

GENOMIC DNA EXTRACTION METHOD FROM MATURE LEAF OF LAI (*Durio kutejensis* Becc.)

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ABSTRACT

Lai (*Durio kutejensis* Becc.) is an indigeneous germplasm of Kalimantan which has some superiorities compared to its close-relative, durian (*Durio zibethinus* Murr.). Genetic exploration of lai is important to support its breeding program. According to rapid development in molecular biology, genetic exploration effort of lai will be easier. One of significant step in any molecular biology activities is DNA isolation to produce high quality DNA for further analysis. Leaves of lai, as other perennial crop, contain of high concentration of polysaccharides and polyphenol which will be co-extracted with the DNA. These compounds can interfere enzymes activities in subsequent molecular analysis. The aim of this study was to establish an optimal and effective DNA extraction method to obtain high-quality DNA from mature leaf of lai. An established extraction buffer and its modification were used in this study. The result showed that modification 4 could produce high quality DNA, and was considered to be the most effective DNA extraction method for mature leaf of lai.

Keywords: CTAB modification; DNA extraction; lai

INTRODUCTION

Durios family (*Durio* spp.) is fruit plant with high economic value. The most popular species of this genera is *Durio zibethinus* Murr. or durian. However, there is another popular durio species in Kalimantan, Indonesia, that is called lai (*Durio kutejensis* Becc.). Lai is an indigeneous germplasm of Kalimantan, and can be found in most of Kalimantan island i.e. East Kalimantan, South Kalimantan, West Kalimantan, Brunei, Sabah

and Serawak (World Conservation Monitoring Centre, 2013).

There are some differences between lai and durian. Durian has strong and spesific smell, while some lai varieties are odorless and the other have smooth aroma (Antarlina, 2009; Santoso, 2010). Additionally, lai has unique and attractive flesh color from yellow to red (Antarlina, 2009; Santoso, 2010; Hariyati *et al.*, 2013; Hadi *et al.*, 2014), blunt fruit spine, and longer storage period than durian (Antarlina, 2009; Santoso, 2010). So lai has more potential as an export commodity in European market than durian.

Exploration of genetic potency of lai is important to support its breeding program. According to rapid development in molecular biology, genetic exploration effort of lai will be easier. One of significant step in any molecular biology analysis is DNA extraction process to obtain high-quality DNA.

The problem of *Durio* genus are woody species (Brown, 1997) that generally contain phenolics, polysaccharides and other secondary metabolites that contaminate DNA and interfere with subsequent analysis (Cheng *et al.*, 1997; Angeles *et al.*, 2005; Sari dan Murti, 2015). According to Latief and Amien (2014), the most preferable and frequently used method to extract DNA from polysaccharide and polyphenol-rich leaves is CTAB (Cetyl Trimethyl Ammonium Bromide) method developed by Doyle and Doyle (1990). But it can not be used to extract DNA from mature leaf of sapodilla (*Manilkara zapota* (L.) van Royen), one of polysaccharide and polyphenol-rich leaves (Sari and Murti 2015). So, some modification in CTAB method is needed.

The modifications that have been carried out were increasing concentration of PVP

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(Polyvinyl Pyrrolidone) to suppress polyphenol oxidation (Doyle and Doyle, 1990; Lodhi *et al.*, 1994; Cheng *et al.*, 1997; Syafaruddin and Santoso, 2011; Sari and Murti, 2015), increasing concentration of 2-mercaptoethanol, CTAB and sodium chloride in the extraction buffer to reduce polysaccharide contamination (Doyle and Doyle, 1990; Cheng *et al.*, 1997; Sari and Murti, 2015), modifying repetition and volume of CIAA (Chloroform Isoamyl Alcohol) adding (Sari and Murti, 2015), using a low concentration of spermine to selectively precipitate and purify DNA in final step (Cheng *et al.*, 1997).

To extract DNA from thick and tough leaves, liquid nitrogen is needed to make grinding process easier. Liquid nitrogen has been extensively used for DNA extraction from fresh leaves and or other tissues on coconut (Angeles *et al.*, 2005), sapodilla (Sari and Murti, 2015), temulawak (Utami *et al.*, 2012), apple, austrian pine, barberry, button-wood, cherry, grape, hazelnut, peach, and pear (Cheng *et al.*, 1997). However, liquid nitrogen is not always easily available or convenient to use, so DNA isolation method without liquid nitrogen which still can obtain high quality DNA is needed.

Widiastuti (2010), Sulassih (2011), and Syahrudin (2012) added sterile quartz sand as liquid nitrogen substitution for easier grinding of mangosteen and durian leaves. While Angeles *et al.* (2005) and Utami *et al.* (2012) added PVP powder in sample grinding instead of mixed it to the extraction buffer. The aim was to make leaf grinding easier, although without liquid nitrogen.

Some protocols of DNA extraction from woody species require young leaf samples to obtain high quality DNA (Angeles *et al.*, 2005; Mariana *et al.*, 2011; Syafaruddin and Santoso, 2011; Syahrudin, 2012) to avoid accumulation of phenolic compound in mature leaf and and to make sample grinding process easier. However, sometimes leaf sample must be collected from remote areas and shipped for several days, thus using young leaf sample is impossible. For some perennial plants, young leaves were not suggested as a source of genomic DNA due to the seasonal nature and its short half-life (Ibrahim, 2011).

Sari and Murti (2015) got an effective method for DNA extraction of sapodilla mature leaf and recommended for DNA extraction of other perennial crops that contain high phenolic

compound, polysaccharides, and other secondary metabolites. This study used modification in way of PVP and 2-mercaptoethanol adding to isolate DNA of lai. It is important to establish an optimal and effective DNA extraction method to obtain high-quality DNA from mature leaf of lai, as the aim of the study. This is an important early step that will influence subsequent molecular biology analysis in lai.

MATERIALS AND METHODS

The experiment had been conducted in Plant Breeding and Genetic Laboratory, Faculty of Agriculture, UGM from November 2014-March 2015. The leaves of lai were collected from Samarinda, East Kalimantan. The fully open leaves were harvested before 7.00 am to avoid increasing of phenolic compound. In shipment process, the leaves were packaged in a plastic bag and wrapped with paper box. DNA extraction was conducted in 3-4 days after leaves harvested.

Extraction Buffer

The buffer components used in this experiment were standard buffer of Doyle and Doyle (1990) and its modification for sapodilla (Sari and Murti, 2015) (Table 1).

Table 1. Components of extraction buffer used in the study

Buffer Components	Doyle and Doyle (1990) Method	Sari and Murti (2015) Method
CTAB	2 %	2.8 %
NaCl	1.4 M	2.5 M
Tris-HCl	0.1 M	0.1 M
EDTA	0.02 M	0.02 M
2-Mercaptoethanol	1 %	3 %
PVP	1 %	2.5 %

DNA Isolation Protocol

Extraction buffer was preheated in 65°C for 30 minutes. Leaf sample (0.1 g) was grinded using mortar and pestle, added with 1.5 ml of preheated extraction buffer, and the homogenate was quickly transferred to a 2 ml microtube. The tube was incubated at 65°C for 60 minutes with frequent swirling. Modification of cell lysis treatment is in Table 2.

Table 2. Treatment in cell lysis and DNA purification step of DNA extraction

Treatment	Cell lysis		DNA purification	
	Liquid nitrogen	PVP+Mercaptoetanol	Sodium acetate	CIAA
Doyle and Doyle	Without	Buffer included	-	2x (@500 µl)
Modification 1*	With	Buffer included	-	2x (@500 µl)
Modification 2*	With	Buffer included	-	3x (500 µl, 500 µl, 300 µl)
Modification 3*	Without	Buffer included	-	3x (500 µl, 500 µl, 300 µl)
Modification 4*	Without	Buffer excluded**	-	3x (500 µl, 500 µl, 300 µl)
Modification 5*	Without	Buffer included	3x (@1/10 vol)	3x (500 µl, 500 µl, 300 µl)
Modification 6*	Without	Buffer excluded**	3x (@1/10 vol)	3x (500 µl, 500 µl, 300 µl)

Remarks: * = Modification 1-6 using CTAB modification buffer component composed by Sari and Murti (2015); ** = PVP was ground with leaf sample using mortar and pestle; mercaptoetanol was added to the homogenate before incubation

An equal volume of 3M sodium acetate and CIAA (24:1) as its modification (Table 2) were added to the tube, and the tube was shaken vigorously to form a complete emulsion. The tube was centrifuged at 12,000 rpm for 15 minutes to separate the phases. The aqueous phase (supernatant) was removed with micropipet, and transferred to a new tube. Purification step was repeated as treatment in Table 2.

Sodium acetate 3M (1/10 volume) was added to the supernatant, mixing gently, precipitated with 2/3 volume of cold isopropanol and incubated in 4°C for 12-24 hours. The precipitated nucleic acids were collected and washed twice with ethanol 70%. The pellets were air dried and resuspended in TE buffer. Loading dye (1 µl) was added to 5 µl of DNA sample and electrophoresed on 1% agarose gel to check DNA quality.

DNA Amplification and Visualisation

Each 10 µl reaction volume of DNA amplification contains 5 µl PCR reaction mix, 2.25 µl nuclease free water, 0.25 µl RAPD (Random Amplified Polymorphism DNA) primer, and 2.5 µl DNA. PCR consists of one cycle of 94°C for 4 minutes, which was followed by 45 cycles of denaturation (94°C, 1 minute), annealing (37°C, 1 minute), and extension (72°C, 1 minute 30 seconds), completed with one cycle of 72°C for 7 minutes and 4°C for 1 minute.

The amplification products were analyzed by electrophoresis using 1.5% agarose gel in TBE buffer 1x for 1.5 hours in 75 Volt. The result was checked by UV transilluminator light and documented by digital camera.

RESULTS AND DISCUSSION

DNA isolation is a primary and critical step for molecular analysis of any plant species. The process becomes more difficult when the plant species contains high amount of polysaccharides and secondary metabolites like polyphenols as contaminants. These contaminants, which are abundant in the foliage of perennial plants, co-extracted with the DNA and interfere with polymerases, ligases, and restriction enzymes (Sarwat *et al.*, 2006; Ogunkanmi *et al.*, 2008). To suppress the interference of the contaminants, some materials like CTAB, NaCl, PVP and 2-mercaptoethanol are used in DNA isolation protocol.

Doyle and Doyle (1990) has used their standard CTAB method successfully on a wide taxonomic sampling of plant families, but it didn't work for *lai*. DNA extraction from leaf of *lai* could not produce high quality DNA when it was extracted by Doyle and Doyle standard buffer, although the purification step with CIAA and washing step with ethanol 70% have been repeated twice (Figure 1A).

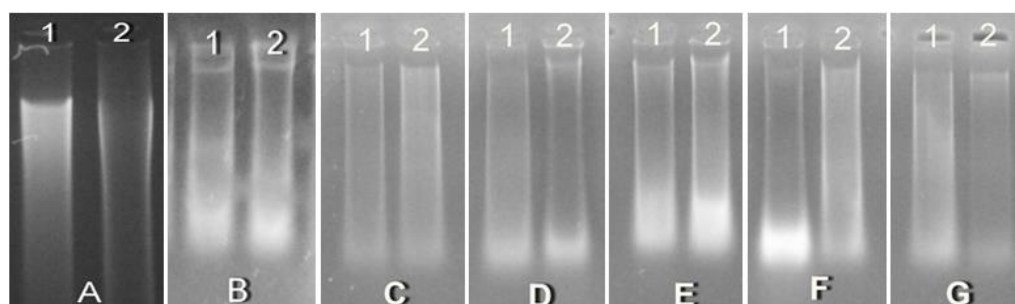


Figure 1. Electrophoresis result of various method of DNA extraction: A. standard method of Doyle and Doyle; B. modification-1; C. modification-2; D. modification-3; E. Modification-4; F. Modification-5; G. Modification-6; (1) sample no.1; (2) sample no.2

Quality of the extracted DNA was evaluated agarose gel electrophoresis and RAPD-PCR. Figure 1A shows the electrophoresis result of the DNA extracted by standard CTAB method of Doyle and Doyle. The extraction treatment produced DNA and a lot of residual DNA that indicated high level of polysaccharides (Sari and Murti, 2015). Polysaccharides contamination are particularly problematic as they can inhibit the activity of many commonly used molecular biology enzyme such as polymerase, ligase and restriction endonucleases. It is because nucleic acid form tight complexes with polysaccharides, creating a gelatinous pellet, and the embedded DNA inaccessible to the enzyme (Sarwat *et al.*, 2006). So the DNA of *lai* extracted by standard CTAB method of Doyle and Doyle (1990) can not be amplified in PCR analysis.

The color of the precipitated DNA extracted by standard CTAB method of Doyle and Doyle was brown, instead of white, indicated the presence of high level oxidized phenols (Angeles *et al.*, 2005). Polyphenols are released when the tissues are wounded. In their oxidized form, polyphenol covalently bind to proteins and DNA, giving the DNA a brown color and making it useless for most research applications (Angeles *et al.*, 2005).

To improve the quality of DNA, the standard CTAB method must be modified. In modification treatment of Doyle and Doyle method (modification 1-6), concentration of CTAB, NaCl, PVP and mercaptoethanol has been increased (Table 1). The increasing concentration of PVP was aimed to suppress phenol oxidation (Doyle and Doyle, 1990; Lodhi *et al.*, 1994; Cheng *et al.*, 1997; Syafaruddin and

Santoso, 2011; Sari and Murti, 2015) by forms complex hydrogen bonds with polyphenolic compound which simplifies their release from DNA strands by centrifugation (Lodhi *et al.*, 1994; Alaey *et al.*, 2005).

While increasing concentration of CTAB, NaCl, and mercaptoethanol was aimed to reduce polysaccharide contamination (Doyle and Doyle, 1990; Cheng *et al.*, 1997; Sari and Murti, 2015). 2-mercaptoethanol also works as an antioxidant agent and forbids oxidation in polyphenol (Lodhi *et al.*, 1994; Alaey *et al.*, 2005; Zidani *et al.*, 2005).

Modification-1 used liquid nitrogen to grind the leaf sample. Its modification produced thicker DNA, but there was still a lot of residual DNA (Figure 1B). The DNA quality produced by modification-1 treatment was not good enough. It also can not be used as template for RAPD-PCR analysis. In modification-2, purification step with CIAA was repeated three times. It resulted brighter final supernatant than the previous one, and so was the precipitated DNA.

In modification-3, liquid nitrogen was omitted while other treatment was identical with modification-2. It produced nearly identical of precipitated DNA color. The weakness of modification-3 treatment was its grinding process which was very hard because of leaf texture that tough and thick. So, according to Angeles *et al.* (2005) and Utami *et al.* (2012), in modification-4 PVP was added in grinding process and was ground simultaneously with leaf sample, instead of mix it to the extraction buffer. It made the grinding process easier although did not use liquid nitrogen.

Meanwhile, using of buffer-excluded mercaptoethanol in modification-4 was because

of safety reason. In Safety Data Sheet of 2-mercaptoethanol product, Life Technologies (2014) asserted that 2-mercaptoethanol causes irritation of respiration duct if it was sniffed. Mixing this material in extraction buffer was risky toward evaporation and spreading of its unpleasant odor when the buffer was used to grind the sample. So, minimize its contact with open air by buffer-excluded using is safer.

DNA electrophoresis of modification 2, 3 and 4 were not significantly different (Figure 1C, 1D, 1E). In addition to DNA electrophoresis, high quality DNA can be proved by using it as template for PCR analysis. DNA obtained from modification 2, 3, and 4 can be amplified in PCR-RAPD using both OPA 13 and OPB 10 primer, except DNA of sample number 2 from modification 3 (Figure 2 and Figure 3). It was linear with Lodhi *et al.* (1994), Cheng *et al.* (1997), Angeles *et al.* (2005), Syafaruddin and Santoso (2011) and Sari and Murti (2015) that increasing concentration of CTAB, NaCl, PVP, 2-mercaptoethanol and modifying repetition of purification step with CIAA will suppress the interference of the contaminants.

Besides polysaccharides and polyphenols, other contaminant which was often contained in initial DNA extract is protein. Most protein is removed by denaturation and precipitation from the extract using chloroform and/or phenol (Zidani *et al.*, 2005). CIAA is an organic solvent that can dissolve protein, bind, and then precipitate it. CIAA adding followed by centrifugation will separate protein from aqueous phase containing nucleic acid.

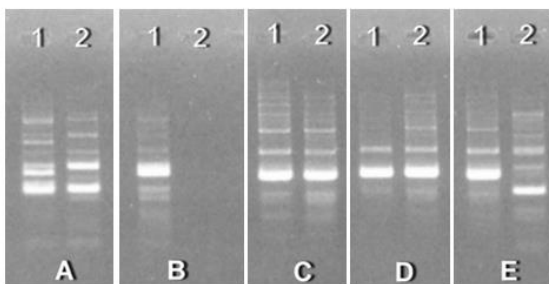


Figure 2. Result of PCR-RAPD analysis using OPA 13; A. modification-2; B. Modification-3; C. Modification-4; D. Modification-5; E. Modification-6; (1) sample no.1; (2) sample no.2

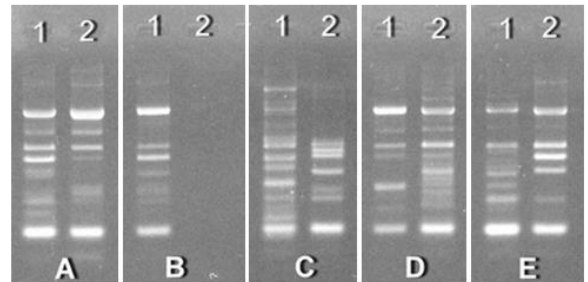


Figure 3. Result of PCR-RAPD analysis using OPB 10; A. modification-2; B. Modification-3; C. Modification-4; D. Modification-5; E. Modification-6;(1) sample no.1; (2) sample no.2

Modification 5 and 6 added 3M sodium acetate prior to CIAA in every repetition of purification step. Besides using organic solvent, the most common type of protein precipitation is salt induced precipitation. According to Fatchiyah *et al.* (2012) that salt, like sodium acetate or sodium chloride in high concentration, can be used to separate DNA from protein. Protein solubility depends on several factors. At high concentration of salt, the solubility of protein drops dramatically. When the salt concentration is increased, water molecules are attracted by salt ions, which decreases the number of water molecules available to interact with the charged part of protein. This is termed salting out, and the protein will be precipitated out.

Combination of high salt concentration and CIAA in purification step will precipitate protein perfectly. In modification 5 and 6, the adding of 3M sodium acetate will separate protein from the aqueous phase. So when CIAA added in further step, protein will be precipitated easily, and produce cleaner DNA. But the fact, DNA electrophoresis of modification 5 and 6 (Figure 1F, 1G) were not significantly different with modification 2, 3 and 4 (Figure 1C, 1D, 1E). There was still residual DNA present, although the DNA produced was high enough in quality, and can also be used as template in PCR-RAPD analysis (Figure 2 and Figure 3).

Among six modifications of Doyle and Doyle standard method described above, modification 4 is the most suitable method to be applied in mature leaf of lai. It was low-relative cost, because did not need liquid nitrogen to grind the leaf, and can produce high quality DNA for subsequent analysis. It was linear with Utami

et al. (2012) that liquid nitrogen using was inefficient because it did not produce better DNA bands, while PVP adding for sample grinding produced clear and thick DNA bands.

CONCLUSION

Increasing concentration of CTAB buffer components and repetition in purification step can obtain high quality DNA for subsequent molecular analysis. Extraction method of modification-4 was considered to be the most effective for DNA extraction of mature leaf of lai. It did not need liquid nitrogen for grinding process, substitute by modification in way of PVP adding.

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