Acacia Research in Malaysia

Proceedings from a Seminar on

Current Updates on Acacia Genomics and Breeding

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PREFACE

Tropical *Acacia* species have proved to be fast growing pioneer species with high adaptability to a wide range of sites and soils. *Acacia* species have been recommended for forest plantations (especially for craft pulp, neutral sulphite semichemical pulp, general utility timber and fuelwood and to a lesser extent for fodder and tannin production) and rehabilitation of marginal and degraded lands.

Malaysia has vast areas of land (1.2 million hectares) reserved for forest plantations to supplement the depleting timber resource from natural forests and large areas of idle and ex-mining lands requiring rehabilitation.

Malaysia can and should be a significant player in tropical forestry through becoming a regional centre of excellence in development and supply of elite planting materials and economically valuable genes and genetic information. Significant potential export market exists throughout ASEAN and Australasia. An aggregate estimate of current demand for 500 million units per year would not be unreasonable.

Universiti Kebangsaan Malaysia (UKM), Forest Research Institute Malaysia (FRIM) and the Malaysia University of Science and Technology (MUST) jointly organised a seminar on **Acacia Research in Malaysia** with the theme **Current Updates on Acacia Genomics and Breeding** on 12 July 2005 at Marriott Hotel, Putrajaya.

This seminar was conducted to report on the various aspects of genetic studies on the *Acacia mangium* x *Acacia auriculiformis* hybrid under a national top-down research grant **IRPA 01-02-02-0000PR0003/03** led by UKM since 2002. The seminar focused on discovery of genes involved in the lignin biosynthesis pathway, development of mapping populations, large-scale micropropagation and construction of genetic and quantitative trait loci linkage maps for marker assisted selection.

The main objective of the seminar was to provide a forum for stakeholders in forestry to explore, exchange and update scientific, technological and industrial findings and information related to acacia research and its utilisation.

A total of 13 technical papers on the genetics, genomics, breeding and propagation of acacia hybrid were presented by local and foreign researchers during this seminar. The seminar also included an active dialogue session to realise strong partnerships for replicated field trials and development of elite planting materials for establishment of fast growing forest plantations in Malaysia and the region. About 50 participants mainly key stakeholders from the industry, forest departments, research institutes and universities attended this seminar.

The editorial committee comprising Assoc. Prof. Dr. Jennifer Ann Harikrishna, Dr. Choong Chee Yen and myself have edited and complied the full papers of this seminar including the recommendations on commercialization of findings for easy reference of the results of the various studies conducted under this top-down research program.

Professor Dr. Wickneswari Ratnam
Program Leader
IRPA 01-02-02-0000PR0003/03

Recommendations on Commercialization of Research Findings

Most forest plantations were established mainly for log production to supplement the timber supply from natural forests in Malaysia. Forest plantations for pulp and paper production did not gain much attention in early years. Problems that have plagued the forest plantation industry in the country were mainly lack of suitable land and good planting materials and relatively poor participation from private sector. The private sector was not keen to invest in forest plantations due to several difficulties, such as availability of suitable land from the state governments, difficulties to secure bank loans and lack of attractive incentives for investment in a venture with slow return of profits.

With this backdrop, the panel and participants discussed some possible ways to rectify these problems and move ahead to establish forest plantations with specific end uses for national needs and our future generations.

It was noted that Terengganu, Pahang, Johor, Sabah and Sarawak state governments have identified suitable land for forest plantations. For example, Sarawak has set aside a total of 1.4 million hectares for forest plantation. Attractive incentives should be made available for investors to actively venture into forest plantations. Incentives should also be considered for individual entrepreneurs. Information regarding the incentives e.g. low land premium and tax exemptions should be made public through the web. Private sector should put in increased efforts to convince the government to provide suitable land for forest plantation establishment. The government on the other hand should continue to play an active role in promoting forest plantations.

This Acacia project can offer good planting stock for advanced tree improvement activities and forest plantations in the country and the region. Acacia has many uses i.e. for production of sawn timber, pulp and extractives and for rehabilitation of degraded lands. Research should be carried out to increase the tree harvesting volume by breeding for sterility to shift the tree's energy from flowering to biomass production. Private companies should form partnerships with universities and research institutions to carry out large scale field trials.

Panel Members:

Datuk Dr. Salleh Mohd Nor, CEO, Tropbio Research Sdn. Bhd. - **Chair**
Dr Abd Rahman Abd Rahim, Director, Forest Plantation Unit, Forestry Department of Peninsular Malaysia.
Juing Ak. Umpit, Plantation Development Manager, Borneo Tree Seeds and Seedlings Supplies Sdn Bhd.
Tengku Adnin Tengku Adnan, Secretary, Division of Timber Industry, Ministry of Plantation Industry and Commodities, Malaysia

Analyses of ESTs Generated from Inner Bark Tissue of *Acacia* Hybrid

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Introduction

The genus *Acacia* is made up of about 1200 species that are widespread but with a large number in Australia (Wagner *et al.*, 1999). *Acacia mangium* and *Acacia auriculiformis* are from the family Fabaceae. *Acacia mangium* and *A. auriculiformis* have been planted in many countries, such as Indonesia, Thailand, Myammar and Malaysia as wood source especially for pulp and paper making industry. The natural hybrid of *A. mangium* and *A. auriculiformis* found in Sabah has shown more superior characteristics than the parental trees. The acacia hybrid is gaining more attention as fibre source for paper making industry. There are three major components involved in wood formation, namely cellulose, lignin and hemicellulose. Lignin is an undesirable component in the pulping process whereas higher cellulose content is desirable. Lignin is formed by intracellular synthesis of the monolignol precursors (ρ-coumaryl, coniferyl and sinapyl alcohol). The polymerization of these monolignols forms the ρ-hydroxyphenyl, guaiacyl and syringyl lignins. There are various different enzymes involved in the biosynthesis pathway of lignin. A better understanding of the genes which encode these enzymes would help towards improvement of lignin content and composition. A study carried out by Hu *et al.* (1999) showed that a reduction in the lignin content would increase the cellulose content in the plant.

Expressed sequence tags (ESTs) are a type of sequenced site tags. The ESTs are sequences of cDNAs that have been transcribed from mRNA templates consisting of only the exons or coding regions of the genes. ESTs are generated by sequencing either one or both ends of an expressed gene. The idea is to sequence a small part of DNA fragments that represent genes expressed in certain cells from different organisms, and use these tags to fish genes out of a portion of chromosomal DNA by base-pair matching. Sequencing the beginning portion of the cDNA produces a 5'EST, this portion of transcript usually codes for a protein. The 5'ESTs tend to be conserved across species and do not change much within a gene family. Sequencing the ending portion of the cDNA produces a 3'EST. The 3'ESTs are likely to fall within non-coding regions or untranslated regions (UTRs). These partial sequences can be used to identify coding regions of genes, to conduct genome mapping and discover new genes.

Materials and Methods

Plant materials and cDNA library construction
A cDNA library of 7000 clones was constructed from the inner bark tissue of the *Acacia* hybrids (Cheong *et al.*, 2003). Total RNA was extracted from the inner bark tissue using RNeasy Midi Kit (Qiagen, Germany). The messenger RNA was then purified using Dynabeads mRNA Purification Kit (Dynal, Norway). cDNA was synthesized using the Ready To Go You-Prime First Strand Beads (Amersham Biosciences, UK). Synthesized cDNAs were then cloned into the plasmid vector pSPORT1 (Invitrogen, USA) and transformed into the competent cell Electromax DH10B (Invitrogen, USA) using MicroPulser (Bio-Rad, USA) at 1.8kv. The cells were then cultured in LB agar plate contaning 100ug/ml ampicilin at 37°C for 16 hours.

DNA sequencing
6816 clones were cultured in LB broth overnight at 37ºC. Plasmid DNA was extracted from these clones using Montage Plasmid Miniprep Kit (Milipore, USA). The extracted plasmid was used as template in the sequencing reaction. Sequencing was performed from the 5' end for 2784 cDNA clones using ABI Prism 3730 and 3100 automated sequencers. The remaining 4032 cDNA clones were single pass sequenced from the 3' end using CEQ 8000™ sequencer.

Sequence analyses
DNA sequences (ESTs) were analyzed with StackPACK version *2.2* software (http://www.sanbi.ac.za) and Sequencer® 4.1 to remove the vector and ambiguous sequences automatically. Raw sequences were assigned base confidence scores using phred. ESTs were assembled into contigs using StackPACK software with a 96% minimum match and 50 base minimum overlap as assembly parameters. Sequences with length more than 100 nucleotides were subjected to similarity searches against the GenBank non-redundant (nr) protein database (www.ncbi.nlm.nih.gov/nr) using Blast-X algorithm with default parameter (Altschul *et al.*, 1990). Blast output was parsed to yield a tabulated output which was imported into Microsoft Access. The constructed database was used for further analysis. ESTs with score >100 and E-value $<e^{-10}$ were accepted as significant hit and classified using the modified MIPS MATDB classification scheme adopted for *Arabidopsis thaliana* (http://www.mips.Bio-chem.mpg.de/proj/thal/db/index.html).

Results and Discussion

4032 cDNAs were single pass sequenced from the 3' end and 2784 cDNAs were single pass sequenced from the 5' end generating 6415 EST sequences with high quality. The average size of the ESTs was approximately 500 nucleotides after trimming off vector and removal of low quality sequences. A total of 930 contigs and 3284 singletons were generated from the 6415 ESTs. This resulted in a total of 4212 assembled sequences representing putative transcripts in the cDNA library and a redundancy of 48.8%.The

redundancy value in this library was lower compared to some other EST projects; for example, 56% in *Citrus sinensis* (Bausher *et al.*, 2003). Most contigs were formed by 2

ESTs. The biggest contig was formed by 21 ESTs with a score of 197 and E-value of $6e^{-14}$ matching to metallothionein-like protein type 2 of apple tree.

Based on the BLASTX analysis, the ESTs can be categorized into five groups:

(a) Group 1: ESTs with significant match and known function,
(b) Group 2: ESTs with significant match and unknown function,
(c) Group 3: ESTs with significant match with putative/unclear classification,
(d) Group 4: ESTs with low significant match, and
(e) Group 5: ESTs with no match.

In this study, Group 1 consisted of 1481 ESTs (23.09%) with significant match (E-value $<e^{-10}$) to proteins of known function from the NCBI database. Group 2 and Group 3 have 503 ESTs (7.84%) and 507 ESTs (7.90%) respectively. On the other hand, Group 4 consisting of 1341 ESTs (20.9%) which were found to have low significant matches (E-value $>e^{-10}$). Group 5 consisted of 2583 ESTs (40.3%) with no match. The absence of these sequences (Group 5) from the public database may indicate a specific role for these proteins in *Acacia* hybrid. Most of the ESTs with significant hits and known function were largely related to metabolism, followed by protein regulation, protein synthesis and defense. Figure 1 shows the distribution of the ESTs according to their putative functions. Twelve distinct functional classes were identified.

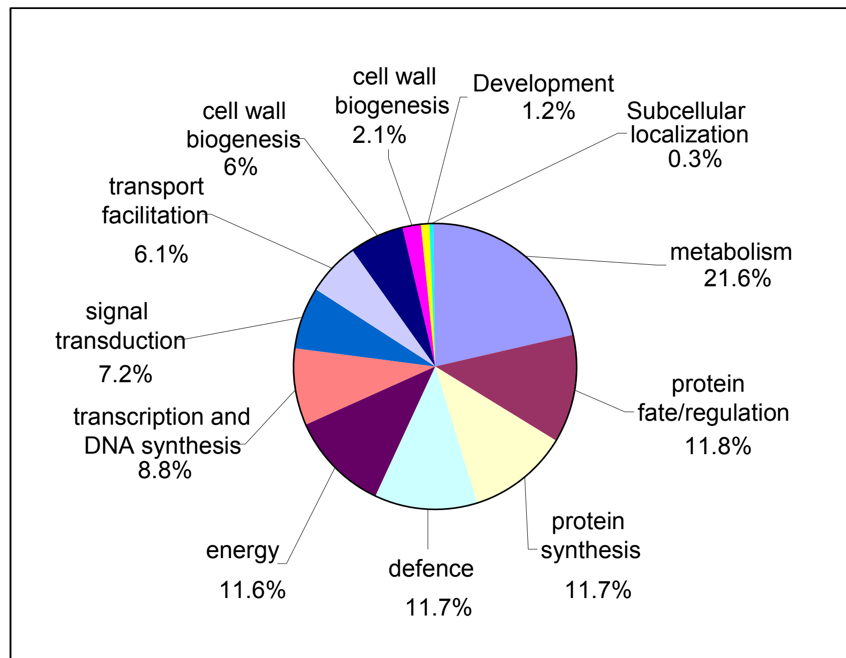

Figure 1: Distribution of ESTs according to their putative functions.

The highly abundant transcripts were mainly acid phosphatase, metallothionein-like protein type 2, trypsin inhibitor precursor and ubiquitin conjugating protein. Table 1 summarizes these highly abundant transcripts with their putative functions and the number of ESTs. These 171 ESTs contributed 2.7% of the total 6415 ESTs analyzed.

Table 1: Frequently occurring transcripts in *Acacia* hybrid cDNA library

Gene	Number of ESTs
Acid phosphatase	38
Metallothionein-like protein type 2	32
Trypsin inhibitor precursor	19
Ubiquitin-conjugating enzyme 8	15
Ubiquitin-conjugating enzyme family protein	11
Non specific lipid transfer protein	11
Aquaporin	11
Histone	10
Polyubiquitin 6	10
Chloroplast/light harvesting complex II protein 5	10
Photosystem II 10 kDa polypeptide	10
Total	177

The cDNA library was derived from the inner bark tissue of *Acacia* hybrid, therefore genes involved in wood formation are expected to be isolated. From the *Acacia* EST dataset, several ESTs showing significant match to enzymes involved in wood formation were identified. Thirty-four ESTs (0.53%) were found to represent enzymes involved in the biosynthesis of lignin. Twenty-one (0.33%) ESTs matched to three enzymes involved in cellulose synthesis and 18 ESTs in hemicellulose synthesis were identified. Table 2 shows the ESTs related to wood formation genes in *Acacia* hybrid cDNA.

The number of ESTs related to wood formation obtained from this cDNA library are considered high compared to some other EST datasets. For example, 1 EST for enzyme involved in cellulose synthesis, 1 EST for lignin biosynthesis and 3 for hemicelluolose synthesis were discovered by Paux *et al*. (2004) in *eucalyptus* species; 6 ESTs for cellulose and hemicellulose synthesis, 12 ESTs for lignin biosynthesis in *Cryptomeria japonica* (Ujino-Ihara *et al*. 2000). In addition, other genes involved indirectly in the cell wall biogenesis were also identified in the *Acacia* ESTs, such as glycine rich protein, beta-1, 3-glucanase-like protein and UDP-glucose pyrophosphorylase. The *Acacia* EST database is a valuable resource for forestry research directed towards understanding the genes involved in wood formation and their function for improvement of wood quality.

Table 2: *Acacia* ESTs related to wood formation genes

	Putative function	Number of ESTs
Hemicellulose	xyloglucan endotransglycosylase XET2	8
	Xyloglucan endotransglucosylase/hydrolase protein A precursor	3
	xyloglucan endo-1,4-beta-D-glucanase	7
Cellulose	cellulose synthase	1
	cellulose synthase A4	1
	cellulase precursor	1
Lignin	Phenylalanine ammonia-lyase	5
	4-coumarate-CoA ligase-like protein	1
	Caffeoyl-CoA O-methyltransferase (Trans-caffeoyl-CoA 3-O-methyltransferase)	6
	caffeic acid O-methyltransferase	2
	cinnamyl-alcohol dehydrogenase	5
	probable cinnamyl-alcohol dehydrogenase	1
	putative cinnamoyl-CoA reductase	5
Total		47

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Isolation and Characterization of *C4H*, *CCoAOMT* and *CAD* from Interspecific Hybrid *Acacia mangium* X *Acacia auriculiformis*

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Introduction

The natural hybrid of *Acacia mangium* and *A. auriculiformis* is very promising for plantation in Southeast Asia. This hybrid appears to have higher cellulose, lower lignin content and give better pulp yield than both parental trees (Yamada *et al*. 1990).

Lignin is a plant phenolic biopolymer made up of three monolignols, namely ρ-coumaryl, coniferyl and sinapyl alcohols, which accounted for 20-30 % of the dry mass of wood. During the lignification process, these monolignols are polymerized to give rise to hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units respectively (Boerjan *et al*. 2003). Lignin is crucial for structural support of the plants, transportation of water and solutes through the vascular system and plant defense system (Whetten & Sederoff 1995). However, for the production of high-quality paper, lignin has to be removed from the cellulose by energy-requiring and polluting methods. The rate of delignification is propotional to the S/G (syringl/guaiacyl) ratio (Piquemal *et al*. 1998).

Cinnamate 4-hydroxylase (C4H), caffeoyl-CoA *O*-methyltransferases (CCoAOMT) and cinnamyl alcohol dehydrogenase (CAD) are three enzymes that play an important role in the beginning, middle and the end of the lignin biosynthesis pathway. C4H is a cytochrome P450-dependent enzyme catalyzing the hydroxylation of cinnamate to 4-coumarate. It was suggested that this gene exerts control over metabolic flux to S unit. CCoAOMT methylates caffeoyl-CoA and 5-hydroxyferuloyl-CoA to produce feruloyl-CoA and sinapoyl-CoA. CAD is a multifunctional enzyme that catalyzes the final reduction of the cinnamaldehydes to ρ-coumaryl, coniferyl and sinapyl alcohols (Lewis *et al*. 1999).

In this study, the full length cDNAs of these three genes from the inner bark tissue of *Acacia* hybrid were isolated and characterized. The information obtained in the study will be much of use in the development of molecular markers for producing high quality wood pulp through marker-assisted selection and genetic modification of lignin biosynthesis to obtain trees more adaptable to industrial uses.

Materials and Methods

Inner bark tissues of an interspecific *A. mangium* x *A. auriculiformis* hybrid were obtained from Plot W, Plant Biotechnology Laboratories, Universiti Kebangsaan Malaysia, Bangi, Malaysia. The tree was pollarded at 0.5 m height. The bark was peeled and inner bark tissues were scraped into a clean plastic bag and dropped directly into liquid nitrogen. The sample was stored at -80°C until needed.

RNA Isolation

Total RNA was extracted by using RNeasy Midi Kit (Qiagen, Germany), and mRNA was isolated from total RNA using Dynabeads oligo-$(dT)_{15}$ (Dynal, Skoyen, Norway) according to the manufacturer's instructions. RNA concentration was determined by UV spectrophotometry.

RACE- PCR, Cloning and DNA Sequencing

RACE-PCR was performed by using *SMART™ RACE cDNA Amplification Kit* (Clontech, USA). cDNA was prepared according to the manufacturer's instructions. Primers were designed based on the partial sequences for *C4H*, *CCoAOMT* and *CAD* (Pang *et al.* 2003) by using *Primer3* (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3). The sequences of the primers are shown in Table 1. The PCR was performed according to the suggested touch-down PCR cycles in the manufacturer's manual. The fragments were purified and cloned into pGEMT-Easy cloning vector (Promega, USA). The cloned cDNA fragments were sequenced in both directions and checked and edited by using Chromas Lite 1.0 (http://www.technelysium.com.au/chromas14x.html).

Table 1. Primers designed for RACE-PCR.

Gene	Primer	RACE	Sequence
C4H	r1112R	5'-RACE	5'-GGA GGT TCA TGT GTG GGA CCA GCA A-3'
	r1112F	3'-RACE	5'-CTG GGT ATC ACT TTG GGG CGT TTG G-3'
CCoAOMT	r78R	5'-RACE	5'-TGG CGA GCA AGG AGT AGC CAG TGT A-3'
	r78F	3'RACE	5'-AAG GTT GGG GGT GTG ATC GGG TAC G-3'
CAD	r1718R	5'-RACE	5'-GAC GAG CCT ATC ACC TGG CAC ACT G-3'

Sequence Analysis

Open reading frames (ORFs) for each cDNA were identified by using ORF finder (http://www.ncbi.nih.gov/gorf/gorf.html). BLASTP (Altschul *et al.*. 1997) was used to identify homologues of the C4H, CCoAOMT and CAD protein by comparing the nucleotide sequences translated into open reading frames against protein databases (http://www.ncbi.nih.gov). Analyses of the molecular weight and theoretical isoelectric point were performed by using Compute pI/Mw tool at

http://us.expasy.org/tools/pi_tool.html. Patterns and motifs of the amino acid sequences were analyzed by using ScanProsite available at http://us.expasy.org/tools/scanprosite/.

Results and Discussion

For the first time in *Acacia* species, cDNAs that are complementary to the C4H, CCoAOMT and CAD enzymes have been studied. 5'- and 3'-RACE primers designed based on the partial cDNA sequences for the corresponding genes (Pang *et al.* 2003) yielded fragments of different sizes (Figure 1).

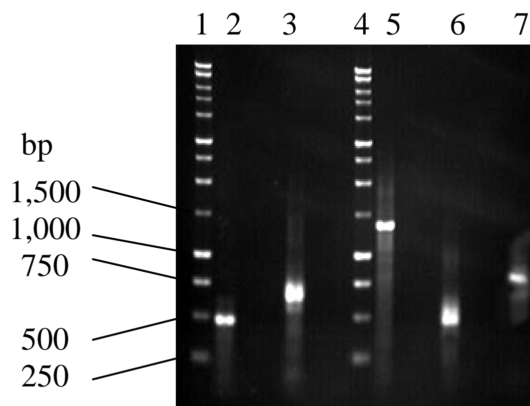

Fig 1. RACE-PCR products. Lane 1: 1kb DNA ladder (Promega, USA), lane 2: 5'-RACE *CCoAOMT*, lane 3: 3'-RACE *CCoAOMT*, lane 4: 1kb DNA ladder, lane 5: 5'-RACE *C4H*, lane 6: 3'-RACE *C4H*, lane 7: 5'-RACE *CAD*.

These fragments were cloned and sequenced. The 5'- and 3'-RACE sequences for each gene were aligned to the partial sequences for the corresponding genes (Pang *et al.* 2003) to get the full length sequences. The full length *C4H*, *CCoAOMT* and *CAD* sequences were 1790 bp, 1087 bp and 1346 bp long. Open reading frames (ORFs) for each cDNA identified by ORF finder revealed that *C4H, CCoAOMT* and *CAD* contained 1353 bp, 750 bp and 1089 bp. Therefore, the polypeptide encoded by C4H was 450 amino acids, CCoAOMT was 249 amino acids and CAD was 363 amino acids. The deduced proteins for these three cDNAs had calculated molecular weights of 51.9 kDa, 28.8 kDa and 40 kDa, with isoelectric points of 9.35, 5.46 and 5.90 respectively.

The encoded polypeptide for C4H exhibits sequence similarity to C4Hs from different plants with the highest identities matching to C4Hs from *Populus kitakamiensis* (88 %) and *Gossypium arboretum* (88 %). For CCoAOMT, the highest polypeptide sequence similarity was to *Broussonetia papyrifera* (89 %) and *Medicago sativa* (86 %); for CAD, the highest similarity was to *Medicago sativa* (69 %) and *Nicotiana tabacum* (67 %). Putative conserved domains, methyltransf_3, p450 and zinc-containing alcohol

dehydrogenase were found in the CCoAOMT, C4H and CAD full length cDNAs of *Acacia* hybrid.

The high degree consensus of the isolated C4H, CCoAOMT and CAD amino acid sequences with the corresponding sequences of the three enzymes from higher plants strongly suggests the *Acacia* hybrid full length cDNA corresponding to mRNA encoding C4H, CCoAOMT and CAD enzymes have been successfully isolated.

Acknowledgement

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Full length cDNA for Cinnamoyl-CoA Reductase (*CCR*) and Caffeic Acid O-Methyltransferase (*COMT*) in *Acacia mangium* Willd. x *Acacia auriculiformis* Cunn. ex Benth. Hybrid

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Introduction

In the early 1960s it was realized that the consumption of pulp and paper products in the country was mainly imported and this was projected to increase tremendously. This prompted the Malaysian government to plan for the establishment of local pulp and paper industries. Most tropical timbers come from the families of Dipterocarpaceae and Fabaceae or Leguminoseae.

Acacia mangium from the Fabaceae family is a plantation species, grown extensively since the 1960s for pulp and chips. It was first introduced to Sabah, Malaysia from Australia as a fire break species in 1966. The species was later found to be suitable for plantation establishment in Sabah due to its growth performance and versatility to grow on a variety of sites (Tham 1976). These traits have put *Acacia* in favour as a plantation species over many other fast-growing species. However, *A. mangium* is susceptible to heart rot disease (Lee 1985). On the other hand, the adaptability of *A. auriculiformis* to Malaysian conditions has been noted since the early days of its introduction to this country. It is more durable and less susceptible to heart rot disease than *A. mangium*. On account of its fast growth, low site selectivity and high resistance to disease and insect attack, it has been recommended as a useful reforestation species (Mitchell 1963).

Spontaneous hybrids of *A. mangium* and *A. auriculiformis* have been reported from plantations in Sabah, especially when these two species are planted in close proximity to one another. Generally, such hybrids tend to grow vigorously and have better form than *A. auriculiformis* and lighter branching, more self pruning ability and smoother bark than *A. mangium* (Tham 1979; Sim 1987). These hybrids are extremely adaptable and able to regenerate naturally on disturbed sites. There is large potential in the establishment of hybrid plantations through the selection of high quality parents especially with characteristics favourable for some end products like sawn timber and pulp and fibre (Rufelds 1987; FAO 1982).

Lignin is an important component of wood and constitutes a quarter of the total wood biomass (Whetten & Sederoff 1995). However, it is an undesirable component in the conversion of wood into pulp and paper. Both lignin content and composition (syringyl:guaiacyl (S:G) ratio) influence the chemical pulping process, with the rate of

delignification being directly proportional to the S:G ratio (Piquemal et al. 1998; Singh et al. 1982). Trees altered in their lignin profile, with reduced amounts of lignin or with a more extractable syringyl-rich composition, are more desirable for pulping. More rapid delignification allows for less severe pulping conditions, in turn giving decreased cellulose degradation, reduced chemical consumption and higher pulp yield and strength (Hibberd et al. 1999).

Cinnamoyl CoA reductase (CCR) and caffeic acid O-methyltransferase (COMT) are two enzymes likely to regulate lignin content and composition, being solely in the monolignol specific branch of the lignin biosynthesis pathway: CCR catalyzes the conversion of cinnamoyl-CoA esters to their corresponding cinnamaldehydes, the first specific step synthesis of the lignin monomers. COMT catalyzes the methylation of both caffeic and 5-hydroxyferulic acids.

In this study, full length cDNA clones encoding CCR and COMT were isolated and characterized. The full length cDNA of CCR and COMT could be used as DNA probes to understand the expression of these genes in different tissues and individuals of *Acacia* hybrid. Specific DNA polymorphisms present in these genes among *Acacia* hybrid individuals could be further exploited for production of elite planting materials with reduced lignin content and high S-lignin levels.

Materials and Methods

Acacia mangium x *Acacia auriculiformis* hybrid inner bark tissues were obtained from *Acacia* Plot W at the Plant Biotechnology Laboratory, Universiti Kebangsaan Malaysia. The inner bark tissues were immediately frozen in liquid nitrogen after harvest and then stored at -80ºC. Total RNA was isolated from young inner bark tissues using a modified RNeasy Midi Kit protocol (Qiagen, Germany). The isolated Total RNA was used for synthesis of first strand cDNA using a BD SMART^{TM} RACE cDNA Amplification Kit from BD Biosciences Clontech (USA). The synthesized cDNA was then used to generate 5' cDNA ends. The 3' EST sequences for *CCR* and *COMT* were obtained from the *Acacia* hybrid cDNA library constructed by Wickneswari et al. (2004). The sequences were used to design gene specific primers (GSP) using Primer3 software (Rozen & Skaletsky, 2000). GSP 5'-GTGACGGATGGCTTCCACGCACAAGTC-3' and 5'-CCACCTGGGTATGAGCAGCATGATGACG-3' were chosen for *CCR* and *COMT* respectively. Both of the pimers were used in 5' RACE amplification reactions. The 5' RACE PCR reactions were set up in 50µl volume according to the SMART^{TM} RACE cDNA Amplification Kit protocol from BD Biosciences Clontech (USA). A touchdown PCR program was used to obtain robust amplication in RACE using a Mastercycler (Eppendorf, Germany). 5' RACE fragments were isolated from a 1.2% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen, Germany). The fragments were cloned into the pGEM-T Easy Vector (Promega Corporation, USA) and screened for the desired insert with a PCR Colony assay. Positive clones were cultured overnight at 37ºC and plasmid extraction was carried out using a Qiagen Plasmid Extraction Kit. Extracted plasmids were digested with *Eco*RI restriction enzyme at 37ºC for 3 hours to

confirm the presence of desired insert. Sequencing was carried out on the extracted plasmids.

Results and Discussion

5' RACE PCR amplification using GSP resulted in a single product for *CCR* and *COMT* respectively, confirming primer specificity. The expected size of the 5' RACE PCR product for *CCR* was 900 bp and 1.1 kb for *COMT*, as shown in Figure 1. Four positive clones each for *CCR* and *COMT* (Figure 2) were sequenced. Sequence analysis revealed that the length of 5' RACE product was 847 bp for *CCR* and 1049 bp for *COMT*. The combination of the 5' cDNA sequences with the 3' cDNA sequences from the existing ESTs and the sequence analysis of the full length cDNA clones gave sequences of 1305 bp for *CCR* (GenBank accession number DQ001168) and 1408 bp for *COMT* (GenBank accession number DQ001169). Translation analysis of the full length cDNA sequence of *CCR* predicted a 960 bp open reading frame encoding 319 amino acids with a predicted molecular mass of 35.1 kDa and a theoretical isoelectric point (pI) of 5.87. The full length cDNA also consisted of a 85 bp 5' untranslated region (UTR), a 230 bp 3' UTR and a poly(A)+ tail of 30 bp. The full length cDNA sequence of *COMT* contained a 1098 bp open reading frame encoding 365 amino acids with a predicted molecular mass of 39.8 kDa and a theoretical pI of 5.61. The full length cDNA also revealed a 71 bp 5' UTR, a 224 bp 3' UTR and a poly(A)+ tail of 15 bp. The nucleotide compositions of the full length cDNA sequences of *CCR* and *COMT* were G+C rich at 48.58% and 50.36% respectively. The full length cDNA sequences were compared with GenBank database entries using BLASTX and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST). The BLASTX result revealed that the full length cDNA sequence of *CCR* has high similarity with cinnamoyl-CoA reductase-like protein (*Arabidopsis thaliana*, AAM64538), with an identity of 67% and E-value of $7e^{-127}$. The BLASTP result showed a match to the same entry with an identity of 67% and E-value of $4e^{-127}$. The BLASTX and BLASTP results for COMT revealed that its full length cDNA sequence has high similarity with caffeic acid O-methyltransferase (*Stylosanthes humilis*, 2119166A), with an identity of 87% and E-value of 0.0. The deduced amino acid sequences of CCR and COMT were aligned with those of other CCRs and COMTs from various plants reported in GenBank to date using ClustalW (http://ww.ebi.ac.uk/clustalw). The deduced amino acid sequence of CCR revealed substantial similarity to the putative CCR from *Arabidopsis thaliana* and *Solanum demissum*, with values of 66% and 62% respectively. The deduced amino acid sequence of COMT exhibited a high level of similarity with the COMT from *Stylosanthes humilis* (87%), *Medicago sativa* (87%) and *Prunus dulcis* (83%). The full length cDNA of *CCR* and *COMT* will be used to screen the genomic phage library of *Acacia* hybrid which is currently under construction. This study opens new avenues for determining the complete gene sequnces for *CCR* and *COMT* in *Acacia* hybrid. Further studies are necessary to characterize these genes in order to understand their function and regulation in the biosynthesis of lignin.

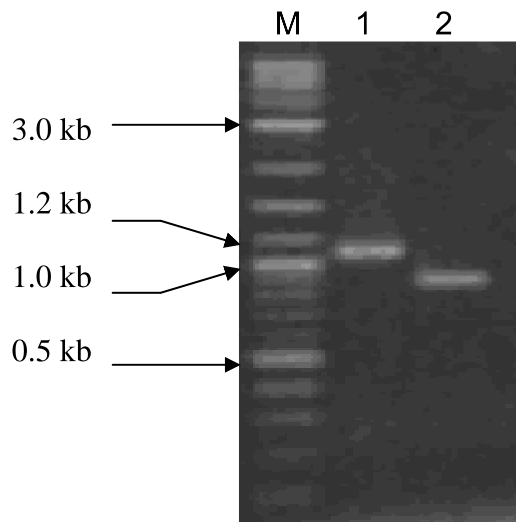

Fig. 1. 5’ RACE-PCR amplification products of *CCR* and *COMT* from *Acacia* hybrid separated on a 1.2% agarose gel. M = 2-Log DNA molecular weight marker (0.1-10.0kb), 1 = *COMT* 5’ RACE fragment (1.1 kb), 2 = *CCR* 5’ RACE fragment (0.9 kb).

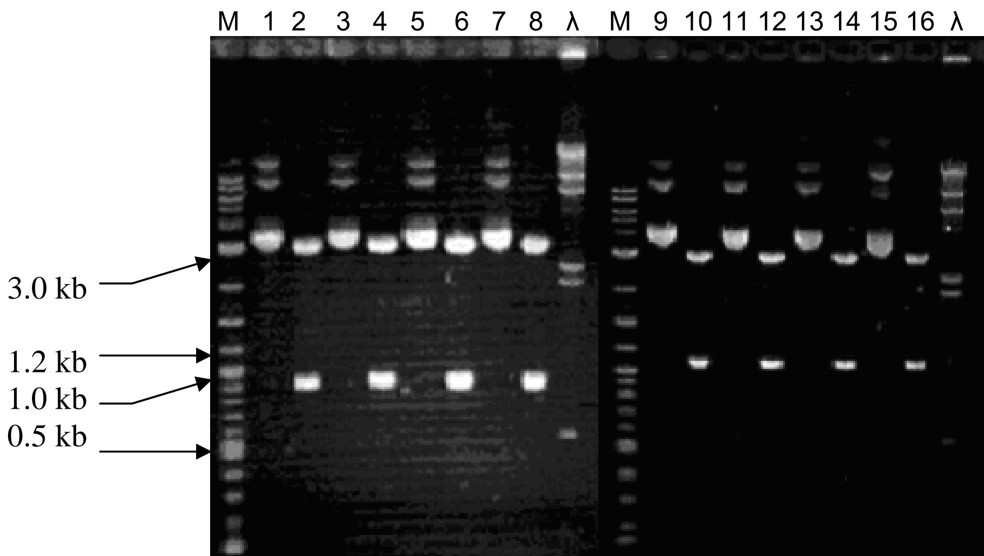

Fig. 2. Plasmid digestion to determine positive clones of *CCR* and *COMT*. M = 2-Log DNA molecular weight marker (0.1-10.0kb), λ = Lamda *Hind*III molecular weight marker, 1 = Undigested CCR1, 2 = Digested CCR1, 3 = Undigested CCR2, 4 = Digested CCR2, 5 = Undigested CCR3, 6 = Digested CCR3, 7 = Undigested CCR4, 8 = Digested CCR4, 9 = Undigested COMT1, 10 = Digested COMT1, 11 = Undigested COMT2, 12 = Digested COMT2, 13 = Undigested COMT3, 14 = Digested COMT3, 15 = Undigested COMT4, 16 = Digested COMT4.

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Construction of Genomic Library for Screening Full Length Genes Related to Wood Formation in *Acacia* Hybrid

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Introduction

Tropical *Acacia* species have been proved to be fast growing species with high adaptability to a wide range of habitats and soils. *Acacia mangium* and *A. auriculiformis* have been planted for wood industry, charcoal production, pulp and paper production, furniture industry and rehabilitation purposes. The natural hybridization of the two *Acacia* species produces a hybrid which posses better characteristics than both the parental trees. For example, the hybrid has better stem form and self pruning ability (Yamada *et al*., 1990). The *Acacia* hybrid has a growing demand especially for fibre source in the pulp and paper making industry. There are three major components involved in the wood formation process, namely cellulose, lignin and hemicellulose. These components play a major part in determining the quality and quantity of the fibre source for pulping process. The higher the cellulose content in the fibre, the higher the pulping yield. On the other hand, lignin is an undesirable component in paper production. The large amount of chemicals used to discard the lignin can cause pollution to the environment and higher cost in the paper production industry. With the increasing demand for wood pulp and paper, combined with the concerns about the environmental effects of chemical intensive paper production, it is clear that nontraditional efforts to increase pulp yield and the incorporation of novel value-added traits are needed. These can be done by bringing together the genetic markers and breeding programme to produce planting materials with better quality.

A genomic library is constructed from the total DNA. The main advantage of a genomic library is that it contains all the sequences of the species including exon, intron and regulatory sequences which enable a more complete understanding about the genetic make-up of an organism. A phage genomic library is a library constructed using phage as the vector. Phage vector is capable of accepting fragments with range of 9kb-23kb. On the other hand, a cDNA library is derived from mRNA. cDNA library provides information on the partial sequences of the genes. To date, a cDNA library has been constructed for the *A. mangium* x *A. auriculiformis* hybrid using the inner bark tissue (Cheong *et al*., 2003). Partial sequences for some of the genes involved in wood formation had been identified in the acacia EST dataset. For example, phenylalanine ammonia lyase, cinnamyl alcohol dehydrogenase, cinnamate 4-hydroxylase, caffeic acid 3-*O*-methyltransferase which involve in the lignin biosynthesis; cellulose synthase and xyloglucan endotransglycosylase which involved in the synthesis of cellulose and hemicellulose respectively (Cheong *et al*., 2003).

Materials and Methods

Library construction

Young leaf sample was collected and kept in -20°C freezer until use. Genomic DNA was extracted from the leaf sample using a modified CTAB method from Murray and Thompson (1980). Purification was carried out using the conventional phenol-chloroform method modified from Murray and Thompsom (1980) as well. The purified DNA was then partially digested with *Bam*HI (New England Biolabs) and fragmented on a 0.8% agarose gel using CHEF-DR III Pulse Field electrophoresis systems (Bio-Rad, USA) at 6v cm-1, 14°C and ramping times of 3 seconds and 7 seconds for 16 hours. DNA fragments with size range of 12kb-19kb were purified from the gel using QIAEX II Gel Extraction Kit (Qiagen, Germany). Ligation was carried out using the Stratagene DNA I Ligation kit. Genomic library was constructed using the Lambda EMBL3/*Bam*HI Vector Kit (Stratagene, USA).

Titering

Titering of the library was done by plating out the mixture of the final packaged reaction and the host cells. This was carried out using a serial dilution of the final packaged reaction. 1 ul of the final packaged reaction was added to 200 ul of host cell (XL1-Blue MRA P2), and the mixture was incubated at 37°C for 30 minutes. 3 ml of LB top agarose was added to the mixture. The mixture was poured immediately on a prewarmed LB agar plate. The plate was incubated at 37°C for 8-12 hours. The number of plaques was counted and the titer of the plaque-forming unit per milliliter (pfu/ml) was determined.

Amplification of the library

Library was amplified once to make a large and stable quantity of a high-titer stock of the library. Twenty aliquots of packaged mixture each containing ~5 x 10^4 pfu of bacteriophage were combined with the 600 ul host cells each into 20 Falcone tubes. The mixtures were incubated at 37°C for 30 minutes. Then, 6.5 ml LB top agarose was added into each mixture and spread evenly onto a freshly poured LB agar plate. The plates were incubated at 37°C for 8-12 hours. After incubation, 10 ml of SM buffer was added to each plate and stored at 4°C overnight. Bacteriophage suspension was recovered from each plate pooled into a sterile polypropylene tube. Plates were rinsed with additional 2 ml of SM buffer and pooled into the same polypropylene tube. Chloroform to a 5% (v/v) final concentration was added to the suspension. The mixture was mixed well and incubated at room temperature for 15 minutes. The tube was centrifuged at 500xg for 10 minutes, and the resulted clear supernatant was transfer into a sterile polypropylene tube. Chloroform to a 0.3% (v/v) final concentration was added and the aliquots of the amplified library were stored at 4°C. Four aliquots of the amplified library were stored in 7% (v/v) DMSO at -80°C for long term storage. The titer of the amplified library was determined using host cells and a serial dilution of the library.

Results and Discussion

Genomic DNA yield extracted from the leaves was high because young leaves were used in the extraction (Figure 1). Three grams of leaves were used for each sample. The DNA concentration was quantified using electrophoresis. The concentration of the extracted DNA was approximately 1ug/ul DNA.

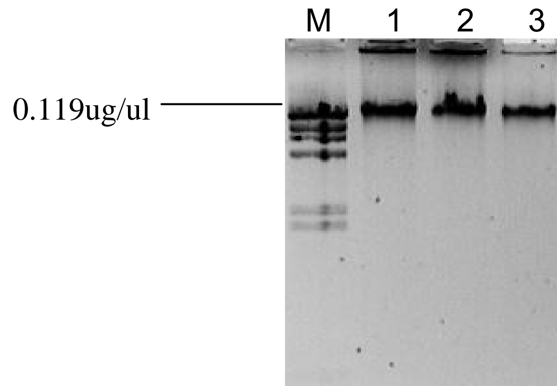

Figure 1: DNA extracts from young leaves. Lane M is Lambda *Hind*III (500ug/ml) used as size marker and lanes1-3 show extracted genomic DNA.

The Lambda EMBL3 vector is a genomic replacement lambda phage vector capable of accepting *Bam*HI compatible fragments ranging in size from 9kb to 23kb. The vector arms were double digested with *Bam*HI and *Eco*RI. This would produce arms with *Bam*HI ends and the stuffer fragment with *Eco*RI ends. Target DNA digested with *Bam*HI was ligated with the vector arms through sticky ends. Genomic DNA digested with enzyme *Bam*HI was fragmented on 0.8% agarose gel using pulse field system. Figure 2 shows the genomic DNA partially digested using *Bam*HI. For ligation, DNA inserts with size ranging from 12kb to 19kb were chosen, and the concentration of the DNA used was ~0.4ug /ul. The ligated DNA was then packaged into vector and transformed into host cell. Only recombinant phages are able to grow in this host cell which enable the selection of the positive plaques.

The library was plated on LB agarose plates and the titer of the library was 10^4 pfu/ml. After one round amplification, the pfu/ml was 10^6-10^7. This number is lower than 10^9 pfu/ml which is the expected titer. This might be due to the fact that predigested vector was used for the ligation. Uncut wild-type lambda DNA packages with higher efficiencies than predigested vector. However, the uses of predigested vector enables the selection of DNA inserts with known sizes.

The aliquots of the library in 7% DMSO were stored at -80°C for long term storage. The titer of the library will remain for long time in this storage. The aliquots of the library stored in 0.3% chloroform will be used for further study. Titer of the library stored in 0.3% chloroform should be checked from time to time. Plaque lifting and gene screening for the desired genes will be carried out using the hybridization approach. With the constructed genomic library, the full length of the genes involved in wood

formation in the acacia hybrid could be determined and characterized. The genomic library of acacia hybrid may also facilitate the construction of genetic linkage map of the acacia hybrid for the breeding programme.

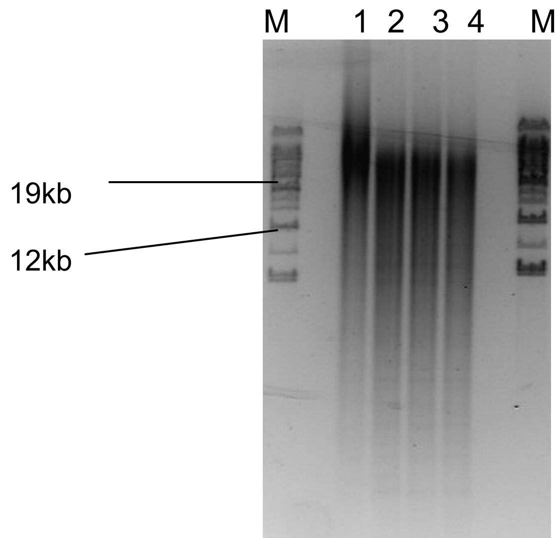

Figure 2: *Bam*HI partially digested genomic DNA resolved on the pulsed-field gel electrophoresis. Lane M is Lambda Mix used as the size marker. Lanes 1-4 show the DNA digested for 1 hour, 2 hours, 3 hours and 4 hours respectively.

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Development of Microsatellite Markers for *Acacia* Hybrid (*A. mangium* x *A. auriculiformis*)

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Introduction

Acacia mangium and *A. auriculiformis* (Leguminosae: Mimosoideae) are two tropical species native to areas extending from northern Australia to Papua New Guinea and the Maluku of Indonesia (Doran & Skelton 1982). Both are fast-growing multipurpose species widely used for timber, fuelwood, tanning, soil improvement, ornamental horticulture and agroforestry (Turnbull 1986). Spontaneous hybrids of these two species have been reported from plantation grown trees in Sabah, Malaysia (Sim 1987). The hybrid shows superior characteristics in growth, adaptation to a wide range of soil and resistance to pests and diseases over the parental species (Pinso & Nasi 1992). The objective of the current study is to develop microsatellite markers for *Acacia* hybrid to be used in linkage mapping.

Materials and methods

Microsatellite primers were isolated from *Acacia* hybrid using nylon membrane hybridization (Edwards *et al.* 1996) and the 5' anchored polymerase chain reaction (PCR) technique (Fisher *et al*. 1996). Loci were initially screened for polymorphism by running PCR products on 1% agarose gels. For those loci showing multiple alleles, low stutter and robustness of interpretation, forward primers labelled with fluorescent dyes were synthesized and further used to confirm the polymorphic loci using two parental samples. Each PCR reaction consisted of 5 ng of template DNA, 0.5 μM of each primer, 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 U of *Taq* DNA polymerase (Promega). Cycling parameters were as follows: 94°C for 2 min, 35 cycles of 30 s denaturation at 94°C, 30 s at optimised annealing temperature and 20 s extension at 72°C. The final extension was conducted at 72°C for 10 min. For genotyping, the PCR products were electrophoresed along with a GeneScan ROX 400 internal size standard on an ABI PRISM 377 DNA sequencer (Applied Biosystems). Allele sizes were assigned against the internal size standard and individuals were genotyped using GENESCAN analysis software and GENOTYPER software version 2.5 (Applied Biosystems).

Results and Discussion

A total of 267 markers were isolated based on library-enrichment methods. After primer optimization on agarose gels, 211 primers were labelled with fluorescent dyes to confirm the polymorphic loci in two parental samples from the mapping population developed for linkage study. The results showed that with the population tested, 106 markers were monomorphic and 53 markers were homozygous polymorphic. These two categories of markers would not show segregation in our mapping population, however, these markers could be used in other mapping populations as long as they show heterozygous genotypes in the parental samples of the respective mapping population. A further 26 markers were obtained which were heterozygous polymorphic, thus could be used for linkage analysis in our F_1 progenies. The low number of heterozygous markers found in this population might be due to the narrow genetic diversity found in the selected parent samples. Both *A. mangium* and *A. auriculiformis* have shown low levels of genetic diversity (Moran *et al*, 1989a, 1989b). One of the ways to improve the detection of heterozygous genotypes in parent samples is to select parent materials from two different provenances. However, both the parent trees must be at a similar growing stage to permit controlled pollination to be carried out between the two species.

Acknowledgements

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Development of EST-SSR Markers for *Acacia* Hybrid (*A. mangium X A. auriculiformis*)

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Introduction

Microsatellites, or simple sequence repeats (SSRs), are sequences made up of a single sequence motif, usually not more than six bases long, that is tandemly repeated and arranged head-to-tail without interruption by any other base or motif. They are ubiquitous in eukaryotic genomes and have been found in every organism investigated so far (John 1999). SSR markers are characterized by their hypervariability, abundance, reproducibility, Mendelian inheritance and codominant nature. Polymorphism is revealed in the form of differences in length, which in turn depend on the number of repeat units (Fraser et al. 2003). SSR markers consistently showed the highest level of polymorphism when compared to AFLPs and RAPDs in the study of barley and potato (Milbourne et al. 1998). They have been recognized as the marker of choice in plant genetics research and for breeding purposes because of their hypervariability and high abundance in the genome. However, traditional methods of developing SSR markers from anonymous DNA fragments of genomic DNA libraries are laborious, intensive, time consuming and expensive, which is a barrier to the widespread use of these molecular markers.

As a free by-product of currently expanding EST databases, EST derived SSR are now being utilized as an alternative source of microsatellite markers. EST-SSRs have been investigated in higher plants such as *Arabidopsis thaliana*, maize (*Zea mays*), soybean (*Glycine max*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Cardle et al. 2000; Toth et al. 2000; Morgante et al. 2002). As EST-SSR are found within transcribed regions of the genome, these markers may be less polymorpohic than those from untranscribed regions, however, possible conservation of the primer sites could make them more transferable across species, an aspect that could increase their value in a breeding programme (Fraser et al. 2004). This higher transferability has been shown in grape (Scott et al. 1999) and sugarcane (Cordeiro et al. 2001) at different taxonomic levels.

EST-SSRs also have some intrinsic advantages: they are quickly obtained by electronic sorting, are unbiased in their repeat type, are present in gene rich regions of the genome and are abundant (Rajeev et al. 2002). Due to all these attractive features, we have used our *Acacia* hybrid (*A. mangium X A. auriculiformis*) EST database to assess the occurrence of SSRs and to develop SSR primer pairs for amplification of

polymorphic markers. We have also tested these markers to evaluate for their suitability for genetic linkage mapping of our *Acacia* hybrid population.

Materials and Methods

6415 non-redundant EST sequences from an *Acacia* hybrid EST database were searched for microsatellites using the SSR Finder program (http://61.50.158.108/molecularbreeding/index.jsp). Since the minimum length of repeats was defined as 14 bp and with sufficient sequence flanking the SSR to enable primer design, 68 dimeric SSRs and 58 trimeric SSRs were identified. 53 SSR primer pairs were designed using PRIMER 3 software (Rozen and Skaletsky, 2000).

Four parents from crosses for wood density (AM 20 X AA 6) and fibre length (AM 22 X AA 3) were used to screen these 53 primer sets. DNA was extracted from fresh leaf tissues using a modified CTAB method (Murray & Thompson, 1980). PCR reactions were as follows: 10 ng of DNA, 1 x PCR Buffer, 1.5 mM $MgCl_2$, 0.2mM dNTP, 0.4 μM of each primer and 0.5 unit of *Taq* polymerase. Reaction volumes were 12.5 μl. The following PCR profile was used in a DNA Mastercycler, Eppendorf, Germany. A single cycle of 94^0C for 2 min led into a programme of 35 cycles of denaturing 94^0C for 30s, annealing at an optimal temperature for a particular primer pair for 30s and 72^0C for 30s. PCR reactions ended with an elongation step at 72^0C for 10 min. The amplification products were analyzed using 6% polyacrylamide denaturing gels followed by silver staining.

Results and Discussion

From 6415 non-redundant EST sequences, there were 1042 (16.24%) ESTs which contained SSRs. These results clearly illustrate that ESTs are a valuable source for mining SSR sequences. Non-redundant ESTs were used because they are unique sequences which can be expected to yield informative SSR markers for the mapping of non-redundant genes (or unigenes). In our study, no mononucleotide repeats were identified. Hexanucleotide repeats were the most abundant class of SSRs in the EST sequences (Figure 1). These findings are in contrast to previous observations in other species where hexanucleotide repeats were the least frequent (Cardle et al. 2000). This difference may be explained due to a variation in the quantity of sequence data analysed and differences in defining the criteria for SSR mining in the EST databases (Rajeev et al. 2002). For every class of SSRs, the frequency of SSRs decreases with increasing repeat length (Figure 2). The heptanucleotide repeats found had insufficient length to be of use, whilst our set of defined SSRs composed of di-, tri-, tetra-, hexa- and pentanucleotide were present in decreasing proportions of 47%, 40%, 8%, 4% and 1%, respectively (Figure 3).

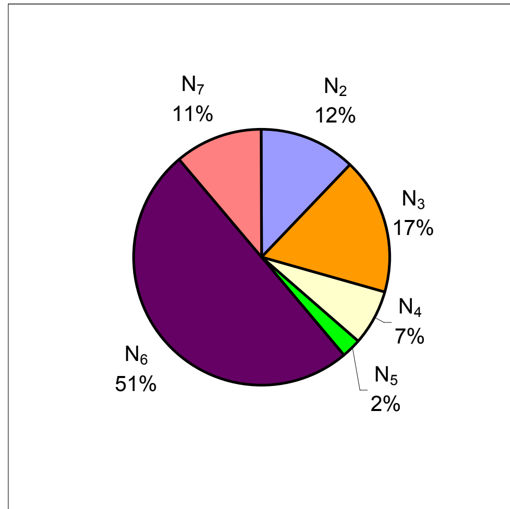

Figure 1. The distribution of EST-SSR in *Acacia* hybrid.

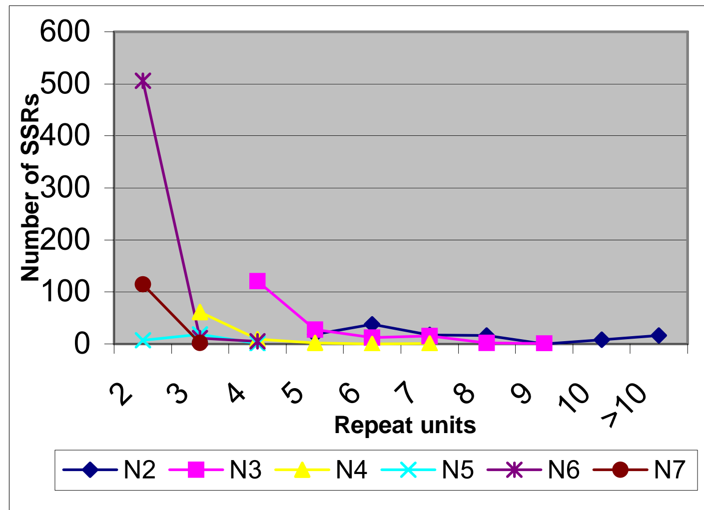

Figure 2. The distribution of EST-SSR of different repeat motif.

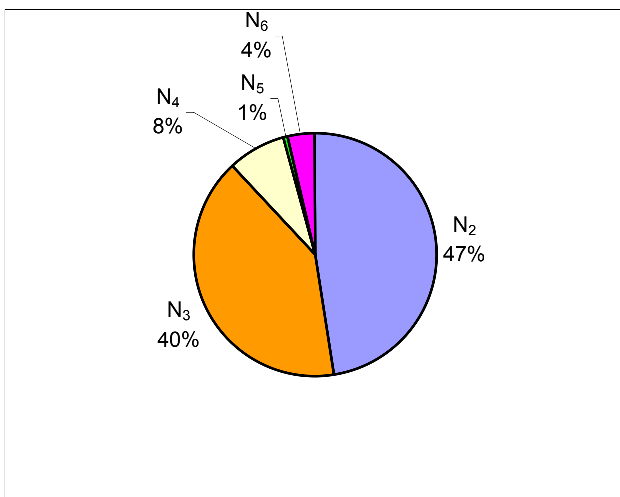

Figure 3. The proportion of defined EST-SSR.

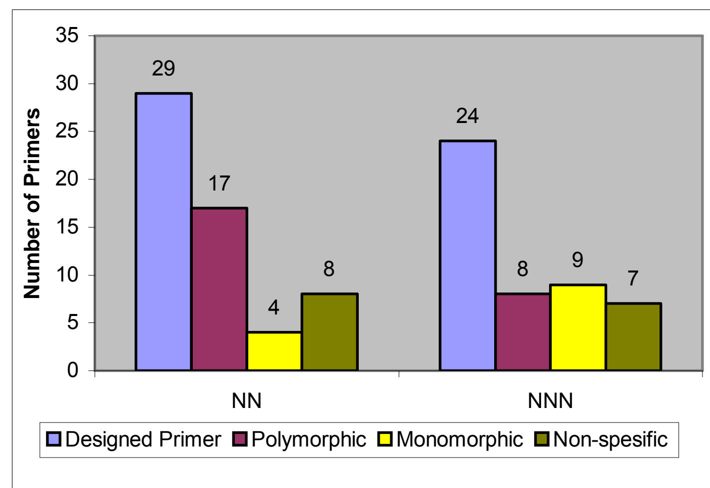

Figure 4. The evaluation of the potential EST-SSR primers.

Therefore, only 143 of these SSRs were identified to fulfill our criteria for use as molecular markers in this study. Of these, 126 potential EST-SSRs with di- and trinucleotide repeats were used to design primers, because of their higher number of repeated units. The number of core repeats is variable and has been attributed to strand slippage during DNA replication, or unequal exchange in meiosis. Experimental evidence suggests that the rate of slippage is dependent on the size of the repeat unit, being greatest for dinucleotide repeats. The rate of slippage is also dependent on the unit sequence, GC repeats being the most stable in rice (Schlotterer and Tautz 1992; Valdes et al. 1993). In this study, we found that poly TA/AT was the most common

among the dinucleotide repeats, while poly AAG/TTC was the most common among trinucleotide repeats. This observation is in agreement with Stallings (1992) and

Lagercrantz et al. (1993), who found that GA and AT repeats were more common than CA repeats in plants, whereas AAG repeats were the most common trinucleotide repeats in EST-SSRs (Gupta et al. 1996).

Of the 53 primer pairs tested to date, all could be amplified under the optimized conditions, 25 pairs (47.17%) were polymorphic, 13 pairs (24.53%) were monomorphic and 15 pairs (28.3%) gave non-specific products. The evaluation of the potential primers is shown in Figure 4. The product size range was between 153 bp – 299 bp. Annealing temperature for all tested primers was in the range of 30^{0}C – 60.5^{0}C. Non-specific primers may hybridise with more than one priming site resulting in multiple bands. Some primers amplified larger fragments than expected, reflecting the possible presence of introns within the genomic DNA sequence.

In conclusion, EST-SSRs are a viable source of polymorphic, highly transferable SSR markers, which have potential for genetic mapping of *Acacia* hybrid.

Acknowledgements

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Development of Cleaved Amplified Polymorphic Sequence (CAPS) Markers for *Acacia* Hybrid (*Acacia mangium* x *Acacia auriculiformis*)

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Introduction

Acacia hybrids have shown superior characteristics in growth, adaptation to different types of soil, resistance to diseases and higher pulp yield over the parental species (Pinso & Nasi 1992) showing their importance to the forest industry. Marker-aided selection has the potential to provide rapid selection many years earlier in the growth cycle of such hybrids. To study the genetic linkage maps of *Acacia* hybrid, we have developed cleaved amplified polymorphic sequence (CAPS) markers utilizing amplified DNA fragments digested with restriction endonucleases, to display restriction site polymorphisms.

Materials and Methods

Total genomic DNA of *Acacia mangium*, *A. auriculiformis* and *Acacia* hybrids (from controlled pollination) was extracted from leaf tissues using the DNeasy Plant Mini Kit (Qiagen, USA). A total of 309 primer pairs, based upon expressed sequence tags (ESTs) derived from an *Acacia* hybrid cDNA library (Wickneswari *et al.* 2004), were designed using Primer 3 software (Rozen & Skaletsky 2000). These primers were tested for PCR amplification using a GeneAmp PCR System 9700 (Applied Biosystems) in a 10 mL reaction volume, with 10 ng DNA, 50 mM KCl, 20 mM Tis-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.5 mM of each dNTP, and 0.5 U of *Taq* DNA polymerase (Promega). The following temperature profiles were used for the PCR: denaturating at 94°C (3 min) followed by 35 cycles of: 94°C (1 min), 50-60°C (1 min), 72°C (2 min) and final elongation at 72°C (10 min). PCR products amplified from the two parental trees were subsequently digested with 36 endonucleases (*Hpa*II, *Mbo*II, *Scr*FI, *Rsa*I, *Msp*I, *Hha*I, *Ase*I, *Hind*III, *Hae*III, *Eco*RV, *Bgl*I, *Bgl*II, *Sty*I, *Hinc*II, *Ban*II, *Nsi*I, *Eco*RI, *Nci*I, *Dra*I, *Ava*II, *Ssp*I, *Hinf*I, *Sau*96I, *Alu*I, *Hph*I, *Bam*HI, *Sal*I, *Pst*I, *Nhe*I, *Spe*I, *Sac*II, *Pvu*II, *Eco*0109I, *Mlu*I, *Bst*BI and *Xho*I) in single enzyme reactions. The products were then observed for restriction patterns following electrophoresis in 2% agarose gels stained with ethidium bromide. Primer pairs which produced heterozygous restriction patterns were verified using 16 F_1 progenies.

Results and Discussion

From the 309 primer pairs designed, 109 produced specific amplification products. In screening for restriction-site polymorphism, there were 128 homozygous polymorphic markers and 29 revealed heterozygous genotypes following digestion with restriction endonucleases. The group of homozygous polymorphic markers would not show segregation in our mapping population but might be useful in other mapping populations if heterozygous genotypes were detected. The segregation of the heterozygous polymorphic marker (locus CA0394) in the progenies from an *A. mangium* x *A. auriculiformis* cross is shown in Figure 1. Ten out of the 29 heterozygous markers were characterised for mode of inheritance using 16 F_1 progenies. Qualitative observation showed all to follow a co-dominant mode of inheritance. An example using the restriction enzyme *Hinc*II is shown in Figure 1. These preliminary results show that the markers developed have the potential for the construction of a linkage map for *Acacia* hybrid. The haploid genome size (1C-value) for *Acacia* hybrid has been estimated as 0.675 pg (=650 Mb, Arumuganathan, personal communication), which is small compared to 21-23 pg for pine genomes (Wakamiya *et al*. 1993). Based on one centimorgan (cM) being equivalent to one million base pairs, the estimated total length of the *Acacia* hybrid genome is 675cM. In order to have a sufficient map density i.e., at least one marker in each 5 cM segment, a total of 135 markers will be required for linkage mapping in *Acacia* hybrid (Ben Hui 1998).

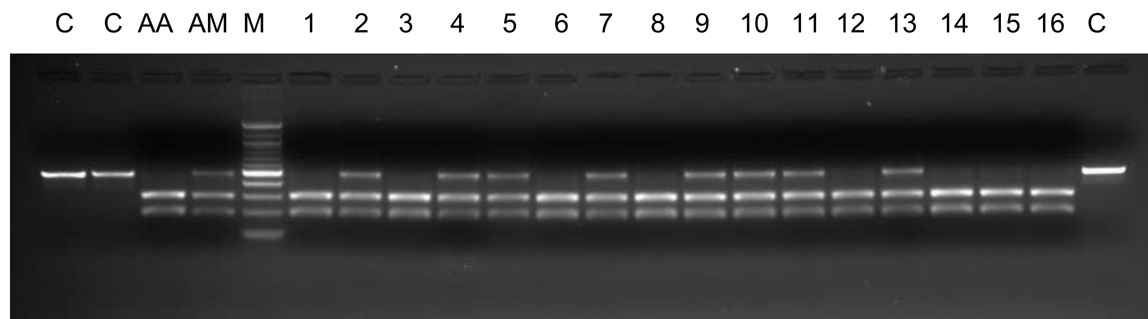

Figure 1. Segregation of the locus CA0394 in F_1 progenies (Numbered 1 – 16) from *Acacia mangium* x *Acacia auriculiformis* cross after digestion with *Hinc*II (C=control before digestion, AA=*Acacia auriculiformis*, AM=*Acacia magium*, M=100 bp DNA ladder)

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Mating System and Intraspecific Variation in *Acacia* Hybrid (*A. mangium* x *A. auriculiformis*) Using RAPD and Allozyme Markers

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Introduction

Acacia is a large woody genus includes over 1200 species occurring naturally in all continents except Europe (Guinet and Vassal, 1978). Two of the tropical acacias, namely *Acacia auriculiformis* and *A. mangium* have been introduced to Malaysia and shown promise as multiple use plantation species, especially on poor soils in the humid tropics (Turnbull, 1986). Spontaneous hybrids combine desirable properties of the parental species: tend to grow vigorously, have better form than *A. auriculiformis*, and have lighter branching, better self pruning ability and smoother bark than *A. mangium* (Bowen, 1981). The aims of this study are to examine the variation among the seeds within pod using RAPD markers and to evaluate the mating system using allozyme markers. The rationales are to see whether the seeds within a pod are clonal materials and how many progenies of hybrid will remain as hybrid.

Materials and Methods

Samples were collected from 5-year-old *Acacia* hybrid trees growing in Forest Researh Institute Malaysia, Kepong. Four inflorescences, two pods in each, from two families were sampled for intraspecific variation study. For mating system analysis, seeds were collected from 12 *Acacia* hybrid half-sib families.

Six RAPD primers were used to screen 127 individual seeds (Table 1). Reactions were performed in 10 µl consisting of 30 ng of template DNA, 2 mM Mg^{2+}, 0.3 µM primer, 0.5 unit of *Taq* polymerase (Promega, USA), 1 X *Taq* polymerase buffer and 200 µM of each dNTP. The PCR programme was 2 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 34°C and 2 min at 72°C. Amplification fragments were scored as 1 (present) or 0 (absent). RAPD profiles were compared among seeds within each pod to determine whether they were different. Variation in RAPD profile was analysed with analysis of molecular variance (program AMOVA, version 1.55, Excoffier *et al.*, 1992).

Table 1: RAPD primer sequences

Primer Code	Primer sequence 5'-3'
OPA-13	CAGCACCCAC
OPC-02	GTGAGGCGTC
OPC-05	GATGACCGCC
OPC-08	TGGACCGGTG
OPU-15	ACGGGCCAGT
OPU-19	GTCAGTGCGG

Enzyme extraction and starch gel electrophoresis of 40 germinating embryo tissues of each tree were carried out following Wickneswari and Norwati (1991). Mating system parameters were analysed using six allozyme loci: alcohol dehydrogenase (ADH), uridine diphosphogluconate pyrophosphatase (UGP), aspartate aminotransferase (AAT), shikimic dehydrogenase (SDH) phosphoglucomutase (PGM) and glucose phosphate isomerase (GPI). Maximum likelihood estimates of multilocus (t_m) outcrossing rates were calculated using MLTR version 3.0 (Ritland, 2002). For inheritance studies, the observed segregation ratio of *A. mangium*, hybrid individuals, and *A. auriculiformis* was tested for goodness-of-fit to expected Mendelian ratios using chi-square analysis. The null hypothesis is that the population sampled has a 1: 2: 1 ratio.

Results and Discussion

The RAPD primers revealed polymorphic bands with the size ranged between 450 and 2600 bp. A total of 44 RAPD profiles were generated among the 127 individual seeds from 16 pods. Two to four RAPD profiles were observed in each pod. An analysis of molecular variance (AMOVA) indicated that most of the genetic variation was attributable to differences among seeds within pod (65.7 % to 70.2 %), followed by variation contributed by the differences among pods within inflorescence (28.9 % to 37.1%). This result indicated that the seeds within pod were not clonal materials with different RAPD profile represents different individual. The finding of variation in seeds within a pod will allow more than one seed from a single pod to be used for the establishment of mapping population.

Mating system study inferred the hybrid to follow the mixed-mating model (outcrossing rate = 49% to 100%). Outcrossing is encouraged by the presence of pollinators such as *Apis mellifera* and *Ceratina* sp. (carpenter bee) because they are the most common visitors and carry a heavy load of hybrid polyads (Sornsathapornkul and Owens, 1998). The goodness-of-fit test shows that the progenies segregated according to a 1: 2: 1 ratio (*A. mangium*: *A.* hybrid: *A. auriculiformis*). This might be contributed by pollens from the hybrids planted along in the experimental plot. This implies that the seeds harvested from a hybrid plantation will consists 50% hybrid seeds and the remaining 25% will be each of *A. mangium* and *A. auriculiformis*. Therefore if a mass

production of the hybrid is needed, they could be vegetatively propagated by root cuttings or tissue culture.

Acknowledgements

Laboratory assistance was provided by Mariam Din, Sharifah Talib and Ghazali Jaafar, whose help is greatly acknowledged. This research was supported by IRPA Grant 01-04-01-0043 PR0003/03-03 from Ministry of Science, Technology and Innovation, Malaysia.

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Development of *Acacia* Hybrid Mapping Populations

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Introduction

The natural hybrid of *Acacia mangium* x *A. auriculiformis* was first reported to occur in *A. mangium* plantation in Sabah (Sim 1987). The putative hybrid has also been detected infrequently in Papua New Guinea (Gunn *et al*. 1989), which is the native area of its parental species, *A. mangium* and *A. auriculiformis* (Pedley 1975, Maslin & Pedley 1982). Since then the putative hybrids have been reported to occur more frequently in *A. mangium* plantations in Malaysia and Taiwan (Darus & Ab. Rasip 1989, Kiang *et al.* 1989).

It is difficult to identify hybrids positively using morphological traits due to the great degree of phenotypic variation in them (Rufelds 1988). Generally, an over estimate of 5-10% was reported using seedling morphological guides (Gan & Sim 1992). Hybrids can be identified positively at seed or early seedling stage (i.e. 2-weeks-old) using isozyme markers (Wickneswari 1989).

The population structure and breeding systems of both the parental species have been well studied. The genetic diversity of *A. auriculiformis* (expected heterozygosity, H_e = 0.081) is higher than that of *A. mangium* (H_e=0.002) with both species exhibiting high outcrossing rates (Moran *et al.* 1989a, b, Wickneswari & Norwati 1993). Hence, there is an opportunity for *A. mangium* to be improved by hybridizing with *A. auriculiformis*. Selection for complementary traits, e.g. disease resistance, growth, form, and wood density should be emphasized in the hybrid-breeding programme.

A preliminary study showed that hybrids are suitable as general utility lumber with wood characteristics slightly superior to *A. mangium* (Shukari *et al*. 2002). It was also found that the *Acacia* hybrid produced better quality pulp and has higher pulp yield (in excess of 55 %) than either *A. mangium* or *A. auriculiformis*.

The objective of this study is to produce *Acacia* hybrid seeds through controlled pollination, in order to develop mapping populations, which would later be used for the construction of genetic linkage and QTL maps of the species.

Materials and Methods

Background of the study area

The study site is located at FRIM sub-station in Bidor, Perak (40°06’N latitude & 101°16’E longitude). The area was an ex mining site interspersed with sand, slime and sandy slime tailings. The plot was established in 1999. Trees of both selected species for this study ranged from 8 to 10 m height. *Acacia auriculiformis* was planted on sand tailings while *A. mangium* was planted on slime tailings. All the selected trees were at age 4 years old.

Selection of parent trees

Two *A. auriculiformis* were used as female parents and two *A. mangium* as the male donor parents in crosses based on their contrasting value for wood density and fibre length (Table 1). No reciprocal crosses were carried out due to weak attraction by *A. mangium* ovules for *A. auriculiformis*, which showed disoriented growth and few penetrations.

Table 1: Comparison of wood density and fibre length traits of the parents

		A. auriculiformis	*A. mangium*
1.	Tree label	A6	M20
	Wood density (g/cm^3)	0.9202	0.4576
2.	Tree label	A3	M22
	Fibre length (mm)	0.7253	0.9947

Methods of pollination

Fresh pollens from *A. mangium* were collected in the morning and air dried before sieving. The pollination was carried out using two methods i.e. emasculation method and direct method. In the emasculation method, all the stamens were discarded before the pollens were transferred onto the stigma. In the direct method, pollens from *A. mangium* were transferred to unemasculated *A. auriculiformis* flowers using a paintbrush.

Results and Discussion

Controlled hand pollination is an artificial method that could be used for genetic improvement. It is a very tedious and difficult procedure, particularly the emasculation of spike. Table 2 shows the results of pollinated flowers and pods harvested. A total of 90 pods were harvested from tree A6 in the wood density cross. For the fibre length cross, a total of 52 pods were harvested. All the harvested pods were sent to the tissue culture laboratory for *in vitro* germination. The number of seeds ranged from one to 12 per pod

with an average of four seeds per pod. Seeds that failed to germinate were considered as hard seeds.

Table 2 also shows that the success rate of pod production was between 2.7 and 3.2%. Some of the crosses from both methods appeared to be successful at the early stage. However, after several days the floral buds shriveled and fell off. This was probably due to pollen viability and incompatibility, environmental interference such as extreme changes in the weather condition and insect damage.

Table 2: Success rate of controlled pollination through emasculation technique

Cross	Pollination method	Number of flowers pollinated	Number of pods harvested	Success rate %
Wood density AB6 x MB20	Emasculation	3284	90	2.7
Fibre length AB3 x MB22	Emasculation	1609	52	3.2

According to Zakaria and Kamis (1991), the timing of anthesis in *Acacia* varies amongst species, mostly occurring during late night to afternoon. Most flowers begin to open about 2400 h and fully open about 0500-0600 h. The peak pollen availability, floral fragrance and stigmatic receptivity occur during the first half-day (0600-1200 h) after anthesis and decrease thereafter. This seems to be the most suitable period for effective pollination. However, high temperature will shorten the duration of stigma receptivity and the duration and viability of pollen. The hot temperature is unfavorable for pollination and fertilization.

In this situation, the weather at FRIM sub-station in Bidor, Perak was always hot and this have might led to the frequent failure of pod formation experienced in the project. The minimum and maximum temperatures were 21.7°C and 34.2°C, while the mean relative humidity was between 61% and 68%. Another major problem was the availability of the fresh pollen. There are no synchronous flowering season between *A. auriculiformis* and *A. mangium* during the project period.

Conclusion

From this study, it could be concluded that the success rate of producing *Acacia* hybrid is very low. Therefore, further study needs to be carried out to improve the technique.

Acknowledgments

We would like to thank Dr. Ang Lai Hoe for permitting access to his *A. auriculiformis* and *A. mangium* plots. We also would like to give high appreciation to Ms. Azzurawati, Mr.

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Micropropagation for the Development of Mapping Populations for QTL Analysis in *Acacia* Hybrid

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Introduction

Tissue culture is a sunrise technology for the multiplication of superior plants in large scale on a perpetual basis, for production of novel and improved plants and for biosynthesis of products of industrial and medicinal value from natural plant resources. Tissue culture in simple terms refers to the cultivation of the cells and tissues free from the mother plant in appropriate nutrient substrates under sterile conditions. The cells in culture divide, multiply and produce compounds specific to the plants and also differentiate (plant cells are known to possess "totipotency"). Since these plants are identical to the mother plant, tissue culture has been effectively used, mainly by the agricultural and horticultural industries, for commercial production and delivery of plants by many private enterprises.

Micropropagation has an immediate application, however, in integrated clonal propagation systems featuring the commercial planting of cuttings harvested from rapidly multiplied, micropropagated stool plants of the selected clones. This approach is of value only in very advanced breeding programmes incorporating the identification of outstanding clones. In many tropical timber species, breeding programmes are either not available yet, or are at an infancy stage, and therefore, individuals indicating superior developmental features are selected from a natural population or from a population for which the growth data are readily available.

However, in a recent R&D programme, which centred around the development of controlled crosses between parents selected for a particular trait, clonal propagation has been used as a means to produce sufficient genetically identical progenies to permit replicated field trials. In this case, two sets of controlled crosses of *Acacia* were made, to examine both fibre-length and wood-density. *Acacia mangium* and *A. auriculiformis* are exotic timber species known for both timber and pulp in Asia, particularly in Southeast Asia. Moreover, these species are already being successfully micropropagated in large-scale for plantation forestry establishment, by several private pulp and paper companies (especially in Southeast Asia, including in Indonesia, Vietnam and Malaysia). Over the past few years, there has been a dramatic change in the production of planting stock from traditionally seed based to an efficient, large-scale production of micropropagated plantlets. As part of our concerted effort towards studying the functional genomics of these species (i.e. *A. mangium* & *A. auriculiformis*), we have produced a large number of seeds from controlled crosses between the two species.

In this paper, we shall attempt to discuss the strategy involved in developing clonal representatives of two mapping populations from the various clones (hybrid seeds) obtained. This was necessary not only for replication, but also to conduct site specific responses for future analysis (i.e. QTL mapping).

Materials and Methods

In this study, the breeders were responsible for the production of over 2000 hybrid (i.e. *A. mangium* x *A. auriculiformis*) seeds from over 380 seed pods from over 70,000 crosses. Both immature and mature seeds were transferred to the tissue culture laboratory for *in vitro* germination and subsequent production of clonal representatives of each hybrid line.

Seeds harvested from mature seed pods were removed from their respective pods, air-dried, packed into paper-bags, labelled and transported to the tissue culture laboratory as soon as possible. At the laboratory, seeds were examined, detailed information recorded and seeds were prepared for the decontamination process prior to *in vitro* germination. Seeds were surface sterilised by first soaking in hot water (80°C) for 30 min; then agitating in 40% Chlorox solution (domestic bleach) containing one drop of detergent (e.g. Tween-20), for 20 min. Subsequently, seeds were washed thoroughly in sterile distilled water (SDW) until all traces of detergent were removed. Seeds were then blotted-dry on sterilised tissue towels and cultured onto MS basal medium. Seeds were observed regularly for signs of both germination and contamination.

In the case of immature seeds, the entire seed pods were harvested, packed in plastic bags, labelled and transported in ice on the same day to the laboratory. At the laboratory, seed pods were examined, detailed information recorded and pods were prepared for *in vitro* germination. Unopened seed pods were dipped in absolute alcohol and flamed before opening to isolate the young (immature) seeds. Seeds were cultured onto MS basal medium and observed regularly for signs of both germination and contamination.

For both mature and immature seeds, the resulting shoots were subsequently multiplied in MS basal medium supplemented with 0.5mg/L BAP. All cultures were incubated under ambient growth room conditions (i.e. 16h photoperiod, 26±2°C). Shoots confirmed to be hybrid, using isozyme markers, were then multiplied by culturing in MS basal medium supplemented with 0.5mg/L BAP. Fully-grown shoot cultures were rooted in MS basal medium supplemented with 1.0 mg/L IBA and subsequently transplanted onto moist compost mixture for hardening in the greenhouse. These plants are intended for use in clonal trials, as well as for the establishment of the mapping population for QTL analysis.

Results and Discussion

The tissue culture protocols developed for any plant species are usually conceived in as simple a manner as possible in order to cope with the constraints of scaling-up (i.e. low costs with high productivity rates). The technology enables the mass (micro) propagation of most plant and tree species by either shoot/stem cuttings (micro-cuttings) or through somatic embryogenesis (formation of asexual embryos from asexual cells), with exponential multiplication rates of three to five new shoots (plantlets) every four to six weeks.

In our case, with *Acacia* hybrid seeds, germination of seeds was evident as early as one week after culture initiation. Overall, we were able to obtain over 70% germination of total seeds introduced into culture. Although initially, contamination rates were fairly high (> 50%), we were able to reduce this drastically (to <10%) by improving our seed preparation and transportation methods. This is critical, especially in our case, because of long distances between the field and the laboratory, coupled with the harsh climatic conditions of an ex-mining area.

Germinated seedlings were ready for further multiplication within four weeks from germination and were able to produce just over two new shoots per sub-culture. During each sub–culture cycle (from the third sub-culture onwards), leaf and stem tissues were made available for isozyme analysis for hybrid confirmation. Non-hybrid plants were discarded and confirmed hybrids were further multiplied on a regular sub-culture cycle, with 6 weeks interval, to produce the desired numbers for clonal trials. A total of 400 clones (genotypes) were selected for both the traits being studied (i.e. 200 clones for fibre-length and another 200 clones for wood-density).

According to the experimental design for the clonal field trials, there is a need for 50 clonal representatives, to allow sufficient replication. Therefore, a comprehensive plant production plan is in place for the production of 20,000 clonal plants from the 400 selected individual genotypes.

Conclusion

The principal approach in mass propagation (i.e. via micropropagation) is axillary budding (actually a miniaturisation of propagation with cuttings), and the main attraction of micropropagation lies in its ability to multiply elite clonal materials very rapidly. More than 1,000 plant species have been micropropagated, including more than 100 forest tree species (Bajaj, 1991). For most industrial forest plantation species, the costs of planting stock and insufficient data regarding field performance remain the major obstacle to be overcome before a broader use of micropropagules as direct planting stock may be contemplated (Haines, 1992).

In this study, we have clearly demonstrated the usefulness of plant tissue culture techniques, especially micropropagation, for the establishment of a breeding population for use in QTL analysis and gene mapping studies.

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Applications of Genetic Markers in Tree Improvement of *Acacia* species in Vietnam

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Introduction

The forest cover in Vietnam was 12,094,518 ha (occupied 36.1% of total land area) in 2003, of which natural forest was 10,004,709 ha and forest plantation was 2,089,809 ha. On the other hand, the production plantation forest was about 1.24 million ha, and acacia plantation occupied 192,200 ha. Among them, 37, 000 ha of land was planted with *Acacia mangium* and *A. auriculiformis,* and about 46, 000 ha with acacia hybrids up to year 2003 (MARD 2004).

Acacia is one of the important tree species in Vietnam. Its timbers are used for pulpwood in domestic paper mills, raw material for manufacturing medium density fibreboard (MDF), woodchips for export, furniture and material for construction as well as represent one of the few economic potentials for land use in the central hills.

Acacia was first introduced in Vietnam for testing in the early 1960s. However, from the early introduction — 16 species in total — only *A. auriculiformis* was widely adopted for planting, mostly in the southern provinces (Turnbull *et al*., 1998). Tree improvement for *Acacia* species have been carried out in Vietnam since 1990 by the Research Centre for Forest Tree Improvement (Forest Science Institute of Vietnam - FSIV) using species/provenance trials, tree selections, establishing seed orchards and seed production areas and hybridization. These trials identified some promising species for different sites as follows: *A. mangium, A. auriculiformis*, *A. mangium* x *A. auriculiformis* hybrid, *A. crassicarpa* and *A. aulacocarpa* for low land areas; *A. difficilis, A. tumida* and *A. torulosa* for dry-zone areas and *A. mearnsii* for high land areas with great potential for commercial production in Vietnam (Le, 2003).

Genetic markers have been applied in *Acacia* species in Vietnam with the aim to strengthen and support breeding programs. Population structure and genetic diversity of acacia breeding populations were examined and monitored.

Research results

Monitoring breeding programs

Estimation of outcrossing rates in seed orchards

Information on population structure such as genetic diversity, genetic variation within and between populations, gene flow, seed dispersal, pollen contamination are prerequisite for selection in breeding programs. The outcrossing rate in seed orchards has important implication on extent of gene flow among trees. If trees in seed orchards exhibit high outcrossing rate, genetic diversity of the seed produced will be high. Theoretically, providing heavy widespread flowering occurs and effective pollinators are present, high levels of outcrossing are expected in a seed orchard as randomised planting of many unrelated families removes the neighbourhood inbreeding effect often found in natural populations (Elderidge *et al*. 1993).

Tran and Maurice (2002) studied the outcrossing rates in the *A. auriculiformis* Ba Vi seed orchard (mostly original seed sources derived from Sakearat - Thailand and Queensland - Australia) by isozyme markers. The results showed that outcrossing rate was relatively high (t_m = 0.89; SE = 0.07) with larger range among individual trees in the orchard (t_m = 0.32-1.00) compared to levels detected in natural populations by Wickneswari and Norwati (1995; mean t_m = 0.79, SE =0.04). The outcrossing rate was also within the range reported in natural populations of *A. auriculiformis* from Queensland and Papua New Guinea by Moran and Bell (1989; t_m = 0.93, SE = 0.04 and t_m = 0.92, SE = 0.03 respectively).

Trees with low outcrossing rates could result from either natural variation between families for self-compatibility, a lack of suitable pollination vectors or poor synchrony of flowering times. Variation between families for self-compatibility is not likely given the lower single-locus estimate compared to the multi-locus estimate (t_m–t_s = 0.06), which suggests occurrence of inbreeding rather than selfing (Shaw and Allard 1982). Pollinating vectors must exist in the orchard (at least in the year studied) for high level of gene flow to occur given that half of the trees assayed had outcrossing rate of 100%. It is more likely, therefore, that poor synchrony of flowering times among trees is operative in the trees with low outcrossing rates at Ba Vi. This is quite possible, because studies at the Sakaerat seed orchard in Thailand (Jiwarawat *et al.* 1996) showed that the northern territory provenances in this orchard have flowering peak some 2 months prior to Queensland and PNG provenances.

Orchard management would benefit if observations on the differences in flowering times among trees were made. Seed quality in the Ba Vi seed orchard could be further improved by removing trees which are consistently out of synchrony in their flowering time, relative to the main flowering peak of the orchard.

Outcrossing rates in the Ba Vi seed orchard indicated genetically diverse seed could be produced if the stands were established from a genetically diverse seed source (Tran and Maurice, 2002).

Seed orchard management

Acacia mangium is one of the valuable tree species in *Acacia* genus and was planted widely in Vietnam. A series of the Seed Production Areas and Seedling Seed Orchards were set up across sites in Vietnam with the aim to supply good seeds for plantation.

However, seed quality in term of genetic basis of seed orchards/seed production areas has not been investigated and compared to growth performance. Highly polymorphic markers (microsatellite markers) were applied to assess mating system and growth performance of six typical seed orchards/seed production areas across Vietnam (Table 1).

Table 1. Latitude, longitude, seed source, area of seed stand, stand density, age and the estimated proportion of trees with seed crops in sampled seed orchards in Vietnam.

Seed Orchard	Latitude (°N)	Longitude (°E)	Seed Source	Area (ha)	Stand density (trees ha^{-1})	Age (years)	Percentage of trees with seed crops
Bau Bang	11°15’	106°38’	Daintree (Qld)	0.5	200	12	>60 but sampled at end of seed crop
Ba Vi Fortip	21°08’	105°28’	PNG, Claudie River (Qld)	2	400	4	18
Ba Vi Pongaki	21°10’	105°20’	Pongaki (PNG)	2	250	8	not available
Dong Ha	16°47’	107°03’	PNG, Claudie River (Qld)	3	400	5	60
Ham Yen[A]	22°02’	106°02’	Queensland[A]	2	200	14	>80
Phong Chau	21°30’	105°13’	Cardwell (Qld)	2	200	>10	>80

[A]The seed sources for this seed orchard were from Queensland but the provenances are not known.

A minimum of 20 seed pods per tree were collected from each of twelve trees within each of the six orchards, except the Ba Vi Fortip where only ten trees were sampled. Seedling families (i.e. progenies raised from individual mother trees) were raised in a nursery at the Forest Science Institute of Vietnam in Hanoi. Orchard, tree and pod identities of all seedlings were retained. Leaves were sampled from one seedling from each of 20 pods per family to extract DNA for the study of outcrossing rates reported by Butcher *et al.* (2002). To determine whether progenies were outcrossed or selfed, they were genotyped, together with the mother, using six microsatellite loci: Am018, Am041, Am164, Am173, Am387 and Am465. Outcrossed progeny contained an allele not present in the mother. The seedlings were raised for planting out in field trials.

The objective of the field trials was to compare the growth performance of outcrossed and selfed plants and compare the performance of progenies from different orchards in order to identify the best orchard seed sources. After 24 months of planting,

height and diameter at breast height were measured and analyzed for different orchards as showed in Table 2.

Table 2. Growth of *A. mangium* after 24 months of planting.

Orchards	**Outcrossing rate**	**Height (m) (12 months)**	**Height (m) (18 months)**	**Height (m) (24 months)**	**$D_{1,3}$ (cm) (24 months)**
Dong Ha – FORTIP	100%	2.36	3.07	5.08	6.34
Ba Vi- PNG	93%	2.37	3.06	4.88	5.39
Phong Chau	93%	2.04	2.61	4.08	4.81
Ham Yen	94%	1.85	2.47	3.99	4.58
Bau Bang	49%	1.74	2.22	3.28	3.66
Ba vi – FORTIP	11%	1.61	2.04	3.12	3.56
F-prob*	0.06 (n.s)	0.06 (n.s)	0.06 (n.s)	0.06 (n.s)	0.04 (P < 0.05)

* *F*-probability of differences between orchards based on ratio of orchard mean square to main plots mean square, with 5 degrees of freedom for both numerator and denominator.
n.s. = not significant, $P < 0.05$ = *F*-probability < 0.05.

The orchards based on PNG provenances that have high outcrossing rate (Dong Ha-FORTIP and Ba Vi – PNG) also displayed best growth after 24 months of planting; 5.08 m and 4.88 m respectively. Whereas, the slowest-growing orchard – Ba Vi FORTIP - with mean height 3.12 m had lowest outcrossing rate (11%).

The results in field experiment indicated that selfing leads to reduced growth in *A. mangium* after 24 months. In our study, two orchards: Dong Ha - FORTIP and Ba Vi – FORTIP have similar genetic background (the same seed sources from a mix of PNG provenances plus about 30% representation of Claudie River, Queensland) but different growth performance due to different outcrossing rates. Flowering rate in orchards is one of the issues which affects outcrossing rate. Our observation in 6 orchards showed that Ba Vi FORTIP orchard only had 18% flowering trees at the time of seed collection whereas the Dong Ha – FORTIP had 82% flowering trees. These results suggest that seeds of *A. mangium* are only of good quality if the flowering trees are more than 50%; better quality if more than 70%.

Conclusions

Molecular genetic markers (isozyme and SSR markers) have been applied in forest tree improvement activities in Vietnam recently and showed good results. Genetic markers should be integrated with conventional breeding programs with the aim to shorten generation intervals and enhance forest breeding.

In the future, the genetic markers in Vietnam would be focused on the following aspects:

- To evaluate genetic population structure (mating system, seed dispersal, pollen movement etc.) of breeding populations and natural populations

- To construct genetic linkage map and study QTL, MAS for some priority tree species (Acacia, Eucalyptus and Pine) for some economical traits (wood quality, lignin content, stress, disease resistant etc.).
- To study gene expression for some necessary traits such as lignin content for early selection.

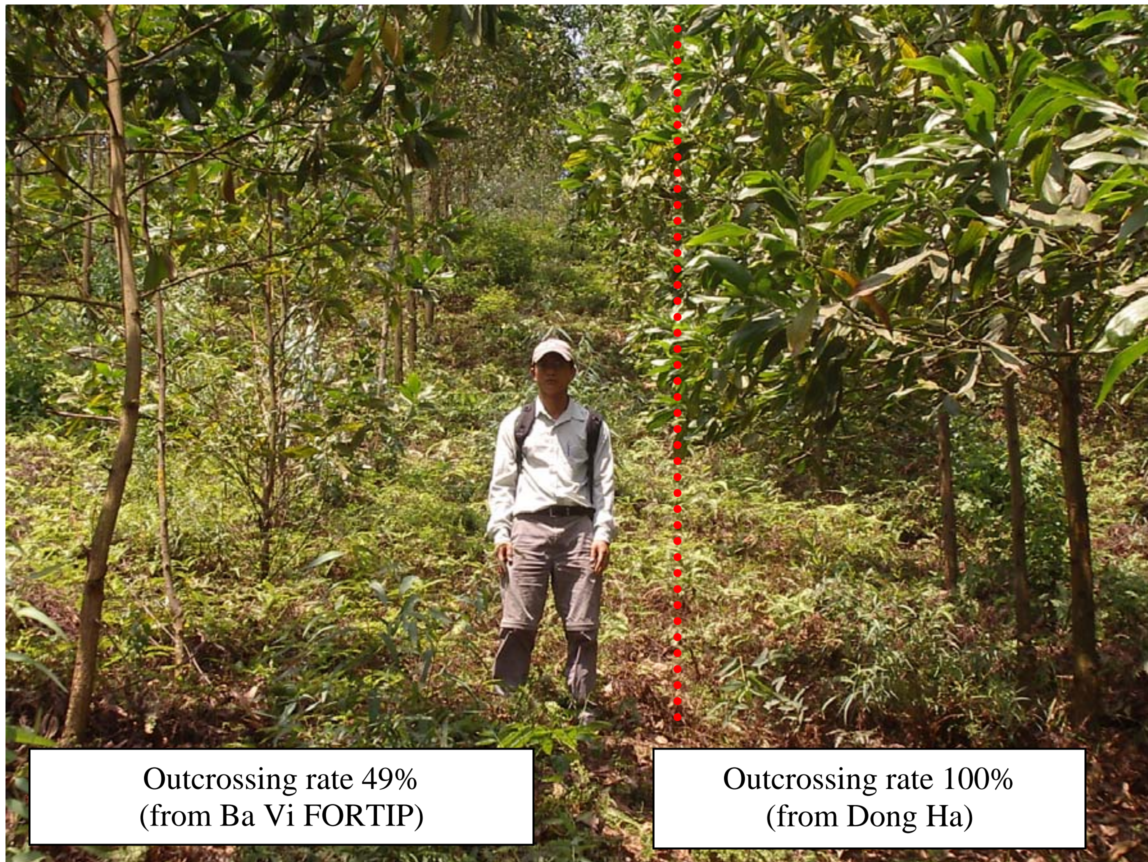

Figure 1. *Acacia mangium* growth after 24 months of planting.

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The Breeding Strategy of *Acacia mangium* and *Acacia crassicarpa* at PT Riau Andalan Pulp and Paper

Wong, C.Y.✉ and Wijoyo, F.S.

PT Riau Andalan Pulp and Paper is one of Indonesia's leading company in pulp, paper and forestry industries. The main reforestation species are *Acacia mangium* and *A. crassicarpa*. Starting in 1996, the company has implemented tree improvement program to ensure self-sufficiency in fiber production in the quantum of 2 million tons annually by year 2009.

This paper summarizes the development of the tree breeding strategy for *A. mangium* and *A. crassicarpa* that is currently being implemented in PT RAPP fibre plantation in Riau Province of Sumatra. The breeding objectives, breeding population, mode of reproduction and management of co-ancestry as the background of the current achievements will be discussed.

Introduction

PT Riau Andalan Pulp and Paper (PT RAPP) is a part of APRIL (Asia Pacific Resources International Holdings Limited) that produces fibre, pulp and paper with forestry and mill operations in Riau Province, central Sumatra, Indonesia. The company operates a pulp mill and a paper mill with a design capacity of 2 million and 350,000 tons per year respectively. This large operation is supported by an aggressive fibre plantation program. PT RAPP has a total concession of 330,000 hectares of which 240,897 hectares have been planted by the end of May 2005. The main species were *A. mangium* and *A. crassicarpa* with an annual planting of 40,000 ha. By 2009, acacia from the fibre plantations will be sufficient to produce 2 million tons of pulp.

Acacia mangium and *A. crassicarpa* have become the primary species in the fibre plantation program – *A. mangium* on mineral soils and *A. crassicarpa* on low-land sites. Since 1996, a tree improvement program of both acacias has been initiated to help growing a profitable and sustainable supply of fibre. Secondary species are also in research and testing stage to provide both potentially good growth and fibre properties. On mineral soils, since 1999, *Eucalyptus urophylla* x *E. grandis, E. grandis* x *E. pellita* and pure *E. pellita* as well as acacia hybrids (*A. mangium* x *A. auriculiformis*) have been evaluated for their genetic properties and effectiveness of vegetative multiplication.

Defining Tree Breeding Objectives

The objectives and goals of tree improvement research must be clearly defined and applied so that the results will produce real gains to the company. As a pulp and paper company, it is very obvious that the breeding objective is to improve delivered ADT pulp per hectare. However, when the program started in 1995, the initial goal was to provide an adaptable species to the local soil-site condition and build up a diverse base population for robust and long term breeding program.

In general, the planting sites are divided into mineral soil that mainly consist of ultisols soils, and low-land sites that are dominated by histosols soils (USDA, 1999). Following a series of species trials, it was found that *A. mangium* and *A. crassicarpa* are adapted to mineral and low-land sites respectively. The next goal was to provide information on which provenance or seed sources for use in operational planting. At this stage, it was realized that tree breeding required operational (production) and developmental (research) phases as described by Zobel and Talbert (1991). The program was then implemented as described in Figure1.

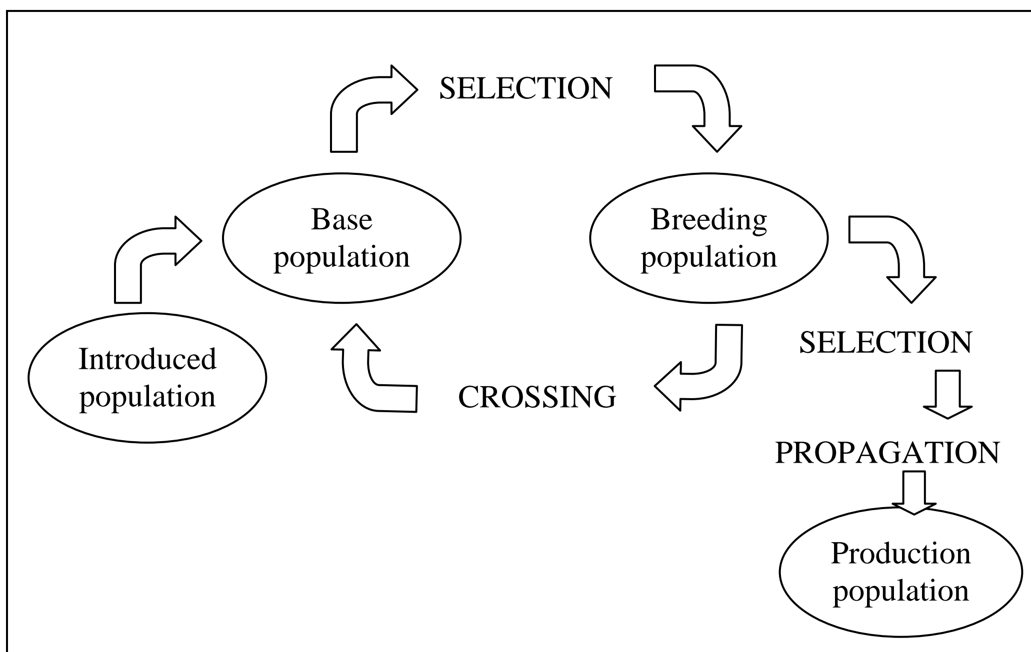

Figure 1. The development stage in tree breeding goals

In mid 2004, the tree breeding objectives of PT RAPP were formulated to maximize fibre yields per hectare by providing planting material with the highest genetic gains. The objectives will be achieved by concentrating into the following keys:

- The selection criteria will focus on volume, wood density and pulp yield

- Disease resistance traits will be attempted to breed for resistance only after carefully determining the real economic impact, available genetic variation, availability of screening method and cloning possibility.
- Each developmental phase would include a plan for deployment

Availability of Breeding Population

PT RAPP started the reforestation program by planting the best matched provenances of *A. mangium* on mineral soils. Most of these early plantings were able to trace back their seed identification. This information together with the results of provenance testing allowed us to select the best phenotypic performance of trees from mature (3 years of age or above) plantation of acceptable provenances from Papua New Guinea and Far North Queensland. More than 600 superior phenotypic trees (plus tree) had been selected between 1996 to 2000. The selection intensity was approximately 1 in 2000. The selected plus trees were then multiplied into sublines. The subline is also called as CBO (clonal breeding orchard). By the end of 2004, 15 CBO had been established.

The planting of *A. crassicarpa* was started in a much smaller area in the mineral soil in 1995. However, the operational planting of this species had only started in year 2000. This situation did not provide as much selection as in *A. mangium*. By the end of 2003, there were only 2 CBO that came from selected best phenotypes.

In order to increase the diversity of breeding population, a series of seed collections were conducted in Papua, in the eastern part of Indonesia during 2003 to 2004. *A. mangium* and *A. crassicarpa* are native to both Papua and Papua New Guinea and to Queensland, Australia. The seed collection took place in the areas as shown in Figure 2.

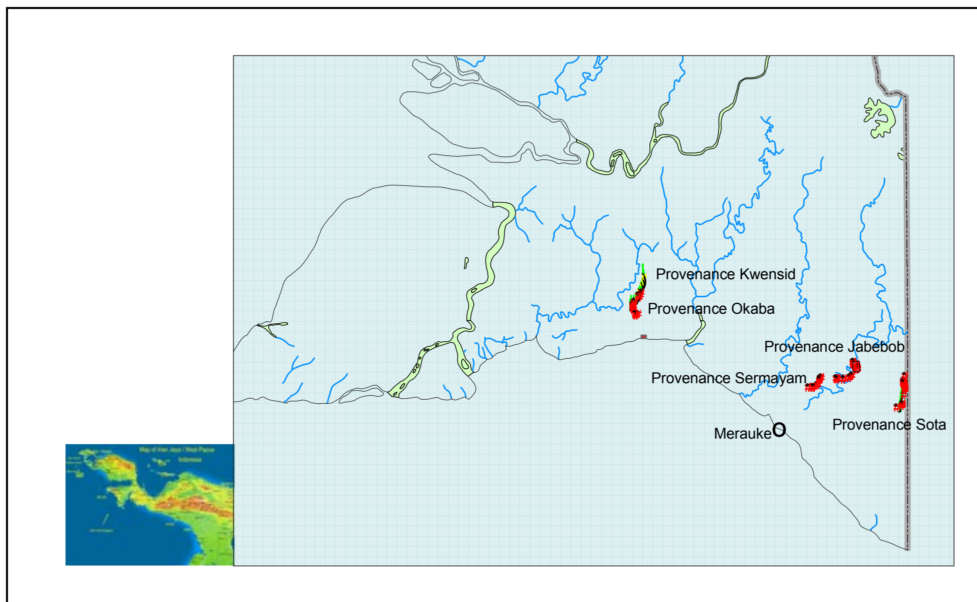

Figure 2. The seed collection sites in 2003 and 2004

Meeting Immediate Improved Stock

One important aspect in tree improvement is the development of improved trees followed by mass production of the improved stock. It is very important to ensure that the improved trees to be delivered to nursery for production of plants for operational planting. In order to meet the immediate seed requirement for operational planting, superior stands of known better performing provenances were converted into seed production area (SPA). By the end of 2004, there were more than 100 hectares and 250 hectares SPAs of *A. mangium* and *A. crassicarpa* respectively.

Most of the CBO have already been producing seeds. A CBO is actually also a seed orchard. On the other hand, the same genetic materials of CBO were also established in a series of Clonal Seed Orchards (CSO). There were 8 CSOs of *A. mangium* and 3 CSOs of *A. crassicarpa* established by the end of 2003. In 2003, the seed production from both CBO and CSO were able to supply improved seeds for operational planting of *A. mangium*. However, the quantity of the improved seed for operational planting of *A. crassicarpa* was still not enough to fully support operational planting. Vegetative propagation can be used to multiply these best materials in form of cloning to be used in mass-production of operational planting material. On the other hand, there are other constrains of using vegetative propagation in *Acacia* species. Clones need to be evaluated to determine their utility in an operational propagation. The main concerns were the onset of maturation effect and rejuvenation process. More studies are being initiated to address the issues.

Managing the Breeding Population

The current breeding population of PT RAPP is in the form of sublines. While each subline is also served as a seed orchard in terms of seed production, it is still quite expensive and complex to manage. Using the current approach, the number of sublines will be increased and even more in the advance generation. In the mid of 2004, the breeding population was divided into a main population (MP) and an elite population (EP).

The main population will include families in the testing process. In other word, the main population is the existing progeny tests of a generation. The MP will be advanced by open pollination. Seed will be collected from selected trees in progeny tests. As a part of risk management, the MP will be divided into 2 un-related sublines.

The elite population is the best selection from the available materials. The selection will also be divided into 2 un-related materials for the same reason as the MP. The EP will be established by the use of vegetative propagation. They will be set up as breeding orchard. The advancement of the EP will be managed through controlled pollination technique. This technique is required to be able to gain full control over pedigree and maximize the selection pressure on both male and female parents. It will also allow making specific combination as a just to improve the additive performance.

One of the concerns in restructuring the breeding population was the application of controlled pollination technique. *Acacia mangium* as well as *A. crassicarpa* have a unique flower structure. Flowers are in loose spikes to 10 cm long, solitary or paired in the upper axils. Flowers pentamerous, the calyx 0.6-0.8 mm long, with short obtuse lobes, the corolla twice as long as the calyx. Our current study in controlled pollination shows that the process is applicable in acacia as given in Figure 3.

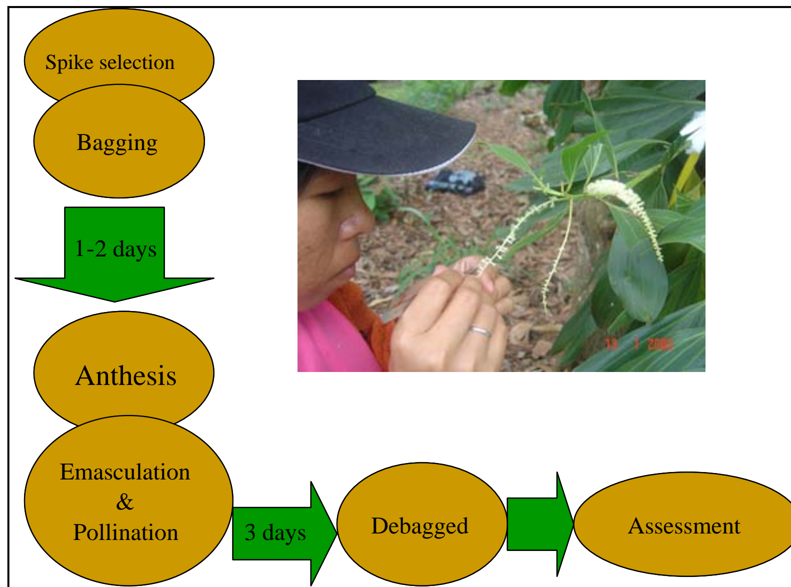

Figure 3. The flow of process of controlled pollination in Acacia

Conclusions

The experience in implementing tree improvement program in PT RAPP showed that a strategy with well defined objectives is required. It is a dynamic rather than static process that always has research and production phases. The breeding strategy should be reviewed by considering the current knowledge and needs, the nature of the species and the available resources.

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Application of GCMS for the Idenfication of Lignin Pyrolysis Products

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Lignin in hardwoods such as eucalyptus and beech consists of guaiacyl-(G) and syringyl-(S) propane units, containing one and two methoxy groups, respectively. It is known that the lignin content and its chemical structure have a significant influence in the pulping process. Pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) provides a rapid way to identify the degradation products from lignin, and to characterize lignin from different wood samples. It is particularly effective for lignins that pyrolyze to simple diagnostic compounds. Here we describe the application of GC/MS for identification of lignin pyrolysis products and give a few example applications.

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