GENOTYPING OF *Eusideroxylon zwageri* Teijsm & Binn. AND *Potoxylon melagangai* Kosterm. (BORNEO IRONWOOD) USING DAMD MARKERS

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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ABSTRACT

Borneo Ironwood or Belian is the endemic hardwood timber in Borneo and Sumatra. There are two species of Belian, i.e. *Eusideroxylon zwageri* and *Potoxylon melagangai*. Both species are difficult to distinguish because of similar morphological characteristics. The detection and analysis of DNA polymorphisms in Belian is an essential component for species identification. The genotyping of Belian was carried using Direct Amplification Minisatellite DNA by PCR (DAMD-PCR) with two minisatellite primers: primer 33.6 (5’-AGGGCTGGAGG-3’) and primer YNZ-22 (5’-CTCTGGGTTGGTGC-3’). The optimum annealing temperatures for each species was optimized, i.e. *E. zwageri* (47.6°C) and *P. melagangai* (50.0°C) from primer 33.6; *E. zwageri* (56.0°C) and *P. melagangai* (51.4°C) from primer YNZ-22. The products of DAMD-PCR from both species of Belian showed a high degree of polymorphism. A total of five distinct diagnostic bands were successfully identified of each primer. However, only two of the diagnostic bands of each primer were excised and purified, i.e. 33.6 Z01 and 33.6 Z02 from *E. zwageri*; YNZ M01 and YNZ M02 from *P. melagangai*. All these purified PCR products were sent for DNA sequencing. However, the sequencing results were in weak signal due to the poor quality and impurities of DNA.

Key words: *Eusideroxylon zwageri*, *Potoxylon melagangai*, DAMD-PCR, primer 33.6, primer YNZ-22
ABSTRAK

Borneo Ironwood atau Belian adalah kayu balak endemik yang terletak di Borneo dan Sumatra. Belian mempunyai dua spesies, iaitu Eusideroxylon zwageri dan Potoxylon melagangai. Kedua-dua spesies ini adalah aman sukar dibezakan dari segi ciri-ciri morfologi. Pengesahan dan analisis Belian polimorfisma DNA merupakan komponen yang asas untuk pengecaman spesies. Kaedah Direct Amplification Minisatellite DNA-PCR (DAMD-PCR) diguna untuk pengecamajaran dengan menggunakan dua jenis pencetus: pencetus 33.6 (5'-AGGGCTGGAGG-3') dan pencetus YNZ-22 (5'-CTCTGGGTGTTGTCG-3'). Suhu penyepuhan optimum setiap spesies telah dipastikan, iaitu, E. zwageri (47.6°C) dan P. melagangai (50.0°C) daripada pencetus 33.6; E. zwageri (56.0°C) dan P. melagangai (51.4°C) daripada pencetus YNZ-22. Hasil DAMD-PCR daripada kedua-dua spesies Belian menunjukkan kadar polimorfisma yang tinggi. Sebanyak lima jalur diagnostik telah dikesan oleh setiap pencetus. Walau bagaimanapun, hanya dua jalur diagnostik telah dieksais dan ditulpak daripada setiap pencetus, iaitu, 33.6 Z01 dan 33.6 Z02 daripada E. zwageri; YNZ M01 dan YNZ M02 daripada P. melagangai. PCR produk yang telah ditulpak dihantar untuk penjujukan DNA. Walau bagaimanapun, keputusan penjujukan DNA menunjukkan isyarat lemah, ini disebabkan oleh kualiti DNA yang tidak baik dan tidak tlen.

Kata kunci: Eusideroxylon zwageri, Potoxylon melagangai, DAMD-PCR, pencetus 33.6, pencetus YNZ-22
CHAPTER I

INTRODUCTION

Belian (Eusideroxylon zwageri and Potoxylon melagangai) are the endemic hardwoods and heaviest timbers in Asia. They come from Lauraceae family. The origin of the ironwood trees was from Borneo and Sumatra (Veevers-Carter, 1984). Generally, these species are found in mixed dipterocarp forest which is lowland and roughly below 300 metres altitude (Wong, 1997).

The two species of Belian, E. zwageri and E. melagangai originally are from the same genus. E. melagangai is a distinct but closely related species of Eusideroxylon which the morphological characteristics of these two species are mostly alike, these have caused confusion to identify the two species of Belian (Browne, 1955; Burgess, 1966; Soerjanegara & Lemmens, 1994; Teo, 1998). But in 1979, Kostermans reported that these two species are distinctive between each other and considered as monotypic species. Therefore, E. melagangai is referred as Potoxylon and named as Potoxylon melagangai Kosterm, which is synonym to E. melagangai Symington (Kostermans, 1979). The two species constitute the subtribe Eusiderocylineae in the tribe Cryptacaryeae (Soerjanegara & Lemmens, 1994).

The wood anatomy of the two species can be differentiated based on their wood composition. E. zwageri has heavier weight, slight coarse texture, the absence of reddish tinge after prolonged exposure and the shorter lines soft tissue between the pores. Meanwhile, P.
melagangai has lighter weight, reddish colour, rather fine texture and longer bands of confluent parenchyma soft tissue linking up the pores (Browne, 1955; Burgess, 1966; Ipor et al., 1999).

This ironwood can be recognized by the local people of Jambi, Sumatra which divided into four varieties, called Sirap, Kapur, Daging and Tanduk (Irawan & Gruber, 2003). The variety of ironwood was recognized based on traditional knowledge of local people (Irawan & Gruber, 2003) which they could easily distinguish these varieties based on the form and size of the fruits (Soerjanegara & Lemmens, 1994).

According to Soerjanegara & Lemmens (1994), Belian is rated as very durable which can be used for 50 years and even more than a century. Generally, Belian is used for heavy construction, boat construction, and agricultural uses. Sabah exported 38,000 m³ of Belian in 1987 with the value of US$ 3 million, and 7,350 m³ with the value of US$ 2.3 million in 1992 (Soerjanegara & Lemmens, 1994). Since this species grows slowly, this requires about 120 years for the stem to reach 30 cm in diameter at breast height (dbh) (Kiyono & Hastaniah, 1998). According to Kurokawa et al. (2003), the reduced growth rate might cause by the high density and carbon-based defensive compounds.

The major threats to this endemic species are over-exploitation and illegal logging (Kostermans, 1979; Irawan & Gruber, 2003). Belian is considered to be endangered species in Kalimatan. The origin areas of Belian in Borneo have been estimated to cover 1,440 km² but recently only about 40% of these areas remain (Kiyono & Hastaniah, 1998). Indonesia has banned the exportation of this species while Sarawak has placed restrictions on export. Due to the
inadequate supply of seeds and seedlings for larger scale Belian plantations, it is now only planted on a small scale. Therefore, the application of tissue culture has been suggested due to the limitation of seeds. Thus, it is very important for regeneration, propagation and genetic resource conservation of Belian (Irawan & Gruber, 2004). The improvement of silvicultural practices was carried out to preserve Belian resources in Indonesia (Kiyono & Hastaniah, 1998).

Since the growth rate of Belian is too slowly, it does not suitable for large-scale plantation establishment. But according to Asia Pacific Forest Genetic Resources Programme (APFORGEN) (2005), most of the researches are focusing on developing propagation techniques as the technique for regeneration of Belian using seedlings and saplings. APFORGEN (2005) also reported that the Centre of Forest Biotechnology and Tree Improvement (CFBTI) had been established ex situ conservation plots for Belian and provided vegetative planting stocks for rehabilitation.

Generally, Belian leaves contain high quantities of polysaccharides and phenolic compounds (Ahmad, 2002), the young leaves samples from the two species must be taken for DNA extraction. A good quality of genomic DNA was successfully extracted using the modified Cetyltrimethylammonium Bromide (CTAB) method (Ariffin, 2003; Yii, 2005) and Sodium Dodecyl Sulfate (SDS) method (Tan, 2005). In fact, a good DNA profile of Belian was obtained by using M13 universal primer (Yii, 2005) and Random Amplified Polymorphic DNA (RAPD) (Tan, 2005).
The most current applications of molecular genetic techniques used for detection is known as molecular marker. Molecular markers recently are being used basically for assessment of genetic diversity, generate a fingerprinting and marker selection (Dayhoff & Eck, 1968). It possesses unique genetic properties and methodological advantages that make them more useful and valuable for genotyping and analyses of genetic structure of populations. It is stable and inherited variation that can be used to detect the presence of a specific genotype or phenotype which is very difficult to detect. Once marker systems are available, these applications are powerful and increasing the efficiency of managing tree improvement programs as it decrease mislabeling errors in forest tree species identification (Carson et al., 1996).

The Polymerase Chain Reaction (PCR) is an essential tool for amplification of specific sequences of genomic DNA (Zhou et al., 1997). The advent of the PCR is the faster way and less expensive to obtain PCR-based markers. According to Dayhoff & Eck (1968), it can amplify repetitive DNA in the form of tandemly repeat sequences such as minisatellite loci, depending on the type of primers used for PCR. As the minisatellite loci exhibit high degree of length variability and dispersed throughout the genome, it is highly reproducible and generate many informative bands per reaction (Somer et al., 1996; Bebeli et al., 1997; Zhou et al., 1997; Bhattacharya & Ranade, 2001).

Directed Amplification of Minisatellite DNA (DAMD) allows PCR to detect the amplification of minisatellite loci by using the core sequence of minisatellite as a primer (Heath et al., 1993). Since minisatellite core sequences are longer than RAPD primers, DAMD can be carried out more effectively at higher stringencies, and can be produced greater reproducibility
than RAPD (Heath et al., 1993). Moreover, it is much faster and requires less DNA in PCR. DAMD-PCR is capable of generating a large number of markers which could be used in constructing genetic linkage maps (Heath et al., 1993) as they are scorable and easily making gel to gel comparisons straightforward.

Since the two species are mostly alike based on their morphological characteristic, it would be easier to identify them based on their genetic fingerprints. In this project, PCR analysis was carried out to genotype Belian by using two types of minisatellite core primers, i.e. primer 33.6 (5'-AGGGCTGGAGG-3') and primer YNZ-22 (5'-CTCTGGGTGTGGTGC-3'). These two types of primers are human derived minisatellite which can be used in fingerprinting analysis. Primer 33.6 was used in developing DNA fingerprinting such as pepper (Piper nigrum L.) (Ho et al., 2005) and genus Oryza (Dallas, 1988; Zhou et al., 1997) while primer YNZ-22 was used in shrimp (Litopenaeus vannamei) (Freitas & Junior, 2002), pepper (Piper nigrum L.) (Ho et al., 2005), Bythotrephes Leydig (Therriault et al., 2002) and detection in Allogeneic Stem Cell Transplantation (Kamel et al., 2002). As primers 33.6 and YNZ-22 yielded more reproducible, scorable and informative fragments, the two primers were selected for development of molecular markers between E. zwageri and P. melagangai using DAMD analysis in this project.

The objectives of the study are 1) to genotype E. zwageri and P. melagangai via PCR using primer 33.6 (5'-AGGGCTGGAGG-3') and primer YNZ-22 (5'-CTCTGGGTGTGGTGC-3'), and 2) to identify the diagnostic bands for Belian typing based on the DNA profiles generated using primers 33.6 and YNZ-22.
CHAPTER II

LITERATURE REVIEW

2.1 Selection of Species

2.1.1 Family Lauraceae

The Lauraceae are also called Laurel, are nearly all woody trees and shrubs which comprising 35 genera and 2500 species (Ng & Phil, 1989; Ng, 1992). According the Ng (1992), all these species are throughout the tropics and subtropics. In Malaysia, 16 genera and 180 species of them are from family Lauraceae (Corner, 1988) and all these species could be found at the lowlands and mountains in Malaysia (Ng & Phil, 1989).

The typical features of the family are the small flowers with their closely packed sepals and stamens (Corner, 1988). The flowers are either bisexual or unisexual on these species. The colour of the flowers either green, white or yellow which are arranged in panicles or in short clusters.

The family Lauraceae is remarkably uniform in the structure of the fruit and seed. The fruits are generally small to large one-seeded berry with fleshly or leathery wall. The fruits are arranged in relatively long-stalked panicles produced from leaf-axils or the ends of the twigs.
The colour of the wood is yellowish brown to reddish brown, but turning darker colour when exposure to the air (Corner, 1988). Some species of Lauraceae have poisonous or irritating substances in the wood content (Corner, 1988). Inner bark usually very thick with strong aromatic smell (Ng & Phil, 1989).

2.1.2 *Eusideroxylon zwageri* Teijsm & Binn.

According to Browne (1955), the common names of *Eusideroxylon zwageri* can be found in various regions. In Indonesia, they are called onglen, ulin and kayu besi (ironwood). *Im muk* (Cantonese) and *yam muk* (Hakka) as Chinese names used in North Borneo, and in Hong Kong, the timber is known by a Cantonese name *ku'an tin*. In the Tawi Tawi islands, Belian is known as Biliran and tabulian. Other recorded vernacular names are telian, telisai, bajujang and tabulin- ulin in parts of Indonesia Borneo. In the timber trade, *E. zwageri* is called billion, belian, Borneo ironwood and Chinese blackwood.

*Eusideroxylon zwageri* is distributed in Sumatra, Borneo, Bangka, Belitung, Sulu Archipelago, and Philippines. In Kalimatan and Sumatra, it is generally found in lowland areas of primary forest up to 400 m above sea level, and also occurs in old secondary forest (Suselo, 1987).

The tree may reach to a height of 30 m, with trunk diameters up to 90 cm. The growth ring of *E. zwageri* is absent and the heartwood is basically light brown in colour while the sapwood colour is distinctive from heartwood colour (Richter & Dallwitz, 2000). The heartwood
of the tree would be darkening to a deep reddish brown on air exposure and the odour of *E. zwageri* is almost lemon-like smell when newly worked (Burgess, 1966). According to Soerjanegara & Lemmens (1994), the density of the species is 830-1040 kg/m$^3$ and moisture content is 9.30% (Ahmad, 2003). The wood of the species is very dense and heavy. The roots are occasionally slightly stilted and on poorly drained soils the bole, up to a height of about 6 feet from the ground, developed numerous thin, dark red aerial roots which do not grow to a large size (Browne, 1955).

According to Burgess (1966), the growth rings are indistinct but might be indicated by layers of confluent parenchyma and the texture moderately coarse. Vessels are evenly distributed, mostly solitary, two to three in groups of vessels, tyloses abundant, and slight tendency to arrangement in oblique lines. The wood parenchyma cells are moderately abundant, predominantly paratracheal, vasicentric to aliform and confluent. Rays are fine which less than 0.05 mm. Oil cells are distinct, oblong or round, small in size and contain a reddish gummy substance.

Leaves alternate, simple, arranged spirally, leathery, variable in shape form ovate or elliptic to narrowly oblong and often wider towards the apex than near the base, the apex blunt or tipped, base rounded, both surfaces hairless and glossy green. Generally, young leaves are red in colour (Browne, 1955; Soerjanegara & Lemmens, 1994).

Flowers small and inconspicuous, bisexual, actinomorphic, borne on racemes in the axils of the leaves (Browne, 1955; Soerjanegara & Lemmens, 1994). Flowering depends on the
location and climate. It begins to flower at an age of 15-20 years, and thereafter they bear fruit, though rarely in perfusion, at irregular intervals, usually every two or three years (Browne, 1955; Soerjanegara & Lemmens, 1994). The flowers bloom in August-November at Palembang; in July at Jambi, and October and November at South Kalimatan. The period from flowering to seed maturity may be as short as three months (Soerjanegara & Lemmens, 1994). The shape and size of fruit variable from more or less cylindrical to spherical marked with weak, irregular, longitudinal grooves and containing one seed (Browne, 1955). According to Browne (1955), the fruit is dispersed by the flood-waters of the north-east monsoon rain, and then the hard-coat of the fruit may take up to 12 months or even more to rot away before the seed can germinate.

The hardwood is resistance to termite attack, though after long periods exposed to termites attack may occur (Meressal, 2003). Moreover, the sapwood and heartwood would resist for many years attack by wood-rotting fungi, insects and marine borers (Browne, 1955; Soerjanegara & Lemmens, 1994). Nevertheless the old trees are frequently hollow would be damaged by longhorn beetles and insects in the sapwood but rarely extend to the heartwood (Browne, 1955, Burgess, 1966). E. zwageri has been classified as a durable timber with a service life of 50 to 100 years under natural condition (Soerjanegara & Lemmens, 1994). Because of the greatest strength and natural durability, this is very useful for all heavy construction, marine piling, wharf construction, roof shingles, street paving, fencing, house posts, fencing posts, telephone posts, heavy duty industrial floors, bridges, poles in pepper construction and furniture (Browne, 1955; Teo, 1998; Soerjanegara & Lemmens, 1994). It is also suitable for printing blocks, survey pegs, vehicle body work, boat building, haulage sleds for hand-hauling logs (Burgess, 1966). According to Soerjanegara & Lemmens (1994), the pepper support posts and
shingles are usually have a life more than 30 years, but the wood used in marine works have a life to 20 years.

Figure 2.1. *Eusideroxylon zwageri*; (a) seedling stage and (b) juvenile stage.

Figure 2.2. Seed of *Eusideroxylon zwageri*. 
Figure 2.3. *Eusideroxylon zwageri*; (a) leaf; (b) inflorescence; and (c) flower (adapted from Soerjanegara & Lemmens, 1994).

Figure 2.4. Natural distribution of *Eusideroxylon zwageri* which are spotted in red (adapted from *Asia Pacific Forest Genetic Resource Programme Priority Species Information Sheets*, 2005).
2.1.3 *Potoxylon melagangai* Kosterm.

*Potoxylon melagangai* could be found in Borneo, mainly on the west coast such as Limbang, Lawas, Miri, and Bintulu (Browne, 1955) and even found on the west coast of Sabah, notably in Kudat and Kawang area (Burgess, 1966). According to Browne (1955), this species is closely related to *E. zwageri*, is also called as *belian melangangai*, *belian simpor* and *belian batu*. In Malaysia, they are called *belian kapok*, *belian kebuau* (Iban, Sarawak) (Sosef et al., 1998). Generally, the species is found in lowland evergreen mixed dipterocarp forest which at 300 m above the sea level (Sosef et al., 1998; Ipor et al., 1999). According to Coode et al. (1996), this species normally can be found at gentle slope in secondary forest.

Although *P. melagangai* is not a particularly large tree as the diameter is around 4 to 6 feet (Burgess, 1966). According to Malaysia Timber Council (2002), the wood density of *P. melagangai* is between 590 and 850 kg/m$^3$ which it is lighter than *E. zwageri* and the moisture content is 10.52% (Ahmad, 2003). Sapwood of *P. melagangai* is not wide, yellowish and sharply differentiated from the heartwood when freshly cut, which is brown with a distinct reddish tinge and the texture is moderately fine (Browne, 1955; Burgess, 1966; Sosef et al., 1998; Malaysia Timber Council, 2002).

According to Burgess (1966), the growth rings of *P. melagangai* are indistinct but fine lines of parenchyma could be marked. *P. melagangai* has vessels with tyloses abundant and simple perforations which are few in number and moderately large, solitary and in the group of two or three vessels distributed. The parenchyma cells present in the form of aliform,
conspicuous and confluent types. Terminal and apotracheal parenchyma occurs as narrow, occasional and straight lines. Rays are fine with moderately in number. Oil cells could be visible in the soft tissue of parenchyma cells.

Leaves arranged spirally, simple and exstipulate. Inflorescence axillary, thyrsoïd, pseudo-recemose with the lateral cymes short-stalked. The flowers are bisexual, with two rows of six sepals. Flowering is irregular and occurs once every 3-5 year, generally in March, April or May. Fruiting is usually in September and the fruits are mainly dispersed by water but some animals such as porcupines and squirrels eat the fruits and may also disperse the seeds (Sosef et al., 1998).

Although *P. melagangai* is very closely related to *E. zwageri* and hard to identify each other, *P. melagangai* can be distinguished from *E. zwageri* based upon reddish colour, lighter weight, finer texture and the longer bands of confluent parenchyma linking up the pores (Browne, 1955; Burgess, 1966). Generally, *P. melagangai* is less durable than *E. zwageri* (Browne, 1955; Meressal, 2003) as the hardwood of *P. melagangai* is more easier attack by termites for weeks, such as *Coptotermes* species (Meressal, 2003). In addition, the flowers of *P. melagangai* are borne on rather stiff spikes, each lateral branch of a spike bearing only one flower while *E. zwageri* has a rather loose inflorescence (Browne, 1955). In the flower of *E. zwageri*, the two outer rows of stamens resemble petals and are sterile whereas in *P. melagangai*, they bear functional anther cells and the twigs are usually rather angular (Browne, 1955).

The population of *P. melagangai* does not reach at a large scale and is very local distributed, so it is not likely to become of commercial importance (Burgess, 1966). But it is