Isolation and genomics DNA amplification of Kapur (Dryobalanops sumatrensis) from North Sumatra

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Abstract. Kamper is one of high value tree species from North Sumatra. Its timber has high economic value and has been targeted for exploitation since decades. Identification of species diversity and further their origin and the evolutionary process is important in the conservation strategy of the targeted species. The aims of this research were to get the information about optimal isolation of DNA genomics procedure and primer amplification for Kamper species by applying various primer tested, those were: ITS 2, matK Kim 1, trnH-psbA, trnL-trnF (c and d), trnL-trnF (c and f) and rbcL. The DNA extraction was conducted by CTAB method with some mild modifications to a few stages. The result showed that the modified CTAB method could yield good quality DNA. rbcL with the primer volume as much F: 1 µl and R: 1 µl produces the best amplified band.

1. Introduction

Champor, Borneo camphor, Sumatran camphor or kapur was locally named for Dryobalanops sumatrensis J.F. Gmelin was one of valuable wood and resin producers in Indonesia. The species was distributed in part of lowland dipterocarp forests of Malaysia, Sumatra, including Riau archipelago, and Borneo [1]. Sumatran camphor was a famous product and has a long history in the international market since 2nd century [2]. Since this product is very expensive more than gold value, Sumatran camphor has become targeted species. Destructive harvesting for crystal camphor which resided in parenchyma cell [3], recalcitrant seed character [4], lower natural regeneration [5] and land conversion for palm oil [6] in North Sumatera contributed to the decreasing of the population. Based on the remnant population, IUCN classifies this species into Vulnerable [7]. It means that a rapid effort is needed to conserve this species.

The disturbed forest area of D. sumatrensis will increase the selfing rate, and an intensive genetic drift has occurred in all of the individuals [8]. Conservation efforts with genetic bases can be done to avoid these species from the extinction. Information about the molecular aspects of D. sumatrensis in Indonesia is still quite limited. Researches on population genetics, DNA isolation processes, and reference markers have also not been obtained. So we need basic research on DNA isolation and reference markers that can be used for further molecular analysis.
DNA isolation is the basic step in the molecular analysis. High purity and quantities of DNA will determine the result of Polymerase Chain Reaction (PCR) process. DNA isolation of tree species is rather difficult than herbaceous species due to the occurrence of polysaccharides, phenols and tannins that can inhibit the activity of DNA polymerase during PCR [8]. This PCR inhibitors must be eliminated to obtain a good quality of DNA.

Some DNA isolation techniques have been developed by previous researchers. But, the most general method for isolation microbes, plant, and animal is CTAB (Cetyl Trimethyl Ammonium Bromide). The successfulness of DNA isolation using CTAB method for higher plant has been reported by some previous researchers [9–11], but there is no report for *D. sumatrensis*. As known that this species contains some secondary metabolites substance such as borneol so that an appropriate method for DNA isolation is still needed.

Five barcoding standard marker, which were ITS 2, *mat* Kim 1, *rbcL*, *trnH-psbA*, *trnL-trnF* (c and d) and *trnL-trnF* (c and f), were used in this research. The five chloroplasts region was chosen in this research because it has been successfully amplified in some dipterocarp species [12–15]. ITS 2 was generally used for taxonomical study showing promise as signature regions for molecular assays. Therefore, the objective of this research was to get information on the successfulness of DNA isolation using CTAB methods and its amplification using barcode gene.

2. Materials and methods

Ten fresh leaves samples of kapur were collected from five natural populations in Aceh and North Sumatra. All samples were stored in plastic bags with silica gel and kept at room temperature until DNA extraction. Leaves samples were dried with silica gel and used for DNA extraction. Total genomic DNA was extracted from leaf samples using a modified CTAB method [16]. For quantification of the stock DNA solution, we run horizontal electrophoresis in 1.5% agarose gel with 2 μL of DYE (loading buffer) combined with 5 μL of DNA. SERVA DNA Standard 1000 and 5000 bp DNA ladder were used. After 50 minutes at 100 V, the gels were stained with GelRed and visualized in an ultraviolet light chamber (GelDoc-It, UVP Imaging system).

Sixth barcoding standard markers which were ITS 2, *mat* Kim 1, *rbcL*, *trnH-psbA*, *trnL-trnF* (c and d) and *trnL-trnF* (c and f) were used in this research. The five chloroplasts region was chosen in this research because it has been successfully amplified in some dipterocarp species [12–15]. ITS 2 was generally used for taxonomical study showing promise as signature regions for molecular assays. Therefore, the objective of this research was to get information on the successfulness of DNA isolation using CTAB methods and its amplification using barcode gene.

| No. | Primer | Sequence | t<sub>m</sub> | Reference |
|-----|--------|----------|-------------|------------|
| 1   | *rbcL*-F | ATGTCCACAAAAACAGAGACTAAAGC | 56 °C | [17] |
|     | *rbcL*-R | GTAAAAATCAATCCACCRGC | 56 °C | [18] |
|     | *rbcLajf634R* | GAAACGGGCTCTCACAAGCAT | 56 °C | [19] |
| 2   | *mat* K 1RKIM | ACCAGCTCCTACCTGGAAATCTTTTC | 50 °C | [20] |
|     | *mat* 3FKIM | CGTCAGTACTTGTGTTATCGAG | 50 °C | [20] |
|     | *mat* 390f | GATCTATTCATTCAATTTC | 50 °C | [21] |
| 3   | *trnL*-c (forward) | CGAAATCGGTAGACGCTACG | 50 °C | [22] |
|     | *trnL*-f (reverse) | ATTTGAACCTGCGACTCAGG | 50 °C | [22] |
| 4   | *psbA* 3_f | GTTATGCGATGACTGGCTTC | 50 °C | [23] |
|     | *trnH* 05 | CGCGCATGGGTGATTCCAATTCC | 50 °C | [23] |
| 5   | *ITS*-DINO | GTGATTTGCAAGACTCCGTTG | 50 °C | [24] |
|     | ITS2Rev2 | CCTCGGCTTCTATTATGTT | 50 °C | [25] |
3. Result and discussion

3.1. DNA isolation
Kapur leaves contain some secondary metabolites, those were 35% terpenoid compound, 10% alcohol, 20% sesquiterpene and 35% resin [26]. Eighty-three chemical components were found in extracted kapur leaves [27]. 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 [28]. Residual polyphenols, alkaloid, polysaccharides and secondary metabolites interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases [29]. The presence of polysaccharides has been shown to inhibit Taq polymerase activity [30] and restriction enzyme activity [31]. Higher concentrations of CTAB and the addition of antioxidants such as polyvinyl-pyrrolidone (PVP) and β-mercaptoethanol to the extraction buffer can help to remove phenolics in DNA preparations from plants.

The result of DNA isolation using CTAB method shows that this method gives better DNA yield and satisfactory result (figure 1). Based on our research, modified CTAB (Cetyl Trimethyl Ammonium Bromide) protocol enables to produce good DNA yield. Several researches have reported on secondary metabolites tree producer like M. tenuiflora, tanin producing trees [32], Dimorphandra mollis, high polyssacharide content [33] and Artemesia annua, antimalaria plant [30].

![Figure 1. Genomic DNA isolated from individual kapur leaves using CTAB method under 1% of Agarose Gel.](image)

Despite our efforts to get a very high DNA purity, several impurities such as proteins, polysaccharides, phenol, tannins and salts were very probably present in the stock solution. For standardized dilution for amplification, we tested four dilution ratio; those were 1:10, 1:20, 1:30 and 1:50. DNA dilution before PCR we realized that a moderate dilution (1:30) would be optimal for all four DNA primer pairs.

At this dilution, the PCR products were uniform and showed the same intensity in all samples. At lower dilutions of DNA (e.g., 1:10, 1:20), very likely, the enzyme inhibitors still have a high concentration. Another plausible explanation is that the proportion of DNA is too high, and primers and free-nucleotides are not in a sufficient quantity for completing the reaction [34].

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 (PCR). The result showed that only rbcL with the primer volume as much F: 1µl and R: 1 µl produced the best-amplified band. The other primer gives discrete amplification and no amplification although several investigators have used ITS, rbcL and marK sequences for barcoding or species identification [35, 36] as well as for phylogenetic analysis [37].

The successfulness of DNA amplification using the barcode region of rbcL on Dipterocarp species has also been reported [38–40]. rbcL is easy to amplify, sequence, and align in most land plants and provides a useful backbone to the barcode dataset, even though it only has modest discriminatory power [41]. Furthermore, it is stated [42] that rbcL is suitable to barcode all of the tested Dipterocarpaceae species, whereas trnH-psbA could not be used alone for this purpose.
The discrete and fail of amplification using ITS 2, matK Kim 1, trnH-psbA and trnL-trnF might be caused by some factor such as inhibitor, length of primer, annealing temperature, genome size, and GC ratio. The polysaccharides and another substance in DNA were the common inhibitor in PCR process. The inhibitor will interact directly with a DNA polymerase to block enzyme activity. DNA polymerases have cofactor requirements that can be the target of inhibition. Magnesium is a critical cofactor, and agents that reduce Mg2+ availability or interfere with the binding of Mg2+ to the DNA polymerase can inhibit PCR [31]. Length of primer and annealed matches increase the specificity of the reaction, but it may not always be an authentic reason to get desired amplicons [43]. The choices of annealing temperature [44], template concentration and cycle number have been thought to have a significant effect on biases caused by selective amplification [45]. Primer mismatch is an inherent characteristic of PCR with ‘universal’ primers, while, owing to single nucleotide variability even in the evolutionarily highly conserved regions of the rRNA genes, the designation of a perfectly matching ‘universal’ primer is not possible [46]. Moreover, genomic properties such as genome size, copy number of 16S rRNA genes, and G/C content influence the PCR product ratios [47].

4. Conclusion
The result on DNA isolation using modified CTAB method gave a good yield of DNA kapur and dilution 30% produced the best DNA performance. Amplification using five barcoding markers showed a discrete and fail amplification by using ITS 2, matK Kim 1, trnH-psbA and trnL-trnF. Only rbcL with the primer volume as much F: 1µl and R: 1 µl produced the best-amplified band. The lower amplification result using ITS 2, matK Kim 1, trnH-psbA and trnL-trnF might be caused by the presence of inhibitor, inappropriate length of primer, annealing temperature, genome size and GC ratio.

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