5.0 DISCUSSION

Currently, most plantation forests are established from seeds collected from superior tree stock. In most cases, only the mother trees are known. The populations are therefore considered as open pollinated half-sib. Controlled pollination is rarely practiced due to limitations such as size and form of the tree, and long generation period. For example *A. excelsa* which was found to be the fastest growing trees in the arboretum of FRIM (Ng and Tang, 1974) has a gestation period of 7 years. Thus the normal breeding practice of backcross selection which require six to seven generations to introgress a desirable trait(s) into a population is difficult to achieve in forest trees. Progress in forest tree breeding has been slow. Can integration of marker technology such as AFLP helps to make the breeding process more efficient? Could the marker technology provide the breeders with more genetic information? In any breeding programme it is essential to gather as much genetic information as possible regarding the breeding population. In the *A. excelsa* experimental plot, a half-sib population was established. The genetic information was extracted from this population using a conventional method of morphological trait measurements as well as AFLP molecular marker technique. Selection of desirable or plus trees is recommended. Meanwhile, tissue culture techniques were investigated for eventual clonal propogation of these plus trees.

5.1 Genetic information derived from morphological traits measurement

5.1.1 Direct growth related traits

It is commonly believed that parental trees of larger sizes will produce larger sizes of progeny trees. Since most forest tree species are in very early stages of domestication in breeding programs, progeny populations were established from seeds collected from larger sized parent trees in the wild. In half-sib populations (where the paternal parent is not known), selection can only be made based on the phenotypic characters of maternal trees. In this *A. excelsa* experimental plot, seeds were collected from various sizes of mother trees from a trial plot. The setup of the *A. excelsa* half-sib population in the form of garden design was to eliminate variation components caused by environmental factors.

In Dbh measurements, it was found that mother trees with large mean Dbh do not necessary produce progenies with overall larger mean Dbh (section 4.4.1). The pattern was similarly occurred in total height trait. Since Dbh was found to be highly correlated to tree volume (see Table 4.20), mother trees with large volume do not necessary produce large size progeny trees. However, since the trees were still at an early stage of growth (2.5 years in the field), it is too presumptive to conclude that there is no genetic gain in raising progeny trees from seeds collected from large sizes mother trees in an open pollinated half-sib population. Moreover, we are dealing with a very small number of experimental samples. Nevertheless, there is a trend showing that the sizes of mother trees have a relatively strong effect on their

respective progeny trees (Table 4.7 and 4.12). This is also reflected in the high correlation (0.99) obtained between the mean tree volume of mother trees and progeny trees.

The occurrence of plus trees was found in all A category tree group based on Dbh measurements (Table 4.24). However, the highest frequency of plus tree occurrence may not be in A category. For example, B1 has the highest frequency of plus tree occurrence based on Dbh measurements. The results indicate that while overall genetic gain of the progeny population could be captured by selecting parents of desirable characters, maximum genetic gain could be obtained by selecting parents from a wide range of characters.

The heritability of all the three growth related traits were very low in magnitude (section 4.8). This is expected because growth related traits of economic importance are often quantitative traits that are usually less strongly inherited and more subjected to interaction with genetic background and environment (Kearsey, 1998; Strausset al., 1992). The genetic component of variance could be contributed by additive, dominance or epistatic variance, and additive variance is the component that is responsible for the inheritance of characters from the parent to the offspring. It is therefore expected that heritability contributed by additive variance alone or narrow-sense heritability will be low in all the three growth related traits under study.

5.1.2 Indirect growth related traits

The number of nodes represents number of leaflets formed in each tree. Thus a high number of nodes indicated a high volume of vegetative growth that could result in an increase in the photosynthetic rate of the tree. This will in turn lead to an increase in the size of the tree. In this study, it was found that there was no significant variability of sizes among progeny trees. Therefore the number of nodes in each tree could not be an indirect indicator for the sizes of the trees.

Another better measurement for the volume of the vegetative growth of the tree is the canopy diameter. This parameter assumed that the longer the canopy diameter length, the larger the crown thus the bigger the volume of the vegetative growth. There is a significant correlation (Table 4.21) between tree volume and canopy diameter (r^2=0.38) of progeny trees indicating possible influence of canopy diameter on volume of the trees. Puangchit *et al.* (1996) estimated canopy area by measuring leaf area of a sub-sample of the canopy. The results suggested that canopy size may be a good indicator for the growth rate of *Acacia auriculiformis*.

5.2 Molecular marker analysis

AFLP marker technology was chosen because it uses the polymerase chain reaction (PCR) technique to generate DNA fragments for analysis. The advantage of this is that the PCR-based technique requires very small quantities of DNA (5ng to 50ng) and is amenable to automation. AFLP is more robust than RAPD in that it is more reproducible. This attribute has been reported by researchers in several laboratories (Akerman *et al.*, 1996; Jones *et al.*, 1997a; Lin *et al.*, 1996). The higher reproducibility of AFLPs compared to RAPDs could be due to the use of longer primers and more stringent conditions in the PCR step (e.g. higher annealing temperature). A large number of polymorphic AFLP markers generated using *A. excelsa* DNA samples indicated that there is a potential for application of this technology to the genetic analysis of the population.

5.2.1 Genetic variability study

The potential of DNA-based marker technology to generate an unlimited number of markers efficiently with an economical amount of DNA has made it a popular marker of choice for genetic analysis in the forest tree species. RAPD is among the DNA-based marker technologies that has been widely used in population genetic applications (Mosseler *et al.*, 1992; Isabel *et al.*, 1995; Tuskan *et al.*, 1996). Although AFLPs are a relatively recent development, there are already several examples of their application in forest trees (Cervera *et al.*, 1996; Akerman *et al.*, 1996; Beismann *et al.*, 1997). Apart from being more reproducible, AFLPs also can

generate a large number of markers with a single primer-pair combination. In fact, since the genome of forest trees are complex, the basic AFLP assay will produce far too many bands to analyse. The basic AFLP assay consisted of a large number of DNA restriction fragments. The number of bands amplified can be reduced by the addition of extra bases to the 3' end of the selective PCR primers. Usually, 1 to 3 selective bases are added to either or both primers. The addition of one selective base to each of the primers will cause a 16-fold reduction in the number of fragments produced (Vos *et al.*, 1995). The addition of 3 selective bases to each primer, a necessity for the large genome of most trees, will result in 4,000-fold reduction in the number of fragments produced (relative to no selective bases added). Since *A. excelsa* has a relatively large genome (*i.e.* 2.75×10^8 bp per haploid genome), selective amplifications were carried out with the addition of 3 bases to each primers. The AFLP assay produced 50 to 200 fragments (section 4.10.2) which are manageable in terms of electrophoretic separation on a denaturing polyacrylamide gel and analysis. A large number of bands also implies that the power of detection for polymorphism (determined by number of polymorphic bands obtained) among individuals will be high.

In the present study, the power of detection for polymorphism in AFLPs has been demonstrated by fingerprinting DNA samples from mother plants. All the mother plants were originated from the same source, which means that they are of narrow genetic base. The morphological data has shown that large trees tend to produce larger progeny trees (section 4.4.1). This implies that to capture maximum genetic

gain, hybridisation should be carried out between selected superior trees, which is the A category mother trees. However, wide crosses between individuals are advisable to avoid inbreeding depression. There is substantial evidence to show that inbreeding *i.e.* crosses among closely related trees causes deleterious effects for a range of species (Eldridge *et al.*, 1993). This is mainly due to homozygosity of deleterious recessive alleles in the progenies. Falconer (1960) also reported that a 5 to 10 percent reduction in phenotypic values may result in 10 percent increase in the inbreeding coefficient. The information on the relatedness among individuals is therefore useful for selection of appropriate candidate plants for performing controlled crosses. Careful selection of appropriate trees for performing hybridization is especially critical in forest tree species due to the long generation time and difficulties in carrying out controlled crosses. Therefore, based on the dendrogram obtained in Figure 4.19, it is recommended that crosses be performed among the mother plants from the A category except between A1 and A2 because they are closely related to each other.

The progeny tree groups have shown to be more closely related to each other compared to the mother trees as indicated by the similarity index obtained in Table 4.29. The result is expected because the flowers of *A. excelsa* could be pollinated by insects (Pannell, 1992) thus natural crosses can only occur among *A. excelsa* trees in close vicinity.

5.2.2 Study on outcrossing rate with dominant AFLP markers

The *A. excelsa* half-sib population experimental plot was derived from open-pollinated seeds which could be formed from selfing, mating with relatives (neighbourhood inbreeding) or outcrossing with unrelated trees (Griffin and Cotterill, 1988). It is important to know the mating pattern of a breeding population in order to avoid inbreeding depression caused by deleterious recessive alleles. Inbreeding could result in an overall reduction in growth in a population. Hodgson (1976) compared the growth of progenies from controlled cross-pollination (outcrosses) with controlled self-pollination (selfs) in 12 different clones of *Eucalyptus grandis* in experimental plantings in South Africa. He found that the overall mean decrease in height from selfing, relative to outcrossing, was 31%.

Early studies based on the reproductive biology of tropical trees suggested that the breeding systems of most tropical rain forest trees were self-pollinated and inbred (Corner, 1954; Federov, 1966). Subsequent work on studies of self- and cross-compatibility and on observations of pollinator behavior indicated strong barrier to selfing and led to the conclusion that tropical trees are predominantly outcrossed (Zapata and Arroyo, 1978; Bawa, *et al*, 1985).

Outcrossing rates in forest tree population has traditionally been estimated from polymorphism data using isozyme markers (Adams, 1983). In recent years, high-throughput PCR-based technologies such as RAPD and AFLP have increasingly

been used for detailed genetic analysis. However, RAPD and AFLP are dominant markers, which provide less information than co-dominant markers. Discrimination of genotypes into homozygotes and heterozygotes are, however, particularly relevant for the estimation of outcrossing rate. Through simulation studies, Ritland and Jain (1981) demonstrated that this limitation could be overcome by multilocus estimation using a large number of dominant markers with intermediate gene frequency.

The present study shows the use of AFLP markers in a mating-system study of a *A. excelsa* population using the multilocus estimation (MLDT) developed by Ritland (1990). The study demonstrated that although AFLP supply dominant markers with lower information content than traditional co-dominant isozymes, they are very adequate for the study of mating system in populations. As part of the processes of AFLP marker technology (e.g. amplification of fragments, collection of data) is automated, it enables a large number of individuals to be analysed with a large number of markers in a relatively short time. Only a single AFLP primer pair is needed to generate sufficient markers for a robust estimation of outcrossing rate.

The estimates of outcrossing rates obtained indicate that the open-pollinated breeding population of *A. excelsa* is preferentially outcrossing (Table 4.32). The result suggests that the expected level of genetic variation for the open-pollinated families is likely to be maintained. The multilocus estimate was not significantly different from the single-locus estimate with a biparental mating rate ($t_m - t_s$) of 0.125 (SE = 0.03) suggesting no occurrence of biparental inbreeding. The multilocus

outcrossing rate of the *A. excelsa* population was estimated to be 0.81. This value is comparable to the findings reported for other tropical trees of Meliaceae family such as *Carapa guianensis* (t_m=0.97 and 0.99; Hall *et al.*, 1994), *C. procera* (t_m=0.78; Doligez and Joly, 1997) and *Trichilia tuberculata* (t_m=1.08; Murawski and Hamrick, 1991). The outcrossing rate of *A. excelsa* population in this study was estimated from an artificial forest stand established from the seeds collected from a group of mother plants that had shown to be not very diverse (see Section 4.10.3). The value of outcrossing rate might be higher if obtained from a natural population. Lee (2000) compared outcrossing rates estimated from primary forest, logged forest, artificial forest and seed orchard using a tropical species, *Dryobalanops aromatica*. He found the outcrossing rate from primary forest is comparatively higher than the other three types of forest populations due to lack of pollinators and flowering synchrony in the newly established ecosystem.

The Wright's fixation index, F obtained was lower than expected based on the estimate of t_m. Taking t_m = 0.81, the expected fixation index was [F = (1-t)/(1+t)] = 0.105, while the estimated F was 0.034. A lower than expected F suggests an excess of heterozygotes and less inbreeding than expected in the progeny population analysed. In the study, plant leaves samples were randomly collected from one year-old trees from the plot. The average germination rate for the selected seedlots for study was approximately 87% (see Table 4.2) and some of the seedlings did not take off in the field. It is therefore very likely that some level of selection against homozygous plants was carried out. Other likely causes of excessive heterozygotes

include variation in information among loci to detect outcrossing events and estimate the rate, statistical aberration or violations in the assumptions of the mixed mating model. Such violations which bias the estimates, among other factors, include spatial heterogeneity of the pollen gamete pool, segregation distortion, assortative mating, differential selection intervening between the union of gametes and the point of census, and population subdivision.

The χ^2 test indicated that a total of 16 AFLP markers have shown significant deviations from the mixed-mating model (Table 4.30). The deviations could be due to selection against homozygous genotypes, genotype-dependant outcrossing rate or the unbalanced frequencies of pollen in the population (Ritland, 1983). The result therefore supports the hypothesis that selection against homozygous plant could have occurred when sampling plants from the field for study.

5.2.3 Searching for molecular markers linked to morphological traits

Traditionally, morphological markers have been used to track discrete heritable morphological traits with desirable quality. The genetic distance between the morphological markers and traits could be estimated by recombination frequency obtained from testcross or F_2 progeny crosses. These markers can then be placed on a genetic map that shows their relative genetic distance from each other (Jones *et al.*, 1997). However, the morphological markers are scarce in occurrence thus could not contribute much to the progress in plant breeding. Today, several marker technologies such as isozyme, RFLP, RAPD, AFLP and microsatellite have been

developed. Each of these marker technology has its respective strength and limitations in terms of its applicability to plant improvement (see section 2.3). The DNA-based marker technologies such as RFLP, AFLP and RAPD produce a potential unlimited number of markers and thus can be used to construct a molecular marker map with better coverage of the genome. Such molecular maps are a powerful tool to be used in breeding programs. For example, molecular maps can be used for locating quantitative trait loci (QTL) which are likely to be scattered all over the genome. In addition, they can also be used as a reference for selecting progenies with genotype similar to recipient parent when introgressing desirable gene from the donor parent.

AFLP has been chosen for the present study due to its potential in producing a large number of reproducible polymorphic markers in a single assay. In addition, it is also able to detect DNA sequence variation between genetically similar plant populations. However, it suffers from its limitation of providing mostly dominant markers which cause a loss in valuable information such as determination of genotypes of progeny.

5.2.3.1 Strategy for finding the molecular markers

Forest tree species are characterised by long generation times, outbreeding behaviour and a heavy genetic load. Due to long waiting times for trees to reach reproductive age, controlled crosses are not common in forest tree breeding programs. The common pedigrees present in the annual crop breeding programs such as F_2 or backcross populations can rarely be found in forest tree species. This is because such

pedigrees require homozygous inbred lines parents that are very difficult and time consuming to obtain in forest trees. The majority of the available pedigrees, particularly for the angiosperm outbred tree species, generally involve only two parents and their full-sibs or maternal half-sib families. Despite these obstacles, several researchers have developed various strategies over recent years to identify markers linked to traits of economical importance. Grattapaglia and Sederoff (1994) devised a strategy to construct linkage maps in a segregating population consisting of a cross between two heterozygous populations of *Eucalyptus grandis* and *E. urophylla* using RAPD markers. The polymorphic RAPD markers that were heterozygous in one parent, null in the other and therefore segregate 1:1 in their progeny were selected for map construction. Since the design resembles the testcross mating configuration, the strategy was given the name "pseudo-testcross". The molecular linkage maps produced can further be used for locating QTL in the breeding population. More recently, Grattapaglia *et al.* (1996) have developed another strategy to find markers linked to QTL controlling growth and wood quality traits in *E. grandis* using a maternal half-sib family and RAPD dominant markers. In this strategy, *E. grandis* genotype was used as the only female clone surrounded by 25 *E. urophylla* clones that were used as pollinators. All the pollinator tree samples were screened for the RAPD markers used in this study to ensure that the markers used uniquely identified the maternal gametic contribution to the half-sibs. These segregating markers can further be used for identifying markers linked to desirable traits using Bulk Segregant Analysis (BSA) or Selective Genotyping (SG) strategy.

The BSA strategy or DNA pooling technique was originally proposed by Arnheim *et al.* (1985) and later adopted by Michelmore *et al.* (1991) for the identification of markers linked to disease-resistance genes in lettuce. This method involved comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool, or bulk, the individuals are identical for a particular trait or genomic region but arbitrary at all unlinked region. BSA based on the principle that the informative individuals are grouped together so that particular genomic region can be studied against a randomised genetic background of unlinked loci. To put it simply, in order to carry out BSA for a qualitative trait involving single gene controlling 2 phenotypes, the progenies are grouped into 2 pools based upon phenotypes. The "bulk" DNA from about 10 individuals from each pool is then combined and the banding patterns compared. Markers that are polymorphic between the 2 bulks are likely linked to the target genes. BSA may also work in the case of detecting major genes of very large effect in a quantitative trait. It can also be used to find other markers linked to a markers in a region of interest.

Another strategy called selective genotyping (SG), which is similar to BSA, except that in SG, segregating individuals with extreme phenotypes are genotyped with the markers. SG can markedly decrease the number of individuals genotyped for a given power at the expense of an increase in the number of individuals phenotyped. Lander and Botstein (1989) showed that for continuous traits, the power of analysis can be

markedly increased when the analysis is based on the quantitative values of the individuals in the high and low tails of the population.

The strategy used for the present study is modified from a combination of pseudo-testcross and SG. First, the segregating markers for screening individual samples with extreme phenotypic tails of the entire sample populations need to be determined. These markers are heterozygous in the maternal sample and homozygous null in the paternal sample. Therefore they will be segregating 1:1 in the progeny population as in a testcross mating configuration. Three assumptions have been made in this strategy : (1) The mother plant is heterozygous for the marker if occurrence of the marker is rare in the population; (2) The paternal sample is homozygous null when the marker is found rarely in the segregating progeny population; (3) All the seeds are derived from outcrossing event with high probability because of the high outcrossing rate (81%) recorded in section 4.10.4.

The extreme phenotype was identified as the family with the highest variance within the family. Dbh was chosen as the trait for study because it is a relatively stable trait. Moreover, it was found to correlate highly to tree volume (section 4.6). Based on the measurements obtained in section 4.4, the family with the highest variance was found to be B2. By focusing on individual trees with extreme Dbh measurements at both ends of the tail in a B2 Dbh distribution curve, the power of detection for the homozygous alleles responsible for the trait increases (Lander and Botstein, 1989; Darvasi and Soller, 1992). Segregating markers were used to screen the selected

individual trees with extreme phenotype. Markers that are present in one group of extreme individual trees and absent in the other group will be classified as putative markers linked to the trait under study. Further screening was performed using these putative markers on a larger number of B2 tree samples.

The segregating markers were found by screening the 13 mother tree samples with 21 primer pairs. The AFLP markers that were present only in B2 sample or with one or two other mother tree samples will be an indication that the marker is heterozygous for the B2 maternal sample. These markers in turn were used to screen approximately ten random samples of B2 progeny. The occurrence of the marker in only one or two progeny samples shows that + allele is rare and thus maternal genotype is heterozygous and paternal genotype would most probably be homozygous null. Based on this assumption, the selected markers were used for screening samples with extreme phenotypes.

This screening did not manage to find markers linked to the traits under study. Thus, in order to improve the chances of finding the markers, more primer pairs and screening need to be carried out. Another reason for failure to detect the markers could be due to occurrence of recombination between the markers and the targeted trait which lead to a breakdown in linkage disequilibrium *i.e.* close association of markers with trait loci.

5.2.3.2 Linkage disequilibrium

The success of finding markers linked to trait of desirable quality largely depends on strong linkage disequilibrium established between marker-trait association. Linkage disequilibria between pairs of loci are produced by three factors : hybridization, random genetic drift and epistatic selection. A common practice in a crop breeding program to generate linkage disequilibrium is to perform a cross between two genetically different inbred lines. It was reported that strong heterosis in a cross helps to maintain linkage disequilibrium (Lewontin, 1964). However, most forest trees are predominantly outcrossing in their system of mating. Even though *A. excelsa* could be insect pollinated as reported by Pannell (1992), the outcrossing rate was found to be relatively high (*i.e.* 81%)(Section 4.1.3). Therefore natural crosses between inbred lines very rarely occur in forest tree species which results in very low linkage disequilibrium. Moreover, recombination distances are found to differ among genetic backgrounds (Strauss and Conkle, 1986) and sexes (Moran *et al.*, 1983) making it difficult to confirm marker-trait association. One solution to overcome the linkage disequilibrium problem is to find markers tightly linked to the trait. This method, however requires tremendous work in terms of screening a large number of populations and markers.

5.2.4 Application of markers linked to desirable traits

Markers tightly linked to desirable traits can be used to aid in selection in a breeding program. Thus, it was given the name marker-aided selection (MAS). MAS can have several applications in a breeding program such as: (1) early selection for characters

that are expressed late in plant development like fruit or flower features or adult characters in species with a juvenile period; (2) when the expression of the target gene is recessive; (3) when breeding for disease or pest resistance that requires special operation for the gene to express. Due to long generation intervals, the early selection application will be the most appealing for forest tree species. For some forest tree species such as *Pseudotsuga menziesii*, selection for gain in height, volume and wood quality could only be conducted with confidence after half the rotation age (80 years) has been reached (Namkoong *et al.*, 1972). The application of MAS will certainly help to save growing time, labor as well as space requirements. For other forest tree species such as the conifers, selection before half-rotation or even quarter-rotation age is possible in recurrent breeding programs (Newman and Williams, 1991). In these species, MAS will make the selection more efficient by shifting the early selection to seedling stage selection. The relative efficiency of marker assisted selection (MAS) is the relative efficiency of MAS (RE_{MAS}) such that

$$RE_{MAS} = \sqrt{(p/h^2) + (1-p)^2/(1-h^2p)}$$

Where h^2 = heritability of target trait; and p = the proportion of the additive genetic variance in the target trait associated with the marker loci.

Thus, MAS is best for traits with low heritability. For traits with high heritability, phenotypic selection is effective.

5.2.5 Implications of the strategy to forest tree breeding program

Most of the forest tree pedigrees, particularly in the tropics, are open pollinated maternal half-sib. If the proposed strategy in this study could find the markers linked

to the desirable traits, it will be applicable to a large number of breeding populations of various species of forest trees in the tropics. This will help to speed up the breeding program in forest tree species. One of the main obstacles facing MAS is the establishment of linkage disequilibrium as outlined in section 5.2.3.2 above. The proposed strategy will improve the chances of obtaining markers tightly linked to the desirable trait by concentrating on extreme phenotypic ends of the trait. The strategy also will help to save cost by reducing the number of samples required for genotyping. If a large number of markers can be found which are linked to various traits of economic importance, a high-density molecular map can be constructed. Such molecular map can have wide applications in forest tree breeding programs such as locating QTL, MAS and map-based cloning of desirable genes.

5.2.6 Limitations of applying AFLP marker system in half-sib population

Identification of markers linked to desirable traits has been successful in test-cross or F2 pedigree using various DNA marker systems. Some of these useful markers have also been identified in half-sib population using codominant marker systems. In half-sib population, only the genetic information contributed by the maternal parent is known. The paternal genetic information can only be predicted from the genetic information gathered from the progeny population *i.e.* the genetic information not contributed by the maternal parent. If homozygosity and heterozygosity of the alleles cannot be differentiated (*i.e.* the markers are dominant), it will be difficult to ascertain allele contribution by either maternal or paternal parent. Since AFLP markers are predominantly dominant, it is less informative and therefore would be

very difficult to derive much genetic information from a half-sib population. In this study, a strategy has been devised to overcome this limitation based on the fact that a large number of AFLP markers can be generated within a short time and several assumptions were made (see section 5.2.3.1). However, in order to locate markers linked to desirable traits, a large sample size and markers are needed for screening. Such operation could not be carried out in the present study due to time constraint. Besides, AFLP marker system requires high quality of DNA, restriction digestion of genomic DNA, ligation of adaptor and PCR (see section 3.2.2). Thus, to analyse a large number of DNA samples using AFLP technique can be laborious and time-consuming.

5.2.7 Morphological data versus AFLP markers for genetic analysis

In the present study, morphological data has provided some useful genetic information regarding growth performance of the progeny trees in relation to the parent trees. However, this information is based on assessment of the data collected from trees of up to only 2.5 years old. *A. excelsa* reach reproductive age at 6 to 7 years old and the relationship between juvenility and maturity of this species is not yet known. Therefore actual performance of the trees at maturity could not be predicted from the data collected in the study.

The study shows that AFLP marker system was able to provide some additional information of the breeding population due to its power in differentiating individual trees by detecting DNA sequence polymorphism exists in the genome of *A. excelsa*.

The study established relationships among mother trees and progeny trees that were present in the form of dendrograms (Figure 4.19 and 4.20). However, AFLP marker system would be of great application for the genetic study if markers linked to desirable traits can be identified. The performance of progeny population may then be accurately predicted without having to take into consideration the age and environments in which the trees are grown.

5.3 The role of *in vitro* propagation in the improvement program of *A. excelsa*

Clonal propagation has been recognized as an important technique in plant breeding programs because of its potential for mass multiplication of genetically improved material. The genetic gain captured in the improvement program can be utilized at plantation level only if the selected superior tree material can be mass multiplied as genetically identical individuals. For *A excelsa*, rooted cuttings is currently the most promising method of clonal propagation for industrial plantation establishment (Kijkar, 1992). Kijkar (1992) used cuttings of lateral root section from the mother trees to induce shoots growth at one end of the cut. Over 90% rooting was obtained when cuttings were taken from these copicing shoots with an application of 300 ppm IBA. Traditionally, rooting of cuttings is used for obtaining clonal propagules in many forest plantations. This method, however, has several limitations such as the inability to propagate plants at a reasonable production rate and cost, mostly due to rapid maturation of the donor plants (ortets). In most forest tree species, the true-to-type propagation by the rooting of cuttings is possible only when the ortets are in

juvenile state. Therefore, an operational clonal program requires several cycles of serial propagation to meet stock requirements (Mullin and Park, 1992; Mullin *et al.*, 1992).

5.3.1 *In vitro* shoot culture of *A. excelsa*

In vitro shoot culture provides an alternative means through which clonal propagation could be carried out to supply clonal propagules to the forest plantation. In the present study, *in vitro* shoot growth of *A. excelsa* has been induced using MS medium supplemented with 1 mg/L BAP and root initiation with the same medium supplemented with 2 mg/L NAA. However, Ahmad *et al.* (1990) found that root initiation could be obtained in MS medium containing 1 mg/L IBA. The present study also found that a complete plantlet with roots ready for transplant takes approximately six months to produce, which is similar to the results obtained by Changtragoon (1993). The long waiting time to maturation means that multiplication rate to obtain propagules would be slow. However, less space is needed to provide stock plants due to the relatively smaller size of the starting materials needed for propagation.

5.3.2 Somatic embryogenesis of *A. excelsa*

Induction of shoots from leaf cuttings by somatic embryogenesis is another alternative means of producing a potential large number of genetically identical individuals. While in shoot culture, the highest shoot number obtained per shoot explant was two (Table 4.36), the highest shoot number induced per leaf explant was

five (Table 4.37). 40 % of these shoots were developed into plantlets. Higher percentage of plantlets (64.1%) was recorded in shoots induced from *A. indica* leaf explants (Eeswara *et al.*, 1998). This method will be potentially useful for providing the future supply of clonal propagules due to an abundant of leaves available as starting material.

5.3.3 Clonal fidelity

Successful application of clonal propagation depends heavily on the assumption that the genotypes propagated are identical to the selected trees with desirable characteristics. While propagation by cuttings provides genetic stability, *in vitro* shoot culture technique has also been known to produce "true-to-type" planting materials (Vasil and Vasil, 1980; Ahuja and Muhs, 1985). The present study shows that one setback of using *in vitro* shoot culture for producing plantlets is the low multiplication rate (*i.e.* an average of 2 plantlets per shoot) obtained.

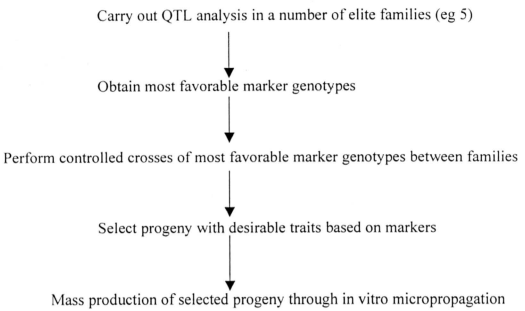
Since genetic aberrations have been reported in clonal propagation techniques involving adventitious budding from callus (Wang and Charles, 1991), it will pose as a major obstacle to the commercialization of somatic embryogenesis system. However, until to date, there is little evidence showing occurrence of genetic aberrations in somatic embryogenesis system (Thorpe 1988; Tartorius *et al.*, 1991). Eastmann *et al.* (1991) tested over 1500 somatic embryos of a single line of *Picea glauca-engelmannii* and found no isozyme pattern variation.

5.3.4 Field performance of propagules

Field performance of propagules is another important factor determining successful application of clonal propagation. Studies have shown that *in vitro* plantlets of douglas fir and loblolly pine displayed a lag phase with respect to both height and diameter growth, and also a more mature morphological characteristics (Ritchie and Long, 1986; Amerson *et al.*, 1985) in the field. Some promising reports have been recorded for plants grow from somatic embrogeneis. For example, in Picea, plantlets have been found to be similar to seedlings with respect to bud phenology (Becwar *et al.*, 1988), growth rate, shoot and root morphology and frost hardiness (Webster and Attree, 1990) in field performance. Due to time constraint, plantlets from the present study could not be tested on the field. However, future study could incorporate clonal trials to test the performance of these plantlets in the field.

5.4 "Pyramiding" of QTLs for next generation of breeding

The improvement of *A. excelsa* could be accelerated through the use of marker technology. Once markers linked to quantitative traits (section 4.10.5.1) are confirmed, QTL analysis needed to be carried out in a number of elite families in order to obtain the most favorable marker genotypes. Subsequently, selection of progenies with desirable traits can be selected based on the markers. The selected progeny can then be mass produced through *in vitro* micropropagation to supply improved planting materials for the plantations. The strategy for incorporation of markers related to growth into breeding population for the improvement of *A. excelsa* can be summarized as follows:


Carry out QTL analysis in a number of elite families (eg 5)
Obtain most favorable marker genotypes
Perform controlled crosses of most favorable marker genotypes between families
Select progeny with desirable traits based on markers
Mass production of selected progeny through in vitro micropropagation

5.5 Recommendations for future studies

The study has laid a foundation for the improvement of *A. excelsa*, an indigenous species with promising qualities for timber. The application of AFLP marker technology can provide more genetic information on the breeding population (e.g. genetic relatedness among individual trees in the population) that is very useful for the breeding program. Strategies applied in this study for *A. excelsa* can similarly be applicable to other forestry species with some modifications. Thus it will benefit tree improvement programs as a whole. However, to obtain genetically improved *A. excelsa* tree stocks that could be clonally multiplied for planting in a large scale would require further research work as outlined in the followings:

1. Trials needed to be conducted on tissue culture plantlets in the field in order to assess their actual performance and stability. Also, by carrying out clonal trials, variance component of environmental effects can be estimated to give more accurate estimate of genetic component of variation.
2. For successful mass production of genetically uniform plus trees, it is important to show that the *in vitro* clonal micropropagated plantlets retain the initial genotype. One way of doing this is to screen the plantlets using AFLP markers to ensure that there is no incidence of somatic mutation.
3. More trial plots needed to be set up in various locations over the country so as to assess the performance of *A. excelsa* trees under different environmental conditions and to study also G x E interaction.

4. The possibilities of obtaining the putative markers linked to desirable traits is higher if more primer-pairs could be used to screen the DNA samples from extreme phenotypes under the proposed strategies outlined in section 5.2.3.1.
5. In order to prove that the putative AFLP markers (section 4.10.5.1) linked to quantitative traits are real, further experiments are needed to carry out AFLP screening using the same primer-pairs on independent verification population derive from same family and environment.
6. Once the putative AFLP markers linked to quantitative traits are confirmed, further research can be carried out to isolate the genes. Several strategies can be adopted to perform gene isolation. For example, map-based cloning or positional cloning in which a gene can be isolated starting from its known position in a linkage map (for single gene trait). In this strategy, a fine scale linkage map close to candidate gene and a library of large overlapping DNA fragments of known order in chromosome region (or physical map) are needed. In addition, confirmation of candidate gene by transformation of appropriate mutant is required. It is important to keep in mind that some problems may arise by adopting this strategy for *A.excelsa*, which are outlines as follows:
    - Fine scale mapping requires very large number of progeny and a simple reliable scored trait
    - 1 cM can be many base pairs in many species
    - Physical map not yet available
    - Lack of appropriate mutants and efficient transformation systems for confirmation

Another strategy that could be used for gene isolation is mRNA differential display.

7. The present study has successfully induced shoots from leaf cuttings of *in vitro A. excelsa* shoots. However, some embryos were also formed. This means that with suitable media formulation, it is possible to induce somatic embryos from leaf cuttings. By encapsulation of these embryos, it would be possible to apply them as seeds for reforestation purposes.

Eventhough the integration of marker technology as a tool to assist in the breeding program is a recent practice, many breeders have already incorporated the technology in their breeding programs. However, its applications in forest tree breeding has been debated (Lande and Thompson, 1990; Strauss *et al.*, 1992; Hospital *et al.*, 1997). It is hoped that with the introduction of such technology, it will help to speed up the improvement programs of forest tree so that sufficient quality planting stocks can be made available for our future forestry plantations.