An Optimized DNA Extraction Protocol For Isolation Of High Quality Genomic DNA From Camphor Containing Timber Tree Species, *Dryobalanops Beccarii* Dyer

Wei-Seng Ho, Kit-Siong Liew, Shek-Ling Pang

Abstract: Isolation of high-quality genomic DNA from Dryobalanops beccarii is obviously difficult due to the existence of large amounts of camphor and other secondary metabolites. These contaminants will co-precipitate with DNA during DNA isolation and purification processes, and therefore, resulting in a brownish DNA pellet that is unsuitable for downstream applications. Many DNA isolation protocols are available for various plant tissues; however these protocols are inefficient in yielding high-quality amplifiable genomic DNA especially from camphor containing timber tree species. A CTAB based protocol has been optimized for isolating genomic DNA from camphor containing timber tree species. Key steps include: 1) using 1% β-mercaptoethanol and 2% PVP 40 (Mr 40,000) in the extraction buffer; 2) sample incubation time, 40 minutes at 65°C, and 3) DNA precipitation at room temperature (25°C). The isolated DNA pellet was transparent colour and the purified genomic DNA is suitable for PCR amplification.

Key words: Genomic DNA, CTAB, Drynobanalops beccarii, Camphor, Secondary metabolites, PCR

The most essential principle in the modern molecular biology is the isolation of high-quality DNA in a reasonable amount. The presence of DNA degrading endonucleases, polysaccharides, polyphenolics and other secondary metabolites in the plant tissue makes the isolation of high-quality intact nucleic acids problematic [1, 2]. In general, specific reagents are required for removing secondary compounds during DNA isolation as plants produce different types of secondary compounds [3]. To date, most standard methods and technologies are available for genomic DNA isolation from various plant tissues. However, these protocols are inefficient in yielding high-quality amplifiable genomic DNA especially from camphor containing timber tree species, *Dryobalanops beccarii* Dyer. Camphor is a white crystalline bicyclic saturated terpene ketone compound with chemical formula $C_{10}H_{16}O$ with formal chemical name (IUPAC) 1,7,7-trimethyl-bicyclo(2,2,1)heptan-2-one. Other names such as 2-camphanone, bornan-2-one, caladryl and 2-bornanone also exists [4].

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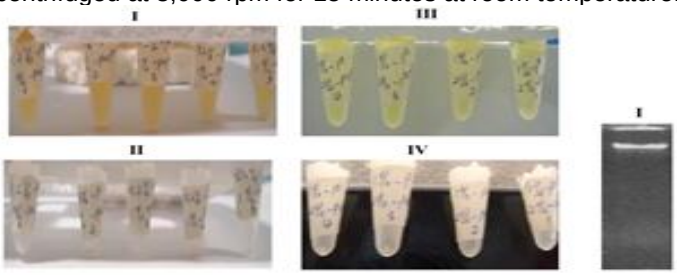
The physical-chemical properties of camphor includes having a pungent odour and taste that is flammable and volatile; melting at 176°C - 180°C, boiling at 204°C, and specific gravity 0.99. It is insoluble in water but soluble in ethanol, ethylether, turpentine, and essential oils [5]. The biosynthesis of camphor involved cyclisation of linaloyl pyrophosphate from geranyl pyrophosphate to becoming bornyl pyrophosphate, followed by hydrolysis to borneol and oxidation to camphor. *Dryobalanops beccarii* or locally known as Kapur Bukit is a moderately heavy timber species of the Dipterocarpaceae family (Fig. 1). It is mainly found in South East Asia, Sumatra and Borneo including Sarawak, Brunei, Sabah and East Kalimantan [6]. It produces large amounts of camphor, one of the polyphenolics found naturally in cavities or fissures in the wood or leaves of the camphor trees either in the form of solid camphor or a light fluid called camphor oil [7]. In oxidized form, phenolic compounds irreversibly bind to protein and nucleic acids [8] and, the isolated DNA becomes unsuitable for downstream applications [9]. Camphor has been medicinally used against coughs, asthma, headache, pains in the stomach or liver and diseases in the urino-generative system as well as against ulcers in mouth and nose, rheumatism, burns and wounded eyes. Kapur Bukit is an important source of quality wood for construction particularly in plywood production, furniture, joinery, beams, toys and decking. In addition, it also can be used for bridges, ship building, vehicle bodies, and railway sleepers [6]. Recently, this species has suffered a massive population reduction due to the human activities and natural catastrophe such as deforestation, pollution, industrial and urban development, and global greenhouse effects [10]. Conservation of forest tree genetic resources is important as it provides a means for breeding, reintroduction programmes or as insurance against possible extinction of species in the wild. According to PROSEA [6], conservation of genetic diversity of forest tree species should be realized *in situ* but occasionally *ex situ* conservation also can play an important role [10, 11]. However, it is difficult to conserve tree species as long as logging is done at trade group level and little attention is paid to inventorying stands of individual species.

(a) (b)

Fig. 1: *Dryobalanops beccarii*. (a) 4-month old seedling, and (b) Leaf.

A CTAB-based DNA extraction method [13,14,15] was proved as an inefficient DNA isolation protocol for *D. beccarii* as the DNA pellet obtained was in brownish colour during precipitation (Fig. 2A). Thus, the original protocol was re-optimized to obtain high-quality genomic DNA from *D. beccarii* with as little as 0.5 g of fresh leaves as starting material. To overcome this problem, we optimized the β-mercaptoethanol (0.2%, 1%, 2%, 3%, 4% and 5%) and PVP 40 (Mr 40,000) (1%, 2%, 3% and 4%) concentrations which are functioning for removal and prevent oxidation of camphor during DNA isolation. In addition, incubation period of sample at 65°C and DNA precipitation temperature were tested. In this report, we described an optimized CTAB-based protocol that consistently yields high-quality amplifiable genomic DNA from *D. beccarii*. DNA extraction solutions were prepared according to the modified extraction method by Doyle and Doyle [13]. 0.5 g of leaf tissue was ground by adding liquid nitrogen until fine powder before transferred into a 50 ml Falcon tube contained 4 ml of preheated CTAB extraction buffer with 40 µl β-mercaptoethanol. The buffer must be preheated to 65°C in order to inactive any nuclease enzyme activities in the ground plant tissues. Temperature above 60°C is effective in terminating any nuclease enzyme activity since the enzymes lose their functioning structure at 60°C. The mixture mixed gently before incubated at 65°C for 40 minutes with occasionally shaking. 750 µl of mixture was transferred into a 1.5 ml centrifuge tube and extracted with same volume of chloroform: isoamylalcohol (24:1) and subsequently centrifuged at 8,000 rpm for 15 minutes at room temperature.



(A) (B)

Fig. 2: (A) Supernatant and DNA pellet. (I) Brownish colour of supernatant. (II) Brownish colour of DNA pellet. (III) Yellowish colour of supernatant. (IV) Transparent colour of DNA pellet. (B) Gel electrophoresis of genomic DNA using a 0.8% agarose gel.

The supernatant was recovered and precipitated by addition of 0.6 volume of cold isopropanol, followed by incubation at room temperature overnight. The DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed with 1 ml cold wash buffer and left on ice for 30 minutes before centrifuged at 12,000 rpm for 10 minutes. The DNA pellet was air-dried and re-suspended in 20 µl of TE buffer. The DNA solution was topped up to a volume of 300 µl with TE buffer. RNase A (0.2 µg/µl) was added into the DNA mixture with a final concentration of 20 µg/ml before incubated for an hour at 37°C. An equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added into the tube and mixed gently by inverting the tube for 15 times before centrifuged at 10,000 rpm for 15 minutes. The aqueous phase was transferred into a new 1.5 ml centrifuge tube and 7.5 M ammonium acetate was added to a final concentration of 2.5 M. Then, 2 volumes of cold isopropanol was added and stored at room temperature overnight. The tube was centrifuged at 4°C, 13,000 rpm for 15 minutes and the supernatant was discarded. 1 ml of 70% ethanol was added, gently mixed and stored at -20°C for 30 minutes. Then, centrifuged at 13,000 rpm for 10 minutes and discarded the supernatant. The pellet was air-dried at room temperature for 15 minutes. TE buffer or ddH_2O was added to dissolve the pellet and stored at -20°C until further analysis. The DNA quality was evaluated based on the colour of DNA pellet obtained, 0.8% agarose gel electrophoresis and RAPD-PCR. The distinct DNA band was obtained as shown in Fig. 2B. As shown in Fig. 2A (IV), 1% β-mercaptoethanol and 2% PVP 40 (Mr 40,000) were investigated as the optimal concentration due to the DNA pellet obtained was transparent colour. PVP was added and forming a complex with phenolic compounds through hydrogen bonding and co-precipitate with cell debris upon lysis [16]. The PVP complexes accumulated at the interface between the organic and aqueous phase during centrifugation in the presence of chloroform [17]. However, the presence of high-molecular weight PVP 40 (Mr 40,000) with large amounts, for instance 3% PVP 40 (Mr 40,000) or higher as observed in this study could be present as contaminant due to co-precipitate with nucleic acids. In addition, inclusion large amounts of PVP often requires lengthy ultracentrifugation and also incompatible with phenol extraction [18, 19]. A small amount of leaf tissue (0.5 g) was used in 4 ml of extraction buffer due to the lower possibilities of contaminants to co-precipitate with DNA. The ratio of extraction buffer to leaves should always be 4:1 (v/w) or greater in order to obtain sufficient amount of clean DNA [17]. In order to obtain good quality DNA, tissue powder was added as rapidly as possible to the pre-heated CTAB extraction buffer contained β-mercaptoethanol followed by incubation at 65°C for 40 minutes [16, 17]. A long time of sample incubation making the solution of tissue powder turned to the yellowish color and resulted in yellowish or brownish color of DNA pellet. Therefore, the sample incubation time was reduced from 60 minutes to 40 minutes. DNA precipitation condition is also crucial in obtaining high quality of DNA [16]. DNA was precipitated at room temperature instead of -20 °C. This is because the color of DNA pellet obtained at room temperature was better than DNA precipitated at -20 °C. However, precipitation overnight at 25 °C resulted in a reduction of total DNA yield [16]. The DNA yields obtained were ranged from 230 to 340 ng/µl per 0.5 g of leaf tissues. A feasible explanation of this phenomenon is in the room temperature precipitation, which diminish the possibility of shorter nucleic acids to precipitate [20]. The quality of DNA was further verified by RAPD-PCR (Fig. 3). OPI-12 primer was selected for RAPD-PCR analysis. This primer produced scorable and informative DNA bands compared to other primers; namely OPZ-07, OPJ-19 and OPD-07 (Operon Technologies, Almeda,

CA, USA) (Fig. 3(A)). PCR was performed using a Mastercycler Personal PCR (eppendorf, Germany). The thermal cycling profile was 1 cycle of 2 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 2 min at 37 °C and 2 min at 72 °C, then a final extension step of 10 min at 72 °C. The PCR products were electrophoresed using a 1.2% agarose gel. The distinct amplification of genomic DNA was detected at the molecular weight ranged from 350 bp to 750 bp (Fig. 3(B)). In summary, the development of an efficient protocol for the isolation of amplifiable genomic DNA from *D. beccarii* rich in camphor was successfully accomplished. The re-optimized protocol gave high-quality of DNA as indicated in this study whereby the pellet obtained was transparent colour and amplifiable by RAPD-PCR.

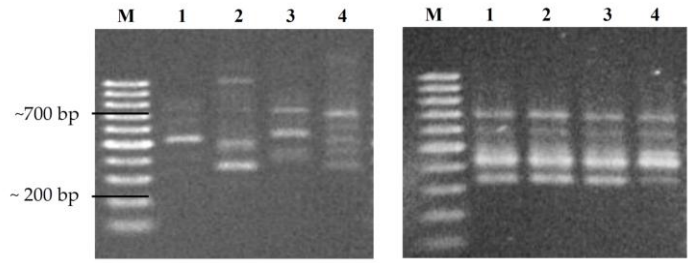


Fig. 3: (A) Gel electrophoresis of RADP profiles on 1.2% agarose gel generated by RAPD primer; lane 1: OPZ-07, Lane 2: OPJ-19, Lane 3: OPD-07 and, Lane 4: OPI-12. (B) Gel electrophoresis of RAPD-PCR amplification products amplified with primer OPI-12 on 1.2% agarose gel. Lanes 1-4: Genomic DNA isolated from *D. beccarii* leaves M: 100bp DNA ladder.

ACKNOWLEDGEMENTS

The authors would like to thank all the laboratory assistants and foresters involved in this research programme for their excellent field assistance in sample collection. This work was part of the joint Industry-University Partnership Programme, a research programme funded by the Sarawak Forestry Corporation (SFC), Sarawak Timber Association (STA), Ministry of Higher Education, and Universiti Malaysia Sarawak (UNIMAS) under grant no.: 02(DPI09)832/2012(1), RACE/a(2)/884/2012(02) and GL(F07)/ 06/2013/STA-UNIMAS(06).

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