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SHORT COMMUNICATION

Comparison of DNA Extraction Methods for Bornean Ironwood Plant (*Eusideroxylon zwageri*)

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ABSTRACT

DNA isolation is a routine procedure in molecular analysis. The method of plant genome DNA extraction has been widely available throughout global laboratories. Several labs made some modifications to obtain optimal results. This research aimed to analyse two types of DNA extraction methods for ironwood plant (*Eusideroxylon zwageri*), one tropical rain forest woody plant known for its high strength and durability. The results indicate that isolation DNA kit produced high DNA purity with lower concentrations while CTAB methods generated lower DNA purity with higher concentrations. This may be used as consideration of DNA isolation for downstream molecular analyses.

Keywords: DNA extraction, ironwood, CTAB, genomic DNA

Introduction

Bornean ironwood or ulin (*Eusideroxylon zwageri* Teijsm. & Binn.) is one of tropical rain forest (jungle) tree species with its giant size and long lifetime. This species is native to Borneo and Sumatra islands. Locally it has several names including belian, tabulin, telian, tulian, ulin, onglen bulian, and bulian rambai (Abdurachman, 2012). This hardwood has a straight fibre texture with high strength and durability. The wood is termite-proof and resistant to stemborers, weather and sea water-resistant (Abdurachman, 2012; Apriyono et al. 2019). Siran et al. (2004) showed that mature ulin has been used and exploited on a large scale, leading to decrease in its population in the natural habitats.

Due to its declining population, ironwood needs to be conserved. More than 20 accessions have been planted in Bogor Botanic Garden, West Java, Indonesia the biggest institution for plant conservation research, education, ecotourism, and environmental services in Indonesia (Ariati and Widyatmoko, 2019). Initial study

would include an assessment of genetic relationships and variations.

Genetic diversity studies can be conducted using accurate and valid methods. Molecular markers have been proven to be able to provide accuracy in conducting genetic diversity analysis of plants. Random Amplified Polymorphism DNA (RAPD) is one of the most widely used DNA genetic markers. RAPD marker has fairly high level of polymorphism, so it is often used to determine genetic diversity within populations and species, even to identify male elders in seed gardens (Goto et al. 2002). RAPD utilizes PCR technique to observe the amplification of DNA segments in the primary and nucleotide sequences randomly which is then visualized by gel electrophoresis. This RAPD marker also has weaknesses such as the inability to detect allele differences in heterozygous. This marker is only capable of detecting the presence or absence of DNA amplification at a specific molecular weight, with no information about heterozygosity (Nadeem et al. 2018).

The quality and quantity of DNA are one of important factors in molecular analysis. The ironic genome DNA extraction method must also be done carefully and efficiently. Although DNA extraction is already a normal part of molecular biology research, some types of plant cells require attention and some have unique biochemical properties that may hinder the extraction process. Enzymes, coenzymes, cellulose or polysaccharides may interfere with DNA extraction (Lange et al. 1998). Therefore, modification in extraction protocol is necessary in order to high quality DNA that can be used for further analysis. This paper aimed to generate most suitable DNA isolation for ulin by modification of three different isolation techniques.

Materials and Methods

Ulin leaves obtained from Bogor Botanic Garden, Bogor, West Java, Indonesia were used as the source of DNA (Table 1). Genomic DNA of 19 accessions were isolated using a commercial DNA isolation kit and CTAB (Cationic Hexadecyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). Table 1 shows the ironwood accession numbers and their specific coordinates within the Garden.

Plant DNA isolation kit

50-100 mg fresh or frozen leaf tissues were powdered using liquid nitrogen and mixed with buffer 1 (containing 0.2% PVP and 2% mercaptoethanol in the modified method) in an Eppendorf tube. Samples were incubated at 60°C for 10 minutes after vortexing. The tubes were reversed several times during incubation. 200 µl hot elution buffer was added and followed by addition of 100 µl buffer 2. After vortexing, the samples were put on ice for 3 minutes. The samples were transferred into assembled filter column-collection tube, followed by 1 minute centrifugation at 1,000x g. Supernatant was transferred into a new 1.5 ml tube and followed by addition of 1.5 vol binding buffer. After vortexing for 5 minutes, the mixture was put into assembled binding

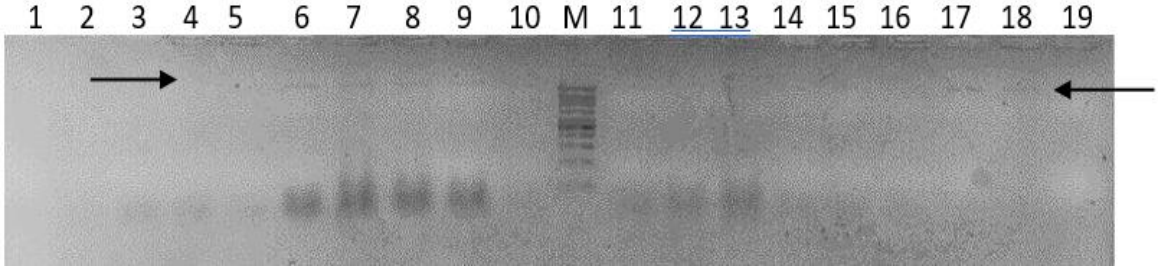

Fig. 1 Total genomic DNA of ironwood (*E. zwageri*) extracted using a commercial isolation kit. The arrows indicate genomic DNA obtained in several samples.

Table 1. Information on ironwood (*E. zwageri* Teijsm. & Binn.) samples and their specific coordinates.

Sample	Vak.No	Accession	Source	Coordinate
1	IX.C.10a.	B1927123	Kalimantan(W): Sambas	6°35'35,39" S 106°47'58,29" E
2	IX.C.130.	B18691126c		6°35'35,36" S 106°47'58,08" E
3	IX.C.7.	B1869111	Kalimantan(W): Sambas	6°35'36,04" S 106°47'58,43" E
4	IX.C.8.	B1869112	Kalimantan(W): Sambas	6°35'35,81" S 106°47'58,39" E
5	IX.D.125a.	B1869114	Kalimantan(W): Sambas	6°35'34,31" S 106°47'59,37" E
6	IX.D.130.	B1869113	Kalimantan: Sambas	6°35'34,88" S 106°47'58,45" E
7	IX.D.191.	B19590948	Spontan in Hortus; Reported 02-09-1959	6°35'36,77" S 106°48'0,89" E
8	V.E.34.	B196105204		6°36'0,65" S 106°47'59,96" E
9	VIII.G.207.	B199411353	Kalimantan(C): Pembibitan TPT.I. Handayani Km.64	6°35'40,44" S 106°48'0,40" E
10	XVI.E.181.	B200012904	Spontan in Hortus	6°35'56,64" S 106°48'5,01" E
11	XVI.E.189.	B200012905	Spontan in Hortus	6°35'56,26" S 106°48'4,43" E
12	XVI.E.197.	B200012906	Spontan in Hortus	6°35'55,50" S 106°48'4,83" E
13	XVII.I.97.	B200012903		6°35'52,72" S 106°48'1,45" E
14	XX.A.18.	B1869115	Kalimantan(W): Sambas	6°36'1,02" S 106°48'14,66" E
15	XX.A.93.	B198111143	Kalimantan(C): Palangkaraya	6°36'1,10" S 106°48'14,80" E
16	XX.B.231.	B20060910	Kalimantan(E): Balikpapan, Htn.Lindung Wain	6°35'56,78" S 106°48'13,84" E
17	XXIV.B.153.	B2015060004	Propagation from XX.A.18 [Kalimantan]	6°35'38,76" S 106°48'5,98" E
18	XX.A.134.	B2017070128	Propagation from V.E.34 [W. Kalimantan]	6°36'0,88" S 106°48'14,74" E
19	XX.B.277.	B2019030001	Propagation from XX.A.18 [W. Kalimantan]	6°35'56,81" S 106°48'14,50" E

column-collection tube. Centrifugation was performed for 2 minutes at 15,000x g. The supernatant was discarded. This centrifugation was repeated until each mixture was finished. 400 µl washing buffer was added to clean the nucleic acid that binds to the column. Washing was performed 2x, and followed by transferring the binding buffer into a new tube where elution was carried out by adding 30 µl TE buffer and centrifugation at 15,000x g for 30 seconds.

CTAB Method

Half a gram of leaves was powdered in 2 ml tube using liquid nitrogen. 700 µl extraction buffer (EDTA 20 mM, Tris-HCl 100 mM [pH 8,0], NaCl 1,4 M, CTAB 2%, with modification of additional 2% PVP and 0.2% mercaptoethanol) was added. The mixture was incubated at 65ºC for 5

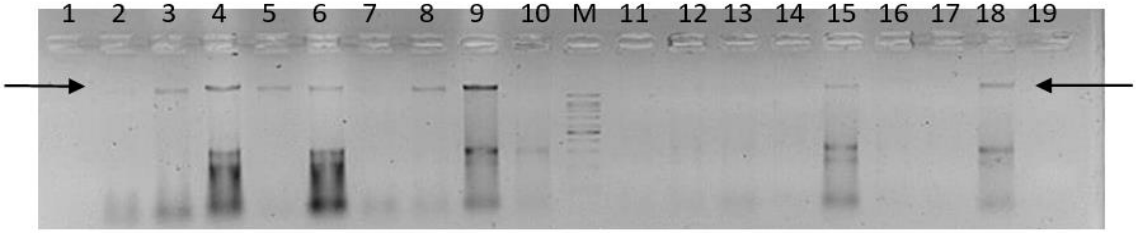

Fig. 2 Total genomic DNA of ironwood (*E. zwageri*) extracted using CTAB method. The arrows indicate genomic DNA obtained in several samples.

minutes. The mixture was inverted several times during the incubation period. 700 µl chloroform: isoamyl alcohol was added, and the mixture was inverted several times. After centrifugation for 15 minutes at 12.000x g, the aqueous solution was collected and transferred to a new tube. 1/10 vol 3M sodium acetate and 0.7 cold isopropanol were added to the supernatant. After several inversions, the tube was centrifuged at 12.000x g for 10 minutes. The pellet was retained by removing the supernatant, followed by washing the pellet with 70% ethanol twice. After centrifugation at 12.000x g for 5 minutes, the pellet was air-dried in the oven for 10 minutes at 50ºC. Finally, the pellet was solubilized in 30 µl TE buffer.

Agarose gel electrophoresis

Electrophoresis was performed at 0.9% low melting agarose in TAE buffer, and run at 80 V. DNA was visualized under UV light in the presence of 1 µl of RedSafe™ in 20 ml gel.

PCR-based RAPD

25 µl PCR mixture was prepared containing 50 ng extracted DNA, 8 mM dNTP mix, 1x PCR buffer, MgCl2, 0.2 µl Taq DNA polymerase, and RAPD primer (OPB-20). PCR was performed using a Biorad 96-well thermal cycler under the conditions of 1 cycle of 94°C for 1 min, followed by 45 cycles of 94°C for 30 sec, 37°C for 30 sec and 72°C for 90 sec and 1 last cycle of 72ºC for 5 min.

Results and Discussion

The results of the DNA isolations were assessed by reading absorbance at 260 nm and 280 nm using Nanodrop. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of both DNA and RNA since nucleotides, RNA, ssDNA, and dsDNA absorb at 260 nm, while other components such as phenol or protein absorb at 280 nm. For DNA, a ratio of around 1.8 is considered "pure", whereas for RNA, a ratio of about 2.0 is considered "pure" (Wilfinger et al. 1997). DNA isolation using the kit showed average ratio of around 1.90, which is only slightly more than pure DNA (Table 1). Although many samples produced the ratio that may still be considered acceptable, there are some exceptions of the value that is considered low (below 1.4) and high (above 2.2).

Tabel 2. NanoDrop™ reading of ironwood (*Eusideroxylon zwageri*) DNA extracted using commercial kit

Sample	DNA conc. (ng/µl))	DNA purity (A260/A280)
1	13.673	1.409
2	31.493	1.393
3	17.787	1.993
4	7.192	1.196
5	8.845	2.250
6	20.775	0.858
7	17.916	1.277
8	19.731	2.080
9	24.300	1.546
10	27.672	1.436
11	5.279	3.944
12	7.407	2.324
13	3.870	2.085
14	3.038	3.571
15	5.810	1.589
16	11.302	1.864
17	5.823	1.825
18	11.101	1.280
19	7.623	2.280

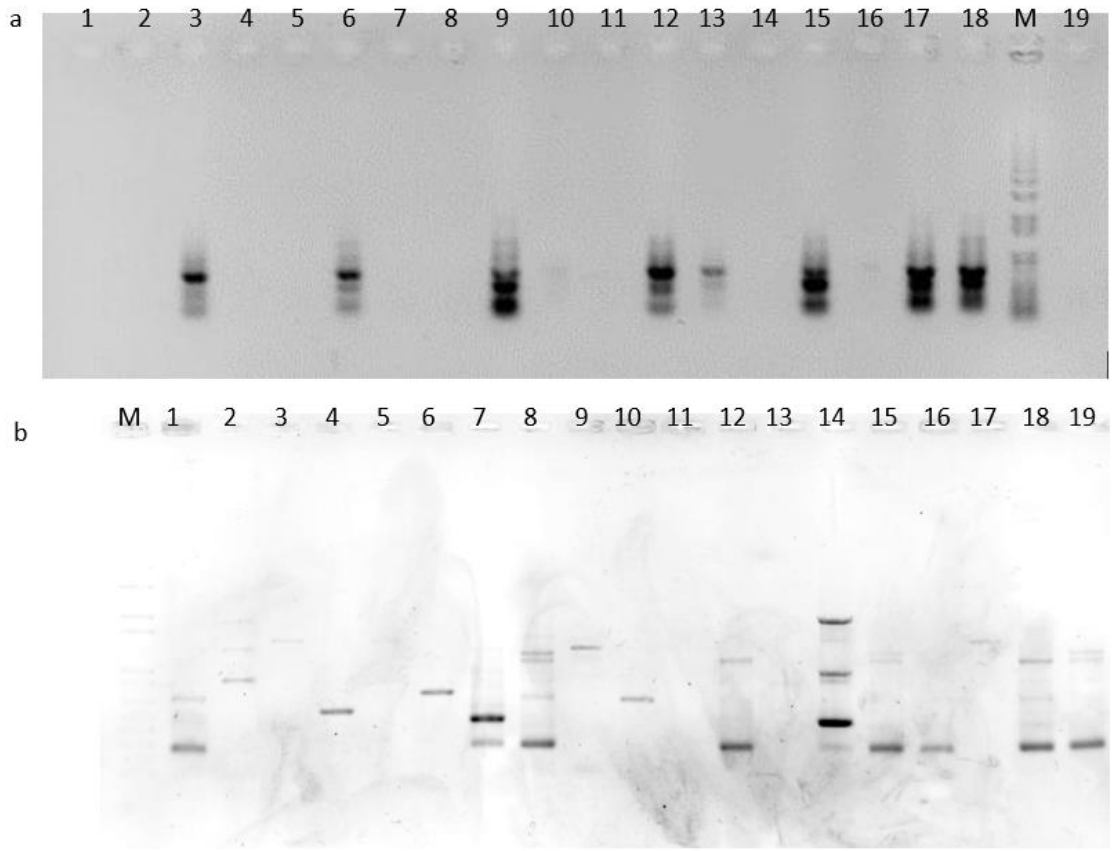

Fig. 3. RAPD profiles of 19 ironwood (*E. zwageri*) accessions after PCR using OPB-20 primers. M = 100 bp DNA ladder. 1 – 19 = sample accession according to Table 1. Templates were (a) DNA extracted using commercial isolation kit or (b) CTAB method.

The concentrations of DNA extracted were regarded as low, ranging from 3.04 ng/µl to 31.49 ng/µl (Table 2). This led to modifications of this method by the addition of 2% PVP (polyvinyl pyrrolidone) and 0.2% mercaptoethanol in the extraction solution/buffer. Both PVP and mercaptoethanol function to absorb polyphenols that may be present abundantly in the solution. During the co-precipitation of DNA, they are separated (Manoj et al. 2007). Polyphenols have been demonstrated to interfere with further extractions and downstream applications (Turaki et al. 2017). The results only showed slightly improved values, but these were not significant (data not shown).

In order to verify the results of Nanodrop^{TM} (Thermo Scientific) instrument, agarose gel electrophoresis was run on low melting. Indeed, the electrophoresis resulted in low concentrations of genomic DNA in some samples with tolerable purity (Fig. 1).

Our second extraction method was using CTAB with modification of 2% PVP and 0.2% mercaptoethanol addition in the extraction solution/buffer. The Nanodrop results are shown in Table 3. The purities of the DNA were mostly above the range (higher than 2.2). High 260/280 purity ratio gives indication that nucleotides were present at high amount and/or the solutions did not absorb 280 nm wavelength. These are not necessarily indicative of a problem. However, one must be precautious of possible contaminants that may hinder the downstream experiments. Absorbance above 2.0 may also indicate high RNA contamination (Khosravinia et al. 2007). These emphasized the need to perform agarose gel electrophoresis.

Although the purities of extracted DNA higher than expected, this method

produced DNA with higher concentrations. Fig. 2 confirmed that there was more DNA extracted using this method when compared to the commercial DNA isolation kit. CTAB method has been widely used for isolation of DNA of plants containing high levels of polysaccharide. This method was modified according to the tissue, organ, or species used to optimize the results (Turaki et al. 2017). In our study, we included PVP and mercaptoethanol in the extraction buffer since ironwood is rich in both polysaccharides and polyphenols (Nawawi et al. 2017; Ahmad et al. 2023). Many studies have modified CTAB method for optimal results (Turaki et al. 2017; Huang et al. 2000; Zhang et al. 2013; Attitalla 2011; Stefanova et al. 2013).

To further trial the quality of extracted DNA, PCR was performed utilizing RAPD primers available. The results showed that 13 samples extracted using CTAB method were amplified (Fig. 3a) when compared to 8 samples extracted using a kit (Fig. 3b). Commercial DNA isolation kits in general are designed to extract DNA from various sources without trying to be specific for certain sources (i.e standardized). This leads to simple processes resulting in high-purity DNA with lower concentrations. CTAB methods are widely used for the extraction of plant nucleic acid that contains high polysaccharide amounts (Kiss et al. 2024). Our DNA extraction results using CTAB method gave higher DNA concentration with DNA purity mostly higher than 2.0 value.

A better A260/A280 ratio obtained using a commercial kit indicates that the protocol is optimized for higher purity, hence less contamination. The protocol may have compatibility issues when used on certain samples. Some sample sources may contain certain complexes that can have inhibitory effects during isolation (Mundotiya et al. 2023), resulting in low DNA concentrations. Low concentrations may also be associated with other factors such as failure to follow the protocols properly, including inability to produce fine tissue powder, and environmental ones, including moisture, pH, and exposure to light (Mundotiya et al. 2023).

It is worth noting that instruments used to measure the absorbance can also play a role in the accuracy of the results. Routine calibration needs to be performed for good reading. Sample reading should be taken twice or three times to measure reproducibility. In addition, homogenous samples may not read accurately, and blank using the sample solvent must also be confirmed.

Conclusion

Isolation of genomic DNA from ironwood was carried out using two different methods, protocol following a commercial kit and CTAB method. While the commercial kit produced higher DNA purity, CTAB method generated a higher concentration of DNA. Several factors may cause these differences, including the ability of the kit to remove the presence of contaminant(s) and different environmental parameters such as pH. These results can be used for consideration in choosing DNA isolation methods for downstream molecular analyses.

Conflict of Interest

We confirm that we have no conflict of interest regarding any financial, personal, or other affiliations with individuals or organizations related to the subject matter discussed in the manuscript.

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