Faculty of Resource Science and Technology

EVALUATION OF GENETIC RELATEDNESS AMONG *ACACIA* SUPERBULK AND *ACACIA* HYBRID (*ACACIA MANGIUM* X *ACACIA AURICULIFORMIS*) TREES USING M13 UNIVERSAL PRIMER

Suliana anak Charles Nyucong

Bachelor of Science with Honours
(Resource Biotechnology)
2008

EVALUATION OF GENETIC RELATEDNESS AMONG *ACACIA* SUPERBULK AND *ACACIA* HYBRID (*ACACIA MANGIUM* X *ACACIA AURICULIFORMIS*) TREES USING M13 UNIVERSAL PRIMER

Suliana anak Charles Nyucong

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

Bachelor of Science with Honours
Resource Biotechnology
2008

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

Suliana anak Charles Nyucong

Resource Biotechnology

Faculty of Resource science and Technology

University Malaysia Sarawak

ACKNOWLEDGEMENTS

Above all, Praise God for He has always provides the best for me since I started this project and for He keeps watching me as the time went on until the completion of this project. I would like to take this opportunity to express my sincere thanks and appreciation to the following persons who have directly or indirectly given generous contributions toward the success of this thesis.

For the most part, I would like to express my greatest gratitude to my supervisor, Dr. Ho Wei Seng for his dedication and guidance throughout the project, and also to my co-supervisor, Asso. Prof. Dr. Ismail Jusoh, I really appreciate his passion in providing me the information on the *Acacia sp.* especially about the plus tree.

Special thanks to every person who help me during the progress of this project. I am grateful to Ms. Pang Shek Ling and Ms. Tan Sze Leng, Mr. Phui Seng Loi and also Mr Lee Kit Siong for their help in lending their time to provide me with the equipment and chemical needed, plus, the knowledge they have shared which for me are worth than gold. In addition, always as my indirect tutor, my other course mates, who are in the same Forest Genomic Lab, thanks for everything.

For the most precious persons in my life, I am very grateful to dedicate the success of this thesis to my family, especially to my beloved parents for their continuous prayers, supports and encouragement throughout my effort.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CIA	Chloroform-Isoamyl Alcohol
CTAB	cetyltrimethylammonium bromide
DAMD	Direct Amplified Minisatellite DNA
dH_2O	Distilled water
dNTP	Deoxyribonucleoside Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Bromide
EtBr	Ethedium Bromide
Mg^{2+}	Magnesium Ion
$MgCl_2$	Magnesium Chloride
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic Acid
RNase	Ribonuclease
TBE	Tris-borate-EDTA
TE	Tris-EDTA Buffer
T_m	Melting Point
UPGMA	Unweighted pair-group Method with Arithmetic Average
UV	Ultraviolet

EVALUATION OF GENETIC RELATEDNESS AMONG *ACACIA* SUPERBULK AND *ACACIA* HYBRID (*ACACIA MANGIUM* X *ACACIA AURICULIFORMIS*) TREES USING M13 UNIVERSAL PRIMER

Suliana anak Charles Nyucong

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

The genetic relatedness among *Acacia* superbulk (plus tree of *Acacia* mangium) and *Acacia* hybrid (*Acacia mangium* x *Acacia auriculiformis*) collected from the UNIMAS arboretum, Kota Samarahan, Sarawak was evaluated using M13 universal Primer. A total of 16 reproducible loci was generated where 56.3% of the fragments were identified as polymorphic band with the size ranging from 500bp to 2.5kbp. An UPGMA dendrogram was constructed based on the Jaccard's coefficient of similarity matrix which then grouped the *Acacia* superbulk and *Acacia* hybrid into two main clusters, i.e. Cluster 1: *Acacia* superbulk trees and Cluster 2: *Acacia* hybrid trees. All *Acacia* hybrid trees showed identical banding profile and therefore, suggested that these trees were originated from the same clone, whereas *Acacia* superbulk trees were originated from randomly selected plus trees seeds of *A. mangium*. The preliminary results showed that M13 universal primer is a potential molecular marker not only for assessing genetic relatedness but also could be used for clonal identification and seedling certification. However, further study by using other DAMD markers such as YNZ-22 and 33.6 can be carried out to validate these results.

Keyword: Genetic relatedness, *Acacia* superbulk, *Acacia* hybrid, UPGMA, DAMD

ABSTRAK

Hubungan genetik antara pokok Acacia superbulk (pokok terbaik daripada Acacia mangium) dengan Acacia hybrid (Acacia mangium x Acacia auriculiformis) dikumpul dari arboretum UNIMAS, Kota Samarahan, Sarawak. Sebanyak 16 lokus dapat dihasilkan dan 56.3% daripadanya telah dikenali sebagai belang polimorfik dengan lingkungan saiz 500bp ke 2.5kbp. Sebuah dendrogram UPGMA telah dijana berdasarkan pekali matriks keserupaan Jaccard yang seterusnya membahagikan Acacia superbulk dan Acacia hybrid kepada dua kelompok utama, iaitu Kelompok 1: pokok Acacia superbulk dan Kelompok 2: pokok Acacia hybrid. Kesemua pokok Acacia hybrid menunjukkan profil belang yang seiras, dan seterusnya mencadangkan pokok ini adalah berasal dari klon yang sama. Manakala bagi pokok Acacia superbulk pula dikatakan berasal dari biji benih pokok terbaik A. mangium yang dipilih secara rawak. Keputusan peringkat awal ini menunjukkan potensi primer universal M13 sebagai penanda perincian molekul bukan sahaja dalam penilaian hubungan genetic, malah digunakan dalam pengecaman klonal dan perakuan anak pohon. Namun begitu, kajian lanjutan dengan menggunakan penanda DAMD yang lain seperti YNZ-22 dan 33.6 juga dapat diteruskan untuk mengesahkan keputusan ini.

Kata kunci: Hubungan genetik, Acacia superbulk, Acacia hybrid, UPGMA, DAMD.

CHAPTER I

INTRODUCTION

The impact of logging situation in Malaysia has been recognized to lead the enforcement being carried out on some exotic and indigenous tree species that has high commercial value such as in *Acacia* species, kelampayan and sawih to be experiment. Some other characteristics and function in the species also cause the species to be chosen in the reforestation. Thus, the said cause of the impact lead the experimental study on *Acacia* species.

Acacia plantations are now common features in the landscapes of many Southeast Asia countries, particularly Malaysia, Indonesia and the Philippines. This *Acacia* plantation is developed specifically to produce industrial wood and initially followed the monoculture approach typical of plantation crops such as rubber and oil palm (Stuebing, 2006). According to Stuebing (2006), the *Acacia* plantation offers significant improvement in biodiversity conservation. A complex mixture of silviculture, natural forests and traditional agriculture are found to be included in those fastidious created landscape mosaics of *Acacia* plantations.

In addition, a research on developing modern beekeeping technique has been done and consequently the sivilculture of *Acacia* species created (PERKASA, 2004). Furthermore, the study stated that beekeeping under *Acacia mangium* forest has the potential in producing *Acacia* honey. This smallest part of *Acacia* trees (the pollen) is uniquely produced at the based of young shoot and abundance throughout the year, thus the bee can produce honey continuously. By this revelation there is a large demand on *Acacia* species in future, maybe in

food production (PERKASA, 2004), and also wood product – pulp and paper (World Wide Wattle, 2007).

Thus, the genetic study is followed by to create and find other functional character from the species and improve the quality of this species. *Acacia sp.* is known because of its own wood properties such as wood density, fiber length and adhesive performance, which have been studied in order to achieve a better value for the utilization of fast growing plantation species. Unfortunately, the heart rot disease started to attack the species (*A. mangium*) in the 1980s, whereby the stem defect with fungal infection of branch stubs, wounds from pruning and singling. Soon after the disease, two new species (*Acacia* hybrid and *Acacia* superbulk) were introduced to Sarawak, whereby the characteristic of both species shows rapid growth rates and greater resistance to heart rot.

Acacia hybrid (hybrid of *A. mangium* and *A. auriculiformis*) is the *Acacia* new species from *A. mangium* and *A. auriculiformis,* which planted side by side and accidentally breed and produce progeny. According to Kha (1996), the hybrid has shown outstanding growth potential with excellent wood properties. Most important, the hybrid species is resistant to heart rot disease unlike *A. mangium,* and tends to be more shrub-like. Moreover, the hybrid has the straight bole and stem of *A. mangium* and the self-pruning ability of *A. auriculiformis* (Ibrahim, 1993). *Acacia* hybrid also shows the higher pulp potential compared to its parental species (PERKASA, 2004).

Recently, Borneo Tree Seeds and Seedlings Supplies Sdn. Bhd. (BTSSSSB) and Commonwealth Scientific Institute Research Organization (CSIRO) in Sarawak had carried out research on *A. mangium* tree improvement. As a result, they had successfully come up with new clone, named it as "super-bulk". In 24 month, *Acacia* superbulk has shows an

impressive growth, with the height of 20.9 meters and is 21.7 cm dbh. This growth of *Acacia* superbulk shows significant result compared to the normal *A. mangium* within 24 months, which only can grow until 16.8 meters in height and 17.7 cm dbh (PERKASA, 2004).

There are also some projects carried out on *Acacia* in Malaysia, for example, project on *Acacia* hybrid under the title "*Acacia* project – *Acacia mangium* x *Acacia auriculiformis*" (*Acacia* Project, 2004). The project is mainly focusing on the genetic to enhance the pulp wood quality. Since the genetic study for *Acacia* superbulk and *Acacia* hybrid still on initial stage, thus this present study is carried out. Both of the species is said to be has history with *A. mangium* as the parental tree, thus the morphological characteristic is most likely to be almost the same. It is proved by previous studies that *Acacia* hybrid inherits mostly from *A. mangium* with excellent wood properties but has the poor form of *A. auriculiformis* (Bowen, 1981). Meanwhile, *Acacia* superbulk is the result from selection of *A. mangium* plus tree; hence the species have all excellent characteristic of *A. mangium*.

The present study was performed by using minisatellite core sequence as primers in Polymerase Chain Reaction (PCR). DNA profiling can be carried out by using Direct Amplification of Minisatellite-region DNA (DAMD), whereby the method is one of the useful method developed by Heath *et al.* (1993) that using PCR with variable number of tandem repeats (VNTRs) core sequences as the primers. According to Zhou *et al*. (1997), PCR analysis is an essential tool for amplification of specific sequences of genomic DNA. Meanwhile, the use of DAMD is to allow PCR to detect the amplification of minisatellite loci by using the core sequence of minisatellite as primers (Heath *et al*, 1993).

The study of using DAMD is said to be more effectively at higher stringencies, and resulting greater reproducibility. Furthermore, it is faster and require less DNA in PCR. In

constructing genetic linkage maps, capability of DAMD-PCR in generating a large number of markers is very useful, as they are scorable and easily making gel to gel comparisons straightforward (Heath *et al*, 1993).

The objectives of the study are (1) to establish the M13 universal primer protocols for *Acacia* hybrid and *Acacia* superbulk, and (2) to determine the genetic relatedness among the selected trees of *Acacia* hybrid and *Acacia* superbulk.

CHAPTER II

LITERATURE REVIEW

2.1 The *Acacia* species

Acacia species such as *A. mangium* Willd, *A. auriculiformis* Benth, and *Acacia* hybrid are major fast-growing plantation species not only for pulp and timber production but also for greening purposes in the tropical Asia region (Md. Hamami *et al.*, 1989; Semsutud & Nitiwattanachai, 1991). According to Pinyopusarerk *et al.* (1993), *A. mangium* is originated in Queensland, Australia, Papua New Guinea, Island of Sula, Ceram, Aru and Irian Jaya, Indonesia. Based on New Forest Project (2007), *A. auriculiformis* is natived in the tropical Northern Coastal Lowlands and Queensland, Australia, Papua New Guinea, and Irian Jaya, in eastern islands of Indonesia. As a plantation species, *Acacia* sp. has shown rapid growth, with good wood quality, tolerance to a range of soil types and pH values (Pinyopusarerk *et al*., 1993).

Acacia mangium's natural habitat is in primary and secondary forest, savanna grassland and on poorly drained floodplains (PERKASA, 2004). Based on Turnbull (1986), the distribution of the species is strongly influenced by rainfall pattern and soil drainage. In addition, this species is able to colonize in difficult site (National Research Council, 1983). *Acacia mangium* which originated from Queensland was first introduced to Sabah and being established in the early 60s as a fire-break for *Pinus caribaea* stands. Subsequently in the 70s, it was then introduced in Sarawak for trial planting at Oya Road Experimental Reserve, Sibu and Niah Forest Reserve (PERKASA, 2004). According to Lee (2004), among exotic species

that being introduced in Malaysia, *A. mangium* shows most potential as the tree helps in nitrogen-fixing in the soil.

Meanwhile, Francis (2006) stated that *A. auriculiformis* is a fast-growing, medium-sized tree that has phyllodes. The study further added that the tree may reach 30 meter in height and 60 cm d.b.h in most favorable environments. Besides, the species is cultivated widely for fuelwood and charchoal. This is approved by the Turnbull's study (1986) which stated that *A. auriculiformis* is more suitable for fuelwood production due to its higher basic density compared to *A. mangium*. The species also yield excellent charchoal that burns without any smoke or spark. On east coast of Peninsular Malaysia, a survey has showed that the species is used for drying the tobacco leaves and the characteristic of vigorous growth and hardiness led the use in providing shade in parks and roadsides (Yap, 1986)

2.1.1 *Acacia* hybrid (*A. auriculiformis* x *A. mangium* hybrid)

Acacia auriculiformis and *Acacia mangium* hybrid occurs when both species grow in close proximity and the flowering time is overlapping (Turnbull, 1986). Recently, the naturally hybrids of *A. mangium* and *A. auriculiformis* in Vietnam has been highlighting for plantation due to the superior characteristics and the hybrid own great characteristic for pulp and paper production (Kha, 1996). Furthermore, the characteristic with straw coloured softwood is useful in making pulp, chipwood, paper production, medium density fiber board and oriented-strand board, meanwhile the dark coloured heartwood is used in general construction.

Based on Darus and Rasip (1989), the natural hybrid plantation in Malaysia can be found in Ulu Sedili, Johor. They grow faster with rounded bark and less branching compared to the other type of *Acacia*. Besides Ulu Sedili, the hybrids also can be found in Sabah (Tham, 1976) and Papua New Guinea (Turnbull *et al*., 1986). Hybrids of *A. mangium* and *A. auriculiformis* also have been developed and the biclonal orchards for mass production of the inter-specific hybrids of this species have been established in Sabah, Malaysia.

Acacia hybrid grows well with the stem in medium-sized and almost looks like *A. mangium*. In two years, the trees can reach 8-10m in height and diameter of 7.5-9.0cm (dbh). This hybrid inherits most of the *A. mangium* wood properties (Kha, 2001) and it can be propagated through stem cutting. However, the hybrids tends to inherits overall poor form of *A. auriculiformis* (Bowen, 1981; Rufelds & Jafirin 1986),

The *Acacia* hybrids have good potential as they have shown superior performance in plantation with economically valuable wood density and pulp quality which are multigenic traits (*Acacia* project, 2005). This may lead to the potential becoming significant economic commodity to nation. In PERKASA (2004) stated that the wood from the tree is suitable for furniture, peeler logs, veneer, plywood, medium density fibreboard, door-skin, particleboard and pulp. Bleached characteristic of the tree to high brightness levels is excellent for making paper. Moreover, the tree has function as firewood, ornamental, for erosion controls and fire-break or wind-break. Meanwhile the leaves may serve as forage for cattle.

(a)

(b)

(c)

Figure 2.1 *Acacia* hybrid, (a) branch, (b) flower, (c) pod

2.1.2 *Acacia* Superbulk

Acacia 'Superbulk' is resulted from *Acacia mangium* seed propagation and had gone through many years of tree improvement programme by natural selection. Until now, this species has not been widely planted in Malaysia, and there is only one company that had *Acacia* superbulk as a mean to achieve genetic improvement of *A. mangium* in its seed orchard (PERKASA, 2004), which is Borneo Tree Seeds and Seedlings Supplies Sdn. Bhd. (BTSSSSB).

The term, tree improvement programme, used mostly in industrial plantation is an important step whereby the most superb characteristic of tree with relatively small interspecific provenance variation is evaluated in selecting plus trees. Selection of the tree will occupy the superb characteristic where the use of improved seeds and strict quality control is to ensure that only the healthy, high growth potential seedlings are planted. In obtaining *Acacia* superbulk, the first step is to screen the plus trees from a large number of trees identified. Next, the clone or family bank will be established from collected material of the plus tree. Finally, it will undergo a progeny testing (Awang and Taylor, 1993).

In Sarawak, the planting materials can be obtained from BTSSSSB Bintulu. BTSSSSB has established a strong synergy with an Australian based Research and Development Institution, one of the international bodies such as Commonwealth Scientific Institute Research Organization (CSIRO), Forest Department of Sarawak and Rubber Research Institute of Malaysia. In February 1998, BTSSSSB and CSIRO had signed an agreement in which both parties agreed to undertake activities to develop and propagate improved tree species (*Acacia* superbulk) in large-scale forest plantation in Sarawak (PERKASA, 2004). The agreement is obtained as the progress of the species impressively shows growth at the height of 20.9 meters and diameter 21.7 cm (dbh) in measurement. Compared to the provenance, which only obtain height in 16.8 meters and diameter 17.7 cm (dbh), *Acacia* superbulk shows quite large divergence.

Figure 2.2 A branch of *Acacia* superbulk

Figure 2.3 Upper leaf - *Acacia* hybrid, lower leaf - *Acacia* superbulk

2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technique invented by Kary B Mullis in 1985 where an *in vitro* method for the enzymatic synthesis of specific DNA sequences and the use of two oligonucleotide primers will hybridize to opposite strands and flank the region of interest in the target DNA and producing millions of copies of DNA in a short period of time (Erlich, 1992).

PCR works best with important ingredient such as, the *Taq* DNA polymerase, target sample (DNA template), DNA primer, and nucleotides (dNTPS) and other chemicals. In PCR, nucleotides (dNTPs) are function to work with DNA polymerase in building blocks on sequence. Besides that, the success of PCR reaction is the DNA thermal cyclers which use computer to control the repetitive temperature changes required for PCR (Bloom, 2007).

The presence of *Taq* DNA polymerase in PCR reaction is very important to initiate exponential amplification of DNA fragment from total genomic DNA template. Common

DNA polymerase could not be apply in this PCR method is because most DNA polymerases work only at low temperatures, where the DNA is tightly coiled and hence, less chance for polymerases to replicate. This important ingredient in PCR (*Taq* DNA polymerase) is originated from *Thermophilus aquaticus*, a thermophilic archaebacterium that endemic in hot springs (at temperature close to boiling point of water).

PCR, a repetitive series of cycles carries 3 processes in each cycle, which are, template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase. As the 3 major steps in PCR reaction continues to repeat cycles, copied DNA templates will results on the large number copies of gene, finally DNA fragments will be amplified. The sequence that will be amplified by two oligonucleotide primers must first be known the before starting the amplification. The reason is, so that the exact size of PCR products can be determined from the primers used.

2.3 Direct Amplification of Minisatellite-region DNA (DAMD)

Deoxyribonucleic acid (DNA) serves as the store of heredity information from generation to generation (Albert *et al.*, 2002). Furthermore, every organism has unique DNA which can be seen in characteristic of the genome. For any complex organism, there are fragments called polymorphic which make the difference between species. DNA profiling is thus essential as the process of separating organism's unique, polymorphic, fragments from complex structure of DNA. Based on Zhou (1997), DAMD-PCR technique allows the isolation of informative molecular probes to be utilized in DNA fingerprinting and genome identification.

According to Heath *et al.* (1993), Direct Amplification of Minisatellite-region DNA (DAMD) was created to produce species-species electrophoretic banding patterns for three species of salmonids, human and birds. DAMD is developed to adapt a simple technique that directs polymerase chain reaction (PCR) amplification to regions rich in variable number of tandem repeats (VNTRs). Minisatellites are repetitive DNA classified as VNTRs, where the numbers of repeats provides good polymorphism at a locus and each repeat contains a highly conserved core sequence (Jeffereys *et al.*, 1985; Weinberg *et al.*, 1993). Meanwhile, DAMD is one of the Non-Arbitrary primed markers, which allows the detection of polymorphisms with the knowledge of nucleotide sequence (Eck, 1999). DAMD patterns are highly reproducible (Santini and Capretti, 2000; Chandelier *et al.*, 2003) and effectively to be carried out at relatively high stringencies (Heath, 1993).

It has been reported that directed amplification of DNA from minisatellite regions using minisatellite core sequences as primer has revealed variation between accession within a species and among individual plants of the same accession (Zhou, 1997). Thus, within the accession primers, different degrees of variation are yielded (within-species polymorphism).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Apparatus and Reagent

Liquid Nitrogen

Mortar, pestle, scarpels

1M Tris HCl pH8.0

0.5M EDTA pH8.0

CTAB extraction buffer (EB)

- 100mM Tris HCl pH8.0
- 20mM EDTA pH8.0
- 1.4M NaCl
- 2% CTAB (SIGMA H-5882)
- 1% PVP (Polyvinyl Prolidane, Mr 40000)
- 0.2% β-mercaptoethanol

Chloroform-isoamyl alcohol (24:1 ; v/v)

Isopropanol

Wash buffer (Ethanol 70%)

TE buffer

RNase A solution

3.2 Methods

3.2.1 Sample collection

A total of 27 samples of young fresh leaves were collected from UNIMAS arboretum [13 samples of *Acacia* superbulk and 14 samples from *Acacia* hybrid]. *Acacia* hybrid trees planting material were originated from propagation of stem cutting, whereas *Acacia* superbulk trees were originated from randomly selected plus trees seeds of *A. mangium.*

(a) (b)

Figure 3.1 *Acacia* in UNIMAS arboretum, (a) *Acacia* hybrid, (b) *Acacia* superbulk

3.2.2 DNA isolation

Total genomic DNA extraction from fresh young leaves tissues were done according to the cetyltrimethylammonium bromide (CTAB) method from Doyle and Doyle (1990). Young fresh leaves of *Acacia* were used and done in small scale.

A total of 4 ml of CTAB extraction buffer and 80 µl β-mercaptoethanol were added into Falcon tube and were preheated to 65^{o}C in water bath for 30 minutes. By using a chilled