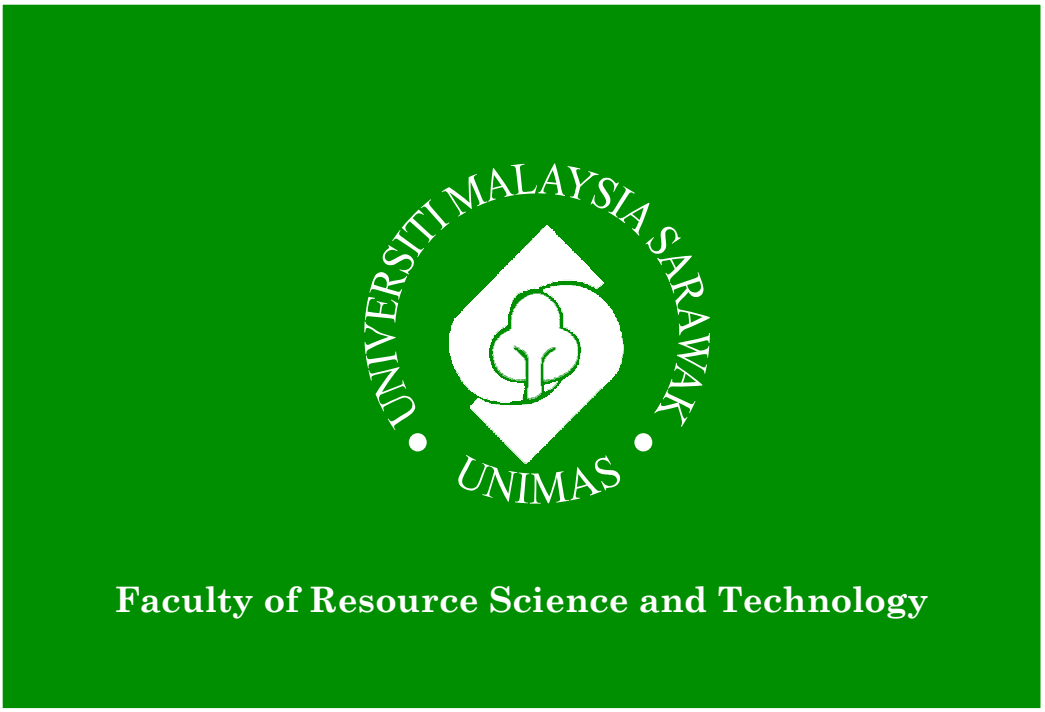

ISOLATION OF HIGH QUALITY GENOMIC DNA FROM *DRYOBANALOPS BECCARII* DYER TISSUES RICH IN CAMPHOR

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LIST OF ABBREVATIONS

CIA	Chloroform-Isoamyl Alcohol
CTAB	Cetyltrimethylammonium Bromide
ddH_2O	Double Distilled Water
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
EtBr	Ethidium Bromide
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
PCI	Phenol: Chloroform: Isoamyl alcohol
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrilodone
RAPD-PCR	Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
TBE	Tris-borate-EDTA
TE	Tris-EDTA buffer
UV	Ultraviolet

ABSTRACT

The isolation of high-quality intact genomic DNA is obviously difficult in a variety of plant species due to the presence of high levels of polysaccharides, polyphenolics 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. Most plant genomic DNA isolation protocols for various plant tissues have been established; however these protocols are inefficient in yielding high-quality amplifiable genomic DNA especially from camphor containing timber species, *Drynobanalops beccarii* Dyer. In this project, the total genomic DNA of *Drynobanalops beccarii* was successfully isolated using a re-optimized CTAB method based on the Murray & Thompson (1980) and Doyle & Doyle (1990). The modifications include: 1) using 1% β-mercaptoethanol and 2% PVP (Mr 40000) 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 in transparent colour and yielded amplifiable genomic DNA for RAPD-PCR.

Key words: genomic DNA isolation, CTAB, *Drynobanalops beccarii* Dyer, camphor, RAPD-PCR.

ABSTRAK

Pemencilan DNA genomik yang berkualiti tinggi dan sempurna daripada pelbagai spesis tumbuhan adalah sukar disebabkan oleh kehadiran polisakarida, poliphenolik dan metabolik sekunder yang lain. Bahan-bahan cemar ini akan termendak bersama-sama dengan DNA semasa proses pemencilan dan penulenan DNA, oleh itu, menghasilkan pellet DNA yang berwarna perang bahawa tidak sesuai untuk applikasi-aplikasi lanjutan. Kebanyakan kaedah pemencilan DNA genomik daripada tisu pelbagai tumbuhan telah dibangunkan; walau bagaimanapun kaedah-kaedah ini tidak berkesan untuk menghasilkan DNA genomik yang berkualiti tinggi serta kebolehamplifikasian terutamanya daripada spesis pokok kapur, Drynobanalops beccarii Dyer. Dalam projek ini, DNA genomik daripada Drynobanalops beccarii telah berjaya dipencilkan mengunakan kaedah CTAB yang telah dioptimalkan berdasarkan kaedah Murray & Thompson (1980) dan Doyle & Doyle (1990). Pengubahsuaian ini adalah termasuk: 1) mengunakan 1% β-mercaptoethanol dan 2% PVP (Mr 40000) dalam larutan pemencilan, 2) masa sampel pengeraman, 40 minit pada 65°C; dan pemendakan DNA pada suhu bilik (25°C). Pellet DNA yang telah dipencilkan adalah berwarna lutsinar dan kebolehamplifikasian melalui RAPD-PCR.

Kata kunci: pemencilan genomik DNA, CTAB, Drynobanalops beccarii Dyer, kapur, RAPD-PCR.

CHAPTER I

INTRODUCTION

Forest trees are very important worldwide and comprise more than 4000 million hectares of land or about one-third of the earth's land surface which 58% of the total forested area is found in tropical countries. As a source of renewable materials, forest trees provide industrial wood products, energy (fuelwood), food, medical products, fibers, resin and etc in order to provide employment opportunities, contribution in revenues and foreign exchange to the State Government. Besides that, forests are also extremely important in ecologically, land and water resources protection, climatic regulation and habitats for wildlife conservation (PROSEA, 1994).

Dryobalanops beccarii Dye*r* or locally known as Kapur Bukit is a moderately heavy timber species of the Dipterocarpaceae family. It is an important source of quality wood for construction purpose particularly in plywood production, furniture, joinery, beams, toys and decking. Besides, it also can be used for bridges, ship building, vehicle bodies and railway sleepers (PROSEA, 1994).

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. In general, two forms of conservation of genetic diversity of forest species have been reported, i.e., 1) *Ex situ* conservation means conservation of genetic resources outside the origin habitats in the form of desiccation, field gene banks, clonal collections, tissue or cell culture and

cryopresevation, and 2) *In situ* conservation which involved maintenance and regeneration of species in their original or natural habitats (Adams & Adams, 1992). According to PROSEA (1994), conservation of genetic diversity of forest species should be realized *in situ* but occasionally *ex situ* conservation also can play an important role. Conservation of forest genetic resources is important as its provide a means for breeding, reintroduction programs or as insurance against possible extinction of species in the wild (Adams & Adams, 1992). 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 (PROSEA, 1994).

The isolation of high-quality intact nucleic acids from tree species is crucial for molecular biology applications including Polymerase Chain Reaction (PCR) amplifications, endonuclease restriction digestion, Southern blot analysis and genomic library construction. 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 (Chakraborti *et al*., 2006; Mischiels *et al*., 2003). In general, specific reagents are required for removing secondary compounds during DNA isolation as plants produce different types of secondary compounds (Loomis, 1974).

In this study, a CTAB DNA isolation protocol (Murray and Thompson, 1980; Doyle and Doyle, 1990) was used to isolate genomic DNA from leaf tissues of *D. beccarii* rich in camphor. However, there is an inefficient DNA isolation protocol for *D. beccarii* as the DNA pellet obtained was in brownish colour. Camphor is one of the phenolic compounds which found naturally from the wood or leaves of camphor

trees. In oxidized form, phenolic compounds irreversibly bind to protein and nucleic acids (Loomis, 1997) and, the isolated DNA becomes unsuitable for downstream applications (Porebski *et al*., 1997).

Currently, a few studies have been carried out on the DNA isolation from timber trees. However, most of the published findings are primarily focused on non-timber species that rich in phenolic compounds such as *Lycopersicon esculentum* (Peterson *et al*., 1997), Cichorioideae plants (Michiels *et al*., 2003), fruit trees and conifer (Kim *et al*., 1997) and Cotton (Permingeat *et al*., 1998).

Thus, the objective of this study is to develop an efficient protocol for the isolation of amplifiable genomic DNA from *Dryobalanops beccarii* Dyer tissues rich in camphor.

CHAPTER II

LITERATURE REVIEW

2.1 Selection of Species Studied

2.1.1 Family Dipterocarpacae

Family Dipterocarpacae comprises of more than 500 species in about fifteen genera, which is divided into three subfamilies, Dipterocarpoideae, Monotoideae and Pakaraimoideae. Their distribution is span tropical from northern South American to Africa, Seychelles, Sri Lanka, Philippines, India, China, Thailand Indonesia and Malaysia (Gamage *et al*., 2006). There are four tribes of Asiatic subfamily Dipterocarpoideae: 1) Dipterocarpeae, 2) Dryobalanopseae, 3) Shoreae, and 4) Vaticeae (Ashton, 1982).

There are two genus under the Dipterocarpeae tribe which are, *Dipterocarpus* and *Anisoptera*. Dryobalanopseae consists of one genus which is *Dryobalanops*. Shoreae tribe consists of four genus, namely the *Hopea, Neobalanopcarpus, Shorea* and *Parashorea*. Vaticeae tribe consists of genus *Cotylelobium* and *Vatica* (Ashton, 1982).

Currently, the dipterocarps predominate the international tropical timber market and play an important role in the economy of many of the Southeast Asian countries (Appanah and Turnbull, 1998).

2.1.2 Genus *Dryobalanops*

According to PROSEA (1994), *Dryobalanops* consists of 7 species and is found in Peninsular Malaysia, Sumatra, Borneo and intervening islands. For the export trade, the species are combined under the name kapur (Miller, 1999). Other name such as Keladan, Kapoer (Indonesia) and Borneo camphorwood (Great Britain) also exists. Two species (*D. oblongfolia* and *D. aromatica*) occurs throughout this area and five others (*D. beccarii, D. fusca, D. keithii, D. lanceolata,* and *D. rappa*) are distributed to Borneo (PROSEA, 1994).

Genus *Dryobalanops* located at an intermediary position between tribe Dipterocarpeae and Shoreae. Grouped of resin canals, basic chromosome number = 7, and the thickness fruit sepal base support the placement of *Dryobalanops* within tribe Shoreae (Ashton, 1982; Dayanandan *et al*., 1999). On the other hand, the presence of solitary vessels and valvate fruit sepals indicates an affinity to the tribe Dipterocarpeae (Maury-Lechon and Curtet, 1998).

2.1.3 *Dryobalanops beccarii* Dyer

In Malaysia, *D. beccarii* is locally known as Kapur Bukit, Kapur ranggi (Sabah, Sarawak) and Kapur Merah (Sabah). Other name such as Kapur Sintuk and Kapur Keladan (Kalimatan) also exists. It is mainly found in South East Asia, Sumatra and Borneo including Sarawak, Brunei, Sabah and East Kalimantan. The tree is very large size, forms 100% of the canopy with trees 60m or more in height. The trunks are

mainly hollow and with a diameter of 1.5m to 2.0m with slightly tapering boles some 30m long above the buttresses (PROSEA, 1994).

The thickness of the sapwood is 4 - 8cm (CIRAD Forestry Department, 2003). The sapwood is whitish to yellowish brown but heartwood is reddish brown without streaks (Richter and Dallwitz, 2000; USDA Forest Service, n.d). The tree barks are shaggily, flaked, fissured and dark brown. The new bark is often in yellow-brown with small lenticels (Wong *et al*., 1993). The wood texture is moderately coarse (CIRAD Forestry Department, 2003), and there is an absent of growth ring (Richter and Dallwitz, 2000).

The leaves are ovate to lanceolate and the size is 5 - 8cm x 1.5 - 3cm with up to 17mm long acumen and glabrous. The fruit calyx lobes are up to 6.5cm x 0.8cm (PROSEA, 1994). Its calyx in ripe fruit is subvalvate (Maury-Lechon and Curtet, 1998). The grain is straight to shallowly interlocked (CIRAD Forestry Department, 2003). Furthermore, *D. beccarii* also produces camphor (PROSEA, 1994). The strong camphor-like smell is gathered after freshly cut down of the tree (USDA Forest Service, n.d).

(a) (b)

Figure 2.1 *Dryobalanops beccarii* Dyer. (a) 4-month old seedling, and (b) Leaf.

Figure 2.2 Matured tree of *Drynobanalops beccarii* Dyer.
(Retrieved from http://www.ibanorum.netfirms.com/santuflora-aug.htm)

D. beccarii often grows well as canopy tree in lowland dipterocarp forest, mixed peat-swamp forest and sometimes also in kerangas (heath forest) vegetation. It grows better in leached sandy soils on hills and ridges below 700m altitude, also along streams and in seasonal swamps (PROSEA, 1994). In general, the physical and mechanical properties are based on heartwood and can vary greatly depending on origin and growth conditions. The density of the wood is 600-710kg/m^3 at 15% moisture content (PROSEA, 1994). The physical properties consist of monnin hardness 4.1g/cm^3, coefficient of volumetric shrinkage 0.62%, total tangential shrinkage 9.1%, fibre saturation point 26% and is stable. For mechanical properties, crushing strength is 60 Mpa, static bending strength is 110 Mpa and modulus of elasticity is 16150 Mpa (CIRAD Forestry Department, 2003).

2.2 Plant Secondary Compounds

Plant cells produce a vast amount of secondary compounds (Stahl, 2006). Secondary plant compounds are biosynthetically derived from primary compounds, restricted to a particular taxonomic group (species, genus, family or closely related group of families) and synthesized in specialized cell types and at distinct developmental stages. Many of these compounds are highly toxic and often stored in specific vesicles or in the vacuole. Secondary compounds are frequently accumulated by plants in smaller quantities than primary compounds (Balandrin *et al*., 1985).

According to Peters & Crateau (2004), secondary compounds are defined as organic compounds with no essential role in growth and development but has been

rationalised by the observation that these natural products play important ecological roles in plant defence, allelopathy and tritrophic interactions as well as acting as attractants for pollinating and seed-dispersing animals.

Although secondary plant products are very common and nearly always found only in certain specific plant organs but some compounds restricted to single species (Stahl, 2006). According to Peterson *et al*. (1997), different plants species can produce vary in number and types of secondary compounds. The concentrations of these compounds vary between species which may contain as little as 1 per cent or as much as one-third of their dry weights as secondary metabolites; between tissues where higher amounts occur in bark, heartwood, roots, branch bases and wound tissues and, tropical and sub-tropical species typically contain greater amounts of extractives than do temperate zone woods. Variations also occur among species from tree to tree and from season to season (Obst, 1998).

Example of plants secondary products such as flavonoids, lignans, terpenes, phenols, alkaloids, sterols, waxes, tannins, sugars, resin acids and carotenoids with having a different of chemical, physical and biological properties (Obst, 1998). During the last 20 to 30 years, the analysis of secondary metabolites has progressed tremendously by using significantly various modern analytical techniques such as chromatography, electrophoresis, isotope techniques and enzymology (Stahl, 2006).

Plants secondary metabolites have long been and will continue to be important sources and models of spices, flavors and fragrances, vegetable oils, soaps, natural rubber, gums, resins, drugs, insecticides, and other industrial, medicinal, and

agricultural raw materials and consequent of economical value (Balandrin *et al*., 1985).

2.2.1 Camphor

The word camphor derived from the Malay word Kapur, meaning "camphor tree". 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 name such as 2-camphanone, bornan-2-one, caladryl and 2-bornanone also exists (Harrison, 2004).

The physical-chemical properties of camphor includes having a pungent odor 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 (Wickstrom, 1989). It is 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 (Shiva & Jantan, 1998).

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.

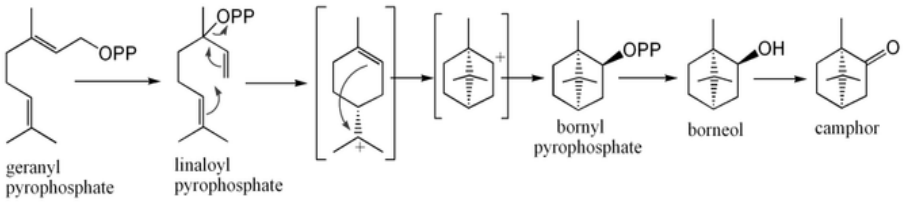

Figure 2.3 Biosynthesis of camphor.
(Retrieved from http://en.wikipedia.org/wiki/Camphor)

The camphor tree is felled, cut into blocks and split into wedges to remove the camphor. One hundred trees rarely yield more than 8-10kg solid camphor. In solid form it occurs in white crystalline translucent fragments, sometimes in long, 5kg pieces (Shiva & Jantan, 1998).

It has been medicinally used against coughs, asthma, headache, pains in the stomach or liver and diseases in the urinogenerative system as well as against ulcers in mouth and nose, rheumatism, burns and wounded eyes (PROSEA, 1994).

2.3 Isolation of Plant Total Genomic DNA

All molecular genetic screening techniques start with the DNA isolation from organism under study. The isolation of large quantities with highly pure intact nucleic acid is crucial for molecular biology applications including Polymerase Chain Reaction (PCR) amplifications, endonuclease restriction digestion, Southern blot analysis and genomic library construction (Mischiels *et al*., 2003). According to Puchooa & Venkatasamy (2005), DNA isolation is a time-consuming and expensive

component of molecular marker analysis which constituting about 30-60% of the total time required for sample processing. This becomes more difficult when working with plants species because they are rich in impurities, such as terpenes, polyphenols and polysaccharides. The presence of these secondary compounds will affect the yield and quality of isolated genomic DNA (Bhattacharjee *et al*., 2004).

As a consequence, many plants species require more complex DNA isolation method. A prerequisite for a successful DNA isolation protocol include: 1) requires only small amounts of plant material (Haymes, 1996), 2) quick, simple and cheap and if possible, 3) avoid the use of dangerous chemicals and, 4) yield adequate and intact DNA of reasonable purity (Puchooa, 2004). QIAGEN (2000) reported three main critical factors for extraction and purification of maximally undegraded and pure genomic DNA: 1) disruption of plant material, i.e. disruption of cell walls, plasma membranes and organelles membrane; 2) the composition of the grinding buffer and, 3) elimination of secondary metabolites. In addition, growth conditions of plants species such as plant age, light intensity, spectrum and duration, and stress (pathogen infection, nutrient deficiency, desiccation or wounding) is closely related to the production and accumulation of plant secondary metabolites. Most of the published genomic DNA isolation protocol recommended using healthy, fresh young tissue (Kim *et al*., 1997). Young tissue is preferred because containing more cells and lower secondary metabolites than the same amount of older tissue (QIAGEN, 2000).

Nowadays, many different methods and technologies are available for the isolation of plant genomic DNA. Basically, separation of DNA from cellular components can be divided into three stages; cell disruption and lysis of the starting

materials, removal of proteins and other contaminants, and finally recovery of the DNA. According to Puchooa (2004), DNA extraction protocol involves breaking or digesting away cell walls in order to release the cellular constituents. This is followed by disruption of the cell membranes to release the DNA into the extraction buffer and normally achieved by using detergents such as SDS or CTAB. The released DNA is protected from endogenous nucleases by including an EDTA in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases. The initial DNA isolates is often associated with large amount of RNA, protein, polysaccharides, polyphenolics and pigments which is difficult to separate and may interfere with the yield and quality of DNA. Therefore, denaturation and precipitation steps are required for initial isolated DNA by using different types of reagents such as chloroform and phenol. Finally, recovery of DNA is usually accomplished by precipitation with isopropanol or ethanol.

CTAB method has been used successfully in the laboratory on a wide taxonomic sampling of plant species such as *Lithci chinensis* Sonn. (Puchoo, 2004), *Cicer arietinum* L. (Chakraborti *et al*., 2006), *Pennisetum glaucum* (Zidani *et al*., 2005), *Trohetia boutoniana* (Puchoo and Venkatasamy, 2005) and Cichoriodeae plants (Michiels *et al*., 2003). The CTAB protocol originally published by Doyle and Doyle (1987) has been slightly modified (Krizman *et al*., 2006). These modifications were focused on PVP or activated charcoal or combination of both and β-mercaptoethanol (Krizman *et al*., 2006; Khan *et al*., 2007; Kim *et al*., 1997; Puchoo, 2004; Zidani *et al*., 2005; Puchoo and Venkatasamy, 2005) in order to remove and prevent oxidation of polyphenolics. High salt concentration in the extraction buffer has been used in polysaccharides removal (Fang *et al*., 2001). Other modifications

include reduction in the incubation time, wash in 80% ethanol, use of PVP 10 (Mr 10000) instead of PVP 40 (Mr 40000) (Puchooa, 2004); increase the concentration of CTAB (Khan *et al*., 2007; Syamkumar *et al*., 2003) and precipitate in ethanol instead of isopropanol (Syamkumar *et al*., 2003). These modifications is required since different plant taxa often may not permit optimal DNA yields from one isolation protocol (Padamalatha and Prasad, 2006) as different plant species produce different types of secondary compounds (Loomis, 1974).

2.4 Isolation of Total Genomic DNA from Species Containing High Levels of Phenolic Compounds.

Currently, a few studies have been carried out on the DNA isolation from timber trees. The published findings are primarily focused on DNA isolation from non-timber species rich in polyphenolic compounds. A new protocol has yielded polyphenol-free nuclear DNA from tomato (*Lycopersicon esculentu*) tissues and well suited in molecular biology studies was developed by Peterson *et al*. (1997). The developed protocol include, 1) utilizing fresh leaf tissues without freezing, 2), treating tissues with ethyl-ether in order to dissolves waxes and cutins that makes the DNA become more easily released from the cells, 3) including Triton X-10 to lyses chloroplast and mitochondria. The isolated DNA pellet was in clear or white colour. In addition, the DNA isolation protocol can be used to isolate genomic DNA from a variety of other plant species.