



## Conference Paper

# Optimizing Genomic DNA Isolation and PCR Amplification For Pasak Bumi (*Eurycoma longifolia*)

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## Abstract

Pasak bumi (*Eurycoma longifolia* Jack) is a shrubs growing wildly in the forests of Southeast Asia and widely used throughout the region because of its medicinal properties. Uncontrolled harvesting of wild-grown trees has led to rapid decrease of its natural populations, as well as causing a potential decrease in genetic diversity. Information about population genetic of pasak bumi still not determined yet, including the optimal DNA isolation and its reference marker. Therefore, our research was conducted to get information about optimal DNA isolation using CTAB methods and reference marker for population genetic study. DNA isolation was conducted through CTAB (*Cetyl Trimethyl Ammonium Bromide*) method. The yield DNA was PCR amplified using four barcoding standard marker those were ITS, *matK*, *rbcl* and *trnL-trnF*. The result showed that CTAB method was able to yield good quality DNA and 30% dilution produced the best band. The *trnL-trnF* primer was able to amplified DNA of pasak bumi with 50°C annealing temperature, *rbcl* with 55°C annealing temperature and ITS with 50°C annealing temperature. While the *matK* primer failed to amplified.

**Keywords:** DNA, genetic, isolation, amplification, pasak bumi

## 1. Introduction

Pasak bumi is famous medicinal plant in Indonesia. Pharmacological testing found the utilization of pasak bumi for anti-cancer [1], anti-malarial [2], anti-leukemia, anti-HIV [3], and aphrodisiacs [4]. The increasing of illegal harvesting, low natural regeneration and limited habitat preference in Sumatra and Kalimantan Island, causing the population conditions to decline. The natural germination percentage of this species is very low and requires a long time due to immature embryos at the time of dispersal [5]. Based on these considerations, Indonesian government since 2006 has listed the species into

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Received: 19 February 2019

Accepted: 5 March 2019

Published: 16 April 2019

Publishing services provided by  
Knowledge E

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Selection and Peer-review under the responsibility of the ICBSA Conference Committee.



protected plant species. Genetic based conservation effort is needed to avoid species extinction from its wild habitat.

Information about molecular aspects of pasak bumi in Indonesia has been lacking. Previous research showed that pasak bumi maintain a moderate diversity [6, 7]. Research on population genetics, DNA isolation processes and reference markers have also been limited. Research on DNA isolation and reference markers that can be used for further molecular analysis therefore needed for underlying basic knowledge in establishing appropriate conservation effort of the species.

DNA quality was one of the significant factor to determine the successfulness of molecular analysis. The high quality and purity of DNA stock will be affecting amplification of DNA on Polymerase Chain Reaction (PCR) process. Different with herbaceous plant, many part of trees containing of some DNA inhibitor such as polysaccharides, tannin, phenol and other secondary metabolites substances. These substances will inhibit DNA polymerase activity during PCR process [8].

Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants and finally recovery of the DNA. CTAB (*Cetyl Trimetyl Ammonium Bromide*) method was used in this research. The method was generally used as choice for extraction of plant material with high polysaccharides and other inhibitory substances and has been used successfully for some higher plant and forest trees [9].

*Internal Transcribed Spacer (ITS)*, maturase K (*matK*), *trnL-trnF* intergenic spacer and ribulose-1, 5-bisphosphate carboxylase oxygenase (*rbcl*) was used in this research as standard marker for barcoding. The utilization of this three chloroplast DNA regions and spacer due to the high ability for determining plant species and producing high quality sequences [10]. The chloroplast genome also non recombining inheritance unit, single, have stable structure and primarily maternally in angiosperms [11, 12]. ITS coding regions have a critical role in the development of functional ribosomal RNA, with sequence variations among species showing promise as signature regions for molecular assays. Therefore, the objective of this research were optimal DNA isolation using CTAB methods and reference marker for further used in various molecular studies.

## 2. Material and Methods

Ten fresh leave samples of pasak bumi were collected from Padang Lawas, North Sumatra. All samples stored in plastic bags containing silica gel and kept at room temperature until DNA extraction. CTAB method [13] was using for extracting total genomic DNA of pasak bumi leaf. To check of DNA quality solution stock, 5  $\mu$ L of DNA combined with 2  $\mu$ L of loading dye was running on 5% agarose gel using horizontal electrophoresis. For marker, 1000 and 5000 bp DNA ladder from SERVA DNA was used. The gels were stained with GelRed. Visualization of DNA product was conducted by using an ultraviolet light chamber (GelDoc-It, UVP Imaging system).

Four barcoding standard marker those were ITS, *mat K*, *rbcL* and *trnL-trnF* were used in this research (Table 1). The PCR was performed using solution containing 20  $\mu$ l (10 ng) genomic DNA pasak bumi, forward and reverse primer (5 p mol) and Go Taq® Hot Start Colourless Master Mix (10  $\mu$ l).

TABLE 1: Primer used in this study.

No	Primer	Sequence	References	Length	$t_m$
1	<i>rbcLa-f</i>	ATGTCACCACAAACAGAGACTAAAGC	[14]	1289 bp	56°C
	<i>rbcLa-r</i>	GTAAAATCAAGTCCACCRCG	[14]	1289 bp	56°C
2	<i>trnLc-f</i>	CGAAATCGGTAGACGCTACG	[15]	447 bp	50°C
	<i>trnLf-r</i>	ATTTGAACTGGTGACACGAG	[15]	447 bp	50°C
3	ITS 1	TCCGTAGGTGAACCTGCGG	[16]	565-613 bp	50°C
4	<i>matK-1RKIM-f</i>	ACCCAGTCCATCTGGAAATCTTGGTTC	[17]	889 bp	50°C
	<i>matK-1RKIM-r</i>	CGT ACA GTA CTT TTG TGT TTA CGAG	[17]	889 bp	50°C

The process of PCR consists of four step those are initial denaturation, denaturation, annealing and final extension. Initial denaturation was conducted at temperature 95°C for 2 min. Denaturation step was perform at temperature 95°C for 1 min, while annealing step was performed at temperature 52°C followed by polymerization at 72°C for 2 min. The last step was final extension which performed at 72°C for 7 min.

## 3. Result and discussion

### 3.1. DNA isolation

DNA isolation methods should ideally be straightforward, quick, efficient, and reproducible while minimizing the potential for cross-contamination. It should also be suitable for extracting multiple samples and generate minimal risk for the operator. Safety, time

and costs are also main considerations [18]. Some species are characterized by a high content of tannins, alkaloids, and phenols in their leaves. These secondary metabolites are released during DNA extraction and influence polymerase chain reaction. It became problem of DNA extraction for some tree species [19].

Leaf of pasak bumi contain some phenolic and terpenoid compound [20]. Polyphenols released from the vacuoles during the cell lysis process are oxidized by cellular oxidases and undergo irreversible interactions with nucleic acids causing browning of the DNA [21]. Residual polyphenols, polysaccharides and secondary metabolites interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases [22]. To avoid the oxidative effect of polyphenols, the extraction buffer should contain anti-oxidant agents, such as PVP (*polyvinylpyrrolidone*), BSA (*bovine serum albumin*), or  $\beta$ -*mercaptoethanol* [23].

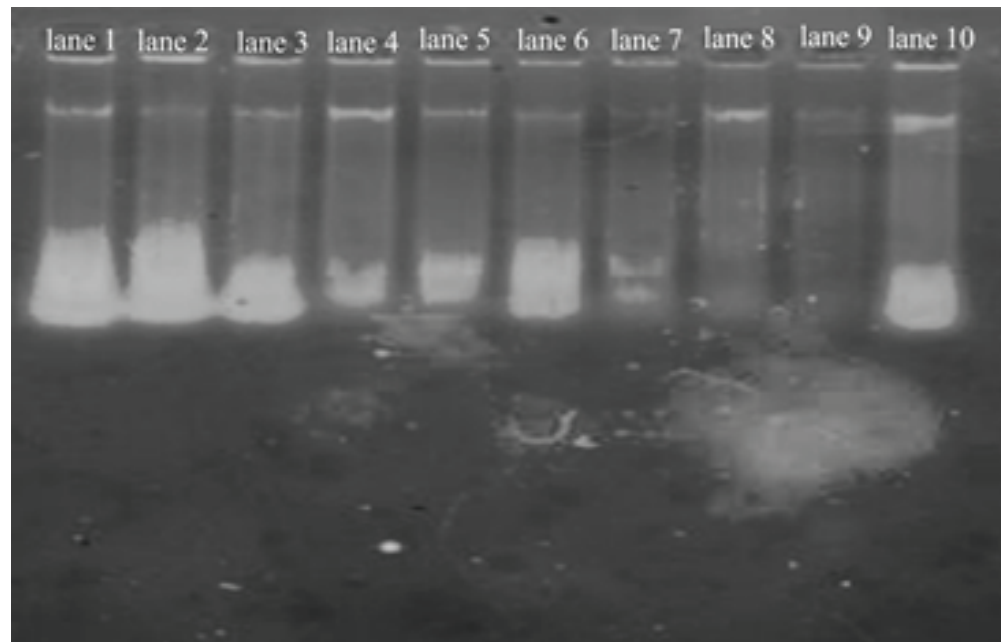
High quality DNA is characterized by DNA of high molecular weight, without contaminating substances, such as proteins, polysaccharides, phenolics, or other secondary metabolites. Result of DNA isolation using CTAB method showed that this method gave better DNA yield in terms of quality and quantity (Fig. 1).

Based on our research modified CTAB (*Cetyl trimethyl ammonium bromide*) protocol enables to produce good DNA yield. CTAB able to produce high quality genomic DNA amenable to RAPD (*Random amplified Polymorphic DNA*), restriction digestion, and amplification of plant barcode genes (*matK* and *rbcl*) with reduced cost [24]. The same result also found in *M. tenuiflora*, tannin producing trees [8].

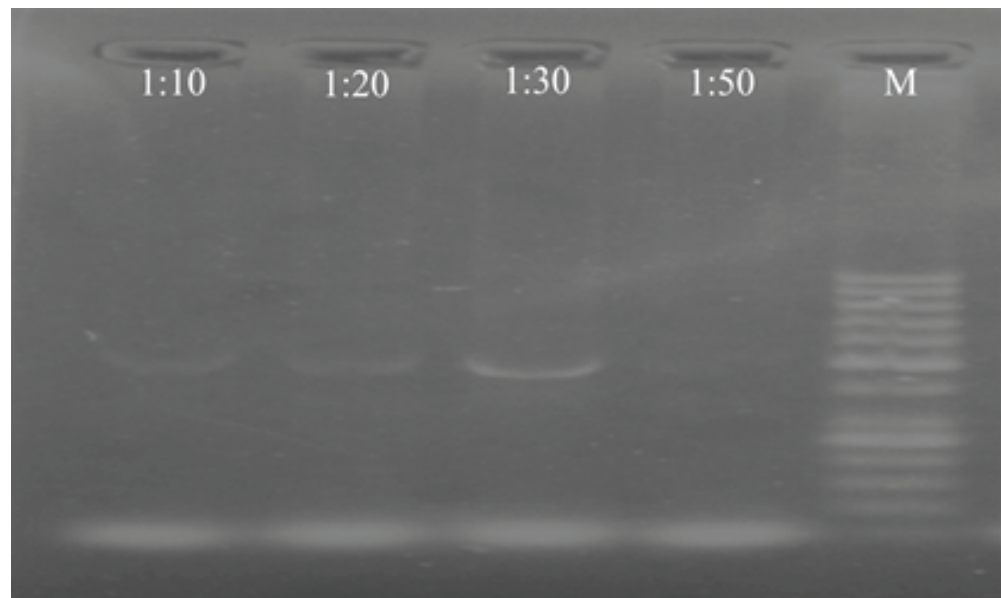
Several impurities from secondary metabolites substance, protein, tannins, phenol and polysaccharides often present on stock solution despite some process for elucidate the substance. For standardized dilution for amplification, we tested four dilution ratio, those were 1:10, 1:20, 1:30 and 1:50. Moderate dilution (1:30) produce the best band for our four DNA primer pairs (Fig. 2). At 1:3 dilution, PCR product showed more uniform for all samples. At lower dilutions of DNA (e.g. 1:10, 1:20), the PCR product still unclear and it probably caused by the high concentration of inhibitor enzyme. Another reason was the high proportion of DNA in PCR product so that the free-nucleotides and primer in not sufficient quantity for completing PCR reaction [25].

### 3.2. PCR amplification

Chloroplast and ribosomal-genomic target sequence were selected to test the suitability of genomic DNA extracts for sequence amplification in polymerase chain reactions



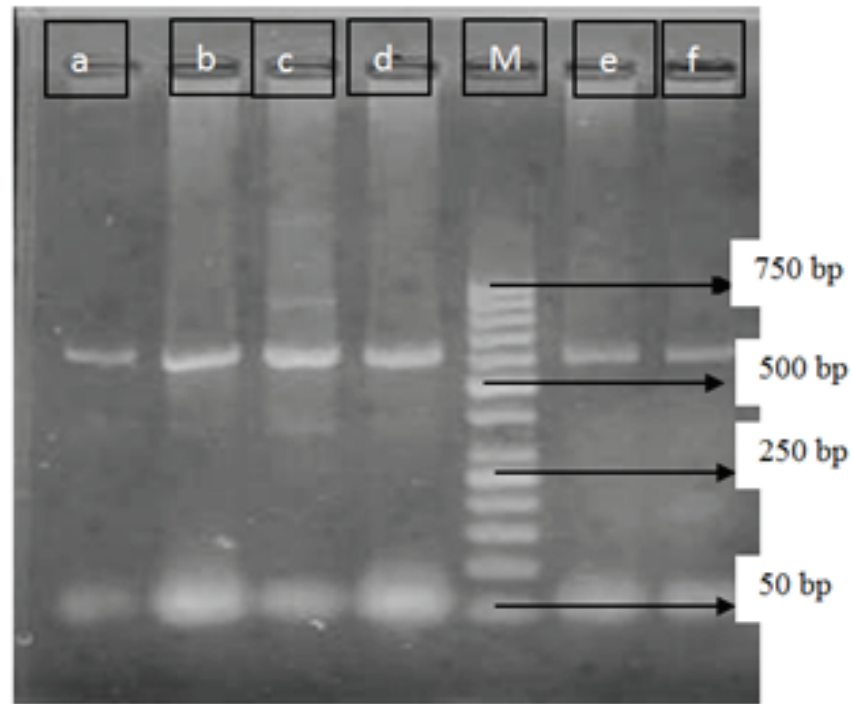
**Figure 1:** Genomic DNA isolated from individual pasak bumi leaf using CTAB method under 1% of Agarose Gel.



**Figure 2:** DNA dilutions testing on PCR product.

(PCR). The four selected sequence were ITS, *MatK*, *rbcl* and *trnL-trnF*. In this study, the amplified of PCR product was determined. ITS, *rbcl* gene and *trnL-trnF* regions of DNA isolated from pasak bumi were amplified by the PCR reactions (Fig. 3).

ITS, *rbcl* and *matK* sequences commonly used for plant barcoding [26, 27] and phylogenetic reconstruction [28]. The ability of primer on DNA amplification on PCR process was determines by primer-template mismatches at the 3' end of the primer sequence [29]. Our research showed appreciable success in amplification of ITS, *rbcl*



**Figure 3:** Amplification pattern of *rbcl* T annealing 56°C (a,b), *rbcl* T annealing 55°C (c,d), *trnL-trnF* T annealing 50°C(e,f), M:marker.

gene and *trnL-trnF* regions. This result was in accordance with [30] which stated that, in many cases the positive amplification *rbcl* but negative of *matK* might be also caused by lower DNA quality and quantity. The other explanation was due to the variation of binding site of *matK* gene. Similar case also occurred in fern using *matK* sequences [31]. The problem on *matK* amplification also reported on *Styrax* in North Sumatra [32]. The successfulness of amplification using *trnL-trnF* have been reported in previous research for *Aquilaria*, *Styrax sumatrana*, *Shorea*, *Taxus sumatrana*, and *Dipterocarpus* [33–37]. Furthermore, Fig. 4, showed good isolation genomic DNA which resulted in good amplification during PCR process would yield good sequence.

#### 4. Conclusion

The result showed that CTAB method was able to yield good quality DNA and 30% dilution produced the best band and further selected for PCR process. PCR amplification found that only ITS, *rbcl* and *trnL-trnF* confirmed produce the best result and can be utilized for further molecular analysis.

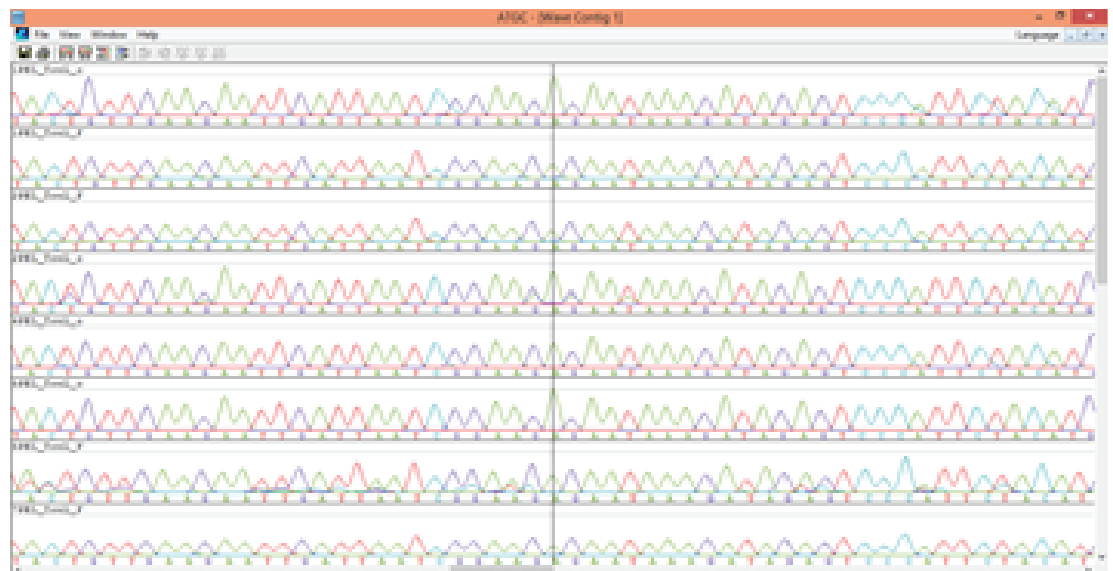


Figure 4: Sequencing result for *trnL-trnF* region of pasak bumi.

## Acknowledgement

We are grateful to Dr. Fifi Gus Dwiyantri for her laboratory assistance. This work was part of Pasak bumi research funded by TALENTA USU 2018 through the Grant ID-2590/UN5.1.R /PPM/2018.

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