10	2.09	KKU13	467.10	2.09
KKU14	371.53	2.09	KKU14	874.87	2.09
KKU15	387.23	2.08	KKU15	1400.57	2.08

Molecular analysis

Due to the limited information regarding the genetic pattern of *D. cochinchinensis* in Thailand, few conclusions can be reached concerning genetic diversity, screening for superior germplasm, or indeed for resource protection. PCR amplification is important to prove the quality of DNA in this case; reproducibility is one of the most important for the marker of choice. Full digestion using a two-enzyme GBS protocol (*Pst*I/*Msp*I) which offered enhanced complexity reduction in comparison to the use of the original protocol based on *Ape*KI (Poland et al., 2012) confirmed the purity of the isolated DNA (Figure 4). In this study, we use of the polymorphism and reproducibility of a pair of multi-locus markers based on PCR technique, SCoT and SRAP by the PCR amplification of extracted genomic DNA template, isolated from 15 *D. cochinchinensis* individuals according to the developed protocol M5 (Figures 2 and 3). Data shown that these extracted genomic DNA by protocol M5 could work well in this dominant marker systems and present the high polymorphism within the *D. cochinchinensis* population without existing genome sequences (Aneja et al., 2012; Robarts and Wolfe, 2014). The generation of SCoT markers had their basis in a short-conserved region which flanked the start codon (ATG) in the gene sequences of *D. cochinchinensis*, which employs 18-mer long primers (Collard and Mackill, 2009) that were successfully amplified in other plants to explore their genetic variability (Agarwal et al., 2019; El-Aziz et al., 2019). Thirty-six SCoT primers and 30 combinations of SRAP primers were screened for analysis on the genomic DNA of selected *D.*

cochinchinensis genotypes. Finally, 4 SCoT primers and 3 SRAP primer combinations were chosen for further genetic analysis based on the clear amplicons (Table 2). Nearly 100% reproducibility of both SCoT and SRAP fingerprints with distinct banding patterns of more than 50 loci were presented by using the extracted genomic DNA as templates from leaves and inner barks of 15 *D. cochinchinensis* genotypes which extracted by our developed protocol M5 (Figures 2 and 3). Under the SCoT and SRAP-DNA molecular marker techniques, it was possible to identify whether each sample was identical or similar to any of the 15 individuals. Also, restrict enzymes digest indicated that the genomic DNA was free from contaminants. However, the fingerprint remarks of extracted genomic DNA from 15 individuals by using the modified method according to the description by Novaes et al. (2009) failed to be repeatable (Figures 2 and 3). Interestingly, both SCoT and SRAP primers were found to give efficiency and informative polymorphic variety, which could be used for genetic diversity evaluation within *D. cochinchinensis* populations (Figures 2 and 3). Both PCR and restriction digest further proved that the extracted genomic DNA by protocol M5 was suitable for the molecular studies.

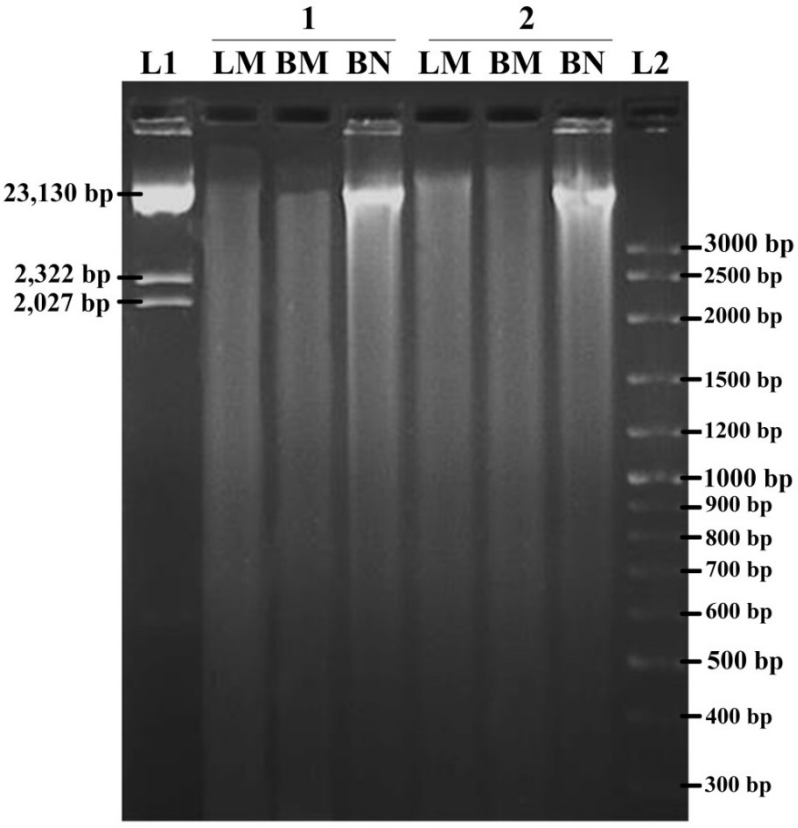

Figure 4. Agarose gel electrophoresis of the restriction digest of the genomic DNA obtained from the dried inner bark and fresh leaf matter of *D. cochinchinensis* via protocol 5 (LM and BM, respectively) compared to the modified method of Novase et al. (2009) (BN). M1 and M2 were Lambda Hind III (Promega) and 100 bp DNA ladder plus (Vivatis), respectively. LM, DNA extracted from leaf; BM, DNA extracted from dried inner bark; BN, DNA extracted from inner bark based on the modified Novaes et al. (2009) method.

CONCLUSIONS

Here, we present a newly-developed SDS-based genomic DNA extraction protocol with comprehensive analysis of DNA yield and quality, PCR amplification, and restriction enzyme digest compared to commercial kits and CTAB-based method. Protocol M5 was the most effective. By using this protocol, the DNA extracted from the inner bark of *D. cochinchinensis* could be used for restriction enzymes digests and PCR reactions. Our research could help to obtain sufficient high-quality genomic DNA from the inner bark and other tissues for genetic structure studies and conservation of *D. cochinchinensis*.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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