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Short Communication: DNA extraction from stored wood of *Falcataria moluccana* suitable for barcoding analysis

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Abstract. *Shabrina H, Siregar UJ, Matra DD, Kamiya D, Siregar IZ. 2019. Short Communication*: *DNA extraction from stored wood of* Falcataria moluccana *suitable for barcoding analysis. Biodiversitas 20*: *1748-1752.* Sengon or *Falcataria moluccana* (Miq.) Barneby & J.W.Grimes is the main crop in community tree plantations in Java, Indonesia, favored because of its fast-growing property and the promising economic return. The wood itself is mostly used for light construction, furniture, plywood, packing materials, and recently as feedstock for bioenergy. Sometimes the wood is used as DNA source for example in the wood identification analysis. However, extracting DNA from woods is considered difficult due particularly to very small quantities of DNA. The objective of this research was to optimize and modify the common CTAB protocols to extract DNA from Sengon wood without liquid nitrogen which sometimes unavailable in some laboratories. The extracted DNA was quantified with nanophotometer and gel visualization and amplified with primer coding *psb*A-*trn*H intergenic spacer for testing. The highest concentration of DNA extracted was from 100 mg of wood stored for 24 hours in -30 °C (257.80 ng/µL or 14.18 µg in total) and even the lowest concentration produced by this method able to produce sufficient amount of PCR product for sequencing. Compared with results from 200 mg samples and longer freezing time (72 hours) and extraction using liquid nitrogen, this method considered gave the best results.

Keywords: DNA isolation, Sengon, liquid nitrogen, wood

INTRODUCTION

Falcataria moluccana (Miq.) Barneby & J.W.Grimes (Indonesian: Sengon, Sundanese: Jeunjing, International name: Batai) is the main crop in the private land tree plantations (PLTPs) in South East Asia, especially in Java, Indonesia. It comes as second most source of log from West Java in 2015 (BPS 2015). Sengon was a native species in Moluccas and Papua and believed spread widely across Indonesia after 1871 (Corryanti and Novitasari 2015). The increasing number of Sengon plantation especially in Java have contributed to elevate community income (Siregar et al. 2007) and short time investment return within four years (Alipon et al. 2016). Sengon plantation in Indonesia was considered as new source of wood, as now we are facing the scarcity of wood from natural forest. The market of Sengon plantation is quite prospective, of which a Sengon with 20 cm in diameter is priced about Rp 500 000 (US$ 38), or equally Rp 1 200 000 (US$ 92) per m^3. The wood industries using sengon or batai wood are also growing fast and start entering international markets. The wood itself is mostly used in light construction, furniture, plywood, packing materials, and recently as one of the woods used as feedstock in bioenergy.

Wood is sometimes used as the DNA source when the fresh tissues are not available. Wood is also useful in studies like transcriptomics, wood identification and DNA based timber tracking. However, DNA extraction from hard tissue like wood that had been stored for particular length is considered difficult due degraded nature of the wood and contamination from wood extractives (Jiao et al. 2012). It is difficult primarily because of only small amounts of DNA present in the xylem of even living trees (Abe et al. 2011).

Sometimes DNA extraction kit used to extract the DNA to obtain better results but somehow it was more expensive and difficult to automate (Bashalkhanov and Pajora 2008). Those situations lead to the necessity to optimize extraction method for stored wood samples with common methods such as CTAB or SDS.

There are many factors that lead to DNA degradation during the isolation process. One of the main problems encountered is due to endonuclease activities that damaging the DNA (Weising et al. 2005). The mechanical treatments applied to disrupt wood tissue also can cause overheating, leading to irreversible DNA degradation

(Rachmayanti et al. 2006; Hamalton 2016). One attempt to inhibit endonuclease activities and overheating is using liquid nitrogen during the disrupting process. However, in some laboratory, the availability of liquid nitrogen is often limited.

The objectives of this study were to optimize a rapid DNA isolation method of sengon wood samples stored for 1 year, and then testing it whether the DNA is sufficient for PCR application by amplifying using *psb*A-*trn*H intergenic spacer primer.

MATERIALS AND METHODS

Sample collections

Samples were collected from trees in plantations in Bogor and Ciamis districts, West Java, Indonesia. The wood tissues used in this research was the sapwood near the bark. The wood tissues were collected from the standing tree by slicing the outer part of the sapwood using a sharp knife. The depth of the wood collected was around 2-3 cm. The wood samples were then cleaned by spraying alcohol before storing it in a plastic bag filled with silica gel and chilled in an ice box during transportation to the lab. The wood samples were then sliced into size 2-5 mm using hand scalpel and kept frozen in-20 °C in a freezer for a year.

DNA isolation

DNA was isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) method developed by Doyle (1991) with some modification. In this research, we also tried to find out the optimum amount of wood sample to obtain the best result. Two sample weights, i.e., 0.1 and 0.2 g were placed in screw-capped extraction tubes along with the metal beads. The samples in the tubes were then stored at -30 °C for either 24 or 72 hours. Later the samples were ground using Qiagen TissueLyser II with 2 mm beads for 2 minutes, 5 times at 30 Hz. Grinding process using liquid nitrogen was also conducted as control. Powdered samples were transferred to 2 ml tube filled with 1 mL extraction buffer (1M Tris-HCl, 5M NaCl, 0.5 M EDTA, 10% w/v CTAB, 1% w/v PVP) and 5 µL β-mercaptoethanol and incubated in heat block for 2 hours at 60 °C. Samples were centrifuged for 5 minutes at 15000 rpm and the supernatant was transferred to other tubes. Chloroform: isoamyl alcohol (24: 1) was added and mixed before centrifuged for 5 minutes at 15000 rpm, after which the supernatants were transferred to other tubes, and this process was repeated until we get the clear supernatant. The final supernatants were transferred to 1.5 mL tubes and added equal volume of isopropanol with the supernatant, and 3 M NaCl 0.25 x supernatant volumes. The samples were incubated at-30 °C for 1 hour and then centrifuged for 5 minutes at 15000 rpm. The watery parts were discarded carefully so the pellet remained on the tubes and air dried. The pellets were diluted by adding 55 µL of TE buffer and vortexed.

The quality and quantity of DNA were checked by running a 3 µL DNA samples mixed with loading dye in electrophoresis separation using 0.8 % agarose gel, for 15 minutes, at 100 V. The agarose was then stained in EtBr solution for 30 minutes and visualized under UV light and photographed. The DNA analyzed using Implen Nanophotometer NP80 (Implen GmbH, Munich, Germany) to quantify the results.

DNA amplification

The primers used in this process were *trn*H (GUG) (5'-ACTGCCTTGATCCACTTGGC-3') and *psb*A (5'-GCAAGCTCCATCTACAAATGG-3') intergenic spacer (Hamilton 1999). The amplification reaction contained *GoTaq* Mastermix 10 µL, 1 µL of each primer, 1µL of DNA template, and water added up to 20 µL. The amplifications were carried out using the ABI thermal cycler 9700 with the following protocol: initial denaturation for 2 minutes at 95°C; followed by 35 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C, and extension for 1 min at 72°C; then continued in a final extension step for 10 minutes at 72°C. The amplified products were separated on 2.0% agarose gels followed by Ethidium Bromide staining and visualization under UV light.

RESULTS AND DISCUSSION

DNA isolation

Samples were collected from sapwood tissue close to the bark considering the convenience of collection process. Another reason for picking the sapwood was the amount of DNA in the tissue is considered higher than in the deeper part of the wood (De Filippis and Magel 1998). The sapwood near barks also had the thinnest cell wall about 10.55 µM (Hussin et al. 2014), which make the grinding process easier to produce fine powder. During the wood tissue extraction process, any mucous solution was not formed in the supernatant after the chloroform purification, unlike extraction process from leaves tissue (Shabrina and Siregar 2015). That means the amount of polysaccharides produced during the extraction process was much lower than from leave tissues.

The amount of DNA extracted from 0.1 g and 0.2 g samples did not differ much. This study showed that the more amount of samples did not always result in more DNA quantity. Study by Fatima et al. (2018) showed that the optimum ratio of samples and extraction buffer was 500 mg: 5 mL, whereas in our case a ratio of 100 mg: 1 mL was the optimum ratio. The additional step for protein removal with chloroform and isoamyl alcohol was performed as mentioned by Verbylaite et al. (2010) to obtain more purity in the product. However, there was still high amount of contaminant shown in the agarose gel as smears due to the absence of PVP and RNAse addition in the lysis process. The A_{260}/A_{280} ratio showed that the variation happened in all methods used. However, the method with liquid nitrogen gave the lowest score of A_{260}/A_{280} ratio. The A_{260}/A_{280} ratio score lower than 1.8 showed that there was the possibility of protein contamination and the score higher than 2.0 indicated that there was another contaminant such RNA or salt (Figure 1; Table 1).

Amplification with *psb*A-*trn*H intergenic spacer region

The successfully extracted DNA was then processed further by PCR amplification using *psb*A-*trn*H intergenic spacer primer. The amplification resulted in single, clear fragment across samples (Figure 2) that were sufficient for sequencing process. The rate of successful amplification was 100% with the fragment sizes about 500 bp in length. The lower concentration of template, the produced band became weaker.

Discussion

Freezing the samples at-20 °C resulted in clear supernatant and no sign of browning which indicated that the oxidation process of DNA was inhibited during extraction process. Sahu et al. (2012) stated that-20 °C worked in preventing damage to the samples, and the cooler the temperature will result in lower degradation. Another sample with 72 hours freezing time prior to grinding resulted in similar quantity and quality of DNA with those of 24 hours treatments. Considering the length of time required to obtain good quality of DNA, freezing the sample for 24 hours is enough and efficient because the quantity of DNA produced did not differ either. The quantity of DNA produced did not differ either. Ferdous et al. (2012) stated that without liquid nitrogen in the grinding process reduced the isolation cost. Membrane column sometimes used to purify the DNA but it could lead to decrease in DNA quantity (Shabrina and Siregar 2015).

Table 1 The nanophotometer result of DNA extracted from all methods used

Starting material	Freezing method	Disrupting method	Maximum DNA yield (ng/µL)	A_{260}/A_{280} ratio
100 mg	24 hrs; -30°C	TissueLyser	257.80	1.87-2.25
200 mg	24 hrs; -30°C	TissueLyser	140.45	1.89-2.86
100 mg	72 hrs; -30°C	TissueLyser	214.35	1.91-4.38
200 mg	Liquid nitrogen	Hand grinding	174.10	1.48-2.84

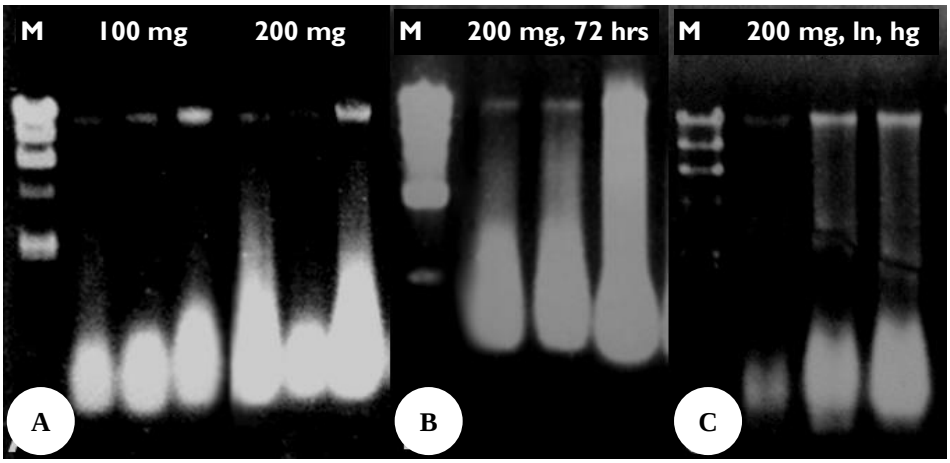

Figure 1. DNA extracted from A) 0.1 (lane 2-4) and 0.2 (lane 5-7) g tissue, frozen 24 hours, B) 0.1 g tissue, frozen 72 hours, and C) 0.2 g tissue with liquid nitrogen and grind using mortar. M: ʎ *Hind*III

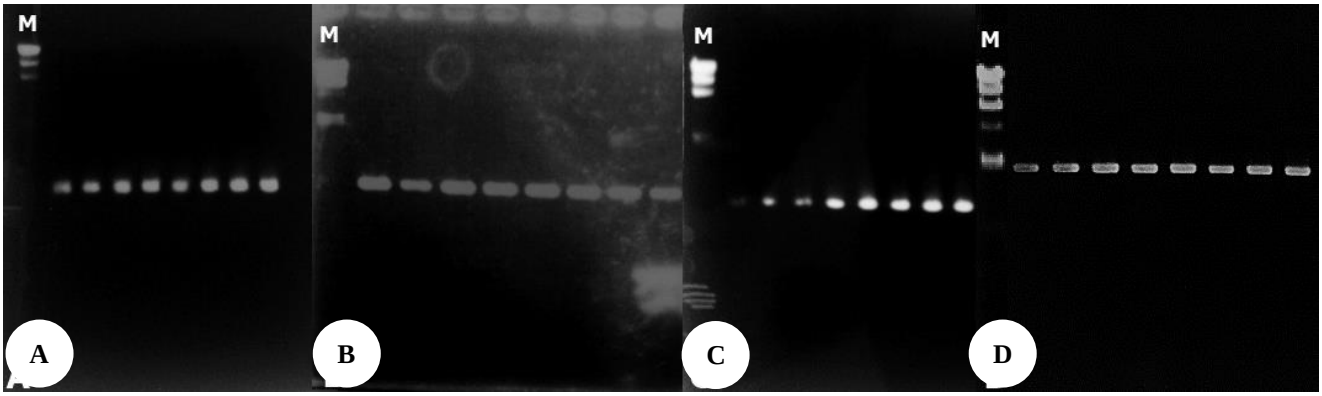

Figure 2. The amplification results using *psb*A-*trn*H intergenic spacer primer with DNA from previous extraction methods as template: A) 100 mg frozen for 24 hours in 30 °C; B) 200 mg frozen for 24 hours in 30 °C; C) 100 mg frozen for 72 hours in 30 °C; D) 200 mg frozen with liquid nitrogen; M: 1 kb DNA ladder.

Rachmayanti et al. (2009) used unprocessed Dipterocarpaceae wood stored for 1-4 years and was still able to achieve 87.85% success of amplification rate using *trn*F, *trn*L, and *ccm*p2 region from the chloroplast genome as primers. Verbylaite et al. (2010) also showed that the DNA obtained from wood was around 1/5 concentration of the DNA isolated from leaves but still able to produce clear band when amplified with *trn*LUAAF - *trn*F GAA primers. The success of the amplification process was also reported in Piccolo et al. (2012) that amplify SCAR markers, ITS region, and 16S rRNA gene from DNA isolated from grapevine plants without liquid nitrogen.

There were a lot of variations regarding the minimum amount of DNA required as PCR templates. A study by Fazekas et al. (2010) in improving sequence quality from PCR products containing long mononucleotide repeats, they used 20 ng DNA as template for 20 µL PCR reaction. However, in our case, as small as 1.8 ng/µL DNA able produced sufficient band for sequencing process and gave good sequence results (data unpublished). In other cases such as PCR with arbitrary primers like RAPD, a study by Stiles et al. (1993) used 15 ng DNA as template so the bands became clear for scoring. Another study by Porebski et al. (1997) modified the CTAB DNA extraction process and as low as 5 ng DNA gave clear result in amplifying DNA from various species that contain high polyphenols and polysaccharides with RAPD primers. Welsh and McClelland (1990) also stated that as low as 30 pg in 10 µL PCR reaction with arbitrary primers gave same band pattern with higher DNA concentration in PCR. Therefore, even if some contaminants like protein or other compound found in the DNA extracted, if the DNA diluted enough so the contaminant will not interfere the PCR process the sufficient band(s) will appear. Study by Shi and Panthee (2017) showed that even diluting DNA from 50 to 0.2 ng (200 folds) able to amplify Ph-3 gene marker in various plants leaves.

In conclusion, this modified CTAB extraction method using 100 mg samples that frozen for 24 hours was the best to extract the DNA from sengon wood in a low-cost and time efficient process. We hope this method will able to reduce the DNA extraction cost and time consumed for laboratory works for molecular analysis, not only for sengon, but also for other woods from fast-growing and polysaccharides and polyphenols rich plants.

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