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Identification of quantitative trait loci influencing early height growth in longleaf pine (Pinus palustris Mill)

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IDENTIFICATION OF QUANTITATIVE TRAIT LOCI INFLUENCING EARLY HEIGHT GROWTH IN LONGLEAF PINE (*PINUS PALUSTRIS* MILL)

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphisms
ANOVA	Analysis of Variance
CCGP	Conifer Comparative Genomic Project
CIM	Composite Interval Mapping
DH	Double Haploid
EHG	Early Height Growth
EST	Expressed Sequence Tag
GAS	Gene-Assisted Selection
IBD	Identity–by–Descent
IM	Interval Mapping
LD	Linkage Disequilibrium
LOD	Log of Odds
LS	Least Square
MAS	Marker-Assisted Selection
ML	Maximum Likelihood
MQM	Multiple QTLs Model
OP	Open Pollinated
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
QTL	Quantitative Trait Loci
QTN	Quantitative Trait Nucleoid
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
RIL	Recombinant Inbred Lines
SA	Simulated Annealing
SCAR	Sequence Characterized Amplified Regions
SNP	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeat
TGOP	Three Generation Outbreed Pedigree

ABSTRACT

The delay in early height growth (EHG) has been a limiting factor for artificial regeneration of longleaf pine (*Pinus palustris* Mill.). Simple Sequence Repeat (SSR) markers have been used to map the genome and quantitative trait loci controlling the EHG in a backcross family (longleaf pine x slash pine) x longleaf pine. A total of 228 locus specific SSR markers were screened against 6 longleaf pine recurrent parents and a sample of 7 longlef x slash pine hybrid parents. In total, 135 polymorphic markers were identified. Based on the genetic variance in EHG, available sample size, and the number of SSR marker polymorphisms, a half-sib family with a common paternal parent (Derr488) and 6 longleaf maternal parents were selected from 27 backcross families as the final mapping population. One hundred and twenty three (123) polymorphic markers showed polymorphisms across the half-sib family. An individual linkage map was built for each full-sib family first, and then the linkage maps from different full-sib families were integrated by common orthologous SSR markers with software JoinMap (ver3.0). There were 112 polymorphic markers mapped to the integrated map which contained 16 linkage groups. The observed map length was 1874.3 cM and covered 79.85% of genome. The estimated 95% confidence interval for genome length was 1781.3-2411.6 cM. Seventeen (17) QTLs were identified by single marker regression using 305 backcross progenies. For the interval mapping, the tallest and shortest 8 percent of seedlings were selected for QTL detection (phase I), and then random selections of 8 percent of the seedlings from the rest of the population and 25 seedlings from both tails of the within family distributions were used for unbiased QTL verification and mapping (phase II). Nine QTLs were detected and verified as associated with the 5 growth traits under P=0.05 chromosome-wide threshold. There was only weak evidence of QTL stability during the three years of growth under this study.

CHAPTER 1 INTRODUCTION

1.1 Early Height Growth of Longleaf Pine

Longleaf pine *(Pinus palustris* Mill.) is a very important softwood species in the southeast United States. It is considered the most valued of the southern pines in many ways (Croker, 1990): it produces the best quality saw timber, the greatest percentage of poles, the highest specific gravity per unit volume, and the best quality pine straw. Besides its outstanding physical characteristics, longleaf pine is also more resistant to insect damage, fusiform rust, wind-throw, wind-breakage, and fire damage than loblolly or slash pine.

Longleaf pine ecosystems once occupied more than 36.4 million hectares of the southeastern United States lower Coastal Plain, from southern Virginia to central Florida and eastern Texas (Frost 1993). However, decades of timber harvest followed by conversion to agriculture, urban development, or other pine species invasion have reduced longleaf pine dominated areas to less than 5% of its original range. Although the reasons for systemic declines in longleaf pine habitat are many, one primary reason is the lack of successful reforestation. Both naturally and artificially, longleaf pine is more difficult to grow than any other southern pine due to the delay in stem elongation known as the "grass-stage", a genetic trait of the species. The grass-stage is characterized by an extended period of root and foliar development in early height growth (EHG) until the seedling root collar reaches about 1.3 to 2.5 cm in size, during which there is no height growth. This phase may take from 1 to 20 years, depending on competition and growth conditions (Layton and Goddard, 1982; Schmidtling and White, 1989; USDA, 1965). The grass-stage trait is thought to be an adaptation to a predictable pattern of ground fires on low to moderate productivity sites (Keeley and Zedler 1998). During the grass-stage, longleaf pine is most susceptible to its major disease, the brown-spot needle blight, caused by *Scirrhia acicola*

(Croker, 1975; Siggers, 1944; Wakeley, 1970). Brown-spot needle blight attacks the longleaf needles and slows their growth. Infected needles develop gray-green spots, which later turn brown, and a yellow band eventually develops on the needle. The affected area then increases in size, resulting in the death of the needle. In the grass-stage, the infected seedling looks brown and the dead needles will fall off; if new needles are repeatedly infected, the seedling will die.

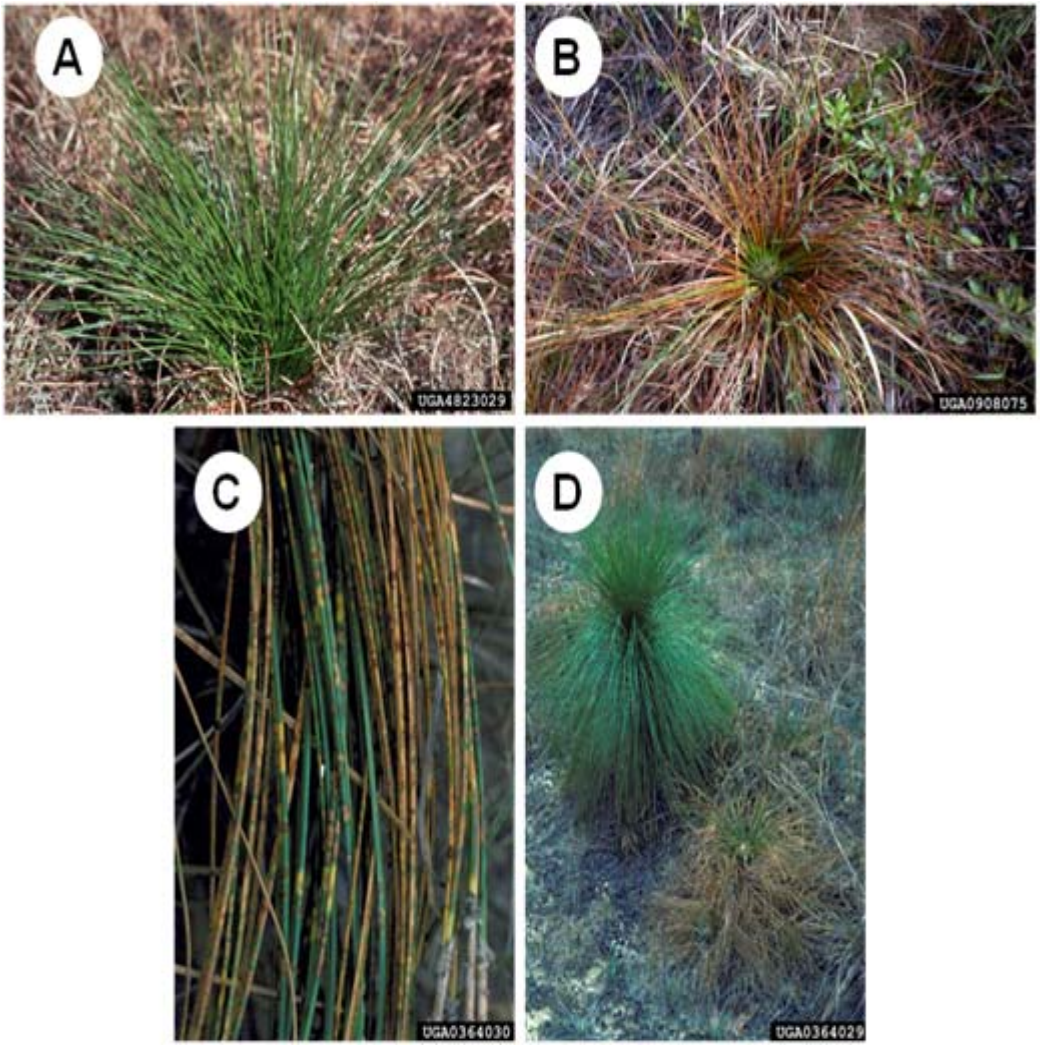

Figure 1.1 Brown spot needle blight (*Scirrhia acicola*) in longleaf grass-stage. A: Normal uninfected longleaf pine seedling in grass-stage (Picuture by: Barnard, E.L.) B: Heavily infected seedling in grass-stage (Picture by: Moorhead, D.J.). C: Close-up view of infected needles. D Lightly infected seedling in foreground and healthy uninfected seeding in background (Picture by: Anderson, R.L.).
(Picture source: http://www.forestryimages.org/browse/subthumb.cfm?sub=904&start=1)

The delay in EHG for the grass-stage has drawn the attention of scientists for a long time. Experiments in improving nursery technique, seedling care, and silvicultural practices have all been shown to have positive effects (Shipman, 1960; Smith and Schmidtling, 1970). Nevertheless, none of these improvements has been widely used in practice due to investment cost, labor and environmental limitations**.**

1.2 The Genetic Improvement of EHG in Longleaf Pine

Breeding programs have been underway for more than 35 years to improve brown-spot resistance and early height growth of longleaf pine (Bey and Snyder, 1978). Longleaf pine is a highly variable species, and a considerable proportion of this variation is genetic. Considering the economically important traits, longleaf pines have as much or more genetic variation than other southern pines (Snyder and Derr, 1977). However, the development of such resources is hampered by the long generation interval, outcrossing mating system, and high genetic load, typical of forest tree species. Furthermore, traditional forest tree improvement methods have exclusively relied on phenotypic selection, expensive long-term field progeny testing for phenotypic traits, and generally elaborate statistical analysis of the data. Summaries of progress using basic tree breeding methods (Jett 1988, Zobel and Talbert, 1984) have shown them to be effective yet slow (Tauer and Hallgren, 1992; Krugman, 1985).

Since this grass-stage condition is a unique characteristic of longleaf pine (Schmidtling and White, 1989); it may be improved by interspecific hybridization. Both slash pine (*Pinus elliottii* Engl.) and loblolly pine (*Pinus taeda* L.) are potential donors of EHG genes because of their early maturity and fast growing characteristics. Natural hybridization is common between longleaf pine and loblolly pine, producing the Sonderegger pine (*Pinus* × *sondereggeri* H.H. Chapm), which is the only named southern pine hybrid. Natural hybridization between longleaf

pine and slash pine is unlikely, based on differences between the species in dormancy and heat requirement for stroboli develeopment (Boyer, 1981). However, artificial crosses between longleaf pine and slash pines can be achieved easily (Boyer, 1990) and the variation in EHG was found to be significant among and within families in several field tests of longleaf pine x slash pine hybrids (Derr 1966; Derr, 1969). Slash pine is one of the fastest growing and earlier-maturing species, but it is also very sensitive to fusiform rust. Lohrey (1990) referred to the longleaf x slash hybrid as showing the most potential because height growth began quickly, almost as fast as slash pine, and it was fairly resistant to both brown-spot needle disease and fusiform rust. Derr (1966) has indicated that the hybridization between longleaf pine and slash pine to improve EHG was practicable; the survival, growth, and disease susceptibility of longleaf pine x slash pine hybrids are improved. For example, the average height for wind–pollinated slash pine and wind-pollinated longleaf at age 4 was 2.4 and 0.8 m, respectively, while the longleaf pine and slash pine hybrid was 2.3 m. Most traits for these hybrids were intermediates between longleaf pine and slash pine. Several generations of backcrosses were needed in order to replace the slash pine portion of the hybrid genome, other than those genes regulating the early height growth. The hybrids that show desired phenotype were selected for recurrent backcrosses. For one generation of backcrossing, fifty percent of the longleaf pine genome was recovered, and 5 or 6 generations of backcrosses gave a reasonable genome recovery.

However, forest tree breeding traditionally has been viewed as an application of quantitative genetics (Zobel and Talbert 1984). Previous studies have shown that EHG in longleaf pine is a quantitative trait, controlled by a small number of major effect genes (Brown 1964; Weng, et al., 1999; Nelson, 2003) with heritability (h^2) ranging from 0.47 to 0.68 (Layton and Goddard 1982; Snyder and Namkoong 1978). Gain from phenotypic selection is

limited when h^2 is small because the limited proportion of genetic variance the breeder can capture at an early stage. Taking into account the long generation interval and linkage drag associated with the selection, to select all the major QTLs using traditional methods would be time-consuming and destructive.

1.3 Marker-Assisted Selection

The use of molecular marker-assisted selection (MAS) are currently utilized in crop and animal breeding, and they also promise to be useful in studies on forest trees that are directed towards obtaining faster genetic improvement in timber quality (Brown, 2003), growth rate (Emebiri, 1997), and stress and disease tolerance (Grattapaglia and Sedero, 1994; Plomion, et al., 1996). The MAS is based on the establishment of a linkage relationship between the easily scorable molecular markers and the characteristics of interest. If markers that are linked to the major QTL can be identified, then these markers can be used to guide the selection of the hybrid and the subsequent backcross generations. The use of DNA markers for indirect selection offers the greatest benefits for quantitative traits with low heritability, as these are the most difficult characters to assess in field experiments. The three essential requirements for MAS in a breeding program are: first, markers should co-segregate or be closely linked with the target gene (within 2 cM or less); and second, an efficient means of screening large populations for the molecular markers should be available; and thirdly the screening technique should have high reproducibility across laboratories, be economical to use and be user-friendly (Mohan, et al., 1997).

Compared with the tradition breeding program, MAS has many advantages. It provides a way to increase the efficiency of within family selection by exploring simultaneous selection for multiple traits by selecting makers that are tightly linked to the QTLs of interest. It allows

selection at the juvenile stage from an early generation and the unfavorable alleles can be eliminated or greatly reduced during the early stages of development. The most straightforward application of molecular markers in MAS includes genetic distance analysis, variety identification, identification of markers tightly linked to specific genes, and MAS backcrossing. I will focus on the last two functions in this project.

The future of MAS aims not only at utilizing perfect markers for improving existing breeding schemes, e.g., backcrossing, but also controlling all allelic variation for all genes of agronomic relevance. In a simulation study of building superior genotypes, Peleman and van der Voort (2003) introduced a concept, "breeding by design", that requires the knowledge of the map position of all loci of agronomic importance, the allelic variation at those loci, and their contribution to the genotype. Although great efforts have to be made to gather all this information of precise genetic stocks, such as introgression line libraries (Eshed and Zamir 1995) for mapping, all relevant traits are available for several crop plants. Additionally, allelic variation at any locus in the genome can be assessed by establishing haplotypes of multiple tightly linked markers. This all embracing approach has to be addressed immediately to make molecular markers an accepted and irreplaceable tool for developing better crop plants.

1.4 Molecular Marker

Since Mendel formulated his law of inheritance in 1865, it has been a core component of biology to relate genetic factors to functions visible as phenotypes. People have been monitoring, inducing, and mapping single gene markers in plants, animals, and human beings. In early research, most of the single gene markers used in plant genetics were those either affecting morphological characters (i.e. morphological markers) or changing the structure and number of chromosomes (i.e. cytological markers). These types of markers generally correspond to

qualitative traits that can be scored visually, such as seed color, leaf shape, or chromosome deletion, duplication, inversion, and translocation. These traits occur naturally, but can also be generated from mutagenesis experiments. These kinds of markers have been found useful in the linkage map construction of forest trees (Chaparro et. al., 1994; Jermstad et al., 1994). Though the markers have served well in various types of basic and applied research, their use in many areas of plant breeding has been very limited (reviewed by Tanksley, 1983). These markers are usually affected by the environment and developmental stage, limited in number. Moreover, the genes controlling these markers can have pleiotropic effect on the character under investigation which eludes the actual location of genes due to distortion of segregation rations.

The development in recent years of molecular markers offers the possibility of finding new approaches to breeding procedures. The molecular markers are heritable molecules that mark loci on chromosomes and reveal polymorphisms at the protein or DNA level. To be a useful molecular marker, it must be polymorphic, reproducible, preferably display co-dominant inheritance (both forms detectable in heterozygote), and fast and inexpensive to detect. The marker methods differ with respect to the type, specificity, volume of genetic data generated, lab time required, and the cost of equipment and materials. Based on the level at which the genes are detected, molecular markers can be divided into two classes: protein markers and DNA markers.

1.4.1 Protein Markers

Protein markers code for proteins that can be separated by electrophoresis to determine the presence or absence of specific alleles. The most widely used protein markers in plants are allozyme. Isozyme are an allelic variant of enzymes encoded by structural genes and provide a relatively simple and inexpensive method of obtaining genetic information. The first linkage studies of *Pinus* were based on the segregation of isozyme extracted from megagametophytes.

More than 10 species have been studied for about 15 loci (Guries et al., 1978; Rudin and Eckberg, 1978; O'Malley et al., 1979; Ekert et al., 1981; Cheliak, et al., 1984; O'Malley et al., 1986; Furmier et al., 1986; Strauss and Conkle, 1986; El-Kassaby et al., 1987; Shiraishi, 1988; Szmidt et al., 1989; Hamrick et al., 1992). A 2D-PAGE of the total proteins of megagametophytes allowed studying of a much large number of loci than had been previously possible with isozyme analysis (Anderson et al., 1985; Bahrman and Damerval, 1989; Gerber et al., 1993). However, their application is limited by the number of enzyme loci, the low levels of variability in some species, poorly understood modes of inheritance and developmental instability (Bahrman and Damerval, 1989), and the fact that they only reveal variation in enzyme genes (Tanksley, et al., 1989). These limitations lead several groups to use other types of molecular markers.

1.4.2 DNA Markers

Scientists are constructing genetic linkage maps composed of DNA markers for a wide range of plant species (O'Brien, 1993). Several types of DNA markers have been widely used: restriction fragment length polymorphisms (RFLPs) (Bostein et al., 1980), random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990), simple sequence repeat (SSRs or microsatellite) (Litt and Luty 1989), amplified fragment length polymorphisms (AFLPs) (Vos et al 1995), and single nucleotide polymorphisms (SNPs) (Wang et al 1998). All types of DNA markers detect sequence polymorphisms and monitor the segregation of a DNA sequence among progenies of a genetic cross in order to construct a linkage relationship. The most commonly used DNA markers are RFLPs and RAPDs. In the last ten years, however, usage of such markers as AFLPs, SSRs, and SNPs has also become widespread.

Each DNA marker method analyzes different aspects of DNA sequence variations and different regions of the genomes. For example, RFLPs were detected using cDNA clones, namely the coding sequence, but were also frequently detected in variations that lay in regions flanking the genes. SSR markers have generally been from non-coding regions, although the recent move to three base repeats and the use of expressed sequence tags (ESTs) as the source of SSR markers is changing this standard. Other markers, such as RAPD and AFLP markers, frequently appear in repetitive regions of the genome. In some cases, the stability of the sequence difference may also be an issue. SSRs are seen as being unstable for some applications since the mutation rate may be high in certain criteria. The decision about the most appropriate marker system to use varies greatly depending on the species, the objective of the marker work, and the resources available.

1.4.2.1 Restriction Fragment Length Polymorphisms

RFLPs are fragments of restricted DNA (usually within the 2~10 kb range) separated by gel electrophoresis and detected by subsequent Southern blot hybridization to a radio-labeled DNA probe. The probe consists of a sequence of unknown identity or part of the sequence of a cloned gene, which is obtained by molecular cloning and isolation of suitable DNA fragments. Polymorphisms are visualized as differences in banding patterns between or among two or more individuals. RFLPs were first used in human genome mapping (Botstein et al., 1980), and it was later adopted for plant genome study.

RFLPs are the most reliable polymorphisms which can be used for accurate scoring of genotypes. They are co-dominant and highly reproducible, which make them useful in identifying a unique locus. RFLP methods are well suited for species maps because the same hybridization probes can be used for comparison among species (Ahuja et al., 1994; Byrne et al.,

1995; Jermstad et al., 1994). Because of their high genomic abundance and random distribution throughout the genome, RFLPs have frequently been used in gene mapping studies of various plant species, although few studies were reported in trees. Devey et al. (1994) presented linkage groups in loblolly pine for 80 RFLPs detected using cDNA probes. Linkage maps using mostly RFLP markers have been recently presented for poplar (Bradshaw et al., 1994; Jorge et al., 2005), Douglas-fir (Jermstad et al., 1994), pine (Nance and Nelson, 1989; Neale, 1991, 1994; Devey et al., 1996, 1999; Jermstad et a., 1998; Sewell et al., 1999; Brown, et al., 2001) and *Eucalyptus* (Byrne et al., 1995; Thamarus et al., 2002).

Although RFLPs are unlimited, they require elaborate laboratory techniques: development of specific probe libraries, use of radioisotopes, southern blot hybridization procedures, and autoradiography, making them labor intensive, time consuming, and costly (Kesseli et al., 1994; Neale et al., 1989). In addition, some tree species, such as pine, have DNA content so high (Wakamiya et al., 1993) that single copy southern hybridization may be impractical as very lengthy exposures are required, and the methylated DNA is usually not well digested (Iwata, et al., 2001).

1.4.2.2 Random Amplified Polymorphic DNAs

RAPDs are DNA fragments amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primers and are usually able to amplify fragments from 3~10 genomic sites simultaneously. Amplified fragments are separated by gel-electrophoresis, and polymorphisms are detected as the presence or absence of bands of a particular size (Welsh et al., 1992; Williams et al., 1990). Polymorphisms for RAPDs may result from single base changes, deletions, or insertions in the template DNA. It is generally assumed to be a very powerful tool

in generating relatively dense linkage maps in a short period of time. The advantages of RAPDs are many: the requirement of small amounts of DNA (5~20 ng), the rapidity to screen for polymorphisms, the efficiency to generate a large number of markers for genomic mapping, and the potential automation of the technique (Neale and Sederoff, 1991; Nelson et al., 1992; Sobral and Honeycutt, 1993). In addition, no prior knowledge of the sequence is required. Since primers can be chosen arbitrarily, and organisms can be mapped with the same set of primers, RAPD markers are far easier to work with than RFLPs, and thus very attractive for breeding applications (Rafalski et al., 1991). As a result, one large impact of RAPD technique implementation has been to increase the species amenable to mapping activities; it is particularly true for forest trees.

Several review papers have compared RAPDs with RFLPs for detecting genetic polymorphisms (Weber, 1989; Ragot and Hoisinton, 1993; Halldén, et al., 1994). There is a general agreement that RAPDs offer a number of important advantages over RFLPs, although their use in genetic studies and improvement programs for forest tree species has only recently become widespread: *Eucalyptus* (Grattapaglia and sederoff, 1994; Verhaegen and Plomion, 1996; Marques et al., 1998; Gen et al., 2003), loblolly pine (Grattapaglia et al., 1992a; Devey et al., 1994, 1999; Sewell et al., 1998), slash pine (Nelson, et al., 1993; Kubisiak et al., 1995; Dale and Teasdale, 1996; Brown, et al., 2001), longleaf pine (Nelson, et al., 1994; Kubisiak, 1995, 1996; Weng et al, 2000), maritime pine (Plomion et al., 1995a, 1995b, 1996; Costa et al., 2000; Ritter et al., 2000; Chagné et al., 2003), Scots pine (Yazdani, et al., 1995; Hurme and Savolainen, 1999; Yin et al., 2003; Komlainen et al., 2003), Monterrey pine (Devey et al., 1996; Emebiri et al., 1998; Wilcox et al., 2001), Norway spruce (Binelli et al., 1994; Lehner, et al., 1995; Bucci et

al., 1997), white spruce (Tulsieram et al., 1992; Gosselin et al., 2002), Douglas-fir (Broome and Calson, 1994) and oak (Moreau et al., 1994).

RAPDs, however, suffer from certain limitations. Because of its high sensitivity (Skroch and Niehuis, 1995), to change in reaction condition, the products can vary, which can lead to inconsistent results between laboratories. A more serious problem is that RAPD markers are typically dominant rather than co-dominant. Many sequence polymorphisms are simply reflected as the presence or absence of a given RAPD marker rather than as a length variation, as in the case of other markers. This problem makes it difficult to distinguish a homozygote from a heterozygote with one 'null' allele (Postlethwait, 1994; Hunt, 1995). Although the use of haploid populations for mapping will circumvent this situation, the current approach still represents an elegant solution to the problem of deriving a genetic map from some tree species that require 15~ 20 years to attain sexual maturity (Tulsieram, 1992). One further drawback to the RAPDs lies in the fact that these markers do not specify sequence-tagged sites (STSs). When a microsatellite marker detects an interesting linkage, the marker can immediately be used to screen a resource such as the BAC library or a sub-chromosomal hybrid cell panel. When a RAPD detects such a linkage, cloning and sequencing of the RAPD band will be required in order to concert it into a conventional STS.

1.4.2.3 Microsatellite or Simple Sequence Repeats

In order to find markers that combine the advantages of both RAPDs and RFLPs that could potentially be used across families, Sequence Tagged Site (STS) markers (Olson et al., 1989) were developed in crop plants (Tragoonrung et al, 1992; Konieczny and Ausubel, 1993) and, recently were widely applied to forest trees (Smith and Devey 1994; Powell et al 1995; Byrne, 1996; Pfeiffer et al 1997; Brondani, 1998; Tanaka et al., 1999; Chen, et. al. 2002). A STS

is a unique, simple-copy segment of the genome whose DNA sequence is known and which can be amplified by specific PCR analysis with STS markers, thus combining the speed of the RAPD markers with the informativeness of the RFLP markers. Three types of STS have been reported in the forest trees. One type contains SSRs, also known as microsatellite sequences, which consist of tandem repeated multi-copies of mono, –di, –tri, and tetra-nucleotide motifs (Bryan et al., 1997; Jacob, et.at., 1991; Litt, et. al., 1989; Weber, et. al., 1989). Slippage of DNA polymerase during DNA replication and failure to repair mismatches is considered a mechanism for creation and hypervariability of microsatellites (Levinson & Gutman, 1987).

Microsatellites, or SSR markers, have been generally recognized as an excellent marker system. Besides having the advantage of being STSs, they have also proven to be ubiquitous, abundant, highly repeatable, widely and uniformly distributed, co-dominant (Morgante et al., 1994 Leopoldino and Pena 2002; Tautz 1989), suitable for automated detection, and, above all, are the most informative markers because of their hypervariability (Goodfellow 1992, 1993; Powell et al., 1996). These properties make them extremely popular molecular markers for applications in some phylogenetic analysis (Alvarez et al., 2001; Matsuoka et al., 2002; Russell et al., 2003; Struss and Plieske 1998) and molecular mapping (Baum et al., 2000; Gupta et al., 1999; Harker et al., 2001; Udupa and Baum 2003) in various crop plants. The initial development of SSRs was quite an expensive and time-consuming task; however, their ease of use and low cost compensate for the primary effort (Rafalski and Tingey 1993).

The identification of SSR markers in species with large genomes, such as conifers, is made more difficult by the high proportion of primer pairs that amplify multiple bands (Kostia et al., 1995; RoÈder et al., 1995; Smith and Devey 1994). However, fully informative, multi-allelic SSR markers, which can unambiguously identify all the alleles transmitted from the parents to

the offspring, are especially desirable (Grattapaglia and Sederoff, 1994) in conifers due to the difficulty, in some instances, carrying out suitable genetic crosses. The first microsatellites developed in forest trees were in *Pinus radiata* (Smith and Devey 1994). They have since been developed from the nuclear genomes of a range of temperate and tropical forest trees, and several linkage maps have been built with microsatellite markers (see summary table for Table 1.1).

However, traditional SSR markers have some disadvantages. First, genomic SSR markers were mostly derived from the intergenic regions, which have no gene function. Second, procedures for developing those markers are complex; the process includes isolating and sequencing clones containing putative SSR motifs, and subsequently designing and testing the flanking primers. The non-amplification of alleles has also been reported from microsatellite data, resulting in apparent heterozygote deficiencies and upwardly biased inbreeding coefficients in population studies (Fisher et al., 1998). Uneven distribution of microsatellite repeat motifs may be another reason for the failure of conifer genetic maps to coalesce into the expected number of linkage groups (Echt and MayMarquardt, 1997; Paglia et al., 1998; Schmidt et al., 2000).

Microsatellites also have some drawbacks as markers. The first problem is a putative reduction or complete loss of amplification of some alleles due to base substitutions or deletions within the priming site (null alleles). A heterozygote carrying one null allele cannot be distinguished on gel from a homozygote for the only DNA fragment which can be scored in the same plant. This can lead to an underestimation of heterozygosity, compared to the expected heterozygosity under the Hardy-Weinberg equilibrium. Segregation analysis in full-sib families helps to identify null alleles. Inheritance and segregation analysis, therefore, are prerequisite for validating SSR variants as markers in population genetics (Gillet, 1999). Another problem is associated to the Taq polymerase which may generate slippage during PCR and therefore

generate problems in microsatellite size determination by means of sequencing (Liepelt et al., 2001).

Table 1.1 Mapping studies in forest trees with microsatellite markers

Species	Pedigree	No. of Linkage Groups	Reference
Castanea mollissima x *C. dentata*	F1	12	Kubisiak et al. (1997)
Castanea mollissima x *C. dentata*	F1	12	Sisco et al. (2005)
Castanea sativa	F1	12	Casasolli et al. (2001)
Eucalyptus globulus	F1	13	Bundock et al. (2000)
Eucalyptus globulus	F1	8	Marques et al. (2002)
Eucalyptus grandis	F1	9	Brondani et al. (1998)
Eucalyptus tereticornis	F1	8	Marques et al. (2002)
Eucalyptus urophylla	F1	10	Brondani et al. (2002)
Populus deltoides	BC1	19	Yin et al. (2004)
Populus deltoides	F1	19	Cervera et al. (2001)
Populus deltoides	F1	19	Jorge et al. (2005)
Populus trichocarpa	F2	26/24	Frewen et al. (2000)
Populus trichocarpa x *Populus deltoides*	BC1	19	Yin et al. (2004)
Quercus Robur	F1	12	Barreneche et al. (2004)
Picea abies	OP	29	Paglia et al. (1998)
Picea abies	F1	12	Acheré et al. (2004)
Picea abies	F1	13	Scotti et al.(2005)
Picea glauca	F1	12	Pelgas et al. (2006)
Pinus elliottii x *P.caribea var. hondurensis*	F1	24/25	Shepherd et al. (2003)
Pinus pinaster	F1	12	Ritter et al. (2002)
Pinus pinaster	TGOP	12	Chagné et al. (2003)
Pinus pinaster	F2	12	Mariette et al. (2001)
Pinus radiata	TGOP	22	Devey et al. (1996)
Pinus radiata	F1	20	Wilcox et al. (2001)
Pinus strobus	OP	12	Echt and Nelson (1997)
Pinus taeda	TGOP	20	Devey et al. (1994)
Pinus taeda	TGOP	15	Zhou et al. (2003)
Pseudotsuga menziesii (Mirb.) Franco	TGOP	22	Krutovsky et al. (2004)

** OP= open pollinated family. TGOP=Three generation outbred pedigree

1.4.2.4 Sequence Characterized Amplified Regions

The other type of STS markers developed in trees are random amplified polymorphism DNAs (RAPDs) that have been sequenced, allowing PCR primers to be made for the ends of the RAPD fragments. These STS-converted RAPD markers are sometimes referred to as SCARs (Paran and Michelmore, 1993) for sequence characterized amplified regions. While SCARs will allow for rapid STS marker development, they may not prove to be highly polymorphic (Bodénès et al., 1996).

1.4.2.5 Amplified Fragment Length Polymorphisms

AFLP is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Vos, et al., 1995). It is similar to RAPD and requires no sequencing or cloning, but the primer consists of a longer fixed portion (circa 15 base pairs) and a short (2-4 base pairs) random portion. The fixed portion gives the primer stability, hence the repeatability (Alonso-Blanco et al., 1998; Haanstra et al., 1999; Vuylsteke et al., 1999; Young et al., 1999). The random portion allows it to detect many loci. Polymorphisms are detected as band presence/absence. AFLP markers are often inherited as tightly linked clusters in centromeric and telomeric regions of chromosomes, but randomly distributed AFLP markers can also occur outside these clusters. The technique is difficult to master and is less appropriate than others for comparative mapping studies (Tanksley et al., 1988).

1.4.2.6 Single Nucleotide Polymorphisms

One of the most popular of the non gel-based marker systems is SNP, which represents sites where the DNA sequence differs by a single base. This polymorphism has been shown to be the most abundant, at least one million SNPs available, only in the non-repetitive transcribed

regions of the human genome. An SNP (single nucleotide polymorphism) marker is a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. For such a base position with sequence alternatives in genomic DNA to be considered as an SNP, it is considered that the least frequent allele should have a frequency of 1% or greater. Although, in principle, any of the four possible nucleotide bases can be present at each position of a sequence stretch, SNPs are usually biallelic in practice. However, the extraordinary abundance of SNPs largely offsets the disadvantage of their being biallelic, making them the most attractive molecular marker system. A wide range of marker techniques is now available for genotyping plant genomes. The characteristics for the commonly used molecular markers were summarized in Table 1.2.

Table 1.2 Comparison of the most commonly used marker systems

Feature	**AFLPs**	**RAPDs**	**RFLPs**	**SCARS**	**SNPs**	**SSRs**
DNA require (μg)	0.5-1.0	0.02	10	0.05	0.05	0.05
DNA quality	Moderate	High	High	High	High	Moderate
PCR-based	Yes	Yes	No	Yes	Yes	Yes
Polymorphisms	High	Med/High	Low/Med	High	High	High
Dominance*	Dom	Dom	CoDom	CoDom	CoDom/Dom	Co-Dom
Reproducibility	High	Unreliable	High	High	High	High
Amenable to automation	Moderate	Moderate	Low	Moderate	High	High
Ease of use	Easy	Easy	Not easy	Easy	Easy	Easy
Development cost	Moderate	Low	Low	Moderate	High	High
Cost per analysis	Moderate	Low	High	Moderate	Low	Low

Dominance: Dom, Dominant markers; CoDom, Co-Dominant markers.

Unfortunately, highly informative marker types, like SSRs and SNPs, have been elaborated for only a few well-studied crop plants. Due to the lack of sequencing and mapping data, genotyping in 'undiscovered' plant genomes still has to be performed using universal marker techniques like RAPDs and AFLPs. However, the strong synteny between closely related species will allow, to a certain extent, the transfer of marker information, thereby increasing the molecular marker pool in genomes of plant families. Finally, reducing genotyping costs for high throughout techniques, e.g. microarrays, is a major challenge for the comprehensive integration of markers into plant breeding programs.

1.5 Linkage Map and Mapping Theory

A layout of the order of genes (loci), as well as the distance between them, is called a genetic map or linkage map. Mapping is defined as the process of deducing schematic representations of DNA. Two genes are said to be linked if they are located on the same chromosome, and they tend to be inherited together in meiosis. However, they have a chance of not being inherited, as in the parental state; this is due to recombination. During meiosis, the chromosome often breaks and then rejoins with the homologous chromosome, such that new chromosomal combinations appear, indicating a crossover. The further the distance between two genes, the more frequently there will be crossovers, and the higher the number of recombination. Thus, the frequency of crossover between any two genes serves as a measure of genetic distance between them (Haldane, 1919; Kosambi, 1944).

1.5.1 Mapping Function

The distance between two genes is determined by their recombination fraction; the map units are Morgans. One Morgan is the distance over which, on average, one crossover occurs per meiosis. When considering the mapping of more than two points on the genetic map, it would be

very handy if the distances on the map were additive. However, recombination fractions themselves are not additive, and it is necessary to redo a genetic map each time new loci are discovered.

To avoid the non-additive problem, the distances on the genetic map are mapped using a mapping function. A mapping function translates recombination frequencies between two loci into a map distance in cM. It will give the relationship between two chromosomal locations on the genetic map in cM and their recombination frequency. To be a good mapping function, two properties are required:

1) Distances are additive, i.e. the distance AC should be equal to AB + BC if the order is ABC;

2) A distance of more than 50 cM should translate into a recombination fraction of 50%

In general, a mapping function depends on the interference assumed. With complete interference, or within small distances, a mapping function is simply:

Distance (d) = r (recombination fraction).

With no interference, the Haldane mapping function is appropriate:

$$d = -\frac{1}{2}\ln(1 - 2r).$$

Kosambi's mapping function allows for some interference:

$$d = \frac{1}{4}\ln[(1 + 2\mathrm{r})/(1 - 2\mathrm{r})].$$

The different mapping functions are depicted in Figure 1.2. From the graph, it shows there is little difference between the different mapping functions below 0.5 cM, and it can safely assume that d= c. However, with the increase of the recombination fraction, the map distances from different mapping functions are also increasing.

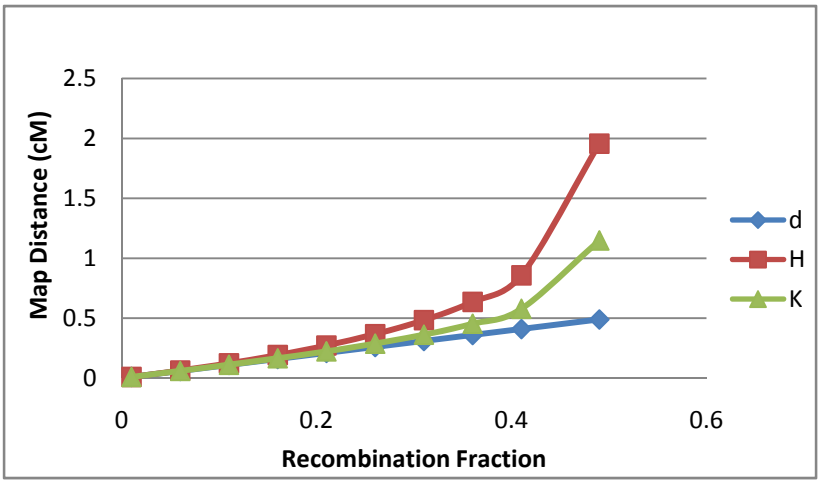

Figure 1.2 The comparison of mapping functions under different recombination fractions. The X-axis is for the recombination fraction, and the Y-axis is for the map distance with unit of cM. d is the map distance obtained directly from recombination fraction, H is the map distance obtained from Haldane's mapping function and K is the map distance obtained from Kosambi's mapping function.

1.5.2 Mapping of Genetic Markers

Genetic markers can be mapped relative to each other by determining recombination fractions or by using a mapping function. For a whole genome map, some markers need to be anchored to their physical position using *in-situ* mapping and several molecular techniques, e.g. Fluorescent *In-Situ* Hybridization (FISH). Recombination fractions between genetic markers can be estimated from mapping experiments. Since the complete marker genotypes can be observed, researchers usually do not fully rely on certain specific designs as often as morphological markers. However, some designs are more efficient for mapping than other designs in determining the percentage of meiosis observed that is actually informative. Recombination fractions are estimated from the proportion of recombinant gametes and this fraction is relatively easy to determine if the linkage phase is known in parents and the haplotype of the gametes transmitted from parents to offspring. Unfortunately, in practice, linkage phases are not always known. This is especially the case in outcross species, as it is hard to create inbred lines. If the

linkage phase is not known, one can usually infer the parental linkage phase, as the number of recombinants is expected to be smaller than the number of non-recombinants. Marker maps can be made from genotyping certain families for a series of markers. There are no strict rules for creating reference families; however, certain designs are better for obtaining information than others. The general rules are:

1) The amount of information available for mapping is based on the number of informative meiosis;

2) An efficient design minimizes the number of genotyping for a given number of informative meioses.

Since the informative meiosis depends on the number of marker alleles and hetero/ homo-zygosity of parents, full-sib families are better than half-sib families because the number of genotyping is lower for the same number of informative meiosis. It is also better to use more families, as two parents may have genotypes at certain markers that will never produce informative meioses.

1.5.3 LOD Score

Maximum likelihood (ML) method is usually used to determine the most likely phase, and therefore, to determine the most likely recombination fraction. Besides estimating the most likely recombination fraction, I also want to test those estimates statistically. In particular, I want to test whether or not two loci are really linked. Therefore, the statistical test to perform is the likelihood of a certain recombination fraction (r) versus the likelihood of no linkage (r=0.5). Different likelihoods are usually compared by taking the ratio of the likelihood.

$$\frac{likelihood\ (r = \hat{r})}{likelihood\ (r = 0.5)}$$

The ^{10}log ratio of this likelihood ratio is indicated by a LOD-score (abbreviation of log of-odds) (Morton, 1955). A LOD-score above 3 is generally used as a critical value. A LOD-score >3 implies a ratio of likelihoods of 1000 to 1, and indicates the null-hypothesis (r = 0.5) is rejected. Though this seems like a very stringent criterion, it accounts for the prior probability of linkage. Morton (1955) suggested that LOD scores from data from additional families, or from additional progeny within a family, could be added to the original LOD score.

1.5.4 Methods and Software Used in Genetic Mapping

Multi-locus genetic mapping can be separated into three problems: grouping, ordering and distance estimation. Grouping is a matter of setting admission rules and requiring any candidate locus ineligible for any existing group to initiate a new group. Usual admission rules are based on upper linkage thresholds and lower limit of detection (LOD) score thresholds for linkage with some other members of the group. These LODs are measures of informativeness, based on r and the number of observations used to estimate it.

Locus ordering is the central problem in linkage mapping. One of the simplest algorithms, seriation (Doerge 1996; Ellis 1997; Crane 2005), involves growing an order outward from the most tightly linked locus pair. It is 'greedy' in the sense that each successive addition is made to optimize the current order without consideration of the loci not yet added or removal of any previously added. A more elaborate greedy algorithm is MAPMAKER (Lander et al., 1987), which finds all three locus orders, then excludes the most unlikely and proceeds by evaluating permissible multilocus orders built from the remaining ones. The method of JoinMap (Stam 1993; Stam and Van Ooijen 1995) is also sequential, adding the most informative markers one at a time, accepting only if a goodness-of-fit test shows an improvement and shuffle-optimizing at each step. Simulated annealing (SA), used by GMendel (Liu and Knapp 1990), employs a

"temperature" parameter that governs the amount of change in a configuration that may be applied at each step, as well as the probability of acceptance of a configuration with a lower (more unfavorable) score than the current one. As the configuration stabilizes at some temperature, the system is "cooled", changes become less extreme, and unfavorable changes are less readily accepted.

Once a locus order has been obtained, the problem remains of computing inter-locus distances. Naive methods retain the original distances between adjacent markers, an unsatisfactory resolution since these were based on ML approximations and partial information to begin with. One improvement described by Jensen and Jorgensen (1975), adapted by JoinMap and reinvented by Newell et al. (1995), consists of calculating the distances using least square error from the two point distances, while giving more weight to distance estimates based on more information. MAPMAKER updates the linkage estimate directly, using an EM algorithm. Both methods increase the likelihood of the final map. GMendel uses a simpler and somewhat less stable method that adjusts the raw distance estimate between two loci to show least absolute deviation from the un-weighted distances between all flanking loci.

However, for obligate outbred species, linkage estimation must distinguish between the coupling and repulsion phase, for both co-dominant and dominant markers, and must accommodate as many as four (i.e. diploid) alleles segregating at a locus. Several statistical models (Ritter et al., 1990, Ritter and Salamini 1996; Maliepard et al., 1997) for handling the outbred data are available, but the current available software do not handle phase-unknown data well. Therefore, it needs to infer the seven possible marker segregation types (Figure 1.3) from two locus genotype frequencies. The next step is either to build separate maps for parents in MAPMAKER and join them by hand with "allelic bridges", markers common to two classes of

segregating alleles from both parents, or by the commercial software JoinMap, which accepts multiple segregation types in a single cross. The JoinMap computes common two point linkage based on the assumption of identical underlying linkages in all populations, supplying LOD scores based on the variation in observed values for a given locus pair.

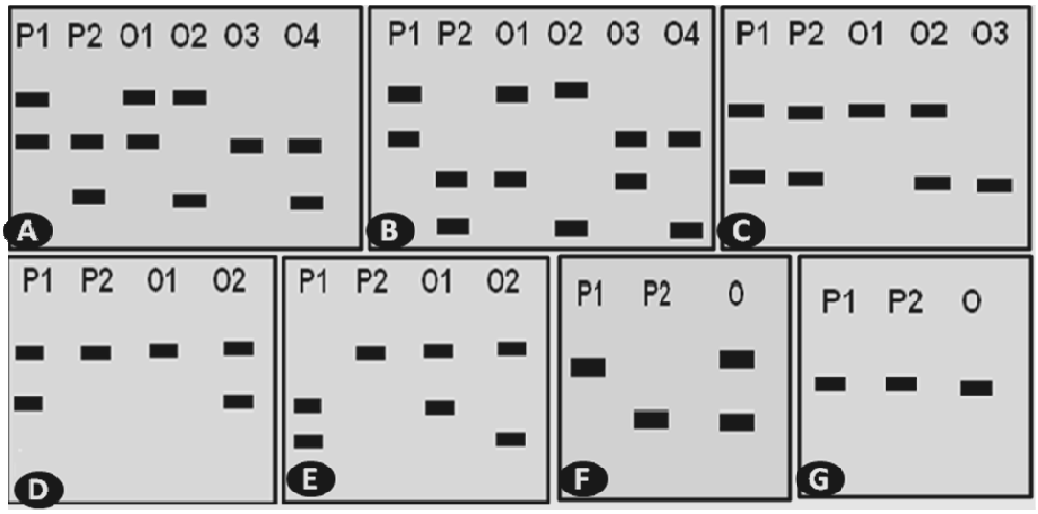

Figure 1.3 Seven possible segregation types for outbred species. A and B: Full informative marker types (segregation 1:1:1:1) C: Both informative marker types (segregation 1:2:1), D and E: Partial informative marker type (segregation 1:1) F and G: Non informative marker type (no segregation). P1 and P2 are for parent 1 and parent 2. O, O1,…, O4 are for different offsprings.

1.5.5 The Application of Linkage Maps in Genome Studies

In the brief period since DNA marker technology was first applied to plants, there has been an explosion in the development and application of genetic linkage maps. Using these new DNA-based maps, researchers have constructed maps in species where only poorly populated classical maps existed before (Bonierbale et al., 1988, Grattapaglia and Sederoff 1994, Gebhardt et al 1991, Landry et al 1987, Menancio- Hautea et al 1993). These linkage maps locate genes for both qualitative and quantitative characters (Concibido et al 1997, Lin et al 1995, Mansur et al 1993) in detail, providing a basis for positional cloning (Tanksley et al 1995). In the *Pinaceae*, intensive genome studies have been conducted on *Pinus teada* (e.g. Sewell et al., 1999; Brown et

al., 2001; Temesgen et al., 2001) and extended to lots of other pine species, such as *P. radiata* (Devey et al., 1999, Wilcox et al., 2001, 2004), *P. elliottii* (Brown et al., 2001, Weng et al., 2002) and *P. palustris* (Nelson, 2003), resulting in the partial construction of comparative maps (Devey et al., 1999; Brown et al., 2001; Chagné et al., 2003; Krutovsky et al., 2004).

Comparative mapping in plants began with the rather simple demonstration that maps in one species could be constructed using RFLP probes from a related species and once such maps were made, they could be compared (Bonierbale et al., 1988; Ann and Tanksley 1993). Loci revealed by RFLP probes are assumed to be orthologous between species, meaning that the gene was present in a common ancestor. Orthologous genetic markers are essential for comparative mapping. RFLPs have been used almost exclusively for comparative mapping. Ahuja et al., (1994) showed that cDNA RFLP probe derived from *Pinus taeda* would hybridize to genomic DNA from other species of *Pinus* and even other members of the conifer family, suggesting that RFLP probes could be shared among labs for mapping purposes and that comparative maps would result from such exchanges.

However, due to the difficulty in performing RFLP analyses in conifers, most genome mapping projects in conifer have used one of the PCR-based marker systems and these markers types do not have the potential for providing orthologous markers, which can be used across different species. Even SSRs can only be used within a narrow range of related species (Echt et al., 1999). The Conifer Comparative Genomic Project (CCGP) had developed and mapped 135 new genetic markers based on EST (Temesgen et al., 2000, 2001; Brown et al., 2001). These primers amplify subgenus *Pinus* DNA at nearly a 100% success rate and at about 50% rate in the subgenus *Strobus*. These markers were used to construct comparative maps between *P. taeda* and several species of *Pinus.*

1.6 Quantitative Trait Loci and QTL Mapping

A quantitative trait locus (QTL) is the location of a gene that affects a trait that is measured on a quantitative (linear) scale. These traits are typically affected by more than one gene and also by the environment. QTL mapping is a means to estimate the location, numbers, magnitude of phenotypic effects, and modes of gene action of individual determinants that contribute to the inheritance of continuous variable traits (Paterson, 2002). Thus, the aim of QTL mapping is to locate the QTLs influencing the traits and to estimate their allelic effects, i.e., additive and dominance effects at individual QTLs and interaction (epitasis) among these effects at two or more QTLs.

1.6.1 QTL Methods and Statistical Analysis

Regardless of the population structure and size, several factors are very crucial for successful QTL identification (Beavis, 1994). The statistical method used significantly influences the accuracy of QTL position and effect estimation. Simple statistical methods such as Analysis of Variance (ANOVA) have opened the way to the development of more powerful QTL detection methods, interval mapping (IM), composite interval mapping (CIM), and multiple-interval mapping (MIM) which integrate the information available at multiple markers.

1.6.1.1 Single Marker Analysis

The simplest QTL method, called single marker analysis, partitions the population into different genotypic classes based on genotype at the marker locus, and then uses correlative statistics to determine whether the individuals of one genotype differ significantly compared with individuals of other genotypes with respect to the trait being measured (Sax, 1923). The principle is that the genotype of a marker should be correlated with the genotype at a linked QTL. The marker should also show a statistical influence on the trait, which declines with increasing

genetic distance from the QTL. This influence can be tested by a contrast of phenotypic means of the marker-genotype classes, using the t test, ANOVA, or regression.

For regression, which requires a numerical explanatory variable, the genotype of an individual at a marker locus may be expressed as the quantity of the reference allele (in a diploid, 0, 1 or 2) that it carries. In designs where all three genotypes are present, the effect can be partitioned into additive and dominance effects. In designs where more than two alleles may segregate at a locus, regression may be replaced by a general linear model or nonparametric equivalent such as the Kruskal-Wallis test. This method has been utilized with various experimental designs, such as backcross and intercross designs. However, this approach has four undesirable properties: 1) The QTL location and QTL effects cannot be separately estimated; 2) the additive and dominance effects are confounded with the amount of recombination; 3) the power of QTL detection is small, especially with wide marker spacing; 4) the individuals whose genotypes are missing at the marker have to be discarded.

1.6.1.2 Interval Marker Analysis

In order to overcome the disadvantages of single marker method, Lander and Bostein (1989) developed interval mapping, which is currently one of the most widely used methods for QTL mapping with experimental crosses. Instead of analyzing the population one marker at a time, a set of linked markers are analyzed simultaneously with regard to their effects on the target trait. With the flanking markers providing a probability distribution for the QTL genotype and assumed normal distribution of the trait within QTL genotype classes, the EM algorithm is used iteratively to find values for the trait means and variances in these classes that maximize the likelihood of the phenotype/flanking-marker genotype combinations observed in progeny individuals. A QTL mapping procedure, implemented in MAPMAKER/QTL (Lincoln et al.,

1992), “walks” along chromosomes, performing the ML calculation at regularly spaced points one or two centiMorgans apart, and the resulting LOD scores are plotted to reveal candidate QTL sites of highest likelihood. Other commonly used software implementing ML-SIM are QTL cartographer (Wang et al., 2004), MAPQTL (Van Ooijan et al., 2002) and MultiQTL (Korol, 2004).

A qualitatively different ML approach to SIM was adapted by Xu and Atchley (1995) from a method from human genetics that requires only the estimation of the identity–by–descent (IBD) proportion of alleles shared by pairs of individuals at a map position. For a QTL at this position, high IBD should be accompanied by low phenotypic difference. Such a random model algorithm, which models the variance rather than the magnitude of QTL effect, has been implemented for plant designs in the web based software QTL Express (Seaton et al., 2002). Its advantages over fixed model methods are that it requires no knowledge of linkage phase or the number of alleles at loci and is readily adapted to complicated pedigree designs.

Least square (LS) methods are much easier and faster to compute than ML methods and allow more straightforward modeling of a large variety of effects, mating designs, and generations with usually negligible loss of estimation accuracy and precision. Haley (1994) was the first person to extend the method to outbreeding species. The computer programs implementing LS-SIM are numerous: MQTL (Tinker and Mather, 1995), MMPTX, QGene (Nelson 1997) and MCQTL (Jourjon et al., 2005).

The failures of SIM in the presence of multiple, especially linked QTLs are the results of its testing the wrong hypothesis at each map position, i.e., that of “QTL at the test position” versus “NO QTLs anywhere”. The correct test (Jansen, 1993) is that of a “multiple-QTL model including” versus “one excluding a QTL at the test position”. Such tests fit well into a multiple

linear-regression framework, and the evolution of multiple-QTL modeling. MQM has progressed from regression on sets of markers (Cowen, 1989), to "hybrid" models containing both QTL expectations at a test point and "background" cofactor markers at other places in the map (Jansen, 1992; Zeng,1994), and finally to models in which all markers are replaced by QTL genotypes (Kao and Zeng, 1999; Sen and Chruchill, 2001).

Composite methods are not interested in the cofactor markers *per se*, which are used only to absorb the approximate trait variation due to presumed QTLs outside the test interval. The QTL search is still a one-dimensional scan across the map. Different methods are used for selecting the cofactor markers, though the more added to increase QTL resolution, the lower the detection power and estimation precision. Error in the modeled QTL genotype can be minimized with weighted regression (Jansen and Stam, 1994) or use of ML instead of LS (Zeng et al., 1999).

The MQM method of Jansen has been implemented in the commercial QTL package MapQTL and the ML-CIM method of Zeng in QTL cartographer. LS implementations are provided by MMQTL, PLABQTL (Utz and Melchinger, 1996), and QTL mapper (Wang et al., 1999). MultiQTL, another commercial program, offers an elaborate suite of QTL analysis that includes CIM. A simpler version of CIM (sCIM) was provided in the program MQTL (Tinker and Mather, 1995), which fits a multiple regression model only once instead of at each QTL test position. The CIM model has not yet been extended to autopolyploid models.

Single-marker and interval mapping methods have been successfully used for several quantitative trait mapping studies in agricultural research (Edward et al., 1987; Lander and Botstein 1989; Lippman and Tanksley 2001; Georgiady et al., 2002). The number of reports of QTL mapping in forest trees for economically important traits, such as growth, phonology, and development, is increasing, including *Eucalyptus* (Grattapaglia et al., 1995; Vaillancourt et al.,

1995; Byrne et al., 1997; Chaparro et al., 1997), Scots pine (Hurme et al., 2000), loblolly pine (Groover et al., 1994; Knott et al., 1997; Sewell et al., 2000, 2002; Gwaze et al., 2003), slash pine (Kubisiak et al., 2000; Weng et al., 2002), maritime pine (Plomion et al, 1996; Brendel et al., 2002; Pot et al., 2005), poplar (Han et al., 1994; Bradshaw et al., 1995; Frewen et al., 2000) and Douglas fir (Jermstad et al., 1998, 2001, 2003; Krutovsky et al, 2004). The results now permit detailed examinations of the fundamental assumptions of the quantitative model as applicable to forest tree breeding, including approximately equal effects of individual polygene, independent assortment of polygene, and minimal epistasis (Tanksley, 1993)

The future of QTL mapping is largely on developing more legitimate methods for genetic parameter estimations for QTL analysis. Methods for QTL mapping in multiple crosses or multiple populations have developed in recent years (George et al., 2000; Walling, et al., 2000; Zou et al., 2001). QTL designs combining information from multiple crosses are more powerful than those involving a single cross (Lynch, et al., 1998). Current methods for complex pedigrees are not completely satisfactory (George et al., 2000). The difficulties arise from unknown marker genotypes and unknown marker phases, especially for data with multiple generations. Moreover, it is challenging to analyze QTL as random effects by introducing mixed model methodology (Xu, et al., 2000). This demonstrates the importance of estimating genetic parameters for QTL analysis in the mixed model framework.

1.6.2 QTL Pedigree and Strategies

Accurate mapping of QTL using these methods depends critically on well-defined mapping pedigrees, such as F2, F3, or backcrosses, initiated with two inbred lines. However, the development of such pedigrees is extremely difficult in outcrossing species. The markers in outbred populations may not be fully informative and may vary in their heterozygosity among

individuals and between families. Two types of strategies have been consistently proposed: F2-like (Beckmann and Soller, 1988; Knott and Haley, 1992) and BC-like strategies. Mapping in F2-like pedigree was carried out in the same manner as for an inbred F2 pedigree, assuming that there was enough marker variation between species that the heterozygous grandparent differed for alleles and could be treated as inbred lines. The F2 generation was treated as though there were three possible genotypes that could occur at any locus: homozygous for parent 1; homozygous for parent 2; or heterozygous, segregation 1:2:1. Later work on this pedigree modified the mapping procedure specifically for an outbred population structure so that a sex-averaged framework map for the F2 was produced. Fully informative markers were preferentially chosen when available. For the BC-like strategies, segregating families come from parents supposed to be heterozygous at the QTL. The families can be one or more half-sibs (i.e. daughter and grand-daughter designs, Weller, 1990), a full-sib family (i.e. double pseudotestcross. Leonards-Schipper et al., 1994), many independent full-sib families coming from a random mating population (Soller and Genizi, 1978) or full-sib families related in a hierarchical structure (Götz and Ollivier, 1992).

Linkage maps have also been produced from F1 or backcross pedigrees using the pseudotestcross strategy (Grattapaglia and Sederoff, 1994). This strategy is mainly based on selection of single-copy polymorphic markers heterozygous in one parent and homozygous null in the other parent and therefore segregates into 1:1 ratios in their F1 progeny as in a testcross. The term “two way pseudotestcross” to define this mapping strategy is generally used to describe the two independent genetic linkage maps that are constructed by analyzing the cosegregation of markers in each progenitor (Wu et al., 2000, Zhang et al., 2004). This method takes advantage of the naturally high level heterozygosity in outbred forest trees. Various studies have taken

advantage of this two generation full-sib design to analyze each parent under a pseudotestcross model (Kumar et al., 2000; Lerceteau et al., 2000; Shepherd et al., 2002; Weng et al., 2002; Yazdani et al., 2003). This model is well suited for dominant markers. However, the main limitation of this pedigree is that the phenotypic effects inherited from each parent are analyzed individually, even though the genetic contributions of each parent simultaneously contribute to the phenotypic variation in the progeny population. Consequently, the genetic information in the four progeny classes of an outbred pedigree is collapsed into only two genotypic classes, thereby reducing the robustness of the analysis. However, if co-dominant markers are used, a consensus map can be built precisely to detect allele effects from both progenitors simultaneously.

Devey et al. (1994) used a three-generation pedigree consisting of four grandparents, two parents, and 95 progeny to develop a linkage map of loblolly pine (*Pinus Taeda* L.). In this three generation outbred pedigree, two crosses are made among four unrelated grandparents, where each mating pair is selected among individuals displaying divergent phenotypic values for the trait. From each grandparental mating, a single phenotypically intermediate individual is chosen as parents. Presumably, these intermediate parents are potentially heterozygous for different allelic pairs that display a divergent phenotypic effect. This three generation full-sib structure is typically designed for QTL analysis under an outbred model and has been used extensively (Groover et al., 1994; Byrne et al., 1995; Kubisiak et al., 1997; Brendel et al., 2002; Sewell et al., 2002; Devey et al., 2004).

In gymnosperms, megagametophytes provide a source for segregating haploid tissue. The megagametophytes are derived from repeated mitotic divisions of a single meiotic product and have the same maternal genetic complement as the embryo contained in the same seed. Since the megagametophytes are haploid of maternal origin, segregation and recombination can be

evaluated in a sample of seeds from a single tree without the need for controlled pollinations. Megagametophytes with DNA markers have been used in slash pine (Nelson, et al., 1993), longleaf pine (Kubisiak, 1994, 1996; Nelson, et al., 1994), maritime pine (Plomion et al., 1995a, 1995b), loblolly pine (Wilcox et al., 1996), and Scots pine (Yazdani et al., 1995).

Until recently, most of the QTL mapping efforts were focused on single family pedigrees. However, given the high rate of polymorphisms encountered in pines and the relative lack of stability of QTLs in different genetic backgrounds, methods aimed at validating markers linked to the traits of interest in more complex pedigrees, or even in unrelated genotypes, are emerging. Furthermore, if the progeny do not segregate at a QTL (i.e., the parents are homozygous at the QTL), then the QTL cannot be detected.

Therefore, the future of QTL mapping is largely dependent on developing more powerful methods of genetic parameter estimation for QTL analysis. Methods for QTL mapping in multiple crosses or multiple populations have also been developed by many quantitative geneticists (George et al., 2000; Walling et al 2000; Zou et al., 2001). QTL designs combining information from multiple crosses are more powerful than those involving a single cross (Lynch et al., 1998).

1.7 Future Perspective: From Linkage Map to QTLs

Genetic mapping will remain a vital research activity for years to come. Only a fraction of species are presently represented among mapped organisms. The key challenge of new work is to investigate strategies for whole genome breeding: how I can use genome-wide information in the form of graphical genotypes, known location of key loci, and marker tags for both desirable and undesirable alleles to design optimal breeding strategies that integrate as much of the available information as possible.

Until recently, QTL analysis has been a search for correlations between genetic markers and phenotypic observations representing the summation, over time, of gene effects. These include interactions with other genes and with the environment (Korol et al., 1998; Cao et al., 2001). An area of growing interest is QTL variation over plant developmental stages (Wu et al., 1999, Cao 2001; Wu et al., 2004); a web interfaced computer program, FunMap (Ma et al., 2004), for identifying these dynamic QTLs has recently appeared. A second area is fine mapping. Currently, a big problem with linkage analysis is that the mapping resolution is poor, around 20 cM. This is not because there are not enough markers, as marker densities can be around 0.5 to 3 cM with the current maps, and this density is still increasing (Georges and Anderson, 1996). The reason for the low resolution is that there's not enough observed meiosis in most experiments in order to distinguish between few and very few recombination events. Subsequently, methods other than linkage analysis have been considered. For example, when using advanced interline crosses (F3, F4, etc), the number of crossovers is increased and gives more resolution to map QTLs. However, advanced inter-crosses will take several generations to be established, which is not practical. Other methods to be discussed hereafter are 1) population wide linkage disequilibrium (Jannink et al., 2001; Wu et al., 2001, 2002; Bink et al., 2002), identity-by-descent (IBD) mapping (Meuwissen and Goddard, 2000). The most active research in the coming years may be in general solutions, e.g., integration of sources of genetic information to predict phenotype, such that the experimental design is only one of the variables. Therefore, not only are unified methods of handling different sources of LD are needed, but meta-analysis to unite disparate experiments (Goffinet and Gerber, 2000; Arcade et al., 2004) and methods for combing QTL models are require (Sillanpää and Corander, 2002).

In a summary, genetic mapping of quantitative trait loci has become a routine tool for the genetic study of plants, animals, and humans. Many fundamental genetic questions including the inheritance mode of quantitative traits, genotype x environment interactions and the genetic basis of heterosis, can be addressed by these tools (Reviewed by Tanksley 1993; Templeton 1999; Wu et al., 2000). The identification of QTLs is the first step towards developing marker-assisted strategies. In families where strong evidence of markers linked to QTLs have been detected, the next step is to use the information to test predictive hypotheses regarding the potential of MAS, and then to verify that potential using an independent experiment.

However, the efficiency of QTL-mapping analysis may be influenced by many factors: population size, genetic differences between parents, heritability of the trait of interest, polymorphism of the molecular markers, and density of the molecular marker map, statistical analysis method and genetic structure characterized by the segregating population derived from different mating designs. The different structures of segregating populations would affect power in the same way no matter which method of QTL detection is used (Muranty, 1996). This research will use the SSR markers initially developed for loblolly pine to build linkage maps for longleaf pine and identify the QTLs controlling EHG in longleaf pine.

1.8 Research Objectives

The primary objectives of this study were to:

1) Select an efficient mapping population for genetic analysis.

2) Establish a microsatellite based linkage map for longleaf pine.

3) Identify the markers associated with the QTLs for EHG and determine the number, chromosome position, and effect of the QTLs.

1.9 Outline of the Dissertation

In this dissertation, this chapter is a general introduction for why and how I can improve the EHG for longleaf pine and the background knowledge for molecular markers, linkage maps, and QTL analysis in pine trees. Chapter 2 focuses on marker screening and strategies used to select population for QTL mapping. Chapter 3 will present the results of assignment SSR markers to chromosomes. Chapters 4 will cover identification of the QTLs controlling EHG. Chapter 5 will discuss general conclusions from this research and future recommendations.

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CHAPTER 2 SSR MARKER SCREEN AND POPULATION SELECTION

2.1 Introduction

The delay in early height growth (EHG) in longleaf pine, known as grass-stage, has been an important limiting factor for its artificial regeneration of this species (Schmidtling and White, 1989). I am studying the genetics of early height growth (EHG) in longleaf pine (*Pinus palustris* Mill.) via interspecific hybridization between longleaf pine and slash pine (*Pinus elliotti* Engelm.), followed by backcrossing to either slash pine or longleaf pine. Previous research showed that EHG in longleaf pine was a quantitative trait controlled by a relatively small number of major effect genes (Brown, et al., 1964; Nelson et al., 2003) and with heritability ranging from 0.47 to 0.68 (Snyder and Namkoong, 1978; Layton and Goddard, 1982). The use of molecular marker assisted selection (MAS) offers a way to explore efficient and reliable simultaneous selection for EHG by selecting makers that are tightly linked to the QTLs controlling EHG. However, the efficiency of QTL-mapping analysis may be influenced by many factors: the type and polymorphisms of the molecular markers used, the density of the molecular markers map, the genetic differences between parents, the heritability of the trait of interest, the population size, the genetic structure which characterized by the segregating population derived from different mating designs and the statistical analysis method.

Microsatellites, or SSRs (Simple Sequence Repeats), are present in the majority of eukaryotic genomes and consist of simple, short tandem repeated di- to penta- nucleotide sequence motifs (Beckman and Soller 1990). The allelic variation in microsatellite loci can easily be detected by PCR using specific flanking primers. A polymorphism based on the variation in the number of repeated motifs is probably due to slippage during DNA replication or unequal crossing-over (Levinson and Gutman 1987). Microsatellites have been widely used in many crop

species due to their abundance, high degree of polymorphism, locus specificity, reproducibility, low amount of DNA required, suitability for multiplexing on automated systems, and, above all, their co-dominant mode of inheritance. These characteristics make SSRs an attractive option for mapping and QTL studies.

In conifers, SSR markers have already been developed and used in genome studies (Table 2.1 in Chapter 2). However, early results suggested that microsatellite variation might be rare in pine (Hutchison et al., 1994), thus restricting their use for mapping when compared with the potentially unlimited number of RFLP, RAPD and AFLP loci. Developing pine microsatellites has proven to be difficult because of the size (approx. 28,000 pg/C) and complexity (approx. 75–86% highly repetitive DNA) of the pine genome (Smith and Devey 1994; Soranzo et al., 1998; Echt et al., 1999; Aukland et al., 2002; Chagné et al., 2004; Guevara et al., 2005). In addition, the ancient divergence time between coniferous species (Price et al., 1998) and the complexity of their genomes mean that transferability of single-copy SSR among genera, and even within *Pinus*, is generally poor, resulting in a large portion of amplification failure, nonspecific amplification, multi-banding pattern, and lack of polymorphisms (Echt and Nelson, 1997; Mariette et al., 2001 Chagné et al., 2004; Plomion et al, 2007).

To circumvent these genome related problems, secondary screening of enriched libraries (Pfeiffer et al., 1997; Scott et al., 1999), either eliminating repetitive regions of the genome (Smith and Devey 1994; Elsik and Williams 2001) or enriching for low-copy genomic sequences using an undermethylated region enrichment method (Elsik et al., 2000; Zhou et al., 2002), have increased the efficacy of pine microsatellite marker development.

However, the cross-transferability of microsatellite markers showed contrasting results depending largely on the evolutionary distance and the complexity of the genome (Barreneche et

al., 2004). Echt et al. (1999) pointed out that the evolutionary conservation of DNA sequences flanking SSR sites allows previously developed SSR primers to be used in various other related species. Given the high cost of developing microsatellite markers, this cross-species transferability is a valuable attribute for genome study in *Pinus*. For example, *Pinus taeda* SSR markers developed by Elsik and Williams (2001) and Zhou et al. (2002) transferred well between American hard pines (Shepherd et al., 2002) but was less transferable in the phylogenetically divergent Mediterranean hard pines (Chagné et al., 2004; González-Martínez et al., 2004). Chagné (2004) also showed that the amplification rate for microsatellite markers mainly developed for *P. taeda* were high in six other pine species and corresponded with the phylogenetic distance between species. Nelson et al. (2007) also showed that some *P.taeda* microsatellite markers were transferable in short leaf, slash, and longleaf pines.

Genetic mapping of quantitative trait loci has become a routine tool for the genetic study of plants, animals, and humans. Many fundamental genetic questions, including the inheritance mode of quantitative traits, genotype x environment interaction, and the genetic basis of heterosis, can be addressed by such a tool (Tanksley 1993; Templeton 1999; Wu et al., 2000). The reliable information on the distribution of genetic variation is a prerequisite for sound selection, breeding, and conservation programs for forest trees. Genetic variation of a species is assessed either by measuring morphological and metric characters in the field or by studying molecular markers in the laboratory.

In outcrossing species, the establishment of inbred lines is not practical because of high genetic load and inbreeding depression. Therefore, mapping QTL in these species cannot be performed by the use of simple segregating populations such as F2, recombinant inbred lines (RIL) and doubled haploid lines. Two types of segregating populations have been aproposed:

The F2-like and BC-like strategies (Muranty, 1996). Until recently, most of the QTL mapping efforts were focused on single family pedigrees. Given the high rate of polymorphism encountered in pines and the relative lack of stability of QTLs in different genetic backgrounds, methods aimed at validating markers linked to the traits of interest in more complex pedigrees, or even in unrelated genotypes, are emerging (Plomion et al., 2007). Furthermore, if the progeny do not segregate at a QTL (i.e., the parents are homozygous at the QTL), then the QTL cannot be detected. Muranty (1996) showed that a mating design with six parents will allow a reasonable power for QTL detection if QTL heterzogosity frequency in the base population is at least 0.2.

Therefore, the objectives in this study are to:

1) Screen and test whether those SSR markers developed for loblolly pine can be transferred to the genome study of longleaf pine;

2) Determine the optimal reaction conditions needed for producing reproducible amplification of longleaf pine template DNAs; and

3) Identify the populations and sample strategies that can be used for QTL mapping controlling EHG in longleaf pine according to the molecular marker information and phenotypic information.

2.2 Materials and Methods

2.2.1 Plant Materials

Several [longleaf pine x (longleaf pine x slash pine)] backcross families were generated to produce a population segregating for EHG. Initially, 17 longleaf pines were crossed with five slash pines in a nested cross design to produce F1 hybrid families (Derr 1966). In 1991, a single tree was selected from each of 17 F1 hybrid families (Table 2.1) and grafted into the clone bank at the Harrison Experimental Forest (HEF) in Saucier, MS (C. D. Nelson and L. H. Lott,

personal communication). More recently, seven of the F1 hybrid trees were used as paternal parents to construct the backcross population using six longleaf pines as maternal recurrent parents. The longleaf pine parents were selected from the U. S. Forest Service Region 8 breeding program from the Erambert Seed Orchard in south Mississippi. Two crosses were made in 2001 and 2002, respectively, and the crosses and family codes are shown in Table 2.2 and 2.3.

Table 2.1 The F1 hybrid pedigree

F1 Hybrid ID	Longleaf (Female)	Slash (Male)
Derr474	3Y	AC1
Derr 475*	11Y	AC1
Derr 476*	4Y	AC2
Derr 477*	13Y	AC2
Derr 478*	21R	AC2
Derr 479	1Y	AC2
Derr 480	15Y	AC3
Derr 481*	17Y	AC3
Derr 482	2R	AC3
Derr 483	19R	AC3
Derr 484	3R	AC40
Derr 485	12R	AC40
Derr 486*	9R	AC40
Derr 487	20Y	AC51
Derr 488*	8R	AC51
Derr 489	18R	AC51
Derr 490	8Y	AC51

Note: * The 7 F1 hybrids selected for backcross with longleaf pine as paternal parents

2.2.2 DNA Extraction, Purification and Quantification

Fresh needle leaves from each tree were collected, labeled, and stored in a -80 °C freezer. Two grams of leaf sample of each individual tree was ground into fine power in liquid

Table 2.2 The pedigree and backcross code for crosses made in 2001

CrossCode	Female (longleaf)	Male (hybrid)	Test Group
1	Em04	Derr488	488_1
2	Em14	Derr488	488_1
3	Em17	Derr488	488_1
4	Em24	Derr488	488_1
5	Em41	Derr488	488_1
6	Em45	Derr488	488_1
7	Em04xwind		
8	Em04	Derr475	FT_1
9	Em04	Derr476	FT_1
10	Em04	Derr477	FT_1
11	Em04	Derr478	FT_1
12	Em04	Derr481	FT_1
13	Em04	Derr486	FT_1
14	Slash Control		
15	Longleaf Control		

Table 2.3 The pedigree and backcross code for crosses made in 2002

	Female Parents					
Male Parents	**Em04**	**Em14**	**Em17**	**Em24**	**Em41**	**Em45**
Derr475	fam1	fam9	fam18	fam26	fam39	fam34
Derr476	fam2	fam10	fam19	fam27	fam40	n/a
Derr477	fam3	fam11	fam20	fam28	fam41	fam35
Derr478	fam4	fam12	n/a*	fam29	fam42	fam36
Derr481	fam5	fam13	fam22	fam30	fam43	fam37
Derr486	fam6	fam14	fam23	fam31	fam44	fam38
Derr488	fam7	fam15	fam24	fam32	n/a	n/a

* There are no offspring available for these families.

nitrogen, and the total genome DNA isolation procedure followed the CTAB method developed by Murray and Thompson (1980) with modifications to fit the medium scale isolation for pines (see appendix A). The DNA samples were further purified using two different purification kits: PREP-A-GENE and Aquapure (Bio-Rad, Richmond, CA 94804, USA) for the degraded DNA and protein contamination.

An agarose gel method was used to provide information regarding both DNA quantity and quality. The concentration of genomic DNA was estimated by comparing the size and intensity of each sample with those of λ-DNA standards on a 0.8% agarose gel.

2.2.3 SSR Marker Sources and Preparation

A total of 228 locus-specific simple sequence repeat (SSR) markers were screened against the six longleaf pine recurrent parents, seven F1 parents and 2 of the progenies. The SSR markers included 80 PtTX loci (developed by C. G. Williams, see Auckland et al., 2002), 56 sifg loci (developed by C. Echt and C. D. Nelson in collaboration with D. G. Peterson and S. Saha, Mississippi State University), 8 RPtest loci (developed by C. Echt, see http://dendrome.ucdavis.edu/dendrome_genome/echt_ssr_primers.html), 66 ript loci (developed by C. Echt and C. D. Nelson) and 18 SsrPt loci (Chagné et.al., 2004). Oligonucleotides were synthesized by Invitrogen (www.invitrogen.com). Forward primers were synthesized with a M13 tail (CACGACGTTGTAAAACGA) to take advantage of "tailed-priming" with M13-IRdye™ (Li-Cor Biosciences). Reverse primers were synthesized with a 'PIG tailing' (Prostate Investigation Group of the National Center for Human Genome Research) (GTTTCTT) attached to modulate the non-templated nucleotide addition by Taq DNA polymerase. The lyophilized primer pellets were reconstituted as100 uM stock solution in TE buffer and stored at -20 ºC in labeled tubes.

2.2.4 Preliminary Testing and Optimization of PCR Reaction Conditions

Before proceeding to detailed evaluation of the markers, the primers were verified to amplify the desired region successfully from genomic DNA, and the optimal reaction condition for PCR amplification was also determined. For each primer pair to be tested, I prepared a 10 ul reaction mixture, 2 ul 5x reaction buffer, 20 ng genomic DNA (3.2 ul x 6.25 ng/ul), 100 nM each primer pair, 1 unit of Taq DNA polymerase in storage buffer B (20mM Tris-HCl, pH 8.0 at 25ºC, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol , 0.5% Tween20 and 0.5% Nonidet-P40, Promega) or 0.25 unit GoTaq Flexi DNA polymerase (Promega), 100 nM dNTP solution, and corresponding distilled water. A factorial design was used to test the different $MgCl_2$ concentrations (1.5 uM, 2.0 uM, 2.5 uM , 3.0 uM, 3.5 uM, 4.0 uM and 4.5 uM) and touchdown temperature profiles (50 °C, 55 °C, 60 °C, and 65 °C). The PCR reaction cycle started with a hot start at 95 °C for 4 min, followed by a touchdown series of cycles with different initial annealing temperatures for each marker (i.e., primer pair) and decreasing by 0.5 °C per cycle for 20 cycles, followed by 20 cycles at the final annealing temperature, and ending with a 15 min extension at 72 °C. After the PCR reaction, 4 uL blue stop solution (Bromophenol Blue, 95% Formamide, Li-Cor) were added to each of the reaction mixes. The thermcycles used were 96 wells PTC-100 ™ programmable thermal controllers (M.J. Research Inc.).

Electrophoresis was conducted on an automatic DNA sequencer (Li-cor 4200 series DNA sequencer). Amplified DNA fragments were separated on a 6% denaturing polyacrylamide gel mix (Li-Cor). The gels were cast at least 90 minutes before use and pre-run for 30 min just before loading the samples. Pre-running and running electrophoresis steps were performed using 16-bit data collection, 1500 V, 40 W, 40 mA, 45 °C, and 4 X scan speed as recommended by Li-Cor. The 1X TBE (89 mM Tris, 89 mM borate, 2.2 mM EDTA pH 8.3) was used as the running

buffer. After the wells were completely flushed to remove urea precipitate or pieces of gel, 0.8 μL of each denatured sample (denaturation conducted at 94 °C for 3 min immediately before loading) was added to a well. Four molecular sizing standards (50-350 bp or 50-700 bp) were used in designated lanes. The real-time TIFF images were automatically collected and recorded during electrophoresis. Loading the same gel twice, each run needed about 2 hours to collect both channel images (700 and 800) resulting in a maximum of four images collected in a single day. The gel images were automatically scored by Saga Generation 2 software with GT & MX modules client version 3.1 (Licor, CA). Alleles were scored based on size relative to known DNA size standards.

2.2.5 Primer Screening

To identify useful polymorphisms, all the primers were screened against 7 F1 hybrids and 6 longleaf pine recurrent parents. Since the primers have been originally tested by loblolly pine B145L, the sample was then used as a standard length control. The primers which did not show polymorphisms or had polymorphisms, but were distorted from corresponding Mendelian segregation (Table 2.4) were not used for later QTL analysis. The cases when a reaction completely failed or the polymorphic bands were not clear were recorded as missing data.

2.2.6 Planting Sites and Experimental Design

The backcross progenies were classified into two groups for each year: Family Test (FT) and 488 Test (488) group and planted in two years (2002 or year 1 and 2003 or year 2) at two different locations: LSU Agricultural Center Lee Memorial Forest near Sheridan, Louisiana, and at the Harrison Experimental Forest (HEF) in south Mississippi. The Family Test planted in 2002 (FT-1), was a half-sib family, which longleaf pine parent Em04 was used as the common maternal parent and crossed with seven hybrid male parents (Table 2.2). There are 18

Table 2.4 Possible marker genotype combinations and segregation pattern

CrossType			Parents			Offspring		
			Cross P1 P2	Observed Band	Informa-tiveness*	Observed Band	Segregation	No. Phenotype
A		1	ab×cd	ab×cd	FI	ac ad bc bd	1:1:1:1	4
		2	ab×ac	ab×ac	FI	a ac ba bc	1:1:1:1	4
		3	ab×co	ab×c	FI	ac a bc b	1:1:1:1	4
		4	ao×bo	a×a	FI	ab a b o	1:1:1:1	4
B	B1	5	ab×ao	ab×a	BI	ab 2a b	1:2:1	3
	B2	6	ao×ab	a×ab	BI	ab 2a b	1:2:1	3
	B3	7	ab×ab	ab×ab	BI	a 2ab b	1:2:1	3
C		8	ao×ao	a×a	BI	3a o	3:1	2
D	D1	9	ab×cc	ab×c	MI	ac bc	1:1	2
		10	ab×aa	ab×a	MI	a ab	1:1	2
		11	ab×oo	ab×o	MI	a b	1:1	2
		12	bo×aa	b×a	MI	ab a	1:1	2
		13	ao×oo	a×o	MI	a o	1:1	2
	D2	14	cc×ab	c×ab	PI	ac bc	1:1	2
		15	aa×ab	a×ab	PI	a ab	1:1	2
		16	oo×ab	o×ab	PI	a b	1:1	2
		17	aa×bo	a×b	PI	ab a	1:1	2
		18	oo×ao	o×a	PI	a o	1:1	2
E		19	aa×bb	a×b	NI	ab	1	1
		20	aa×aa	a×a	NI	a	1	1

Table source: Wu (2002) with minor modifications.
Cross: o: null allele
*Informativeness:
FI: Full information for both maternal and paternal parents (multiple alleles per locus);
BI: Informative for both maternal and paternal parents (two alleles per locus);
MI: Informative for the maternal parent; PI: Informative for the paternal parent;
NI: Not informative

replications in a RCB Design in each location and 1 plant per cross (15 plants) in each replication. For the Family Test planted in 2003 (FT-2), it included all available 38 full-sib backcross families (Table 2.3). There are 6 replications and 30 crosses in each replication with 6 trees planted in the same row per cross. The progenies of 488 test came from a half-sib family where hybrid male parent Derr488 served as the common paternal parents and crossed with six female longleaf parents and followed a completely randomized design (CRD) in both locations and years. However, the 488 test planted in 2003 (488-2) was not replicated in Louisiana.

2.2.7 Field Data Collection

The total height and diameter values were used to address the EHG of the longleaf backcross population. The total height was scored as the height from the ground to the top of the stem. The diameter measurement had three values: the first diameter measurement (d3) was scored as the ground level diameter; the second diameter measurement (d4) was scored as the 15 cm level diameter above the ground; the third diameter measurement (d5) was scored as the 30 cm level diameter above the ground. For these seedlings planted in 2002, four height measurements, ht2, ht3, ht4, and ht5, and three diameter measurements, d3, d4, and d5 were taken in 2004, 2005, 2006, and 2007. For the trees planted in 2003, three height measurements, ht2, ht3, and ht4 and two diameter measurements, d3 and d4, were taken in 2005, 2006, and 2007, respectively (see Table 2.5 for detailed variable information).

Table 2.5 The response variables used in data analyses

Year measured	**Trees planted in 2002**		**Trees planted in 2003**	
Jan, 2004	ht2			
Feb, 2005	ht3	d3	ht2	
Jan, 2006	ht4	d4	ht3	d3
Dec, 2006	ht5	d5	ht4	d4

2.2.8 Statistical Analyses in Genetic Variance Estimation

The variance component estimation was followed by the method of Wu and Stettler (1997). Data analyses were based on analyses of variance (ANOVA) with SAS procedure PROC MIXED. The effects of Location, Parent, Parent x Location, Replication within Location and Parent x Replication within Location for each male or female parent were estimated. However, tests of main effect were confounded by the effects of interaction, making it difficult to conclusively determine the significance and variance components of main effects. To solve the problem, the parent x location interaction was first tested. If the interaction was not significant, the variance component for parents can be estimated by the model directly. If the interaction was significant, the residual variance, which included the genetic variance component and the specific-site environment, would be used. The variance components for the effects were calculated by equating the mean squares with the expected mean squares derived from type III sums of squares.

Two different statistical models were used to estimate the variance component. Since there were multiple measurements for both height and diameter, in the first method, each height or diameter measurement was treated as an individual response variable, thus 7 separate ANOVA analyses for all the measurements were conducted in this model. The second model treated all the height or diameter measurements as repeat measurements, and only two analyses were conducted for the height and diameter as response variables, respectively.

The statistical model for the first method:

$$y_{ijkl} = \mu + \tau_i + \alpha_j + +\tau\alpha_{ij} + \gamma(\alpha)_{jk} + \tau\gamma(\alpha)_{ijk} + \varepsilon_{ijkl} \qquad 2.1$$

where y_{ijkl} was the different height or diameter value for the ith parent, jth location, kth replication, and lth tree. μ was overall mean, τ_i was for the male or female effect (i=1, 2…7 for male parent

and i=1, 2,…,6 for female parent), α_j was for location effect (i=1, 2 Louisiana or Mississippi), γ_k was for the replication, and ε was for residual error term. The male and female effects were analyzed separately because they were not strictly 2-way factorial designs due to availability of the seeds.

The second model was a repeated measurement, that is, the height or diameter in different years were treated as one single response variable, and the time of measurement was used as a time serial variable. The statistical model for the method 2 was:

$$y_{ijklm} = \mu + \tau_{1i} + \alpha_j + \tau_1\alpha_{ij} + \gamma(\alpha)_{jk} + \tau_1\gamma(\alpha)_{ijk} + \tau_{2m} + \tau_1\tau_{2im} + \tau_1\tau_2\alpha_{imj} + \tau_1\tau_2\gamma(\alpha)_{imjk} + \varepsilon_{ijklm} \quad 2.2$$

where y_{ijklm} was the height or diameter measurement, μ is overall mean, τ_{1i} was for the male or female effect (i=1, 2…7 for male parent and i=1, 2…6 for female parent), α_j was for location effect (i=1, 2 Louisiana or Mississippi), $\tau_{2m}$was the repeat measurement for each height and diameter value (m=1, 2, 3 for height measurements and m=1, 2 for diameter measurements), and ε was the residual error term.

2.3 Results and Discussion

2.3.1 Finding the Optimal Condition for PCR Amplification of SSRs

The 'PIG tailing' (5'-GTTTCTT-3') was very efficient for minimizing the effects of enzyme-directed, template-independent additions of an "A" nucleotide to PCR amplification by DNA polymerase. It has shown that the stuttered bands were reduced and the band patterns were clean and uniform (Figure 2.1), which can reduce genotype error significantly, especially for automatic genotyping by software which only read the band signal. Tailing the primers has also allowed altering magnesium concentrations to reduce the noise and increase band signal.

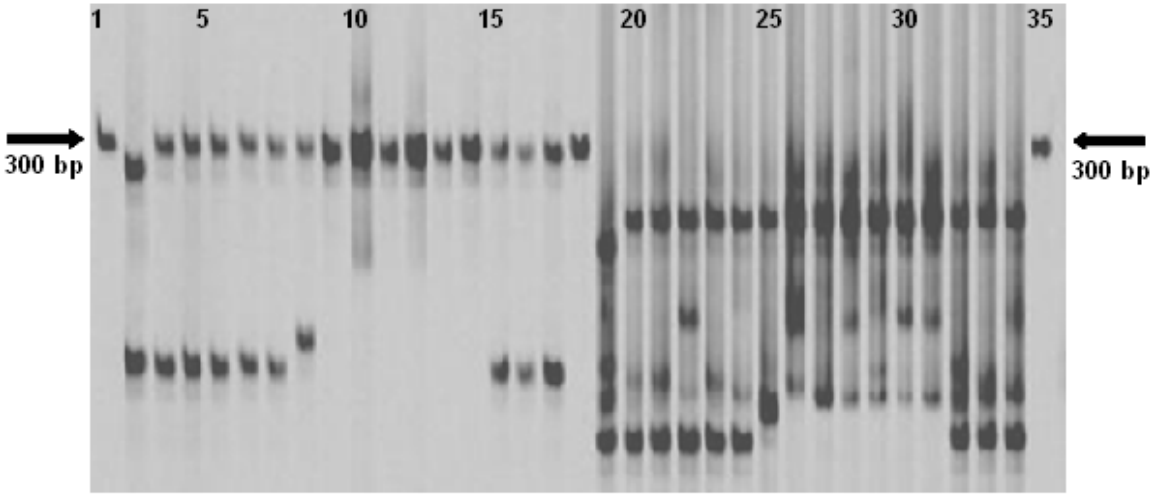

Figure 2.1 The effect of PIG tailing to the primer RPtest09 on PCR amplification. The DNA samples from lane 1 to lane 17 are: standard size marker, loblolly pine control, 7 hybrid paternal parents, 6 longleaf maternal parents, 2 randomly selected progenies and the primer used is pig tailing primer. The DNA samples from lane 18 to lane 34 were exactly the same sequence as lane 1 to lane 17, and the primer used is not pig tailing primer. The lane 35 was standard size marker.

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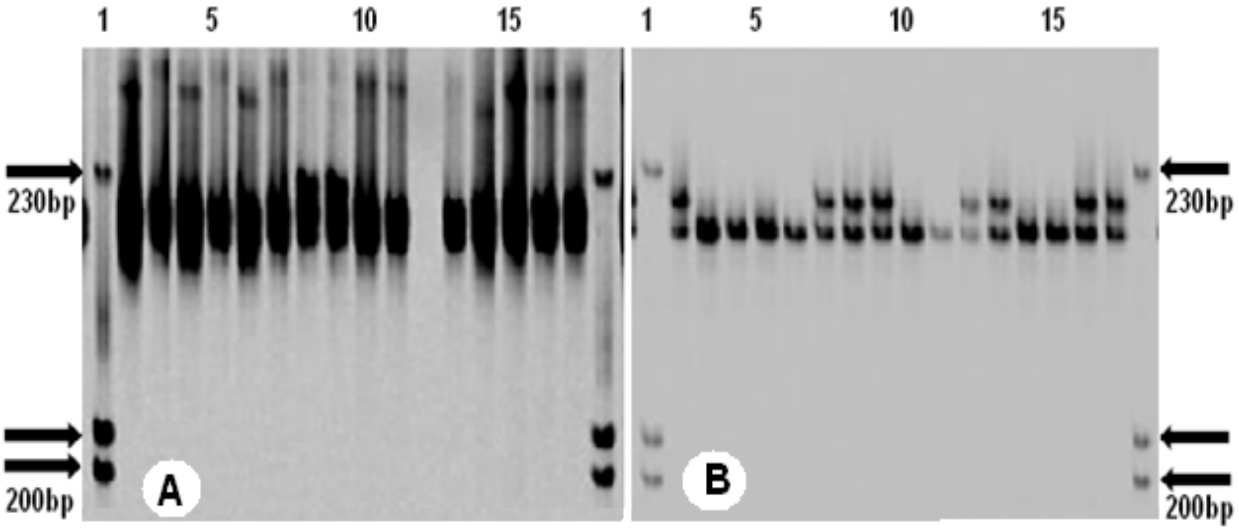

Figure 2.2 The effects of Taq polymerases on PCR amplification (Primer sifg1055).
The Taq polymerase used in picture A was Taq DNA polymerase in storage Buffer B (Promega); The Taq polymerase used in picture B was GoTaq Flexi DNA polymerase (Promega). The DNA samples in both pictures (from left to right) are: standard size marker, loblolly pine control, 7 hybrid paternal parents, 6 longleaf maternal parents, 2 randomly selected progenies and standard size marker.

Taq DNA polymerase used less amounts of DNA polymerase, less magnesium, and produced more uniform and clean amplification (see Figure 2.2). With the new DNA polymerase, 18 primers that failed amplifying or amplified but could not be genotyped have shown perfect polymorphisms. This outcome is probably due to the high glycerol concentration in the stock solution associated with a high concentration of polymerase, resulting in an unbalanced amplification of various loci and a slight increase in the background.

For optimizing the PCR condition, the first step was to determine the concentration of the DNA template. Based on the preliminary test, a DNA concentration of 20 ng was chosen for future amplification. Keeping all other parameters fixed the effects of $MgCl_2$ concentration and initial annealing temperatures were also investigated. These two factors can affect the amplification in different ways for different sources of the primers (Figure 2.3 and Figure 2.4). Since Mg^{2+} ions form complexes with dNTPs, primers, and DNA templates, the concentration of $MgCl_2$ was assumed to be the component with the most dramatic effects on specificity. Thus, the optimal concentration of $MgCl_2$ has to be selected for each experiment. Too few Mg^{2+} ions result in a low yield of PCR product, and too many ions increase the yield of non-specific products and promote misincorporation. In our experiments, with the new GoTaq Flexi DNA polymerase, $MgCl_2$ concentration ranges of 2.5±0.5 mM are suitable in most cases.

The optimal initial annealing temperature depends upon the melting temperature of the primer-template hybrid. If the temperature is too high, the primers will not anneal efficiently, and if the annealing temperature is too low, the primers may anneal nonspecifically. Usually, the optimal annealing temperature is 5°C lower than the melting temperature of the primer-template DNA duplex. Incubation for 0.5-2 minutes is usually sufficient. However, if nonspecific PCR products are obtained in addition to the expected product, the annealing temperature should be

optimized by increasing it stepwise by 1-2°C. In our experiment, with the new GoTaq Flexi DNA polymerase and optimized $MgCl_2$ concentration, an initial annealing temperature of 60 °C was suitable for the majority of the primer pairs. The optimized PCR reaction conditions for all 228 polymorphic markers were obtained. These optimized PCR reaction condition profiles would facilitate the later marker screen and genotyping.

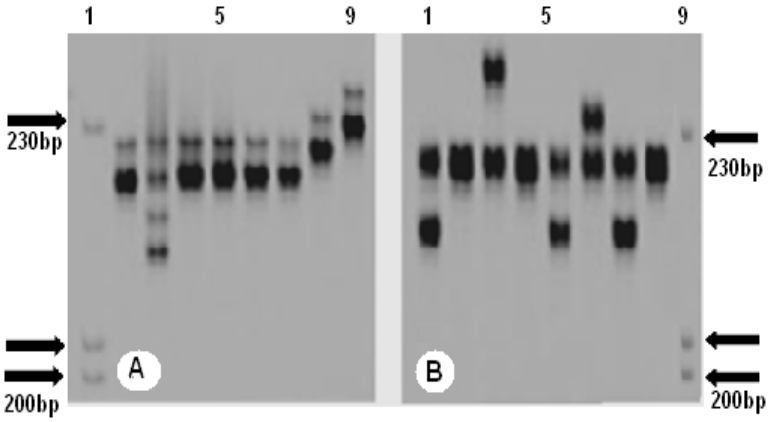

Figure 2.3 The effect of Mg^{2+} on PCR amplification (Primer: PtTX_3117). Picture A was for $Mg2^{+}$ = 1.5 mM and picture B was 2.5 mM. The initial annealing temperature was 60 ºC. The DNA samples for picture A were (from left to right): standard size marker, loblolly control, 7 paternal parents. The DNA samples for picture B were (from left to right): 6 longleaf maternal parents, 2 randomly selected progenies and standard size marker.

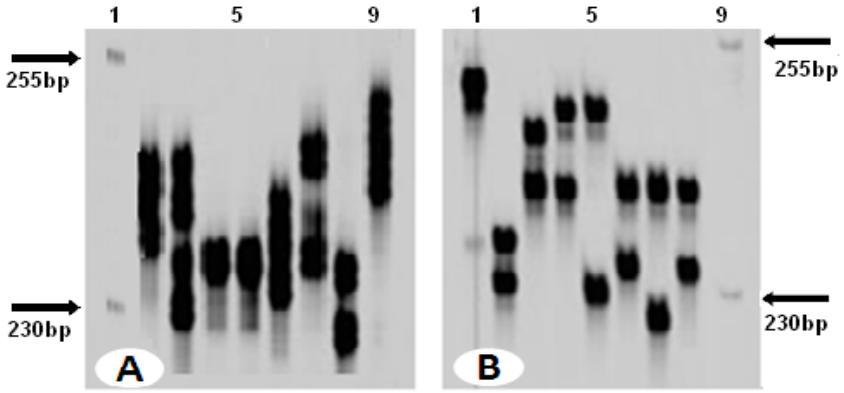

Figure 2.4 The effect of initial annealing temperature on PCR amplification (PtTX_3116). Picture A was for temperature at 50 ºC and picture B was for 60 ºC. The DNA samples for picture A were (from left to right): standard size marker, loblolly control, 7 paternal parents. The DNA samples for picture B were (from left to right): 6 longleaf maternal parents, 2 randomly selected progenies and standard size marker.

Pine microsatellite markers tend to be sensitive to amplification conditions. This is probably due to the size of the genome and to its composition. Even low-copy-number clones rarely occur as a single copy throughout the genome. Vendramin et al. (2005) showed that Southern hybridizations of probes deriving from the hypomethylated fraction of the genome typically hybridize to several bands from independent loci, showing that even DNA fragments that typically occur in low-to single-copies are represented several times in the genome. This may explain why primers designed for SSR amplification tend to amplify more bands than expected. Therefore, care must be taken in the fine-tuning of PCR conditions, downscaling of reaction volumes, and of the concentration of template DNA and Taq polymerase that seem to affect the quality of banding patterns (Scotti et al., 2002). Quality of the DNA seems to have minor effects on the quality of PCR products, since a variety of DNA extraction methods are reported in papers describing microsatellite marker in conifers. Different types of polymerase are reported as well throughout the literature, although it is well known that different polymerase display different degrees of specificity. Therefore, it is advisable to keep the same enzyme, once the protocol for a set of markers has been established.

2.3.2 Molecular Marker Screen for Parents

In order to determine the useful microsatellite in the longleaf pine genome study, all 13 parents' DNA samples were screened against 228 SSR markers. Among the 13 parents, 135 primer pairs out of 228 (59.2%) showed polymorphisms, including 46 of 80 PtTX_ loci (57.5%), 21 of 56 sifg loci (37.5%), 5 of 8 RPtest loci (62.5%) 49 of 66 ript loci (74.2%), and 14 of 18 SsrPt loci (77.8%).

For outcrossing species, such as *Pinus* spp., the alleles at the QTLs and nearby marker loci are usually not fully heterozygous. To avoid the loss of statistical power by using

homozygous individual parents for the mapping population, all parents and families were evaluated for their heterozygosity and number of informative polymorphic markers. The polymorphic marker information for each parent is listed in Table 2.6.

Table 2.6 Summary of polymorphic markers that generate different loci information among parents

Parent	Species	Homozygous loci	Heterozygous loci	Non amplification
Derr475	F1	55	76 (56%)	4
Derr476	F1	57	72 (53%)	6
Derr477	F1	68	60 (44%)	7
Derr478	F1	57	69 (51%)	9
Derr481	F1	58	70 (52%)	7
Derr486	F1	60	68 (47%)	7
Derr488	F1	54	80 (59%)	1
Em04	Longleaf	66	65 (48%)	4
Em14	Longleaf	70	56 (41%)	9
Em17	Longleaf	61	70 (46%)	4
Em24	Longleaf	58	68 (50%)	9
Em41	Longleaf	66	66 (49%)	3
Em45	Longleaf	66	65 (48%)	4

Table 2.7 Number of informative polymorphic SSR markers within each family

Cross	Family size	TI	FI	BI	PI	MI	NI
Em04xDerr488	274	104	27	7	43	27	31
Em14xDerr488	249	99	21	10	48	20	36
Em17xDerr488	150	102	34	10	34	24	33
Em24xDerr488	364	103	28	17	35	23	32
Em41xDerr488	110	104	29	13	38	24	31
Em45xDerr488	61	109	23	13	44	29	26

FI: # full informative markers; BI: # both maternal and paternal informative markers;
PI: # paternal informative markers; MI: # maternal informative markers;
NI: # non informative markers; TI: total number of informative markers.

For the male parents, Derr488 showed the highest ratio of heterozygous loci, least frequency of non-amplification and largest population size which indicated the parent was a potential candidate for parent selection on mapping purpose. Therefore, the number of informative markers within each full-sib family of Derr488 half-sib family was evaluated and the results were listed in Table 2.7.

2.3.3 Genetic Variance Estimation

It is expected that the more the parental lines differ, the more genetic factors will be described for the trait in the segregating population and the easier the identification. However, the height growth data for both grandparents and parents were not available, and the information has to be inferred by the phenotypic and genetic variation in backcross population. The phenotypic variation is determined by the genetic variation, environmental variation and their interaction. The broad sense heritability is characterized by the genetic variation portion in the whole phenotypic variation. I want to maximize the genetic variation part, thus the parents that have large phenotypic variation but small heritability are not favorable.

The main effects of parent, location, and the interaction between parent x location effects were tested first, and the test probability for each response variable was plotted in Figure 2.5. If a test effect was not significant, then it could be omitted from the full model. All the male and female effects were highly significant. The location effect was not significant for both male and female effects, except at the Ht2 variable. For the interaction effect, the female x location effect was not significant at ht2 and ht3, while the male x location effect was not significant at D3. However, for method 2, all the effects were significant.

According to the above results, the new reduced model for each height or diameter variables was created, and the observation number (N), mean (M), and genetic variance

component estimate (G) results for height are listed in Table 2.8. Since within male/female variances were included in the error term, the families with larger error term had more power in QTL identification. Therefore, the ratio of error variance to the sum of all variance components in the model was used as the criterion to estimate the genetic components for each parent. Compared with other parents, Derr488 family had the largest population size, and genetic variance estimation. For the longleaf pine parents, Em04 had the largest population size and genetic variance estimation.

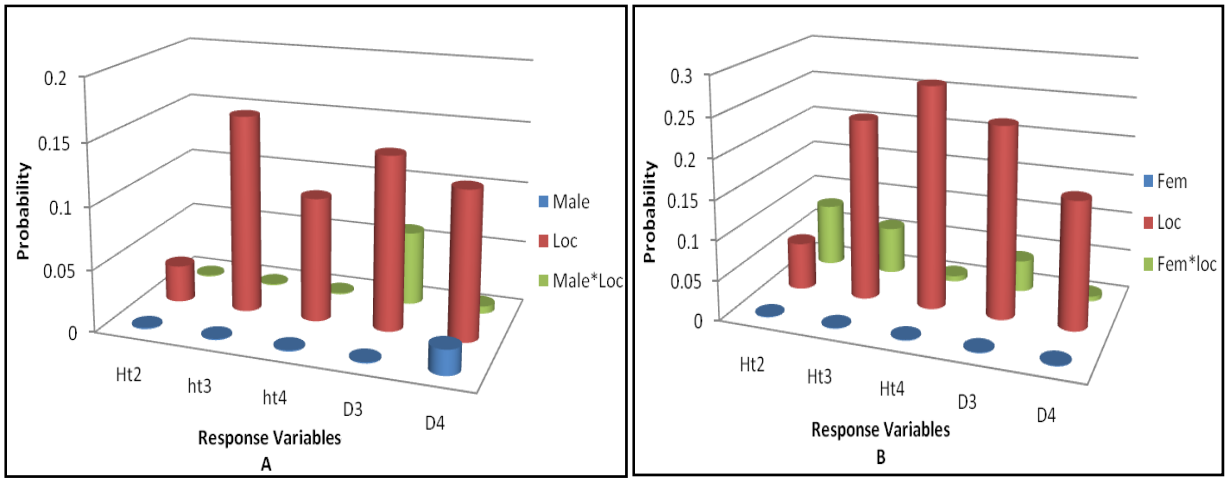

Figure 2.5 The probability plot for parent, location, and parent x location effects. A is for the male effect and B is for female effect. The X axis is for the different traits, and the Y axis is the probability of F test with the method described in formula 2.1.

2.3.4 Population Selection

The families with the highest number of informative markers, largest sample size, and the highest genetic variance for the trait of interest will be most valuable for MAS due to their higher power to detect QTLs. Based on this premise and the data presented in Tables 2.6, 2.7 and 2.8, the largest half-sib family provided by the hybrid paternal parent, Derr488, was selected as the final mapping population to do linkage map analysis and QTL identification. Furthermore, if the progeny do not segregate at a QTL (i.e., the parents are homozygous at the QTL), then the QTL

cannot be detected. Muranty (1996) showed that a mating design with six parents will allow a reasonable power for QTL detection if QTL heterzogosity frequency in the base population is at least 0.2. This result adds support to the decision to select the half-sib family with common paternal parent Derr488 and six different longleaf pine maternal parents to identify QTLs controlling EHG in the study.

Table 2.8 Genetic variance estimation results for Height variables from two different methods

	Method I									**Method II**
	ht2			**ht3**			**ht4**			**Repeat**
Parents	**N**	**M**	**G (%)**	**N**	**M**	**G (%)**	**N**	**M**	**G (%)**	**G (%)**
Derr475	281	34	77	273	104	74	257	187	59	63
Derr476	168	28	73	165	92	70	156	170	65	55
Derr477	157	32	75	148	101	64	141	174	46	45
Derr478	265	30	70	250	49	58	240	177	54	52
Derr481	428	23	57	417	88	74	408	169	70	63
Derr486	256	27	79	254	90	75	251	160	64	34
Derr488	**1257**	27	**71**	**1243**	90	**76**	**1208**	184	**81**	**65**
Ctl(LL)	31	14	20	31	42	17	31	130	79	32
Ctl(Sl)	11	95	89	11	159	92	11	272	84	67
Em04	936	25	87	924	90	83	897	172	63	65
Em14	669	22	59	643	79	65	626	156	56	53
Em17	241	28	36	237	98	56	231	194	49	57
Em24	777	32	47	764	103	72	738	193	52	68
Em41	241	27	68	239	83	62	227	170	38	47
Em45	217	27	70	214	87	70	210	169	37	43

Method I: Ht: height. Each height variable was treated as a separate dependent variable in the model. N: Total number of the observation for the family. M: the mean estimation for the family. G: Genetic component: the ratio of residual to the sum of all variance components in the model, used to estimate within parent variance; Method II: The height variable was treated as repeat measurements. Ctl (LL): Longleaf pine control. Ctl (SL): Slash pine control.

2.3.5 Selective Genotyping

A half-sib family with Derr488 as the common paternal parents was selected as the final mapping population for this study. There are more than 1200 samples in this half-sib family, and it was impossible to genotype all these progenies. Random selection has always been a good way to do the unbiased estimation, however, in this study, a procedure termed 'selective genotyping' (Darvasi and Soller, 1992; Lander and Botstein 1989; Lebowitz et al., 1987) was used. With this method, only individuals from the high and low phenotypic extremes were genotyped. The major limitation of this approach is that if the aim of the experiment is analyzing a set of traits, selecting the extremes of each trait one would select most of the population, and then no reduction in genotyping can be obtained. Selective genotyping is the most appropriate for the cases where only one trait is being analyzed. In this case, the height growth and diameter were highly correlated (results will be showed in Chapter 5), thus the SG was appropriate for the purpose of this study.

A two-step sample selection strategy was used in our research: Within each of the six full-sib families, the tallest and shortest 8 percent of seedlings (220 seedlings total) were selected for QTL detection (phase I). Random selections of 8 percent of the seedlings from the rest of the population (110 seedlings) and 10 seedlings from both tails (130 seedlings total) of the within-family distributions will be used for unbiased QTL verification and mapping (phase II).

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CHAPTER 3 THE DEVELOPMENT OF AN INTEGRATED GENETIC MAP FOR LONGLEAF PINE COMPRISED OF MICROSATELLITE MARKERS

3.1 Introduction

Longleaf pine (*Pinus palustris* Mill) has many desirable characteristics, such as high wood quality and fusiform rust resistance. However, the delay in its early height growth (EHG), known as "grass-stage", has been an important factor that limits the artificial regeneration of the species (Schmidtling and White, 1989). Efforts to genetically improve the EHG through the introgression of genes controlling genes from slash pine by traditional approaches have shown to be effective (Derr, 1966, 1969). However, these efforts are still limited by the time required to evaluate the growth performances. In addition, phenotypic selections remain imprecise due to interaction between environmental effects and genetic effects for quantitative traits. In that context, any tools directed toward selection processes that improves the evaluation of genetic value and reduces the generation time would be of considerable value.

The use of MAS in forest trees is currently a major research effort in tree improvement programs and has shown to be useful in directing changes towards obtaining faster genetic improvement in timber quality, growth rate, stress, and disease tolerance (Grattapaglia and Sedero, 1994; Plomion et al., 1996; Brown et al., 2003; Davey, et al., 2004). The MAS dramatically cuts the time needed to create new genotypes and, ultimately, the new improved varieties. By examining DNA from very young plants, or even cultured tissues, breeders can determine in the lab whether the molecular markers, and therefore the genes that code for desirable traits, have been inherited (CIAT, 2004). Thus, the unfavorable alleles can be eliminated or greatly reduced during the early development stage, making the selection in the field focused on a reduced number of mature plants. The molecular marker information is

usually presented in the framework of genetic linkage maps. The construction of a linkage map is based on the statistical analysis of polymorphic markers in the mapping population, considering that the distance between two loci is related to the probability of observing a recombination event between them. If pure lines are available or can be generated with only a slight change of plant vigor, the mapping populations that can be used consist of F2 populations, recombinant inbred lines (RIL), backcrosses (BC), introgression lines assembled in exotic libraries, and double haploid lines (DH).

However, for highly outbred species, such as *Pinus*, map construction is always complicated by the lack of suitable pedigrees and controlled genetic crosses due to the deleterious effects of high genetic load and the long generation time. Setting up classical F2 or BC progenies derived from inbred lines is nearly impossible. Therefore, different population structures were always found for linkage mapping, such as pedigrees of several generations (Sewell, et al., 1999; Davey, et al., 1999), open pollinated families combined with megagametophyte analysis (Remington et al., 1999; Hayashi et al., 2001), full-sib progenies (Arcade et al., 2000; Hurme and Savolainen, 1999), or half-sib families (Hurme et al., 2000). However, depending on the resulting marker configuration for the analyses, these designs are not always the most informative (Ritter and Salamini, 1996).

In addition, the parents of an outbred pedigree are typically highly heterozygous and can possess different pairs of alleles at each locus (*i.e.*, as many as four alleles can segregate for any given marker). The genetic segregation observed in such mapping populations is the result of meiotic recombination from both parents, and any given marker can segregate in two (1:1), three (1:2:1), or four (1:1:1:1) genotypic classes within a single mapping population. Phase relationships among alleles are usually not known *a priori*, and must be determined either from

the inheritance of alleles within a three- generation pedigree structure or from progeny segregation data.

Consequently, for a map construction with a cross between two highly heterozygous parents, the progeny data need to be subdivided into two independent data sets that separately contain the meiotic segregation data from each parent, and thus two independent linkage maps were constructed for each parent. A sex-average map is then constructed using fully informative and intercross markers to serve as common anchor points between each parental data set (Davey, et. al.1994, Sewell et. al.1999). A number of genetic maps, using above methods and pedigrees, have been constructed consisting primarily of RAPD markers for slash pine, longleaf pine, and their hybrids (Nelson et al., 1993, 1994; Kubisiak et al., 1995, Weng et al., 1998, 2002). However, separated linkage maps are constructed for both male and female parents because of the lack of orthologous markers to combine the maps in these pine species. SSR markers are promising for further integrated analyses in these species (Nelson, C.D. personal communication). Recently, with the development of comparative genetics, researchers have been using multiple populations and lines of the same species. Mapping with multiple populations allows for mapping of a large number of loci, therefore increasing the genomic coverage and/or marker density in specific genomic regions (Butcher and Moran, 2000). This expanded coverage facilitates the marker/trait association analysis for QTL identification and marker assisted selection. Later simulation studies indicated that the use of more than one full-sib pedigree increased the power to detect QTLs, especially where QTLs explained more than 10% of the phenotypic variance (Muranty 1996). Consensus maps have been constructed for a number of plant species, e.g., *Arabidopsis thaliana* (Hague et al., 1993), *Brassica oleracea*, (Kianian and Quiros ,1992), *Cryptomeria japonica* (Tani et al., 2003), *Helianthus annus* (Gentzbittel et al.,

1995), *Hordeum vulgare*(Qi et al., 1996), *Pinus taeda* (Davey, 1999; Sewell, et al., 1999), and *Zea mays* (Beavis and Grant, 1991).

In this chapter, I will use the families and microsatellite markers selected in chapter 2 to construct integrated linkage maps for longleaf pine and longleaf slash pine hybrid. This is the first SSR marker based linkage map for longleaf pine and provides the basis for MAS and comparative studies of genome organization in other members of the genus. The objectives of this chapter are to:

1) Construct the integrated linkage maps based on the SSR markers;

2) Estimate the genome size and degree of coverage.

3.2 Materials and Methods

3.2.1 Plant Materials, DNA Isolation and Gel Electrophoresis

A half-sib family, which included longleaf pine x slash pine hybrid Derr488 as the common paternal parent and six longleaf pines as recurrent maternal parents, was selected for the linkage map construction. Within each full-sib family, the tallest and shortest 8 percent of seedlings and 8 percent of the seedlings from the rest of the population (in total, 330 individuals) were selected as individual samples.

The DNA isolation, purification, and quantification procedures followed the methods described in Chapter 2.2.2. The marker preparation, PCR reaction condition, and gel electrophoresis were followed the methods described in Chapter Pt2.2.3 and 2.2.4.

3.2.2 Allele Frequencies and Parentage Test

In the process of marker genotyping, several individuals were found showing consistently unusual mis-parentage. As a result, a parentage test of the progenies was conducted by the software Cervus, a computer program for assignment of parents to their offspring using genetic

markers where the parentage was in doubt (www.fieldgenetics.com). The software analyzes genetic data from co-dominant genetic markers (i.e. SSRs). Since the parentage testing using likelihood requires allele frequencies, the software can also calculate the frequency of each allele for each locus in the population, along with a range of summary statistics, including the allele frequency, heterozygosity and polymorphism information content (PIC), Hardy-Weinberg equilibrium, and the presence of null alleles.

To calculate an overall likelihood of parentage, Cervus takes the product of the likelihood ratios from individual loci. This step assumes that loci are inherited independently, in other words, that they are unlinked (strictly, in linkage equilibrium). Weak linkage between one or two pairs of loci is unlikely to bias the confidence of parentage assignments. However, if one or more pairs or groups of loci known to be tightly linked are included in the same analysis, confidence will tend to be overestimated, and so parentage assignments should be treated with caution (Cervus help document). In this research, all the markers that showed mis-parentage in the progenies were first chosen, and then the mis-parentage individuals for these markers were coded as the missing value. The linkage relationship of the markers was tested in JoinMap (ver. 3.0), and the closed linkage markers were excluded from the parentage analysis.

Three simulation tests were conducted for parentage: the maternity test, paternity test, and parent test for sex unknown. The parameters used for simulated analyses were by default, except for these following values:

Number of offspring: 10000

Candidate father: 7

Candidate mother: 6

Probability for parents sampled: 100%.

The parameters used for parentage analyses were those criterions by default. The DNA samples which have the same parentage results across the maternity test, paternity test, and parent pair test were re-labeled for their actual parents and re-grouped into the right families.

3.2.3 Linkage Data Analyses

Loci were scored concurrently for progenies of the half-sib family and fragment length similarities used to infer orthologous loci and alleles. Alleles were coded as the actual band size for each full-sib family and transferred to the JoinMap data set for outcross species (CP population, Table 3.1) with a SAS macro developed by C.D. Nelson. For the markers with "null" alleles, a special coded strategy was used:

1) For those that segregate as ao x bc, the segregation genotypes were ab, ac, ob, and oc. The four genotypes could be identified from the fragment length, then <ab x cd> segregation type code was used;

2) For those that segregate as <ao x ab>, the segregation genotypes were aa, ao, ab, and ob. The aa and ao genotypes could not be distinguished from each other, the segregation ratio ab: a: b=1:2:1 was used, and then the <hk x hk> segregation type code was used;

3) For those that segregate as <ao x ao>, the segregation genotypes were aa, ao, oa, oo. The aa, ao, and oa genotypes could not be distinguished from each other, the segregation ratio a:o=3:1 was used, and then the dominant <hk x hk> segregation type code was used. Those SSR markers were assumed as dominant markers and not used as framework markers due to limited informativeness.

3.2.4 Locus Ordering and Map Construction

Linkage analyses of microsatellite loci were performed using the commercial software JoinMap ver.3.0 (Ooijen and Voorrips 2001) for PC. JoinMap is one of the most commonly

Table 3.1 Genotype codes for a CP population

Segregation Type (for software)	Segregation Type (for dataset)*	Possible Genotypes	Segregation ratio
<ab x cd>	ab x cd	ac, ad, bc, bd, -- (no dominance allowed)	1:1:1:1
<ef x eg>	ab x ac ab x bc	ee, eg, ef, fg, -- (no dominance allowed)	1:1:1:1
<hk x hk>	ab x ab	hh, hk, kk, h-, k-, --	1:2:1 or 3:1
<lm x ll>	ab x aa ab x bb ab x cc	ll, lm, --	1:1
<nn x np>	aa x ab aa x bc	nn, np, --	1:1

* 1) Each character, a, b, c… represent a distinct allele. "-" means unknown allele;
2) h-, k- are dominant genotypes, h- means hh or hk, and k- means kk or hk;
3) In the segregation type of the data set for each parent, the alphabet sequence represents the band size. (e.g. for ab x cd, ab is the genotype for parent 1, and the a allele fragment length is smaller than b, cd is the genotype for parent 2, and the c allele fragment length is smaller than d)

used programs for constructing linkage maps for plant populations. More importantly, it appears to be the only software option for building a consensus map from the integrated dataset of multiple populations derived from independent crosses between different pairs of parents. JoinMap uses a LOD score that is derived from the probability in the chi-square test for independent segregation, which is somewhat different from normal LOD scores. The rationale behind using a test of independence rather than normal LOD scores is that the distortion of segregation affects normal LOD scores, but does not affect the test of independence. The use of normal LOD scores can result in spurious linkage of markers with segregation distortion (Stam and Oojen, 1995). Where there is no segregation distortion, these LOD scores are equal to the usual linkage analysis LOD score. Pair-wise data files were then obtained for each linkage group within each family, which is a list of pair-wise recombination estimates and LOD scores. The

other linkage analyses parameters, such as weak linkages, strong linkages, and suspect linkages, were to follow the default parameters of the software.

Segregation of the loci was first tested for deviation from expected Mendelian segregation by chi-square analysis. Those markers that showed highly significant segregation distortion ($P<0.005$) were excluded from further map construction. Linkage groupings were made using a logarithm of odds ratio (LOD) threshold of 4 and maximum recombination frequency $\theta=0.45$. Map distances in cM were calculated using both Haldane's and Kosambi's mapping function. The map distance calculated with Haldane's mapping function was labeled as $cM_{(H)}$ and the map distance calculated with Kosambi's mapping function was labeled as $cM_{(K)}$ as described by Nelson et al. (1994).

Locus ordering and linkage map extension with JoinMap involved three cycles of data shuffling to find the best fitting linear order of markers. The best-fitting order determined by the change or 'jump' in goodness-of-fit of the map after a marker was inserted and controlled by the 'jump' parameter. During the first cycle, only the markers that caused a 'jump' or increase in the chi-square value smaller than the specified threshold (i.e. LOD=4.0) were positioned. Markers that caused a 'jump' greater than 4.0 were not discarded, but were temporarily kept aside because the change in the chi-square value not only depended on the marker itself, but also on the markers already on the map (Stam and Ooijen, 1995). During the second round of map construction, JoinMap attempted to position these markers using the same fitting criterion as in the first round. Thus, the markers ordered during the first and second round of map construction considered as "framework" markers since they were placed with a high degree of stringency. In the third round, all previously removed loci were given a final attempt to be added to the map by ignoring the requirements of maximum allowed reduction in goodness-of-fit and no negative

distances. However, when such markers caused a chi-square jump greater than 6.0, they were considered troublesome and discarded from further analysis.

3.2.5 Map Integration

For map integration, JoinMap used all the segregation data obtained from each full-sib family to estimate recombination frequencies and then used to determine the linear arrangement of genetic markers by minimizing recombination events. It considered the estimates of recombination frequency between a given pair of markers of different origins (data sets / mapping populations), calculated and applied the appropriate weighting, and then generated a single recombination value (Stam 1993). After assigning weights to all available pairwise combinations, JoinMap instituted a numerical search for the best fitting linear arrangement of the marker loci. It calculated a goodness-of-fit criterion corresponding to the two hypothesized levels of interference (positive and negative) allowing for examination of each synthesized map. Initial exploratory mapping analyses included conservative (LOD 4.0 or 5.0) evaluations of initial and merged linkage maps to test the robustness of linkages. A map merging strategy was developed after repeated experimentation with smaller data sets. This strategy involved the sequential joining of paired maps having common anchor markers. A threshold LOD score of 3.0 was used as an acceptance criterion in the sequential merging of each pair of maps to produce an integrated map in this study. After the narrow and wide based merged maps were constructed, attempts were made to merge both maps to produce a single consensus map.

3.2.6 Estimation of Genome Length and Marker Coverage

The estimate of genome length E (G) was done following the method of moment procedure of Hulbert et al. (1988). Let M denote the number of informative pairs of loci and the possible linkage of these loci was tested with the LOD score method. When the LOD score

exceeded a certain threshold Z, the loci were assumed to be linked. Let K denote the number of such pairs and the ratio K/M was then the probability that a pair of loci chosen at random would be declared linked. This probability can also be expressed as a function of G, the genome length, and X, the map distance between two loci for which a LOD score was expected. It was equal to 2X/G. The estimate genome length E (G) was then expressed as:

$$E(G)= 2MX/K$$

where M=m (m-1)/2 and n was the number of framework markers. The value of K was obtained from the linkages tab from JoinMap results panel.

The confidence interval for G, Iα (G) was calculated from the equation

$$I_{\alpha}(G)=E(G)(1\mp n_{\alpha}K^{-\frac{1}{2}})^{-1}$$

where n_{α}=1.96 for an α of 5% (Gerber and Rodolphe 1994; Echt et al., 1997). Only the framework markers were used to avoid an overestimate of genome coverage (Grattapaglia and Sederoff, 1994).

The observed genome map Go was calculated by the formula of Nelson et al. (1994), which takes into account all markers, linked and unlinked:

$$Go=G_F+ X (L-R)$$

where G_F is the total length of framework map, X is the observed maximum distance between two framework markers, L is the total number of linkage groups, pairs and unlinked loci, and R is the haploid number of chromosomes (Nelson et al., 1994; Echt et al., 1997).

The expected genome coverage, E (Cn) % (Bishop et al., 1983), was calculated from pairwise segregation data for marker pairs above a threshold LOD of 3.0.

$$E(C_n)=1-P_{l,n}$$

where

$$P_{1,n}=\frac{2R}{n+1}\left[\left(1-\frac{X}{2G}\right)^{n+1}-\left(1-\frac{X}{G}\right)^{n+1}\right]+\left(1-\frac{RX}{G}\right)\left(1-\frac{X}{G}\right)^{n}$$

where R is the number of chromosome (R=12 for longleaf pine), X is the maximum distance under Z, and G is the expected genome length.

3.3 Results

3.3.1 Allele Frequencies and Parentage Analyses

There are 123 out of 135 polymorphic SSR markers showed polymorphisms in the Derr488 half-sib family. All these polymorphic markers were tested for allele number (k), observed heterozygosity (Hobs), expected heterozygosity (HExp), polymorphic information content (PIC), null allele frequence (FNull), and the deviation of genotypic frequencies from Hardy-Weinberg equilibrium. The mean and standard deviation of these parameters are listed in Table 3.2. The individual marker estimation for all polymorphic markers is listed in appendix B.

Table 3.2 Summary for allele frequencies estimation obtained from CERVUS

Variable	Mean	Std Dev	Minimum	Maximum
k	3.8	1.94	2	9
HObs	0.62	0.17	0.37	1
HExp	0.53	0.15	0.3	0.82
PIC	0.47	0.16	0.25	0.8
FNull	-0.08	0.08	-0.26	0.17

The observed heterozygosity is the number of heterozygotes divided by the total number of individuals typed. The expected heterozygosity is calculated by using an unbiased formula from allele frequencies assuming Hardy-Weinberg equilibrium (equation 8.4, Nei 1987). Expected heterozygosity is a very useful measure of informativeness of a locus. The expected heterozygosity generally differs from the observed heterozygosity because it is a prediction based on the known allele frequency from a sample of individuals. The observed heterozygosity

for the polymorphic markers ranged from 0.37 to 1.00 with the mean value 0.62, and the expected heterozygosity ranged from 0.30 to 0.82 with the mean value of 0.53. For all the markers typed, PtTX_4093, SsrPt_ctg, and ript0079 showed the highest observed heterozygosity (0.99, 1, and 1 respectively), while ript1027, ript0079, and PtTX_3116 showed the highest expected heterozygosity (0.82). Sifg1008, sifg1069, and PtTX_2094 had the smallest observed and expected heterozygosity value (Appendix B).

Polymorphic information content (PIC) is a measure of informativeness related to expected heterozygosity and is likewise calculated from allele frequencies (Botstein et al., 1980; Hearne et al., 1992). It is commonly used in linkage mapping, and preferential selection of markers with high PIC values will increase the marker polymorphism rate in a cross in which allele sizes for the parental strains are not known (Patterson, et al., 1995). The PIC value ranged from 0.25 to 0.82 and with the mean value of 0.47. The three markers, sifg1008, sifg1069, and PtTX_2094, which had the smallest expected heterozygosity, had the smallest PIC. The three highest expected heterozygosity markers also had the highest PIC value (0.25, 0.27, and 0.28). PIC values are usually smaller than corresponding expected heterozygosity and a large number of alleles per locus usually corresponding high value of PIC and expected heterozygosity (Figure 3.1). But this was not always true, e.g., ript0791 has 4 alleles per locus, however, its expected heterozygosity and PIC values was smaller than all those with 3 alleles markers and the majority of those with 2 alleles markers.

A null allele is any allele that cannot be detected by the assay used to genotype individuals at a particular locus. A locus with a large positive estimate of null allele frequency indicates an excess of homozygotes, but does not necessarily imply that a null allele is present. However, the loci with large null allele frequencies were usually suspected for null alleles. Thirty

out of 464 (6.46%) alleles were identified as null allele according to their segregation pattern in genotyping process. All loci containing null alleles were among the highest value for frequency of null alleles. This result confirmed the expectation for the occurrence of a small percentage of null alleles based on population deviations from Hardy-Weinberg equilibrium.

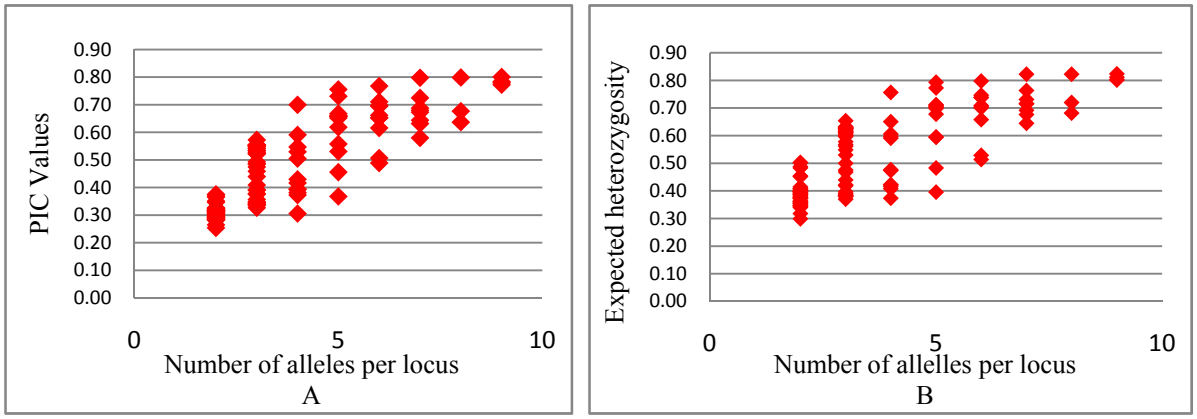


Figure 3.1 The scatter plot of the PIC value (A) and the expected heterozygosity (B) with the change of number of alleles per locus.

With Cervus, a parentage analysis has also been conducted and identified 21 offspring coded with the wrong paternal parent (Derr481 instead of Derr488), and the data was deleted from the original data. Five offspring were coded with the wrong maternal parents, and they were regrouped in the right families. Four offspring did not belong to any of our interested families and were deleted from the population. Therefore, the final sample size used in linkage analysis was 305 instead of 330. The sample size for families Em04, Em14, Em17, Em24, Em41 and Em45 were 39, 53, 55, 77, 44, and 37 respectively instead of 44, 62, 62, 76, 47, and 40.

3.3.2 Segregation of Markers

In total, 123 polymorphic SSR markers were used for map construction for the Derr488 half-sib family. For each full-sib family within the half-sib family, 102, 92, 93, 97, 94, and 91polymorphic markers were identified. A table summarizing the sample size and

informativeness of markers used for final genotyping process and linkage analyses were listed in Table 3.3. These results were different from the one I got in the previous chapter. The sample size for each individual family was changed because of the deleting and regrouping of the samples according to the parentage test. The change of number of polymorphic markers was due to failure of reaction, non-specific amplification, failure of identifying multiple loci, and existence of null alleles. Fifty-eight (47.2%) marker loci were polymorphic across the whole half-sib families. Ten SSR loci (8.0%) showed polymorphism only in one family, which included 2 loci for Em04 family, 4 loci for Em24 family, 1 locus for Em41 family, and 3 loci for Em45 family. Nine (7.3%), 13 (10.6%), 12 (9.8%), and 20 (16.3%) loci were polymorphic across at least 2, 3, 4, and 5 full-sib families.

Table 3.3 Number of informative polymorphic SSR markers within each family

Cross	Sample size	TI	FI	BI	PI	MI
Em04xDerr488	39	102	23	19	36	24
Em14xDerr488	53	92	24	12	40	16
Em17xDerr488	55	93	27	11	32	23
Em24xDerr488	77	97	25	18	29	25
Em41xDerr488	44	94	22	13	33	26
Em45xDerr488	37	91	19	8	38	26

TI: Total number of informative marker; FI: Full information for both maternal and paternal parents (multiple alleles per locus); BI: Informative for both maternal and paternal parents (two alleles per locus); MI: Informative for the maternal parent; PI: Informative for the paternal parent.

The chi-square values, significance, and the distribution of the distorted markers were summarized in Table 3.4. In total, 34 markers showed segregation distortions across the Derr488 half-sib family, and 3 of them were highly significant ($\alpha < 0.005$). There were 6 (5.9%), 6 (6.5%), 6 (6.5%), 16 (16%), 3(3.2%) and 2(2.2%) distorted markers for Em04, Em14, Em17, Em24,

Table 3.4 Chi-square test for distorted markers and their distribution in linkage groups

Marker Name	Family	Chi-square	DF	†Significance	Linkage Group for individual family	Linkage Group for integrated map
PtTX_4092	Em04	7.5	3	*	15	1
PtTX_4221	Em04	3.1	1	*	15	1
ript0064	Em04	3.1	1	*	x	x
ript0165	Em04	10.7	2	****	15	1
ript0852	Em04	3.1	1	*	x	12
ript9058	Em04	19.8	3	****	9	6
PtTX_3055	Em14	3.2	1	*	10	4
ript0064	Em14	3.2	1	*	x	x
ript0852	Em14	3.2	1	*	2	12
ript0968	Em14	7.8	3	*	8	2
sifg1060	Em14	6.4	2	**	x	4
sifg1061	Em14	7.6	2	**	x	x
PtTX_3011	Em17	12.9	3	****	6	15
PtTX_3052	Em17	5.4	2	*	x	x
PtTX_3116	Em17	7.2	3	*	11	3
ript0032	Em17	3.1	1	*	x	13
ript0947	Em17	3.1	1	*	x	x
sifg1061	Em17	11.1	3	**	x	x
PtTX_2080	Em24	6.1	2	**	x	1
PtTX_3029	Em24	8.1	3	**	7	4
PtTX_3030	Em24	5.8	2	*	x	4
PtTX_3034	Em24	6.9	3	*	19	2
PtTX_3045	Em24	6.4	2	**	12	10
PtTX_3081	Em24	6.7	2	**	5	6
PtTX_3118	Em24	3.1	1	*	x	5
PtTX_4137	Em24	10.4	3	**	x	12
PtTX_4205	Em24	8	3	**	x	12
ript0767	Em24	2.9	1	*	x	x
ript0852	Em24	4.7	1	**	x	12
ript0984	Em24	2.9	1	*	x	7
RPtest01	Em24	2.9	1	*	x	12
sifg1060	Em24	8.6	3	**	x	4

† : * significant level at α=0.1 ** significant level at α=0.05 *** significant level at α=0.01 **** significant level at α=0.005 x: the markers are unlinked to any linkage groups.

Table 3.4 Continued

Marker Name	Family	Chi-square	DF	†Significance	Linkage Group for individual family	Linkage Group for integrated map
sifg1064	Em24	9.1	2	**	19	2
SsrPt_ctg4698	Em24	9.5	3	**	x	5
PtTX_2094	Em41	3.3	1	*	x	2
PtTX_4030	Em41	8.2	3	**	x	7
ript0065	Em41	5.8	1	**	11	5
PtTX_3049	Em45	8.3	3	**	x	4
sifg1055	Em45	6.6	3	*	x	4

Em41, and Em45 full-sib families, respectively. Four markers distorted across at least two families, (e.g. ript0064 for family Em04 and Em14; ript0852 for family Em04, Em14, and Em24; sifg1060 for family E14 and family Em24; sifg1061 for family Em14 and Em17). The other distorted markers were specific to each family. These distorted markers were discarded in the first round of linkage analysis for framework markers.

The distorted markers were placed on the linkage map at the second run of the map construction because the distribution of the distorted markers was important for genome structure study. For each full-sib families, 2 of 6 (33%), 3 of 6 (50%), 4 of 6 (67%), 11 of 16 (69%), 2 of 3 (67%), and 2 of 2 (100%) distorted markers could not combined to any linkage groups, even when under LOD threshold 2.0. The other distorted markers tended to scattered in 1 or 2 linkage groups in each full-sib family. Seven distorted markers could not combine to any linkage group for integrated maps, and the other distorted markers were distributed on 11 linkage groups.

Reasons for skewed segregation ratios of molecular markers are still not well understood, but are generally believed to be related to genetic factors such as chromosome loss and structural rearrangements (Williams et al., 1995; Kuang et al., 1999), genetic isolating mechanisms (Zamir and Tadmor, 1986), and other non-biological factors, like sampling in finite mapping population

or scoring errors (Plomion et al., 1995). The null allele (6.46%) also had effects on the segregation distortion. Eleven of 34 (32.4%) distorted markers contained null alleles.

3.3.3 Construction of Linkage Map for the Individual Family

The estimates of observed recombination distance, mean interval, and percentage of framework markers for each individual family were summarized at Table 3.5. At the LOD threshold for linkage of 4.0 and θ=0.40, 68.6%, 70.7%, 66.7%, 74.2%, 55.3%, and 56.0% of polymorphic loci were mapped to 19, 19, 17, 19, 18, and 17 linkage groups for each full-sib family. Note the percentage of linked markers was small for each mapping population. This may caused by the small sample size and number of polymorphic markers used for each family. The family Em24 had the largest family size (e.g.77), and thus the largest percentage of linked markers (74.2%). However, the number of sample size and number of linked markers was not as simple as a linear relationship. The number of polymorphic markers and their linkage relationships also played a very important role. The results also showed that the linkage maps for individual families were not complete because the pines have 12 pairs of chromosomes, while the linkage groups in each individual family were more than that. Family 24 had the largest genetic length and percentage of framework markers. Family Em14 had the largest mean interval (e.g. 17.6 $cM_{(H)}$ and 12.2 $cM_{(K)}$). The results in Table 3.3 and Table 3.5 suggested that increasing the sample size and number of polymorphic markers would be useful for increasing genetic length and decreasing mean interval.

The comparison of different linkage groups for each mapping population is shown in Figure 3.2. For each picture in Figure 3.2, the linkage groups came from different families with common markers were put together for comparison their differences in marker order and distances. Only the linkage groups with common markers across 6 full-sib families were shown

here. For each family, the largest linkage group was 142 cM, 106 cM, 76 cM, 183 cM, 123 cM, and 66 cM for Haldane's mapping function and 98 cM, 69 cM, 57 cM, 124 cM, 89 cM, and 47 cM for Kosambi's mapping function. The largest linkage group came from family Em24, which had 7 markers and covered 183 $cM_{(H)}$ (Figure 3.2 E). The map distances calculated from Kosambi's mapping function were shorter than the distances calculated from Haldane's mapping function.

In Figure 3.2A, three small linkage groups for family Em04 could not combine due to lack of linkage information between maker PtTX_4033 and PtTX_2037 and marker ript0968 and ript1040. However, all these markers linked to one group in family Em14. Marker RPtest05 tightly linked to PtTX_4033 and PtTX_2037 for family Em17 and Family Em41, respectively. RPtest05 was a maternal informative marker and only showed polymorphism for female parents Em17 and Em41. Marker ript0293 was also a maternal informative marker for female parents Em24 and Em45, and it tightly linked to PtTX_4033 in these two families.

For the Figure 3.2 B, family Em04 had the largest linkage group. The order of marker PtTX_4181 and ript1027 was reversed for family Em14 and Em17 compared to the Em04 family. Given the very small distances involved (e.g. 6.2 cM for family Em14, 5.7 cM for family Em17), these differences may reflect statistical inaccuracies in the estimated recombination frequencies (Maliepaard et al., 1997; Butcher et al., 2000), rather than be a result of chromosome rearrangement. Marker ript0984 was a maternal polymorphic marker for female parent Em14 and Em17, but it did not linked to any marker in Family Em17. As a summary, multiple families can offer more information of linkage relationship, especially when some markers did not segregate in a single family.

Table 3.5 Comparison of linkage groups, marker numbers, genetic length and mean interval between markers in the SSR-based linkage map for each full-sib family

Family	No. L.G.	Total No. Markers	No. linked Markers	Genetic $Length_{(H)}$	Mean $Interval_{(H)}$	Genetic $Length_{(K)}$	Mean $Interval_{(k)}$	Percent of framework
Em04	19	102	70	976	13.9	629	9.0	63.8
Em14	19	92	65	1146	17.6	796	12.2	64.2
Em17	17	93	62	658	10.6	528	8.5	62.3
Em24	19	97	72	1190	16.5	743	10.3	67.0
Em41	18	94	52	598	11.5	411	7.9	54.3
Em45	18	91	51	575	11.3	348	6.8	56.0

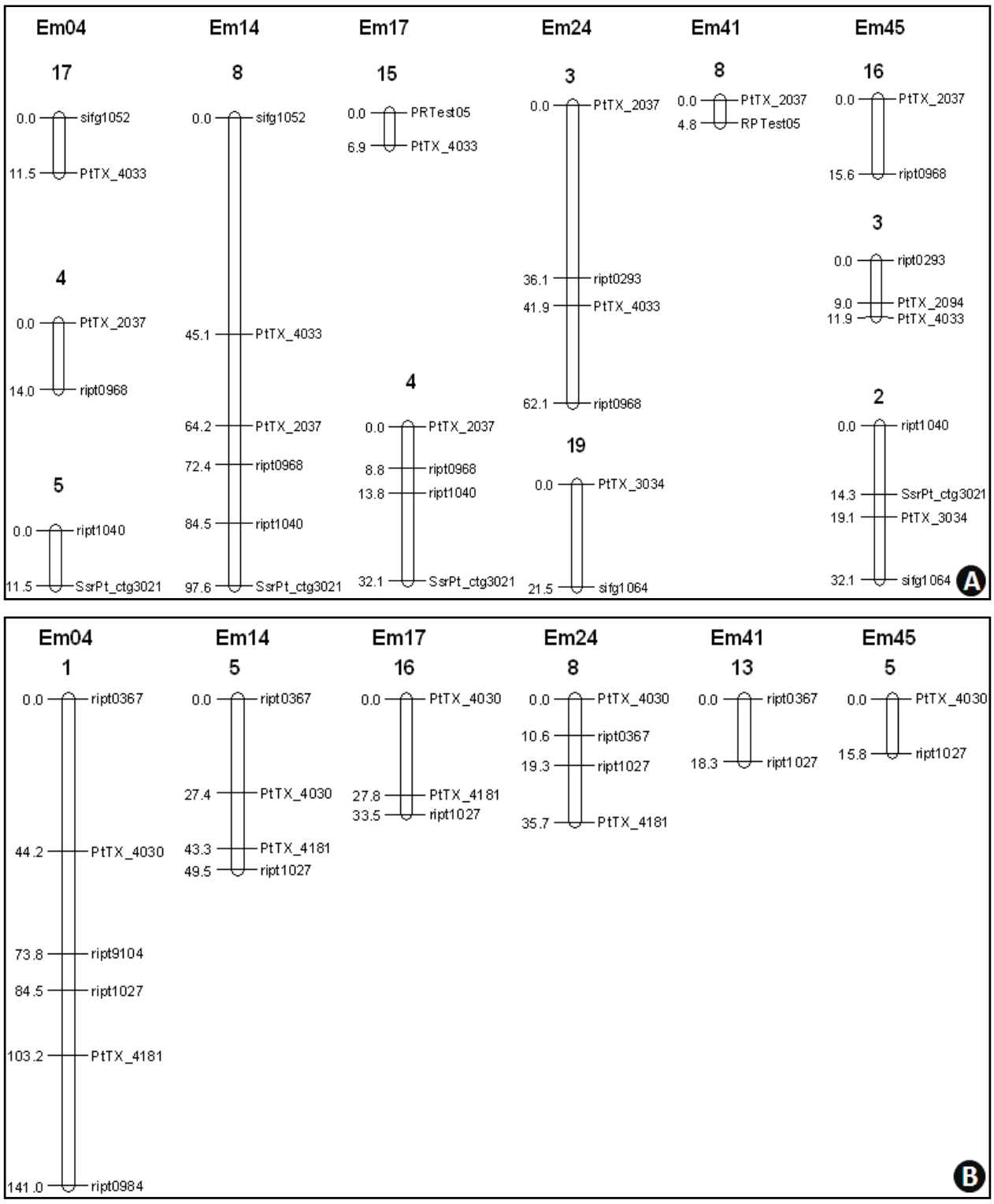
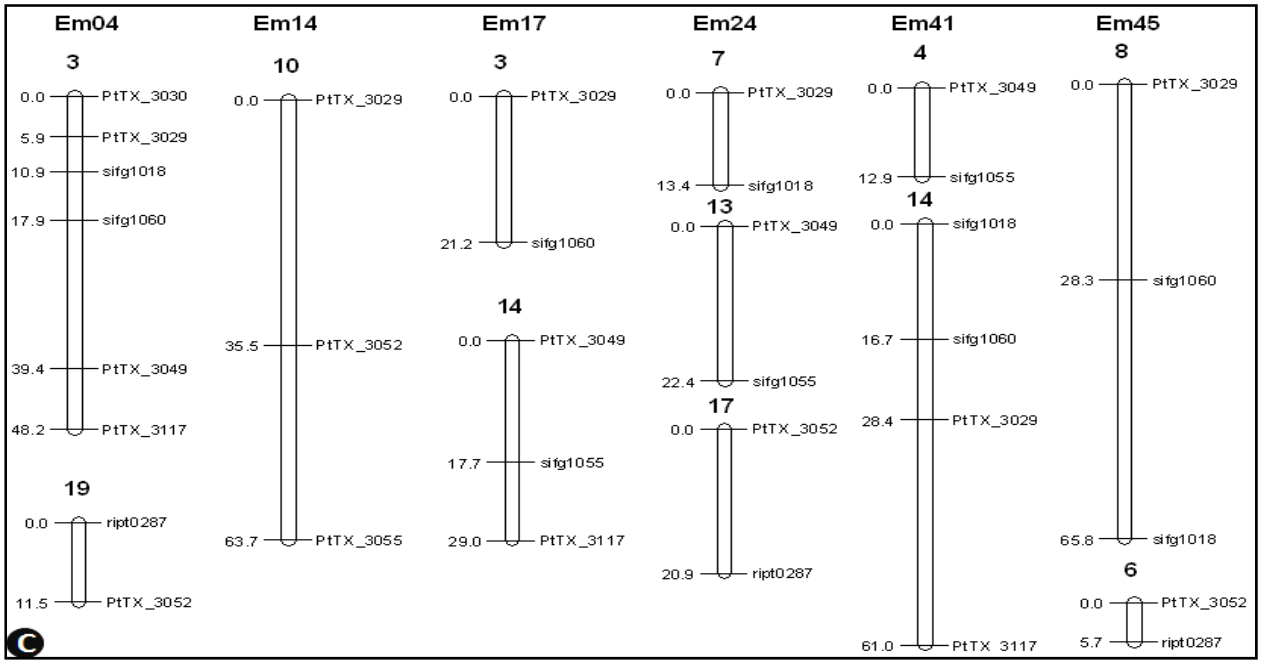


Figure 3.2 Comparison of linkage group for individual full-sib mapping populations. The linkage group in each family that had common marker were grouped together to compare their recombination frequency and order of markers. Picture A, B…G were the seven groups, which have common markers across the six individual full-sib families. Loci listed at the right side with their original name and recombination distance cM $_{(H)}$ on the left.

Figure 3.2 Continued



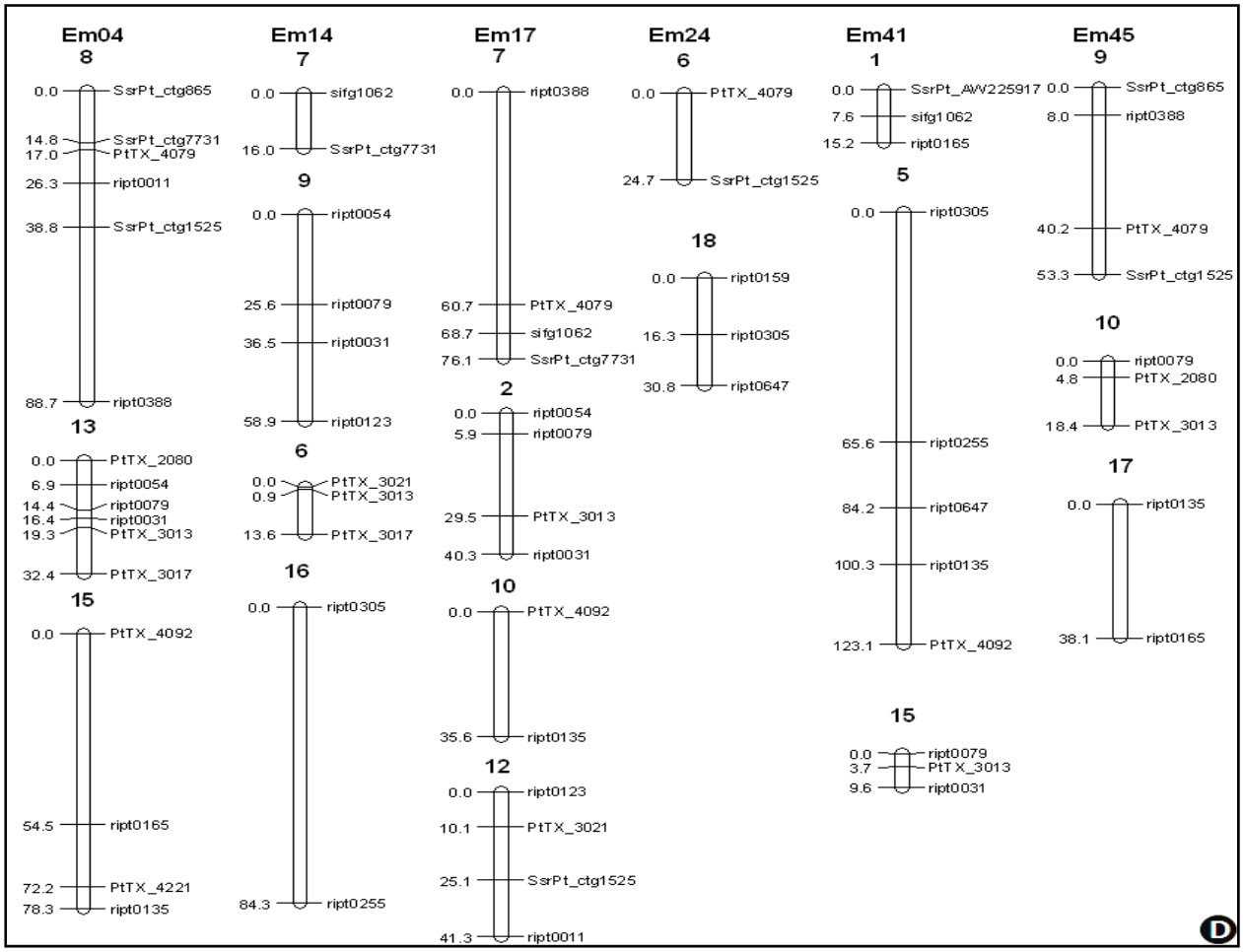
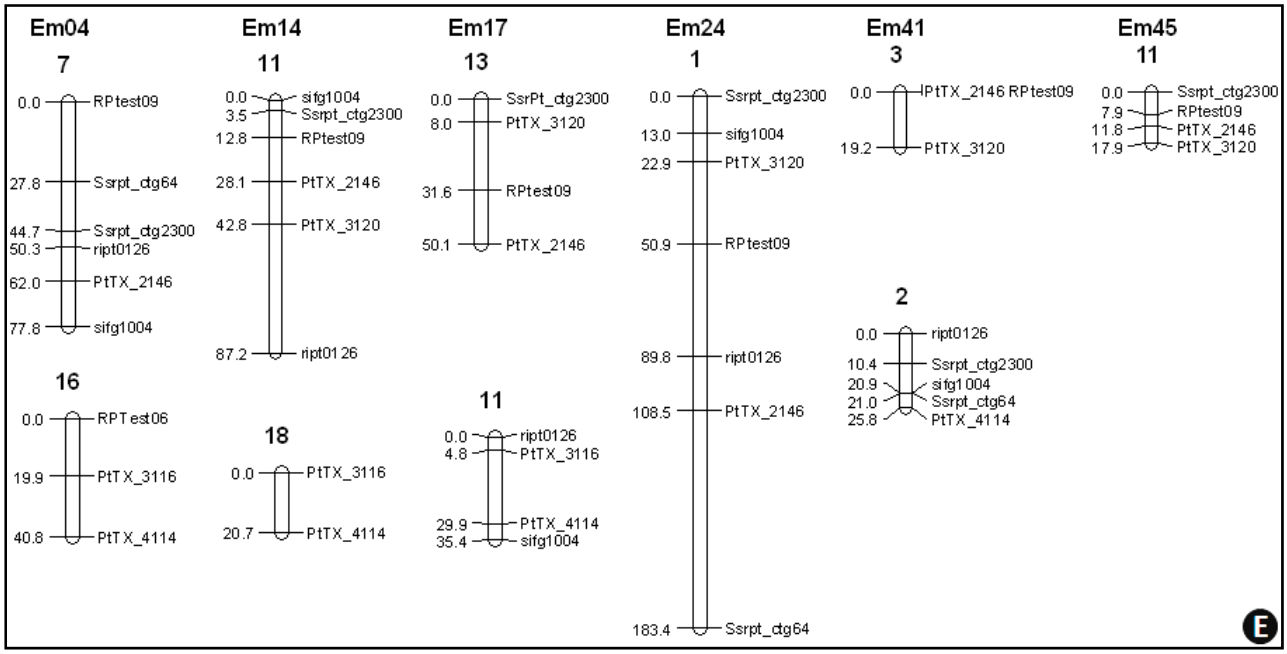


Figure 3.2 Continued



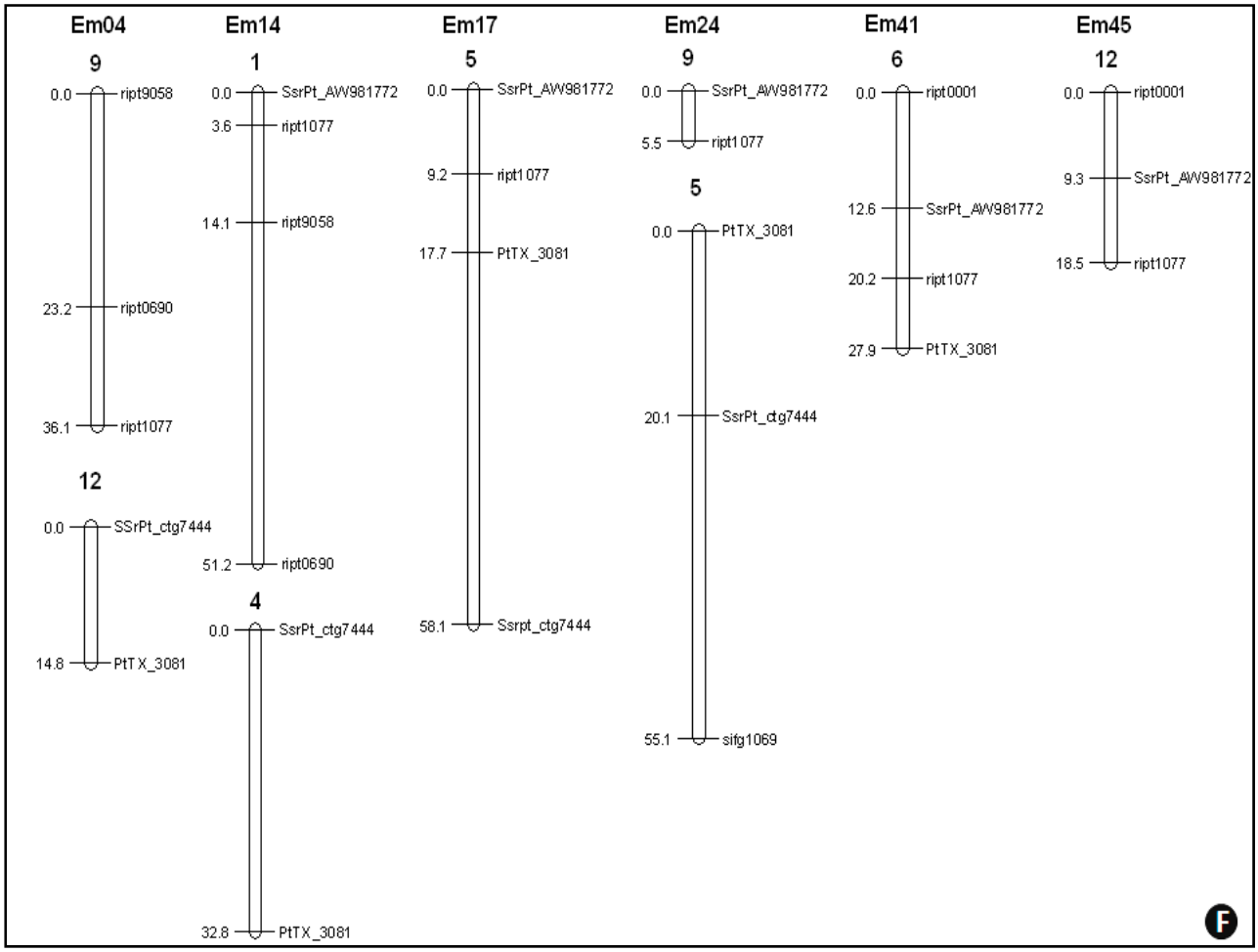
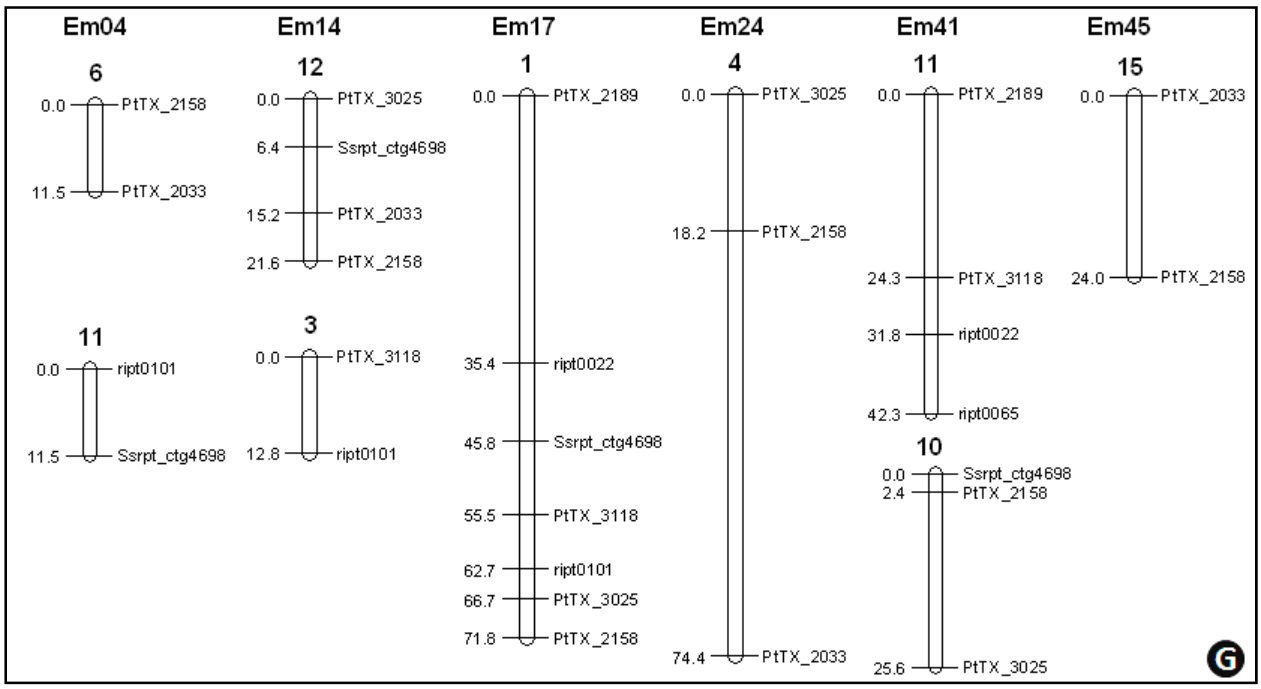


Figure 3.2 Continued



3.3.4 Integrated Maps from Six Full-Sib Families

The family Em24 was treated as the basic map because of its largest sample size and number of linked markers. The other families were integrated one by one according to the order of their sample size and number of linked markers. There were 110 markers mapped to 16 linkage groups, and three of the linkage groups had only 2 markers. The linkage groups 15 and 16 both contain marker PtTX_3011, however, there was not enough linkage information to group them into one linkage group. The linkage relationship between PtTX_3011 and PtTX_3019 was observed in 5 families, while the linkage relationship of PtTX_3011 and SsrPt_ctg3754 was only observed in Family Em45. The marker SsrPt_ctg3754 was a maternal polymorphic marker and only showed polymorphism for Family Em24 and Em45; it did not combined to any linkage group in Family Em24. The 16 linkage groups covered 1290 cM $_{(H)}$ with a mean interval of 11.4 cM $_{(H)}$. The largest linkage group contained 25 markers and covered 199 cM $_{(H)}$. The largest mean interval was 27.5 cM $_{(H)}$ for linkage group 8. The detailed information was summarized in Table

3.6 for Haldane's mapping function and Table 3.7 for Kosambi's mapping function. By comparing the results from individual full-sib families, the combination of the data from multiple crosses provide additional linkage information, enabling small linkage groups to be combined to the big linkage group. The integrated map was listed in Figure 3.3 for Haldane's mapping function and 3.4 for Kosambi's mapping function. Linkage group 1 is the largest linkage group (Figure 3.3, L.G.1); however, it was split into two linkage groups when Kosambi's mapping function was used (Figure 3.4, L.G.1a and 1b).

Table 3.6 Comparison of marker numbers, genetic length, and mean interval between markers in the SSR-based linkage map for integrated map (Haldane's mapping function)

Linkage Group	No. of Markers	Genetic Length	Mean Interval	No. framework	Percent of framework
1	25	199.1	8.0	13	52%
2	11	70.7	6.4	7	64%
3	10	110.2	11.0	8	80%
4	10	97.6	9.8	9	90%
5	9	69.9	7.8	4	44%
6	8	190.1	23.8	7	88%
7	7	112.7	16.1	6	86%
8	5	137.4	27.5	3	60%
9	5	93.9	18.8	5	100%
10	4	69.7	17.4	4	100%
11	4	45.5	11.3	4	100%
12	4	28.8	7.2	4	100%
13	4	4.6	1.2	4	100%
14	2	34.7	17.4	0	0%
15	2	13.4	6.7	0	0%
16	2	11.7	5.9	0	0%
Total	112	1290	196.1	78	-
Average	7	80.6	12.3	-	70%

Table 3.7 Comparison of marker numbers, genetic length, and mean interval between markers in the SSR-based linkage map for integrated map (Kosambi's mapping function)

Linkage Group	No. of Markers	Genetic Length	Mean Interval	No. framework	Percent of framework
1a	18	91.8	5.1	12	67%
1b	7	28.7	4.1	6	86%
2	10	48.0	4.8	9	90%
3	10	72.5	7.3	4	40%
4	10	80.0	8.0	9	90%
5	9	52.8	5.9	6	67%
6	8	94.2	11.8	6	75%
7	7	94.5	13.5	6	86%
8	5	81.8	16.4	5	100%
9	5	82.4	16.5	5	100%
10	4	53.9	13.5	4	100%
11	4	37.3	9.3	4	100%
12	4	21.4	5.4	4	100%
13	4	13.2	3.3	4	100%
14	2	27.5	13.8	0	0%
15	2	12.0	6.0	0	0%
16	2	10.6	5.3	0	0%
Total	111	902.6	149.7	84	-
Average	7	56.4	9.4	-	76%

There were 8 linkage groups that had distances between adjacent markers greater than 25 cM $_{(H)}$, namely ript0647 and ript0159 on group 1 (30.8cM), PtTX_2146 and RPtest09 on Group 3 (44.8 cM), ript0690 and ript9058 (54.5 cM), ript0001 and SsrPt_ctg7444 (58.1 cM), and SsrPt_ctg7444 and sifg1069 (40.6 cM) on linkage group 6, ript0211 and PtTX_4181

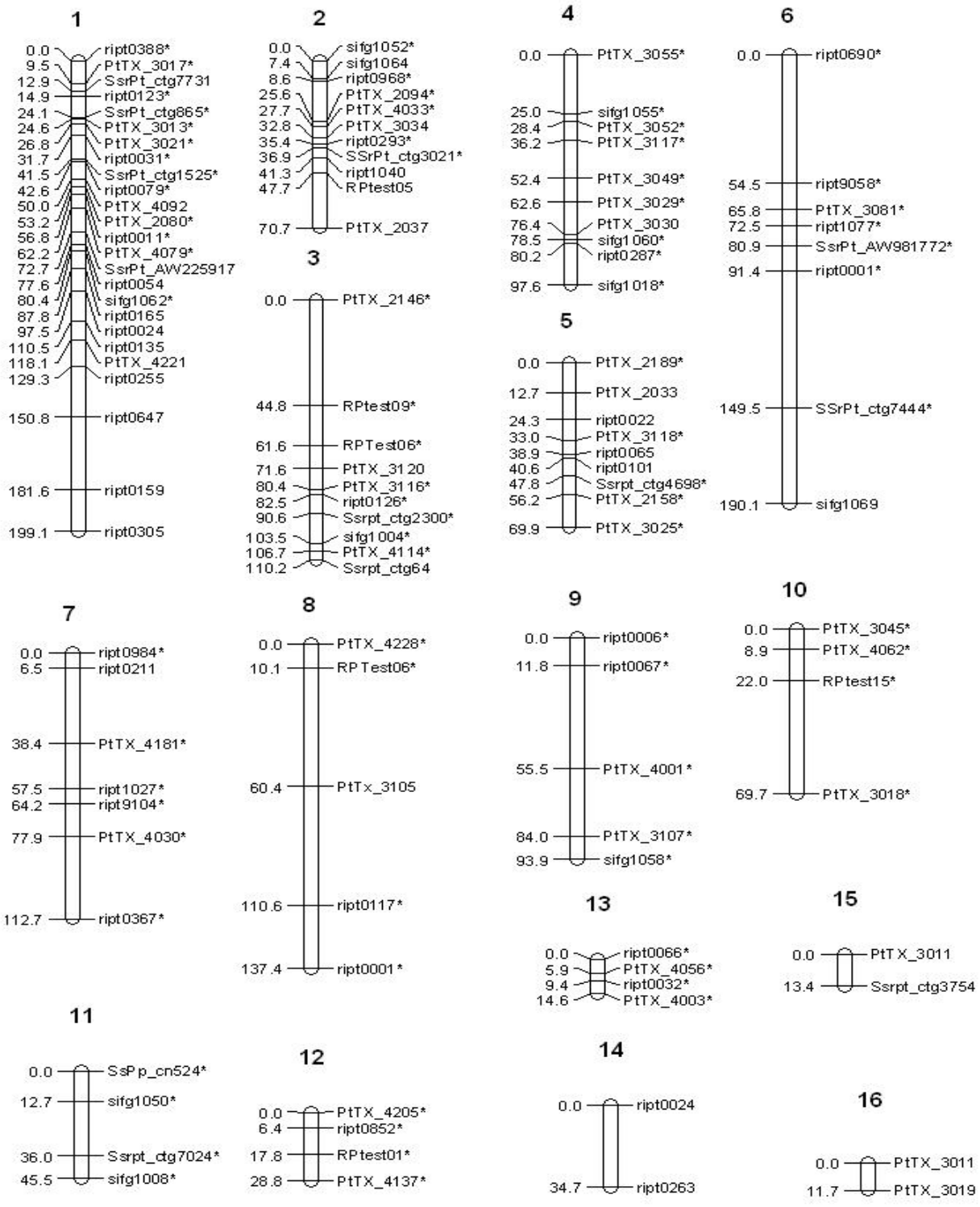


Figure 3.3 An integrated SSR-based genetic map constructed by 112 polymorphic SSR markers with Haldane's mapping function. Three hundred and five samples come from a half-sib family. Loci listed at the right side and cumulative recombination distance ($cM_{(H)}$) on the left. The markers with * at the right side are the framework markers. A was for Haldane's mapping function and B was for the Kosambi's mapping function.

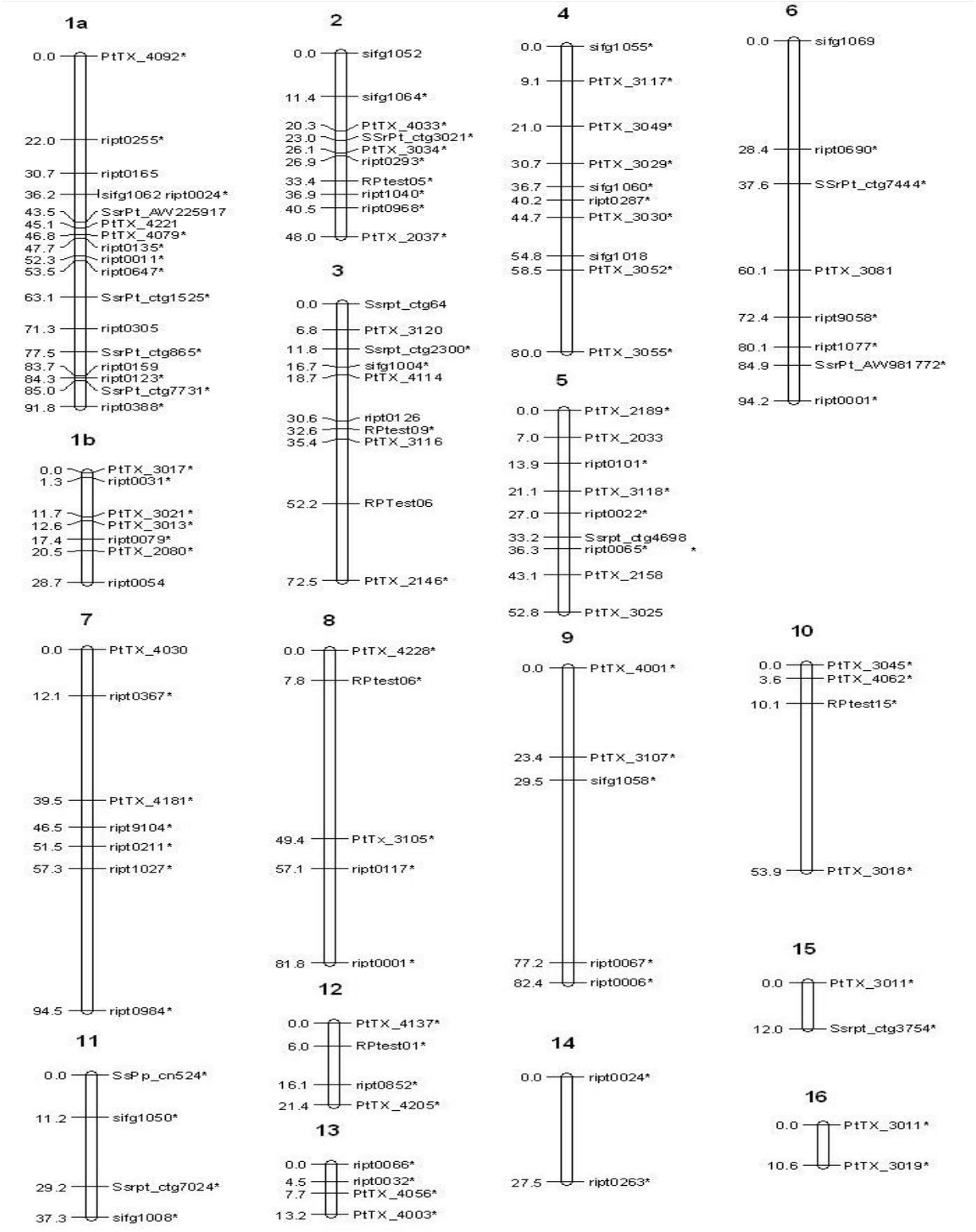


Figure 3.4 An integrated SSR-based genetic map constructed by 111 polymorphic SSR markers with Kosambi's mapping function.Three hundred and five samples come from a half-sib family. Loci listed at the right side and cumulative recombination distance ($cM_{(k)}$) on the left. The markers with * at the right side are the framework markers.

(31.9 cM) and PtTX_4030 and ript0367 (34.8 cM) on linkage group 7, RPTest06 and PtTX_3105 (50.3 cM), PtTX_3105 and ript0117 (50.4 cM) and ript0117 and ript0001 (26.6 cM) on linkage group 8, ript0067 and PtTX_4001 (43.7 cM), PtTX_4001 and PtTX_3107 (28.5 cM) on linkage group 9, RPTest15 and PtTX_3018 (47.7 cM) on linkage group 10, and ript0024 and ript0263 (34.7 cM) on linkage group 14. These linkages were all supported by linkage between more than one pair of markers and LOD scores higher than 3.0. For example, ript0647 on linkage group 1 was linked to both ript0255 (θ=0.08 LOD=5.55) and ript0305 (θ=0.13 LOD=10.26).

The distribution of interval distance was plotted in Figure 3.4. The mode of the distribution was between 9 and 11.9 cM for Haldane's mapping function and 6-8.9 cM for Kosambi's mapping function. For the lower tail of interval distance distribution, there were more numbers of intervals for Kosambi's mapping function, while at the upper tail, Haldane's mapping function had more number of intervals.

3.3.5 Genome Length and Map Coverage

Approximately 75% $_{(H)}$ or 81% $_{(K)}$ of the markers were placed on the framework defining a total of 84 or 90 loci for Haldane's mapping function and Kosambi's mapping function. The largest observed map distance between linked markers at a LOD score of 3.0 was 58 cM $_{(H)}$ and 47cM $_{(K).}$ The observed number of locus pairs with a LOD score 3.0 or greater was 212 $_{(H)}$ and 210 ($_{K).}$ These values were substituted in the Hulbert method to estimate the size of the longleaf pine genome as 2049.1 cM for Haldane's mapping function and 1950.5 cM for Kosambi's mapping function. The 95% confidence interval for genome length was 1781.3-2411.6 cM $_{(H)}$ and 1694.3 -2298.0 cM $_{(K)}$. The expected genome map coverage E (Cn) at LOD=3.0 was 86.4 % $_{(H)}$ and 84.5% $_{(K).}$ The observed genome length was 1874.3 cM $_{(H)}$ and 1582.6 $_{(K),}$ which covered 79.8.5% $_{(H)}$ and 80.2 % $_{(K)}$ of the genome length.

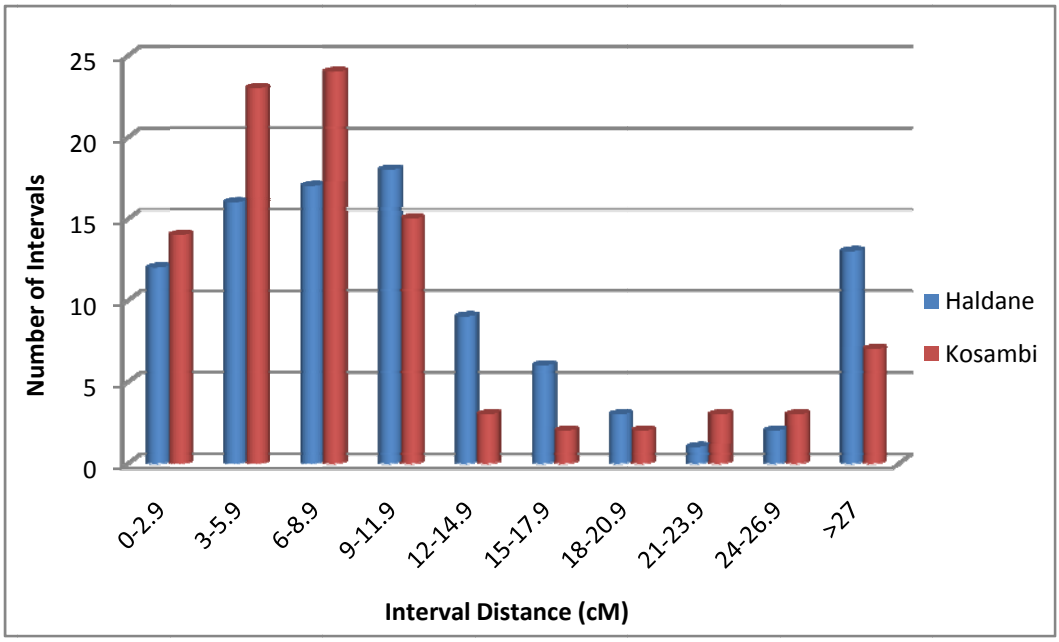


Figure 3.5 The distribution of interval distance between adjacent markers for the integrated map. The X-axis is the interval distance (cM) of two adjacent markers. The Y-axis is the number of intervals in the corresponding intervals.

3.4 Discussion

3.4.1 Heterozygosity and Polymorphic Information Content

For a mating to be informative, at least one of the two parents must be heterozygous for the locus. Therefore, the usefulness of a marker in a model-based linkage analysis depends on its degree of polymorphism (i.e., its alleles and their frequencies) (Guo and Elston, 1999). Quantitatively, the degree of polymorphism of markers is measured by two distinct quantities: one is heterozygosity (Nei and Roychoudhury 1974), and another one is the polymorphism information content (PIC) value (Botstein et al., 1980). For all the markers estimated in this study, the PIC value was smaller than the expected heterozygosity, and the expected heterozygosity was smaller than the observed heterozygosity. The relationship between heterozygosity and PIC value was not direct but was very closely related. The markers that had more alleles in one locus usually had high heterozygosity and PIC value, but these results was

not absolute, i.e. marker ript0791 had 4 alleles, but the heterozygosity and PIC value was smaller than all the 3-allele markers and majority of 2-allele markers. The estimate of mean expected heterozygosity (0.53) was close to the results of Echt et al.(1996) estimation in eastern white pine (0.515), but smaller than the estimation of 0.59 in *Pinus.halepensis* and *P.brutia* (Keys, et al., 2000) and 0.85 for *P. sylbestris* (Soranze et al., 1998). The heterozygosity and PIC value may be related to the length of the SSR marker sequence (Slavov et al., 2004) and also related to the population studied.

3.4.2 Segregation Distortion

There was 5.9%, 6.5%, 6.5%, 16%, 3.2%, and 2.2% markers showed segregation distortion in each individual full-sib family. Segregation distortion of markers in forest trees had been a common problem in forest genome studies (Lemes, et al., 2002; Ritter et al., 2002; Tani et al., 2003; Yin, et al., 2004; Woolbright, et al., 2008; Gill et al., 2006). Segregation distortion may occur due to biological reasons, e.g. chromosome loss (Kasha and Kao, 1970), genetic isolation mechanisms (Zamir and Tadmor, 1986), presence of lethal genes and/or fragment complexes (overlapping fragments consisting of identically sized fragments) (Nikaido et al, 1999; Hansen et al, 1999), and the expression of genetic load via a lethal recessive allele(Bradshaw and Stettler, 1995). Distortion in segregation can influence map construction (Zhang, et al., 2002, Woolbrightwith, et al, 2008) and QTL detection (Bradshaw et al., 1994; Cervera et al., 2001, Yin et al., 2004). These studies also suggest that markers showing segregation distortion due to linkage with genes under selection may have important ecological consequences, and should therefore be included in mapping studies of natural populations. However, a study by Säll and Nilsson (1994) proved that the effect of these sources of segregation distortion on recombination estimates was relatively small, and bias to marker order was negligible (Lin and Ritland, 1996).

Therefore, caution must be exercised when making conclusions involving QTL linked to distorted markers (Woolright et al., 2008).

Non-biological reasons, such as scoring errors (Devey et al., 1994; Xu et al., 1997) and sampling errors (Plomion et al., 1995, Echt and Nelson, 1997), may also lead to segregation distortion in mapping studies. The errors associated with the non-biological reason were assumed to have serious effects on recombination values (Hallden et al., 1996; Säll and Nilsson 1994). Genotyping error is a major concern when molecular markers are used for parentage analysis. Minimizing the rate of mistyping and avoiding markers with high frequencies of null alleles may be crucial for obtaining unbiased estimates of gene flow and pollen contamination (Slavov et al., 2004). With microsatellite loci, a null allele most often occurs because of mutations in one or both primer binding sites, sufficient to prevent effective amplification of the microsatellite allele (Dakin & Avise 2004). This problem is particularly common when the microsatellite locus cloned in one species is applied to a different species using the same primers. A null allele cannot be distinguished on gel from a homozygote for the only DNA fragment, which can be scored in the same plant, which usually leaded to scoring errors. Thirty out of 464 (6.46%) alleles were identified as null alleles according to their segregation pattern in genotyping processes, and these results were matched with the null allele frequency results in CERVUS output. Among the 34 distorted markers, 11 (32.4%) of them contain null alleles. Given the facts that most distorted markers (88.23%) were specific for individual families and scattered to 11 linkage groups, the distorted segregation in this study is considered to be mostly caused by non-biological reasons.

Microsatellite markers containing multiple loci can simultaneously detect two or more loci were attractive and useful for some applications (Fisher et al., 1998; Amarasinge and

Carlson 2002). However, it was very hard to differentiate different loci when the amplification products had similar sizes (Figure 3.5), if this marker also contained null alleles, it was impossible to avoid genotyping error. The multiple locus and null alleles were also the main reasons I used less markers for the linkage study than I screened in the previous chapters. Single locus SSR markers were usually preferred in genome mapping studies.

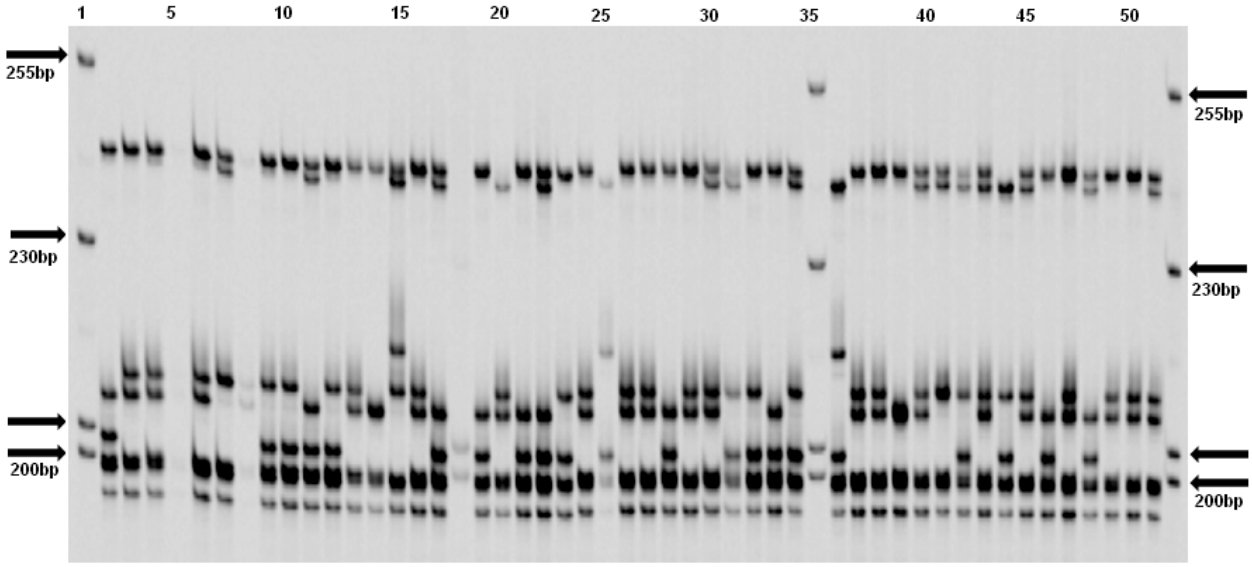


Figure 3.6 Multiple locus SSR markers (sifg1004). The 1st, 18th, 35th, and 52nd are standard molecular weight. The other lines are DNA samples for backcross population.

3.4.3 The Integrated Map

Although several moderately dense linkage maps have been previously constructed for longleaf and slash pine using RAPD makers, this was the first time a linkage map for longleaf pine and longleaf pine by slash pine hybrid was made exclusively from microsatellite DNA markers. The expected advantages of a microsatellite based linkage map, in contrast to a RAPD based linkage map, was stability and portability to other families and pedigrees.

There were some problems associated with construction linkage maps in single family in this study: there were up to 19 linkage groups for each individual family, but the homologous chromosome number for pine trees was 12; each family had several linkage groups containing

only 2 or 3 markers, with some markers segregated in only in one or two families. Therefore, combining segregation information from multiple families was necessary and effective, especially when the marker did not segregate within a single family, e.g.SsrPt_ctg3754 segregate only in Family Em24 and Em45, however, it cannot combined to any linkage group while in family Em45, it was tightly linked to PtTX_3011 (11 cM $_{(H)}$) in Family Em24. After combining all the segregation information from 6 families, 13 big linkage groups were created. The integrated maps from half-sib family contain 112 SSR markers, which covered about 86.92% of the genome, providing a low-density resolution of the longleaf and slash pine genome. This map can be used to compare QTLs identified in different genetic backgrounds (Beavis and Grant, 1991).

Compared with the SSR-based linkage map built by Echt et al. (In preparation) using two reference pedigrees of loblolly pine, discrepancies were found between the two sets of linkage maps. The integrated linkage groups 2, 3, 5, 7, 10, 15 and 16 correspond to the loblolly linkage groups 2, 3, 2, 1, 6, 7 and 7, respectively. However, the other linkage groups are mixtures of the loblolly pine linkage groups. For example, the integrated linkage group 1 was a mixture of loblolly linkage groups 9, 10 and 12, the integrated linkage groups 4 was a mixture of loblolly linkage groups 5 and 11, and the integrated linkage group 6 was a mixture of loblolly linkage groups 4 and 9. The reasons for the discordance are not known, but it may relate to both biological and non-biological reasons. In the process of primer screening, several markers were found have different segregation patterns among loblolly pine control and the longleaf pine and hybrid. For example, sifg1058, was homozygous for loblolly pine, however, it was heterozygous for all the longleaf x slash pine hybrids and longleaf pines. Integrated linkage group 11 included 4 linked markers, all the markers were informative for longleaf pine, but none of them were

identified as heterozygous for loblolly. This different segregation pattern may indicate some major differences between longleaf pine and loblolly pine. The non-biological reasons also play an important role on linkage analysis. Since the linkage relationship was analyzed based on the individual full-sib family and followed by the integration from multiple families, the sample sizes for each full-sib family were critical. Although there were 305 samples available for linkage analyses, the sample size for each full-sib family was smaller compared with the loblolly pedigrees, which reduce the power of linkage analyses. In the process of map integration, two common markers were used in this study to join the linkage groups from different families. The integrated linkage group 1 corresponds to the mixture of loblolly linkage groups of 9, 10, and 12. However, with closer inspection of Figure 3.2 D (the source of integrated linkage group 1), it can be found that if 3 common markers were used to join the linkage groups from the different families, then 3 linkage groups would be created matching the results from the loblolly pine linkage group. Therefore, caution must be taken in joining process to avoid false linkage association.

According to Butcher (2000), the advantages of mapping multiple pedigrees include: a large number of loci are mapped, gene order and map distances are estimated more accurately, and alterations in these values, possibly due to chromosomal rearrangements affecting one of the parents, are easily detected. The differences in recombination frequencies were observed in each individual family and caused distortion of map distances between the relevant pairs of markers on the integrated map. However, small discrepancies in marker order may caused by mapping imprecision rather than real rearrangement (Lombard and Delourme, 2001).The large mean marker interval, unlinked markers, 16 linkage groups, and the existence of 2 marker linkage

groups suggest that the integrated map was not complete and the linkage gaps remained to be filled by adding more markers.

3.4.4 Genome Length and Map Coverage

The method of moment estimator of Hulbert et al (1988) is the most widely used function for deducing genome length because it is easy to calculate from readily available genome mapping data and statistics (Echt and Nelson, 1997). Chakravarti et al (1991) used a modification of Hulbert's method. For a given LOD score, Z, the pairs with the largest estimated θ value was chosen from the pair of loci with LOD scores greater or equal than Z. In the simulation experiment, Chakravarti et al. observed that the Hulbert method overestimated the genetic distance. However, in this study, the Hulbert method always gave the smaller estimation for both Kosambi and Haldane's mapping function. Our results matched the conclusion obtained by Gerber and Rodolphe (1994) in their study on maritime pine.

Genome length estimates in forest trees with different markers, pedigrees and computer programs were obtained (Echt and Nelson, 1997; Barreneche et al., 1998; Devey et al., 1999; Sewell et al., 1999; Lespinasse et al., 2000; Chagne et al., 2002, 2003; Wilcox et al., 2004; Pelgas et al., 2006). Generally, a common assumption is that maps constructed with JoinMap are shorter than those maps constructed with the multilocus-likelihood computer software, e.g., MapMaker and OutMap (Sewell et al., 1999; Butcher et al., 2002; Gosselin et al., 2002; Tani et al., 2003). The multilocus-likelihood method used by MapMaker assumes an absence of crossover interference, and map length was calculated as the sum of adjacent distances using adjacent marker pairs only. The recombination frequencies were transferred to cM according to the selected mapping function directly. However, JoinMap use all pairwise estimates for estimating the map distance (Stam, 1993) and correctly produces shorter maps when an

interference exist even Kosambi's mapping function was used in both programs (Stam, 1993). The estimate of the genome length for longleaf pine and slash pine in this study was 2049 cM $_{(H)}$ and 1959 cM $_{(K)}$ under LOD score 3.0 with JoinMap ver.3.0. This estimate was shorter than the estimation of Echt and Nelson (1997) and Weng (1999), in which the expected genome length was 2618 cM $_{(H)}$, 2000 cM $_{(K)}$ and 2400 cM $_{(H)}$ for longleaf pine. In the work of comparing three pines, *Pinus strobus*, *P. palustris* and *P. pinaster*, Echt and Nelson (1997) estimated the average theoretical length was close to 2000 cM $_{(K)}$.

The theoretical map distance was also found to be greater than the observed genome map coverage for both mapping functions. The process of framework construction (Echt and Nelson, 1997) may cause the differences between expected and observed values, where the framework only accounted for 70%. This result also suggests that additional informative markers would be useful to join smaller groups and increase map coverage.

3.5 References

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CHAPTER 4 QTL MAPPING FOR GENES CONTROLLING EARLY HEIGHT GROWTH IN LONGLEAF PINE

4.1 Introduction

Mapping quantitative trait loci (QTL) is a basic operation for positional cloning and for application of marker-assisted selection or marker-assisted introgression in genetic improvements (Darvasi and Soller, 1994). In forest trees, QTLs for different traits, such as wood quality (Groover et al., 1994; Kumar et al., 2000; Ball, 2001; Sewell et al., 2002; Sheperd et al., 2002; Brown et al., 2003; Markussen et al, 2003; Pot et al., 2005a 2005b), growth traits (Bradshaw and Stettler, 1995; Weng et al., 2002; Gwaze et al., 2003;), and adaptative traits (Hurme et al., 2000; Brendel et al., 2002; Yazdani et al., 2003; Monclus et al., 2005, 2006, Tschaplinski et al,2006) have been detected, showing the usefulness of this approach for dissecting genomic regions controlling complex traits.

However, the long-lived nature, outbred mating system, and high genetic load have hampered the development of QTL analysis in forest trees. Plants undergo significant morphological changes throughout development, and different sets of genes may contribute to phenotypic variation from the juvenile to mature phases. In a study of mapping wood density in *Eucalyptus* (Verhaegen et al., 1997), none of the QTLs detected at one time (18, 24, and 36 months of age) could be repeated throughout the entire experiment, suggesting that different loci contribute to phenotypic variation during different stages of development. Weng et al. (2002) showed in *Pinus palustris* that the variance explained by major QTLs decrease over time, suggesting the increased complexity of quantitative traits with the aging of the tree. The contribution of different sets of genes to quantitative variation during development may lead to

low powers of detecting QTLs, as a phenotype measured at rotation age essentially represents the cumulative effect of many distinct genes.

For outcrossing species, the marker-based linkage mapping of QTLs is generally thought of as requiring an accumulation of data over a number of relatively small half-sib or full-sib families that together make up the mapping population (Song, J. Z, 1999). With few exceptions (Brown et al., 2003; Jermstad et al., 2003), the size of segregating populations used in these studies is often small (90 to 200 individuals). Among factors influencing QTL detection powers, small sample sizes and low trait heritability were shown to cause an overestimation of QTL effects, underestimation of QTL number, and hamper the detection of QTLs with low effects (Beavis 1995). In addition, over the mapping population as a whole, there is a strong tendency for linkage equilibrium of marker alleles and QTL alleles. Small family size and linkage equilibrium reduce the power of full-sib and half-sib populations as much as 10-fold compared with populations derived from crosses between inbred lines (Soller and Genizi, 1978; Weller et al., 1990; Knott and Haley, 1992; van der Beek et al., 1995; Knott et al., 1996).

Statistical methods are well developed for single-family degrees (Lander and Botstein 1989; Haley and Knott, 1992; Jansen, 1994; Zeng, 1994); however, it is undesirable when the two lines initiating the cross are not segregating at a QTL (Xu, 1998). If a QTL is present, but undetected because of fixation to the same allele in both lines, then a type II error, also referred to as a genetic drift error (Xu, 1996), has occurred. A type II error can be reduced by using multiple families (Muranty, 1996), and the simulations have shown that six parents should give a good sample of variance and allow the detection of QTLs with reasonable power if the QTL heterozygote frequency in the base population is high enough.

For these reasons, a single QTL detection experiment in a single-family pedigree with a small population size does not give an exhaustive idea of the genetic architecture of a quantitative trait. One possible strategy to overcome these difficulties is to detect QTLs several times across different families, environments, and developmental stages. In this way, by un-segregating QTLs, the environmental and temporal stability of QTLs can be verified and a more complete picture of the genetic architecture of the complex trait can be drawn.

The major objectives for this chapter are to:

1) Analyze the phenotype traits at the different ages and their correlation;

2) Identify the QTLs controlling EHG in longleaf pine.

4.2 Materials and Methods

4.2.1 Plant Materials, DNA Isolation and Gel Electrophoresis

A half-sib family, which included longleaf pine x slash pine hybrid Derr 488 as a common paternal parent and six longleaf pines as recurrent maternal parents, was selected for the QTL identification. Within each of the six full-sib families, the tallest and shortest 8% of seedlings (220 seedlings total) were selected for QTL detection (phase I). Random selections of 8 percent of the seedlings from the rest of the population (110 seedlings) and 25 seedlings from both tails (135 seedlings total) of the within-family distributions were used for unbiased QTL verification and mapping (phase II). Due to the DNA isolation failure and the results of mis-parentage, there were only 305 samples used for the final data analysis (see Chapter 3). One hundred and sixty (160) samples were used for phase I, consisting of the 85 tallest samples and the 75 shortest samples. One hundred and seventy (170) samples were used for phase II, which including 110 random samples from the rest of the population, 30 samples from previous study

to make up the losing samples due to mis-parentage, and 25 randomly selected seedlings from the phase I population.

The DNA isolation, purification, and quantification procedures follow the method described in Chapter 2.2.2. The marker preparation, PCR reaction condition, and gel electrophoresis followed the methods described in Chapter2.2.3 and 2.2.4. For a more detailed description of experimental design and plant material pedigree, see Chapter 2.

4.2.2 Statistical Analysis for Phenotypic Data

In this study, the height and diameter measurements for each year were used as the response variables to estimate the early height growth of longleaf pine. For each year, a different plot was assigned, thus, the year effect and the plot effect were confounded. When a plot was proved to be significant, it was hard to distinguish whether it was caused by the year effect, location effect, or their interaction. To solve the problem, a new variable called 'environment', a combination of the plot and year effect (i.e., the seven different field tests), was created. Therefore, the linear model used in the study was

$$Y_{ijkl} = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \gamma(\beta)_{jk} + \varepsilon_{ijkl}$$

where μ is the overall mean, τ_i is the female parent effect (i=6 for six female parants), β_j is the environment effect (i=7 for seven different test fields, a combination of year and plot effect), $\tau\beta_{ij}$ is the female parent-by-environment interaction, $\gamma(\beta)_{jk}$ is the replication effect within each environment, and Y_{ijkl} is the trait value for *i*th family, *j*th environment *k*th replication and *l*th tree. The family effect was a fixed effect the environment, the female-by-environment interaction and replication were treated as the random effect.

All the phenotypic data analyses for the population were performed using SAS (ver 9.1). The heights and diameters for the entire half-sib families were first tested for normality using the

Shappiro-Wilk test (SAS Proc Univariate). The correlations between the 5 traits were tested by the Pearson test statistic. The ANOVA was performed using PROC MIXED because of the mixed effects.

The trait value of a tree Y_{ijkl} was determined by the female effect, environment effect, and their interaction. To reflect the true value of the genetic effect for the tree, a residual value $R_{ijkl} = Y_{ijkl} - \beta_j$, was used as a final response value for the QTL analysis. The interaction between family and environment was tested first. If the interaction was not significant for the tested trait, the interaction for the trait was dropped from the full model (model 4.1), and the residual value for that trait included the genetic effect and error term only. If the interaction for a trait was significant, the residual value then included the genetic effect, the error, and the specific female-by-environment effect. The residual process was performed with SAS Proc Glm/solution, which is the regression approach for categorical data. These residual values were used as the trait value for DNA sample selection for selective genotyping and for QTL mapping.

4.2.3 QTL Identification

Associations between segregating genetic markers and phenotypic traits were detected using both the single point method and interval mapping method. Since marker-QTL phase relationships were not known *a priori* because of linkage equilibrium, and the interactions among QTL alleles were possibly dependent on the genetic background for each individual family, the analyses were conducted for each marker in each full-sib family individually by software MapQTL (ver.4.0). The linkage phase used for each pair of molecular markers was obtained from JoinMap (ver.3.0) in the process of linkage mapping analyses. However, MapQTL (ver.4.0) cannot join the data from different families. The significant markers for a QTL determined by MAPQTL across at least 3 full-sib families were then outputted and re-analyzed

by simple regression approach. The regression considered, as the dependent variable, the value of the measured character in each of the 305 half-sib progenies. As the regressor, the allelic composition of each SSR locus was used.

Interval mapping was performed by QTL-Express (Seaton et al., 2002), a World Wide Web-based interface for the least square method (Haley and Knott, 1992; Haley et al, 1994; Knott et al., 1996) for both the detection population (Phase I) and the verification population (Phase II). QTL express was the first application for QTL mapping in outbred populations with a web-based user interface (Seaton, 2002). The analysis was carried out using the half-sib module, in which QTLs were mapped and explained by within-family variations, with the evidence for QTL segregation accumulated across the common parents (Knott et.al., 1996). Trait data were tested for normality using the Shapiro-Wilk test before the QTL identification. Each linkage group was scanned at 5-cM intervals for locations explaining a high proportion of the phenotypic variance using a one-QTL model and a two-QTL model. Chromosome-wide permutation tests with 1000 iterations were carried out to determine P-values and a significant threshold of 0.05 was taken as evidence for the presence of a QTL (Churchill and Doerge, 1994; Seaton, 2002). The confidence interval (CI) for the position of a QTL was defined as the interval in which the F-statistics of the presence of a QTL was at least twofold of its maximum value, and the bootstrap procedure was used to estimate the confidence interval of a QTL location (Visscher et al., 1996).

4.3 Results

4.3.1 Phenotypic Analysis of Height and Diameter

The ANOVA table for the above statistical model is given in Table 4.1. For all 5 traits, the interaction between family and environment was not significant, except ht2 ($p=0.002$), while the environment and female parent effects were all significant ($P<0.001$). To evaluate the

difference among different environments, the mean responses for each full-sib family at different environments are summarized in Table 4.2. Only the variable ht4 (total height after planting 4 years) was shown here. The mean responses differed significantly among different test environments, indicating the quantitative inheritance pattern of the EHG. The families Em17 and Em24 were significantly taller than the other families, indicating that the parents may be potential candidate parents for improving EHG. Frequency distributions for total heights, diameters, and their residual values of the Derr488 half-sib family are shown in Figure 4.1 and Figure 4.2, respectively.

Table 4.1 ANOVA table for different environments for height at age 4 years (ht4)

Effect	Num DF	Den DF	F Value	Pr > F
Female	5	1128	7.43	<.0001
Environment	6	1128	5.87	<.0001
Female x Environment	21	1128	1.01	0.4497

Table 4.2 Mean (cm) estimation of the height (ht4) for individual full-sib family at different environments after being planted for four years

	LA			MS				
	Year2002		Year2003	Year2002		Year2003		Environment
	FT_1	488_1	FT_2	FT_1	488_1	FT_2	488_2	
Em04	222.5	/	193.86	187.83	/	162.52	188.85	186.17
Em14	181.5	176.78	175.06	187.07	132.07	146.17	162.22	162.74
Em17	183.13	204.65	213.62	268.22	206.25	176.1	202.83	203.57
Em24	202.91	196.81	245.83	203.96	164.35	187.52	203.45	197.95
Em41	150.57	169.07	/	159.6	/	/	/	164.82
Em45	187.87	196.29	/	224.44	150.48	/	/	185.58
Over Females	180.95	185.30	209.32	206.91	159.26	171.27	187.44	185.92

Note: / there are no progenies presented for the sites.

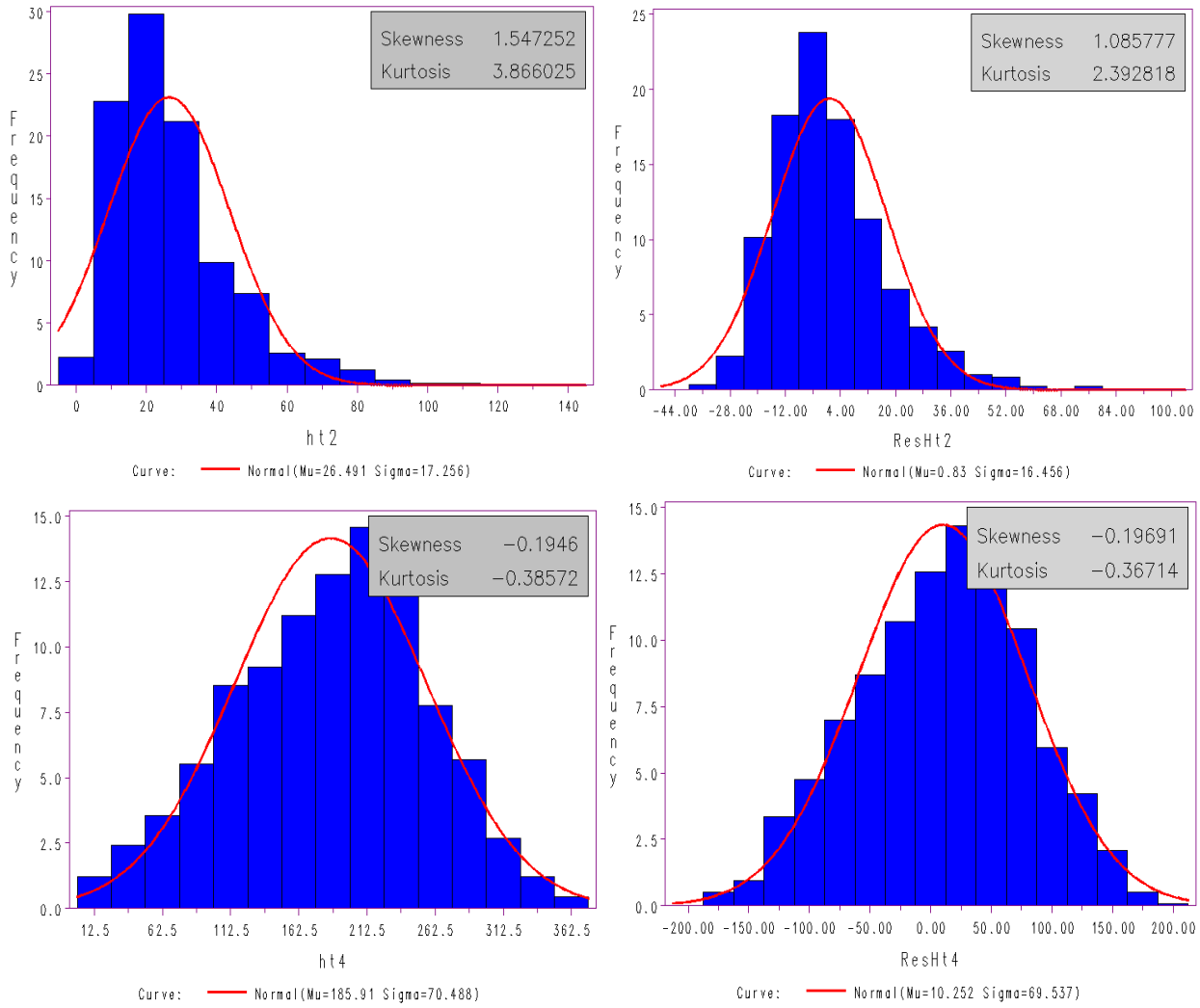

Figure 4.1 Frequency distributions of height growth (left) and the corresponding residual height growth (right).The fitted normal curve was superposed on the finely bin histogram. The mean value, standard deviation, skewness and kurtosis are also displayed.

All the diameter measurements and their residual values are normally distributed, while the skewness and kurtosis of residual values were smaller than the original data. These results were expected because pooling the phenotypic data from different environments could skew the distribution when the environment effect was significant. Residual values gave more accurate estimates when multiple environmental data were pooled. This is especially useful for the QTL identification with small sample sizes and several environments involved. Ht2 was the only trait in which the female parent-by-environment interaction was significant, thus, its residual value

included both within family genetic effect and genetic by specific site interaction. This may help to explain why ResHt2 was not normally distributed. The parent by environment interactions for other traits were not significant, thus the interaction term was dropped from the full model and residual value for these traits, included only genetic effects and random error. For the QTL analyses, the residual values for all phenotypic traits were used instead of the original value. The original data were used for identifying QTL by environment interaction.

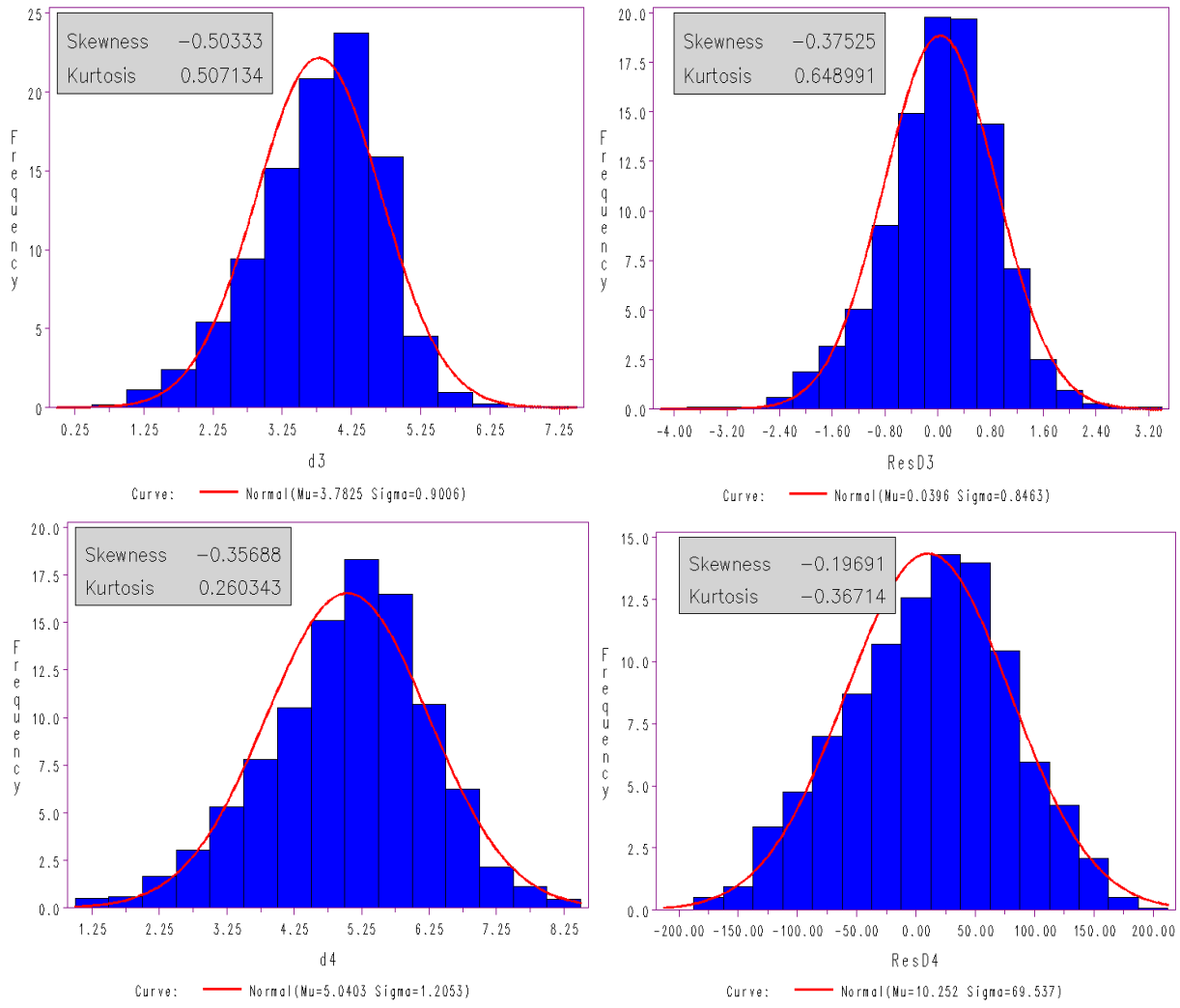

Figure 4.2 Frequency distributions of diameter (left) and the corresponding residual diameter (right). The fitted normal curve was superpose on the finely bin histogram. The mean value, standard deviation, skewness and kurtosis are also displayed.

The correlation coefficients for the 3 height variables and 2 diameter variables were analyzed, using the SAS procedure CORR with Pearson test statistics, and the results are summarized in Table 4.3. The height variables and diameter variables were highly correlated for all ages. The lowest correlation coefficients came from the ResHt2 with ResD3 and ResD4, with a correlation coefficient of 0.69 and 0.70. The correlation between height and diameter was much stronger in the later growing stages, i.e., the correlation coefficient between ResHt4 and ResD3 and ResD4 was 0.89 and 0.98.

Table 4.3 Correlation coefficient for total heights and diameters in the Derr488 half-sib family

	resht2	**resht3**	**resht4**	**resd3**	**resd4**
resht2	1.00000				
resht3	0.85379 <.0001	1.00000			
resht4	0.73291 <.0001	0.91077 <.0001	1.00000		
resd3	0.68904 <.0001	0.96832 <.0001	0.88582 <.0001	1.00000	
resd4	0.69548 <.0001	0.89952 <.0001	0.98298 <.0001	0.98561 <.0001	1.00000

4.3.2 QTL Identification by Single Marker Analysis

Single point marker analyses detected 17 markers associated with the 5 growth traits and the results are summarized in Table 4.4. For ResHt2, there were 4, 3, 4, 7, 8, and 6 markers identified for each individual full-sib family at LOD threshold 3.0. Three markers, PtTX_4092, PtTX_4137, and PtTX_4205, were significant across the 6 full-sib families. Markers PtTX_4137 and PtTX_4205 were both located on linkage group 12, however, they have opposite estimate for the alleles (-5.47 and 5.80). The estimate for each marker locus in Table 4.4 was the coefficient

Table 4.4 Linkage group, marker loci, and single-locus point effects of SSR markers significantly associated with the QTLs using simple regression approach

Trait	Linkage Group	Locus	Locus Position (cM)	P-value	Estimate (cm)	R-Square
ResHt2	1	PtTX_4092	50	0.0039	4.98	2.56
	12	PtTX_4137	29	0.0024	-5.47	3.09
	12	PtTX_4205	0	0.0019	5.80	3.46
Total						9.11
ResHt3	1	PtTX_4092	50	0.0042	13.1	2.63
	1	SsrPt_ctg1525	41	0.0035	-13.5	2.75
	5	PtTX_2189	0	0.0008	16.3	5.62
	12	PtTX_4205	0	0.0003	19.1	5.60
	12	PtTX_4137	29	0.0032	-14.1	3.55
	unlinked	sifg1035		0.0022	15.0	3.47
Total						23.62
ResHt4	1	PtTX_4092	50	0.0018	23.7	3.73
	1	PtTX_4221	118	0.0044	-19.8	2.60
	1	SsrPt_ctg1525	41	0.0013	-23.3	3.55
	4	PtTX_3029	63	0.0007	-24.0	3.81
	5	PtTX_2189	0	0.0037	19.3	2.44
	6	ript0001	91	0.0021	-21.8	6.04
	12	PtTX_4137	29	0.0026	-22.1	3.24
	12	PtTX_4205	0	0.0011	25.4	4.27
Total						29.68
ResD3	1	SsrPtctg1525	41	0.0004	-0.36	5.33
	1	ript0135	110	0.0023	-0.23	4.69
	3	PtTX_2146	0	0.0008	0.22	6.37
	4	PtTX_3117	36	0.0042	0.33	4.16
	4	sifg1018	98	0.0035	0.18	4.37
	5	PtTX_2189	0	0.0019	0.16	5.23
	7	ript0211	6	0.0034	-0.32	4.44
	13	PtTX_4056	6	0.0037	-0.46	9.60
	unlinked	sifg1035		0.0009	0.32	4.46
Total						48.65
ResD4	1	PtTX_2080	53	0.0009	0.60	6.04
	1	SsrPtctg1525	41	0.0007	-0.47	6.91
	1	PtTX_4221	118	0.0037	-0.36	2.86
	3	PtTX_3120	72	0.0038	-0.36	2.81
	3	PtTX_2146	0	0.0002	0.88	8.14
	4	PtTX_3117	36	0.0010	0.56	6.06
	7	ript0211	6	0.0005	-0.54	6.75
	12	PtTX_4137	29	0.0043	-0.35	2.68
Total						42.25

estimate differences between the two alleles for common parent Derr488. Since the linkage phase of markers and QTLs was not known *priori*, the estimate was simply the mean differences between two alleles (smaller allele size vs larger allele size) of the associated marker. There are 6 markers identified as associated with ResHt3. Among these markers, two of them were located on linkage group 1 and two markers were located on linkage group 12. An unlinked marker, sifg1035, was also found to associate with ResHt3. Eight markers were identified for ResHt4: three of them were located on linkage group 1, two markers were located on linkage group 12, three markers were scattered on linkage groups 4, 5, and 6, and one unlinked marker, sifg1035, was also identified. Three markers PtTX_4092, PtTX_4137, and PtTX_4205 were linked with all of the height growth traits.

Nine and eight markers were identified to associate with ResD3 and ResD4, respectively. The markers linked to ResD3 were distributed in 6 linkage groups and the unlinked marker sifg1035 was also linked to the trait. The markers associated with trait ResD4 were distributed in 5 linkage groups. The marker SsrPtctg1525 was found to associate with 4 of the interested traits (ResHt3, ResHt4, ResD3, and ResD4). Marker PtTX_4092 was only linked to those height traits and markers PtTX_2146 and PtTX_3117 were only associated with diameter traits. Most of the markers were distributed on linkage groups 1, 5, 7, and 12.

For the simple marker approach, the software MapQTL (ver 4.0) was first explored to identify the markers associated with the QTLs. Since the software does not handle the linkage data from different parents, and cannot combine the linkage information from multiple families, the significant markers were output and re-analyzed by SAS regression. Therefore, two-phase strategy was not performed for single marker approach due to small sample size within each full-sib family.

4.3.3 Phase I: QTL Detection by Interval Mapping

The QTL identification by interval mapping was following a two-phase strategy: QTL detection by selective genotyping and QTL verification by random sampling. For Phase I, 18 QTLs were detected for 5 traits by QTL express and were located in 8 intervals (Table 4.5).

Table 4.5 Intervals detected that contain QTLs influencing growth traits by QTL Express for QTL detection population

L.G	Trait	Position (cM)	Interval	CI (cM)	Est.	P value	Variation
1	ResHt2	40	ript0031-SsrPtctg1525		7.83	0.0111	
	ResHt2	120	PtTX_4221-ript0225	10-124	14.52	0.0032	7.55%
	ResHt3	40	ript0031-SsrPtctg1525	2-125	-40.12	0.0088	3.01%
	ResHt4	40	ript0031-SsrPtctg1525	24-124	-65.78	0.0072	3.22%
	ResD3	35	ript0031-SsrPtctg1525	12-50	-0.74	0.0085	2.86%
	ResD4	115	ript0135-PtTX_4221	88-124	0.90	0.0093	2.92%
2	ResHt3	5	sifg1052-sifg1064	0-15	27.26	0.0125	2.60%
	ResHt4	5	sifg1052-sifg1064	0-12	38.38	0.0188	2.13%
	ResD3	5	sifg1052-sifg1064	0-18	0.57	0.0115	2.68%
4	ResHt4	80	sifg1060-ript0287	75-95	-39.45	0.0089	3.01%
	ResD4	80	sifg1060-ript0287	75-95	-0.79	0.0091	3.01%
7	ResHt2	75	ript9104-PtTX_4130	60-110	12.93	0.0018	4.85%
	ResHt3	75	ript9104-PtTX_4130	65-110	33.31	0.0028	4.30%
	ResHt4	75	ript9104-PtTX_4130	60-110	69.85	0.0011	4.92%
	ResD3	75	ript9104-PtTX_4130	60-110	1.01	0.0045	3.76%
	ResD4	75	ript9104-PtTX_4130	60-110	1.16	0.0022	4.42%
12	ResHt4	0	PtTX_4205-ript0852	0-28	-11.07	0.0014	5.22%
	ResHt4	25	RPTest01-PtTX_4137	0-28	38.16	0.0009	6.87%

L.G.: linkage group; Position (cM): position to which the QTL mapped in cM; Variation: percentage variation explained by the QTL; CI: confidence interval; P-value: significance of a quantitative trait locus being present; Est.: The estimation of QTL effect.

The interval ript0031-SsrPtCtg1525 on linkage group 1 contained 4 QTLs (ResHt2, ResHt3, ResHt4, and ResD3), and interval ript9104-PtTX_4130 on linkage group 7 contained 5 QTLs, interval sifg1052-sifg1064 located on linkage group 5 contained 3 QTLs (ResHt3, ResHt4 and ResD3) and interval sifg1060-ript0287 contained two QTLs (ResHt3 and ResHt3).

The other two intervals located on linkage group 1 and 12 contained only one QTL. There are 3, 4, 6, 4 and 2 QTLs detected for ResHt2, RestHt3, ResHt4, ResD3, and ResD4, and the variance explained by the QTLs was plotted in the Figure 4.3. The variances explained by the QTLs for ResHt4 was 25.4 % with mean value 4.23%. These numbers were much greater than the other traits. The total accumulative variance explained by QTLs for ResHt3 was similar with the ResHt2; however, the mean value for ResHt3 was 3.2%, which is less than the mean value for ResHt2. The total accumulative variance explained by QTLs for ResD3 was greater than ResD4 while the mean value was less than ResD4.

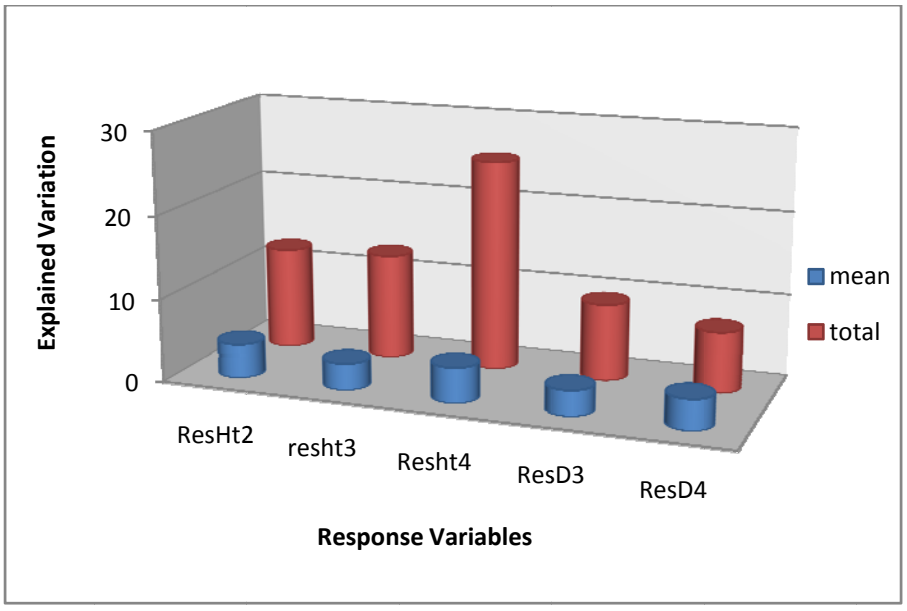

Figure 4.3 The plot for explained variance for 5 growth traits for a QTL detection population. The axis is the five growth traits. The y-axis is explained variance for each trait. The red bars are for the total accumulative explained variance for all QTLs detected for that trait. The blue bars are for the mean explained variance for the trait.

The QTL interval peaks for all traits at each linkage group are also plotted in Figure 4.4 for graphically review of the QTLs. Two QTL peaks were found on linkage group 1 (Picture A). One peak was located around 40 cM and the other peak was around 120 cM. All traits except ResD5 reached the significant F threshold 5.8 at the QTL peak 40 cM. However, for the second peak 120 cM, only two traits, ResHt2 and ResD4, reached the F threshold. Picture B is for linkage group 2. There is only one peak at 0-5 cM and three traits ResHt3, ResHt4 and ResD3 reached the significant F threshold 4.3. For the linkage group 4 (Picture C) , there is one QTL peak located at 80 cM, two traits ResHt4 and ResD4 reached the F threshold 4.5. One QTL peak is also found for linkage group 7 (Picture D) and all traits are significant. Two QTL peaks are found for linkage group 12, and only trait ResHt4 was significant at both peaks.

4.3.4 Phase II: QTL Verification by Interval Mapping

In phase II, 15 QTLs were identified at 8 intervals distributed on 6 linkage groups (Table 4.6) and the accumulative explained variation for each trait was plotted in Figure 4.5. Compared with the results from Phase I, the QTL verification population failed to detect any QTLs on linkage group 2, while QTLs were verified on linkage groups 4, 7, and 12 with some light change on location, and new QTLs were identified on linkage groups 5 and 6. The QTLs detected included 3 QTLs for ResHt2, 2 QTLs for ResHt3, 4 QTLs for ResHt4, 3 QTLs for ResD3, and 3 QTLs for ResD4. The Over-plot for QTLs identified for each linkage group is shown in Figure 4.6. For picture A, the peak at 40 cM contained 5 QTLs for each studied trait and a second peak at 120 cM for ResHt2, but failed to verify the ResD4 at this position. For linkage group 4, only the QTLs for ResHt4 were verified, and for linkage group 12, both ResHt3 and ResHt4 were verified. The accumulative explained variance for height growth was decreased from ResHt2 to ResHt3, while increased for ResHt4. The explained variance from ResD3 and

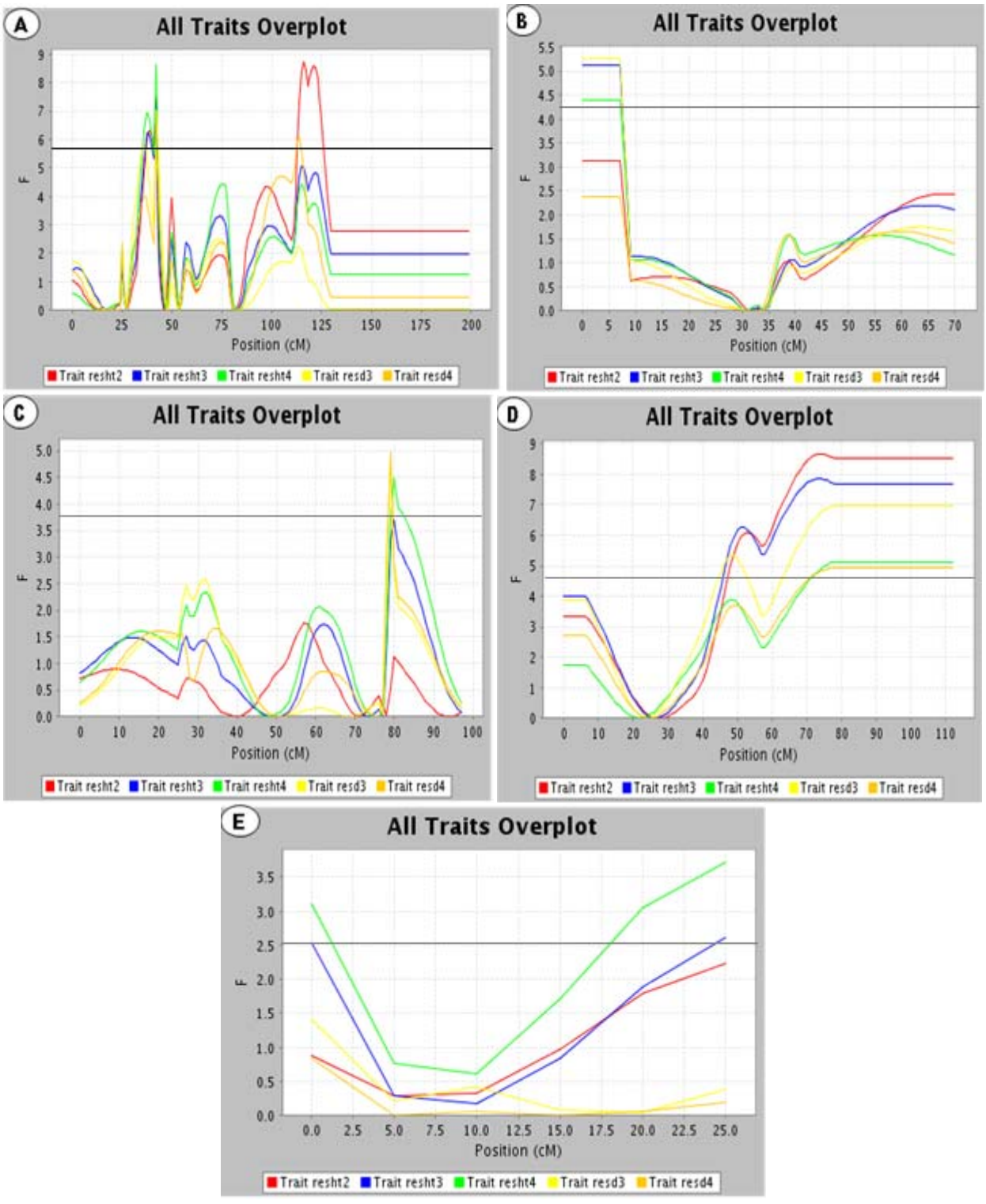

Figure 4.4 The overplot of QTLs for growth traits detected by interval mapping with QTL Express for QTL detection population. The X-axis is the position of linkage group with cM as the unit. The Y-axis is the F test value for each trait. The different colors of the plots are for different traits. A: linkage group 1, B: linkage group 2, C: linkage group 4, D: linkage group 7 and E: linkage group 12.

ResD4 was also increased. However, the mean value was decreasing for the height variables for different growing stages. As a summary, the QTLs identified by two QTL populations were pooled together at Figure 4.7 with software MapChart. Those QTLs that have been detected and verified were labeled as the solid squares and the QTLs detected by one population were labeled as open squares.

Table 4.6 Intervals detected that contain QTLs influencing growth traits by QTL Express for QTL verification population

L.G.	Trait	Position (cM)	Interval	CI (cM)	Est.	P value	Variation
1	resht2	40	ript0031-SsrPtctg1525	15-60	7.93	0.0064	5.98%
	resht3	40	ript0031-SsrPtctg1525	15-60	-21.31	0.0104	2.42%
	resht4	40	ript0031-SsrPtctg1525	20-55	-28.66	0.0114	2.32%
	resd3	40	ript0031-SsrPtctg1525	12-50	-0.37	0.0091	2.54%
	resd4	40	ript0031-SsrPtctg1525	25-50	0.65	0.0016	4.25%
4	resht4	80	PtTX_3052-PtTX_3117	10-60	-23.52	0.0239	1.42%
5	resd3	5	PtTX_2189-PtTX_2033	0-15	0.31	0.0088	3.97%
6	resht4	90	SsrptAW981772-ript0001	70-150	24.00	0.0264	1.52%
	resd3	65	ript9058-PtTX_3081	30-75	-0.34	0.0120	2.27%
	resd4	65	ript9058-PtTX_3081	10-75	-0.38	0.0333	1.30%
7	resd4	60	ript1027-ript9104	40-80	0.43	0.0058	4.07%
12	resht2	0	PtTX_4205-ript0852	0-28	4.98	0.0155	2.06%
	resht3	0	PtTX_4205-ript0852	0-28	16.29	0.0039	4.83%
	resht4	0	PtTX_4205-ript0852	0-28	27.05	0.0010	5.76%

L.G.: linkage group; Position (cM): position to which the QTL mapped in cM; Variation: percentage variation explained by the QTL; CI: confidence interval; P-value: significance of a quantitative trait locus being present. Est.: The estimation of QTL effect.

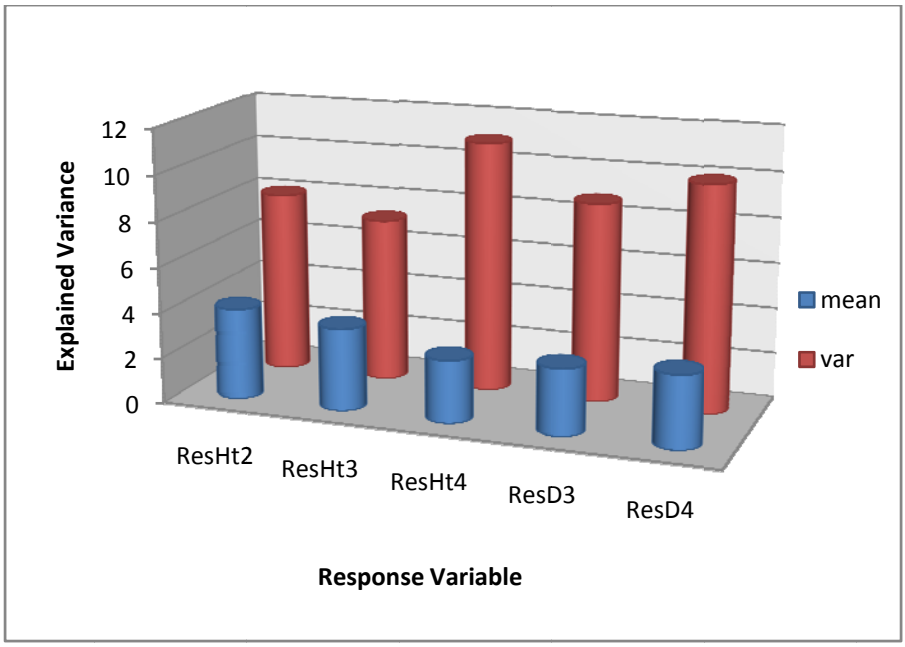

Figure 4.5 The plot for explained variance for 5 growth traits for QTL detection population. The axis is the five growth traits. The y-axis is explained variance for each trait. The red bars are for the total accumulative explained variance for all QTLs detected for that trait. The blue bars are for the mean explained variance for the QTLs.

4.4 Discussion

4.4.1 Phenotypic Data

Forest tree breeding traditionally has been viewed as an application of quantitative genetics (Zobel and Talbert 1984). Most commercially important traits, such as growth and adaptive characters, are quantitative and tend to normal distribution. This is true for the tree population in each environment in this study. However, when the trees from different envrionment were pooled together, they tended to be bi-modal or skewed because the environment effect was significant. Thus, the genetic effect was confounded with the environment effect, that is, when a seedling was identified as an extreme sample, we do not know whether it caused by the genetic effect or was just the response to certain environmental effect. If the samples were selected by original data, most upper extreme samples would come

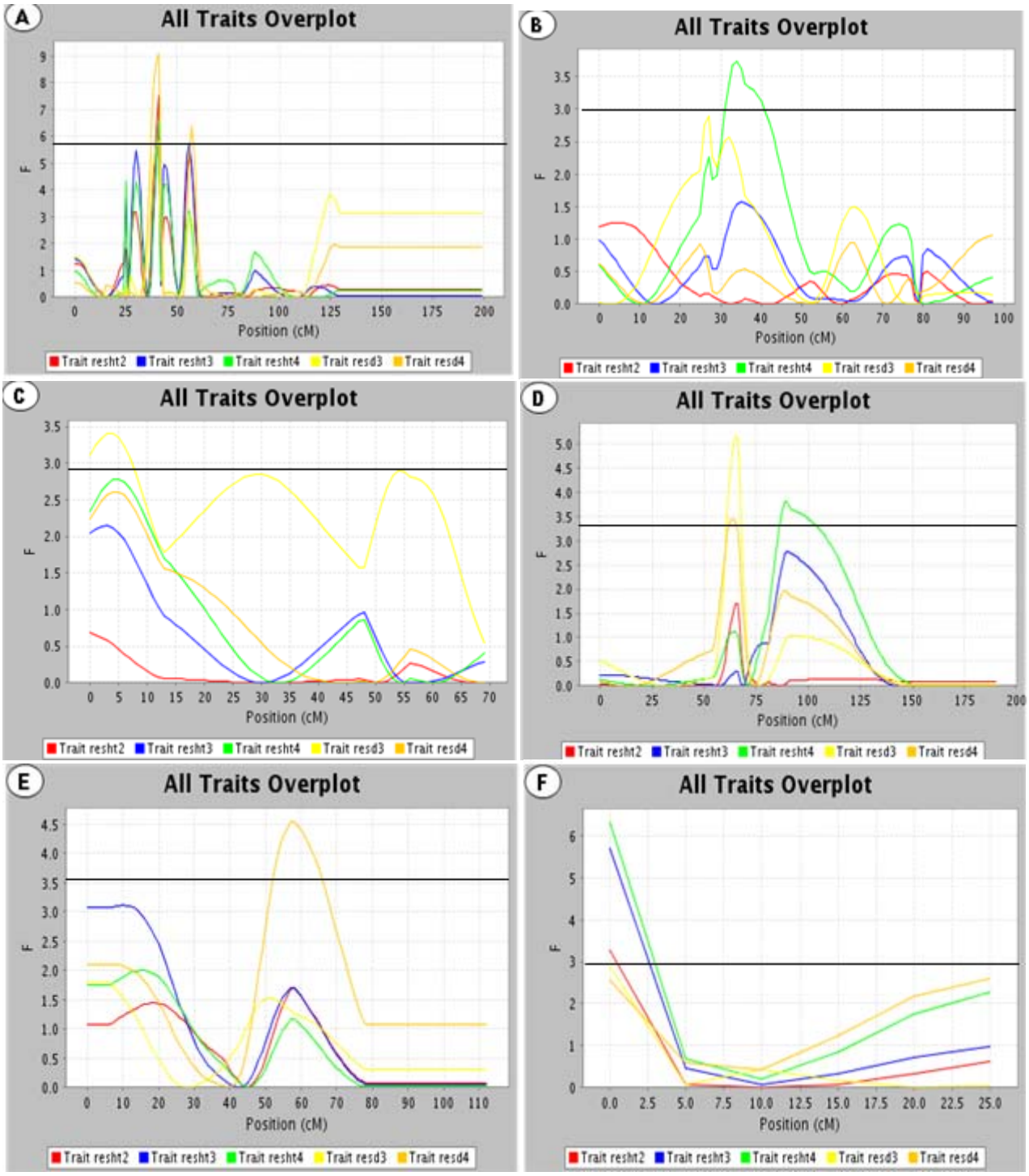

Figure 4.6 The overplot of QTLs for growth traits detected by interval mapping with QTL Express for QTL verification population. The X-axis is the position of linkage group with cM as unit; the Y-axis is the F test value for each trait. The different colors of the plots are for different for different traits. A: linkage group 1, B: linkage group 4, C: linkage group 5, D: linkage group 6, E: linkage group 7, and F: linkage group 12.

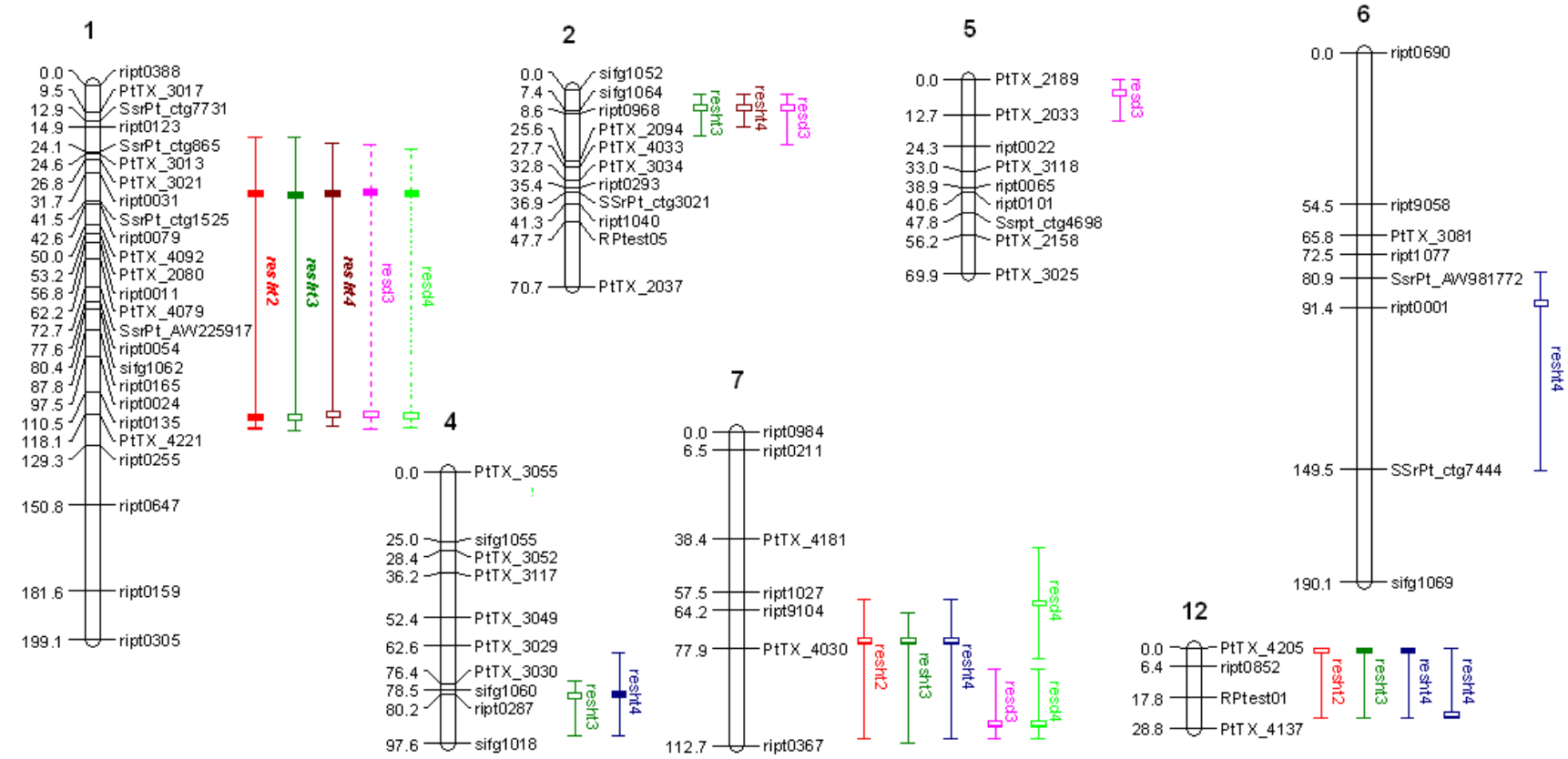

Figure 4.7 The QTLs were detected and verified by 3 populations and 2 methods at different linkage groups. The red color is for ResHt2, the dark green color is for the ResHt3, the Brown color is for ResHt4, the pink color is for ResD3, and the light green color is for ResD4. The solid squares with color filled in are the QTLs detected and verified by all populations and methods. The open squares without filled color are the QTLs detected by the single point method and interval mapping for QTL detection populations, but were not verified. The lines on each square are the confidence intervals for each identified QTL.

from LAFT_2 and lower extreme samples would come from MS488_1. When the environmental effect was accounted for in the phenotypic data, the environment effect for the residual value was not significant (p=0.39), and the residual value for all environments was comparable. Therefore, all the trait values from different environments can be pooled together for data analysis without inflated error.

A simulation test showed that the required sample size would be 800 if two genotypes and three environments were involved (Weng, 1999). In this study, the sample size for each QTL identification population was less than 200 and 7 environments, 5 traits were involved. The QTL power would be extremely small when the original data was explored. Accordingly, using the residual value would a useful solution to reduce error and increase power.

4.4.2 QTL Methods and Statistical Techniques

Two QTL methods were performed for identifying the QTLs association with early height growth in longleaf x slash pine: the single marker method and the interval method. One advantage of single marker analyses is it can identify the association between QTLs and unlinked markers, e.g. sifg1035 in this study, since it does not require a genetic map. In a sufficient marker-saturated map region (Darvasi et al., 1993) or in designs where recombination has been limited, single marker testing is as good as any other method for finding QTLs one at a time. However, a major disadvantage for the single marker test is that the QTL effect and the location are confounded, and unable to be estimated separately. Hence, the single maker method was used as a rough estimate of QTL location and searches the QTLs for unlinked markers in this study.

Compared to the method of single marker analyses, interval mapping methods have been shown to be more powerful, accurate, and robust to the failure of normality assumption (Lander and Botstein, 1989; Knott and Haley, 1992). The original method implemented in interval mapping was maximum likelihood (ML) in which information on the presence of a

QTL was derived from both the mean difference between the flanking marker genotype classes and the distribution of the trait within each marker genotype class. In 1995, a qualitatively different approach was adapted by Xu and Atchley from a method of human genetics that requires only the estimation of the identity-by-descent (IBD) proportion of alleles shared by pairs of individuals at a map position. For a QTL at this position, high IBD should be accompanied by low phenotypic difference. Such a "random model" algorithm, which models the variance rather than the magnitude of QTL effects, has been implemented for plant and animal designs in the web-based software QTL Express. In outbreeding species, marker-QTL phase relationships are not known a *priori* because of linkage equilibrium. Its advantage over "fixed model" methods are that it requires no knowledge of linkage phase or number of alleles at loci and is readily adapted to complicated pedigree designs (Nelson 2005). At a QTL position, simple regression of phenotypic value on the expectation of the genotype given flanking markers, expressed in the term of additive effect "α" and dominance "d", lead to estimates of these effects (Haley and Knott, 1992; Martínez and Curnow 1992). This method was then extended to outbreeding designs by Haley (1994) and was adapted by QTL Express.

In the process of interval mapping, only the regions of the genome that exceed chromosome wide $P<0.05$ were reported**.** Empirically derived significance thresholds for all traits from 1,000 permutations were found to be quite similar (data not shown) for a given chromosome. Therefore, the same threshold was used for all traits on a given chromosome. As suggested by Thomsen et al. (2004) for the parent of origin models, significance tests with the same degrees of freedom had similar significance thresholds, so empirically derived thresholds for 1-QTL vs. no QTL were used for 2-QTL vs. 1-QTL. In addition to chromosome-wise significance thresholds, experiment-wise significance thresholds were also obtained by the option of an experimental-wide module in QTL Express (de Koning et al.,

2001). The threshold obtained by chromosome-wide P<0.05 (suggestive level) and P<0.01 (significantly level) correspond approximately to experimental-wide significant levels of 0.6 and 0.12, respectively, following by the Bonferroni correction (Lynch and Walsh, 1998). Therefore, it is possible that some QTLs identified by chromosome-wide threshold are false positive, but are reported to the mapping community as recommended by Lander and Kruglyak (1995).

4.4.3 QTL Detection Population and Verification Population

QTL location by whatever method involves scanning each chromosome for the most likely position of the QTL. This inevitably implies that a large number of possible positions are tested and those whose likelihood of containing a QTL exceeds some critical value are accepted. To avoid too many false positives, the test probability level is adjusted downwards to allow for the multiple tests, and obviously, this has the concomitant result of increasing the probability of false negatives. The only real solution to the problems of QTL location in segregating populations is to repeat experiments using a completely different sample of genotypes derived from the same population. Thus, two phases of QTL identification strategy was used in this study.

After the QTLs were detected by the first population, another QTL identification procedure was then performed for verifying whether there are QTLs in the second population located at the position identified in the first. Using more than a single family for QTL mapping may reduce a type II error caused by homogeneous parents being sampled. This strategy was also applied by Groover et al., (1994), Knott et al., (1997), Sewell et al., (2000, 2002), and Brown et al.,(2003) in early QTL mapping studies for wood quality in loblolly pine. Devey et al. (2004) also used 6 related radiata pine full-sib families to detect and independently verify QTLs for resistance to Dothistroma needle blight.

In the QTL detection population (Phase I), a non-random selection of samples can increase the statistical power for detecting QTLs responsible for the trait used as the selection criterion. However, caution must be taken about the statistical inference space of the parameter estimation. Because of the non-random selection, estimation of the QTL effect is biased, can only be inferred upon the selective trait, and may not be helpful in detecting QTLs responsible for other traits. The residual value of Ht3 was used as the criterion for selective genotyping. The correlation test of the trait has shown that ResHt3 has high correlation with all the other traits, which may reduce the inaccuracy of detecting QTLs on other traits. Verification of QTL was based on the repeated detection of QTL among populations, as well as among multiple growing seasons for each population (Brown et al., 2003). In the study, a randomly selected sample was used as the non-biased verification population. Compared with the bi-modal distribution of the phase I populations, all the traits for the QTL verification population were normally distributed. Also since the verification population do not infer upon any specific traits, the power of QTL identification for all traits were the same. Therefore, the QTL verification is essential to substantiate a biological basis for observed marker-trait association, to provide precise estimates of the magnitude of QTL effects, and to predict whether a QTL will be expressed at a given developmental age (Brown, et al., 2003).

However, give the allogamous reproductive system and recent domestication, pines are characterized by high levels of genetic diversity and low level of linkage disequilibrium (LD) (Reviewed by González-Martinez et al 2006). Together with its perennial characteristics, pines are generally assumed to contain QTL with low stability across genotypes and environments. In this study, only small fraction (33%) of the detected QTLs is common across two populations. This phenomenon was also reported by Brown et al. (2003)

and Devey et al. (2004 a, b). An unusual observation made by Kaya et al. (1999) was a failure to find any QTLs shared between pedigrees.

4.4.4 QTLs Associated with Growth Traits

In the QTL detection population, 3 QTLs were detected for association with ResHt2 and explained 12.4% of the phenotypic variance, in which 2 QTLs were located on linkage group 1, and 1 QTL was located on linkage group 7. In the QTL verification population, two QTLs located on linkage group 1 were verified, while the QTLs located on linkage group 7 failed the verification. Instead, a QTL located on linkage group 12 was found to be associated with ResHt2 in verification population and the 3 QTLs explained 8.04% of the phenotypic variance. This result from QTL verification population was similar to the results from single marker regression. From Figure 4.4 E, I found there was a peak for all traits at the marker interval PtTX_4205-ript0852 on linkage group 12 for QTL detection population. However, only the QTL for ResHt4 have reached the criterion value, and as seen in figure 4.6 F, all the height growth traits have reached the criterion. The different QTLs identified from different families were observed in other research (Devey et al., 2004a, 2004b, Kaya et al., 1999). In this research, the QTL peaks for ResHt2 existed for both populations on linkage groups 7 and 12, while the test statistic was not significant and did not reach the chromosome-wide criterion, although it was very close to the criterion. It is possible that potential QTLs exsited at these locations. A similar pattern was also found for other traits. This may be related to the small sample size used in our study and relatively low heritability of EHG. Beavis (1995) found that among factors influencing QTL detection powers, small sample sizes (less than 200) and low trait heritability were shown to cause an overestimation of QTL effects, underestimation of QTL number, and hamper detection of QTLs with low effects (Soller and Genizi, 1978; Weller et al., 1990; Knott and Haley, 1992; van der Beek et al., 1995; Knott et

al., 1996). In this study, 305 backcross progenies were used for QTL identification, but they were split into two QTL populations, which lead to less than 200 samples for each population.

Weng et al. (2002) found that the variance explained by major QTLs decrease over time, suggesting the increased complexity of quantitative traits with the aging of the tree. However, in this study, opposite results were observed for height growth. In both populations, both the accumulative variance explained by QTL and the mean value for later height growth (i.e. ResHt4) were greater than those in early height growth (ResHt2 and ResHt3). It may have been caused by increased numbers of QTLs for the later growth trait in this study. In Weng's study, the QTLs identified were 3, 1, and 1 for each height growth variable, while in this study, numbers of QTLs identified for were 3, 4, and 6 for QTL detection population and 3, 2, and 4 for QTL verification population. Another reason for these results may have been caused by the environmental stress. Most measurements of Ht4 and D4 in our QTL population were taken at Year 2006, which was the year after Hurricane Katrina (Year 2005). During Katrina, many trees were bent and broken (Figure 4.7) and it was possible that some genes were activated during the recovery process under the growing stress. There was only weak evidence of QTL stability during the three years of growth under this study. Individual QTLs became active at various stages. However, some consistency (i.e. the interval ript0031-SsrPtCtg 1525) was observed for QTLs involved in both height growth and diameter growth, which was active at the 2nd year and still detectable at the 4th year and was potentially a major effect gene controlling EHG. Gwaze et al. (2003) also reported the detection of a similar QTL region at different maturation stages in loblolly pine. However, the ontogenenic effects is a more common phenomena in pines (Plomion, et al., 1996; Emebiri, et al., 1997; Sewell et al., 2000; Pot et al., 2004) suggests different genomic regions control the quantitative traits and explain the low QTL stability across the matuartion stage.

Figure 4.8 The Hurricane Katrina Damaged Tree Recovery in Jan, 2006.

As a summary, 4, 6, 6, 5, and 4 QTLs were detected and 2, 2, 3, 1 and 1 QTLs were verified for ResHt2, ResHt3, ResHt4, ResD3 and ResD4. The results were close to the numbers of QTLs identified by Weng (2002), but it was less than expected. Previous studies suggested that there may be as many as 5 major QTLs controling EHG (Nelson, et al., 2002). In his paper, Weng gave a detailed description for the reasons for detecting few QTL. Beside them, another reason for the low numbers may relate to the DNA location. It is difficult to distinguish two QTLs that are less than 20 cM apart, even with a QTL of moderate heritability, and consequently, two or more QTLs within this interval may be misinterpreted as one (Lebreton et al., 1998). This can result in a large ghost QTL being located between the two true QTLs if they are linked in coupling (Martinez and Curnow, 1993) and possibly no QTL being identified if they are linked in repulsion. Either way, one is misled both in the location and in the size of the QTL effect. For linkage group 12, two QTLs were identified that were the opposite of estimates for ResHt2, however, the QTLs failed to be detected at the QTL detection population. Similarly, the two QTLs for ResHt3 with opposite estimates detected by the simple marker method also failed to be detected by interval mapping.

As discussed in Chapter 3, the integrated linkage map used in QTL identification has discrepancies with the linkage map built by Echt et al. (In preparation) for loblolly pine. Given the acknowledged genetic conservation between these two closely related pine species, further studies are needed before these QTLs can be applied to MAS.

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CHAPTER 5 CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Conclusions

In this study, 228 SSR markers were first screened against 13 parents, which included 7 hybrid paternal parents and 6 longleaf recurrent maternal parents, and the polymorphism information for each parent were obtained. Then, the available sample sizes and the genetic variances for all the parents were analyzed with two statistical models: separate ANOVA test for each response variable and repeated measurement test for height and diameter measurements. Based upon the results, a half-sib family which included hybrid Derr488 as the common paternal parent and 6 longleaf pines as maternal parents were selected as the mapping population due to its large sample size, number of polymorphic markers, and highest genetic variance.

A SSR-based consensus linkage map was constructed using the data of 135 SSR markers on the 305 backcross progenies using the CP model in the JoinMap (ver. 3.0) program. The individual linkage map from the 6 full-sib families were finally joined by the 'Join' module to build an integrated map for both male and female parents. The results showed that out of the 135 polymorphic markers, 112 of them were grouped to 16 linkage maps and 23 of them were unlinked. The observed genome length was 1874.3 cM $_{(H)}$ and covered 79.85% $_{(H)}$ of the genome length. The 95% expected genome length interval was 1781.3-2411.6 cM $_{(H)}$. The individual QTLs for the 5 growth traits were identified by two QTL methods: single marker regression and interval mapping. For the interval mapping, 305 samples were split into two populations: the QTL detection population and the QTL verification population. There were 2, 2, 3, 1, and 1 QTLs were detected and verified for trait ResHt2, ResHt3, ResHt4, ResD3, and ResD4.

From a breeding perspective, the most reliable QTLs are those that have been consistently detected at different development stages, in different environments, and in

diverse genetic backgrounds (Plomion et al., 2007). In this study, a half-sib family, which included Derr488 as the common paternal parents and 6 longleaf pines as maternal parents, offered the probability to study the QTL in different genetic backgrounds. Two related populations, the detection population and the verification population were used to certify the unbiased verification of the QTLs. The repeated measurements on total height and ground level diameter guaranteed the estimation at different development stages. This is the first SSR-based integrated linkage map and the first application of multiple families QTL identification in longleaf pine genome study.

5.2 Optimum Number of Markers and Minimum Number of Sample Size

The accuracy of locating QTLs is limited by the information, in particular, the number of recombinants gained from observing the genotypic states of the markers. These observed recombinants can be limited by both small sample sizes and missing genotypic data. Statistically, the sample size for a study depended on the magnitude of random error, the magnitude of difference that needs to be detected, the type I error, and the power of detection needed (Kuehl, 1994). Weng (1999) showed that the minimum sample size would be 266 for detecting a QTL effect explaining 5% of the total genotypic variance at 0.005 of type I error and 80% of power of test for the 2 genotypes. If the genotype by environment interaction were involved, the minimum sample size would be 800 for three environments. The sample size of 305 for this study was sufficient for detecting one or two QTL main effects, but far from adequate for detecting QTL by environment interactions.

The density and coverage of linkage maps is another important issue for linkage analysis. The big intervals and 16 linkage groups in this study showed that the linkage map in this study is not complete and more polymorphic markers were needed for filling the interval gap. For a low to moderately dense linkage map, 10-20 cM marker interval is usually assumed as an accepted level and the power of QTL detection and the standard errors of

genetic effect estimates are little affected by the increase of marker density under 10 cM (Lander and Bostein, 1989; Darvasi et al., 1993; Piepho, 2000).

However, increasing both the sample size and numbers of marker would increase the investment on longleaf pine breeding program using MAS strategy. Therefore, a question is "should I genotype more markers on fewer individuals or score more individuals (for genotype and phenotype) with fewer markers?" (Doerge, R. W. et al, 2001). One should find a balance point to satisfy both criterions for a specific experimental design since the optimal numbers of markers determine the distance between the target genes and flanking markers. The optimal distance between target and flanking markers governs the selection intensity that can be exerted (Figure 5.1). With the increase of marker distance, the required sample size is also increasing. Lynch and Walsh (1998) and Liu (1998) present standard closed form calculations for the purposes of evaluating how many markers to genotype relative to the desired QTL location accuracy, and how many individuals to phenotype given a particular significance level, QTL effect and location.

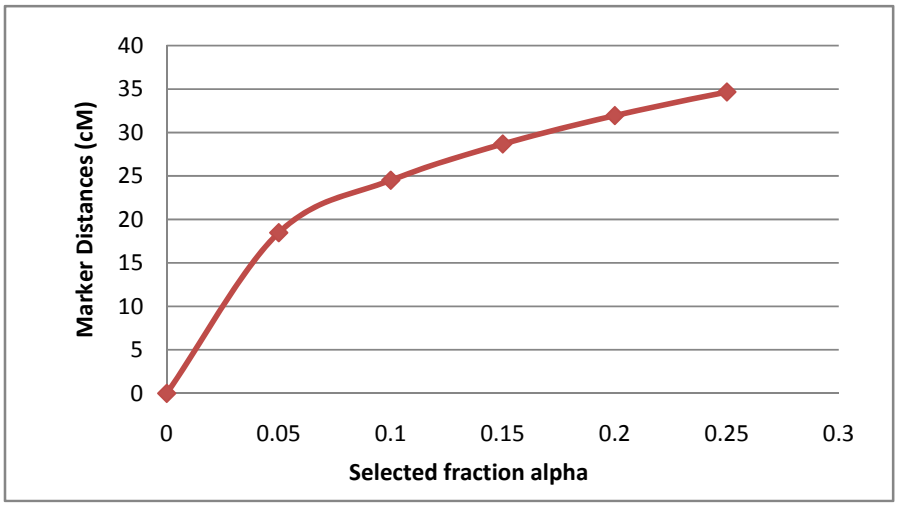

Figure 5.1 Optimal distance between target locus and flanking markers. Model equation: $d = \frac{1}{2}\ln(1 + 2\sqrt{\alpha})$ where α=fraction of selected backcrossing plants and d=distance between flanking marker and target locus (Hospital et al, 1992).The assumption is the population samples is infinity large

In a simulation study for the measurements of outcrossing rate in plant populations, Shaw and Brown (1982) found that the actual level of outcrosing is a major factor in determine the experimental strategy: more loci or more samples. Maximum efficiency for estimation outcrossing in predominantly inbreeding plants comes from large samples assayed for few polymorphic loci, while in contrast, in predominantly outcrossing plants, more loci should be assayed at the expense of sample size for improved statistical efficiency. They also showed that the relative effort required to increasing the number of loci scored is not equaivalent to increasing the sample size and made a conclusion that increasing the number of loci would be more efficient than increasing sample size for outcrossing plants. Therefore, a general conclusion is that the optimum number of marker loci and density of linkage map should be determined first (i.e. about 10 markers with interval 15-20 cM for a linkage group), and then a minimum number of samples can be obtained according to the marker information with the model described by Frisch et al. (1999) if gene by environment effect is not involved. With a fixed probability (i.e. q=95%), the minimum sample size is determined by the distances between the target locus and the two flanking markers when at least one individual should be generated that carries the target gene at both flanking markers. For example, if two distances are 15 cM and 20 cM, then d1=0.15 and d2=0.20, and the sample size for each genotype is estimated with the equation:

$$n = \frac{\ln(1-q)}{\ln\left[1-\frac{1}{8}(1-e^{-2d_1})(1-e^{-2d_2})\right]} = 278$$

5.3 QTL Mapping Approach

Another question involved the QTL method and statistical method used to identify the QTLs. Single-marker analyses is usually used as a means to identify markers by screening large populations for specific traits. Since the QTL effect and location is always confounded by this method, it is usually a rough method to evaluate the QTLs. However, the method can

offer the extra information by identifying markers associated with QTL for unlinked markers. Thus, a quick run of single marker regression before interval mapping analysis is recommended.

Detection of QTLs in outbred half-sib family structures has mainly been based on interval mapping. The results of the tests are expressed as LOD (logarithm of the odds) scores, which compare the evaluation of the likelihood function under the null hypothesis (no QTL) with the alternative hypothesis (QTL at the testing position) for the purpose of locating probable QTLs (Doerge, 2001). However, this method is limited by both the model that defines it as a single QTL method and by the one-dimensional search that does not allow interactions between multiple QTLs to be considered. A regression approach developed by Haley and Knott (1992) is much easier and faster to compute than ML methods and allow more straightforward modeling of a large variety of effects, mating designs, and generation, with usually negligible loss of estimation accuracy and precision. In this research, web-based software QTL Express (Seaton, et al., 2002), was used for interval mapping. A one-QTL model and a two-QTL model were available for QTL identification, which allowed two dimension searches for QTLs.

Statistical approaches for locating multiple QTLs are more powerful than single QTL approaches because they can potentially differentiate between linked and/or interacting QTLs. Multiple-trait QTL analyses will likely become very important for breeding purposes since pine breeding is a multi-trait process (Plomion et al., 2007). Co-localizations and QTL clusters between QTLs for different traits were observed in several forest tree species (Verhaegen, et al, 1998; Brown et al., 2003; Pot et al., 2006) which suggests the effect of pleiotropic genes rather than the existence of physically linked genes controlling different traits. Therefore, in future studies, identifying multiple traits by multiple families in multiple stages and environment are recommended. Considering pine improvement involves the

deployment of many familes or clonal genotype, the genetic stability of marker trait association is a pre-requiste before any extended use of molecular makers is considered in operational breeding program (Brown et al., 2003; Devey et al, 2004; Plomion et al., 2007). Therefore, the QTLs detected in this study must be verified in different experiments, as well as in different genetic and environment backgrounds before the application of MAS in breeding programs can proceed.

5.4 Application of Marker-assisted Selection in Tree Breeding

MAS is one of the most anticipated and frequently cited benifis of molecular markers as indirect selection tools in breeding programs. However, routine implementations of MAS in ongoing plant breeding programs are still scarce. In crop, MAS has been successfully applied in disease resistance (Gebhardt et al., 2004; Hayden et al., 2004), adaptive traits (Saranga et al., 2001; Thornsberrry et al., 2001) and productivity (Dirlewanger et al., 2004). However, the use of MAS to shortcut the long breeding cycles in forest trees was more of a concept than a reality (Bousquet et al., 2007). Improvement of quantitative traits through MAS resulted in variable results ranging from limited success to a few highly successful stories in various plants (Wilcox et al., 1997; Wu, 2002; Semagn et al., 2006). A major constraint to the implementation of MAS in pragmatic breeding programs has been the high relative cost compared to conventional phenotypic selection. For example, in radiata pine, the identification and verification of QTLs involved measurement on nearly 4,435 trees from a single full-sib family (Devey, et al., 2004).

The opportunity for MAS has been evaluated for Douglas fir (Johnson et al., 2000) and radiata pine (Kumar and Garick, 2001), and results indicated that gain from MAS is possible in *P.radiata* for a range of options but marginal for Douglas fir. In their study, Wilcox et al. (2001) showed that even modest gains in physical traits of 3.0 to 3.4% resulted in product value gains in excess of 9% and internal rates of return ranging from 9.1 to 21%.

Given the information above, it is not surprising that relative few breeding programs are actively pursing MAS, particularly for quantitatively inherited characteristics. However, there is still some potential, as some of the above studies have indicated, as well as other possible areas of application yet to be explored (Plomion et al., 2007). In the near future, the new technique, e.g., gene-assisted selection (GAS) which is based on association studies with expressed gene, may supersed MAS (Wilcox, et al., 2007). Compared with MAS which the selection have been largely confined to within-family selection, and neutral markers, the potential of GAS has arisen for identifying and subsequently using expressed polymorphisms that are in strong linkage disequilibrium (LD) with quantitative trait nucleotides (QTN) for both within- and among-family selection (Wilcox et al., 2003, 2006; Wilcox and Burdon, 2006). Because association genetics is still in its infancy stage for forest tree, there are only a few studies have been undertaken which involved detailed specific strategies for incorporation into breeding programs. Wilcox et al. (2006) have a detailed description for the range of application within tree breeding program with GAS. With the advances in expressed genes study over the past 10 years, it is almost possible unrestricted access to any region of tree genome, which makes the GAS very promising in forest tree breeding program. The comparative mapping and LD mapping may be a solution to current breeding problem.

Interest in connifer genomics study continues to increase. Conifer genomics received a major boost recently with the announcement of a $6 million award from the USDA for the Conifer Coordinated Agricultural Project titled “Conifer Translational Genomics Network”. There are many research groups such as federal program (e.g., USDA Forest Service: Pacific Southwest Research Station with Andrew Groover as Project Leader, Southern Institute of Forest Genetics with Dana Nelson as the Project Leader), cooperative tree improvement programs (e.g., the Northwest Tree improvement cooperative at Oregon State University with Keith Jayawickrama as Director, the Western Gulf Tree Improvement Cooperative at Texas

A& M University with Tom Byram as Director, and the North Carolina State University Tree Improvement Cooperative with Steve McKeand as Director) and forest companies (e.g., Weyerhaeuser, International Paper, Arborbon, Cell For).

Information from DNA polymorphisms has a wide range of application, however, only some of the applications have been implemented to date. Some potential applications, such as association genetics and pedigree construction as part of operational testing, are largely in the research and development phase. The lack of uptake across the spectrum of potential of application is likely due to cost, which is particularly important in tree-improvement programs, which usually take years to recoup such costs. Nonetheless, technological advances will increase the scope of applications for tree improvement.

5.5 References

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APPENDIX A: CTAB DNA ISOLATION PROTOCAL

CTAB DNA "Medium" PREP

This procedure was modified by the Stine Lab at LSU for angiosperms from a modification by Rich Jorgensoen (Advanced Genetic Systems, Oakland California) from: Murray, M.G. and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8:4321-4325.

1. Weigh-out 2-3 g of fresh or frozen leaf tissue. Grind tissue in liquid nitrogen until fine powder. Place powdered tissue in a 50 ml beaker containing 20 ml cold Ct Extraction Buffer.

2. Filter through nylon mesh into small (50 ml Oak Ridge) centrifuge tube. Keep tube chilled in ice until next step.

3. Centrifuge in JA-20 rotor: 10,500 rpm (13,200 x g), 15 min, 4°C.

4. Remove supernatant carefully using plastic one-piece disposable pipette. Discard supernatant.

5. Resuspend pellet in 5 ml Ct Wash Buffer.

6. Add 1 ml 5% N-lauroylsarcosine. Mix gently by inversion.

7. Incubate for at least 30 min at room temperature.

8. Add 1 ml 5 M NaCl. Mix gently by inversion.

9. Add 0.8 ml 10% CTAB/0.7 M NaCl solution. Mix gently by inversion. Incubate 10 min at 60°C in the H_2O bath.

10. Add 10 ml cholorform: octanol (24:1). Mix by inversion until emulsion is formed.

11. Centrifuge in JA-20 rotor at 10,000 rpm (12,100 x g), 15 min, and 20 °C

12. Transfer aqueous layer (upper) to fresh 50 ml tube. Place on ice. Add 10 ml cold isopropanol, mix by inverting, and place in –20 C freezer

13. Hook out DNA. If unable to hook DNA, centrifuge in JA-20 rotor at the lowest speed that will pellet the DNA (5,000 rpm, 10 min).

14. Transfer the hooked DNA or precipitate to a new tube (usually a 15 ml Corning tube) containing 5 ml of 76% ETOH/10mM ammonium acetate. Leave for 20 min to overnight, 4°C.

15. Transfer the DNA threads (or precipitate) to a sterile 1.5 ml sterile microfuge tube containing 0.25 ml (250 ul) 1X TE buffer. Refrigerate several hours-to-overnight to allow DNA to fully dissolve in buffer.

16. Check concentration and purity by electrophoresis on 0.8% (w/v) agarose gel.

Table A.1 Solutions for DNA extraction

Content	Amount
CTAB Extraction Buffer	
Tris	0.05 M
Sorbitol	0.35 M
BSA	0.1%
PEG 3350	10%
β-Mercaptoethanol	0.1%
CTAB Wash Buffer	
Tris	0.05 M
Sorbital	0.35 M
β-Mercaptoethanol	0.1%

APPENDIX B: ALLELE FREQUENCY FOR THE POLYMORPHIC MARKERS

Table B.1 The allele frequency test for polymorphic markers form software CERVUS

Locus	FreNull	k	N	HObs	HExp	PIC	HW	OffName
PtTX_2037	-0.0087	8	312	0.705	0.682	0.637	***	PtTX2037
PtTX_2080	0.018	5	274	0.697	0.706	0.658	***	PtTX2080
PtTX_2094	-0.1202	2	312	0.433	0.340	0.282	***	PtTX2094
PtTX_2146	-0.074	2	312	0.442	0.382	0.309	NS	PtTX2146
PtTX_2158	-0.1223	2	312	0.439	0.343	0.284	***	PtTX2158
PtTX_2189	0.1402	3	274	0.453	0.568	0.493	***	PtTX2189
PtTX_3011	-0.0808	4	312	0.689	0.591	0.505	NS	PtTX3011
PtTX_3013	-0.0685	6	312	0.846	0.738	0.695	***	PtTX3013
PtTX_3017	-0.144	2	95	0.505	0.380	0.306	NS	PtTX3017
PtTX_3018	0.1705	5	312	0.372	0.483	0.456	***	PtTX3018
PtTX_3019	-0.1405	2	95	0.495	0.374	0.303	NS	PtTX3019
PtTX_3021	-0.0765	2	312	0.529	0.454	0.351	NS	PtTX3021
PtTX_3025	-0.0511	3	312	0.622	0.562	0.475	NS	PtTX3025
PtTX_3029	-0.0509	7	312	0.801	0.715	0.673	***	PtTX3029
PtTX_3030	-0.0022	6	274	0.708	0.701	0.653	***	PtTX3030
PtTX_3034	-0.0354	7	312	0.769	0.717	0.680	***	PtTX3034
PtTX_3045	-0.0374	9	267	0.869	0.803	0.780	***	PtTX3045
PtTX_3049	-0.0484	3	133	0.707	0.621	0.550	***	PtTX3049
PtTX_3052	-0.0197	2	229	0.507	0.488	0.368	NS	PtTX3052
PtTX_3055	0.014	5	312	0.583	0.595	0.531	NS	PtTX3055
PtTX_3081	0.0137	3	312	0.628	0.610	0.541	***	PtTX3081
PtTX_3105	-0.1092	5	95	0.979	0.794	0.756	***	PtTX3105
PtTX_3107	-0.1501	6	218	0.633	0.514	0.489	***	PtTX3107
PtTX_3116	-0.0785	9	234	0.953	0.824	0.801	***	PtTX3116
PtTX_3117	0.0203	5	218	0.697	0.699	0.653	***	PtTX3117
PtTX_3118	-0.1231	3	175	0.469	0.384	0.347	*	PtTX3118
PtTX_3120	-0.1254	2	312	0.449	0.349	0.287	***	PtTX3120
PtTX_4001	-0.2492	3	39	0.974	0.633	0.555	***	PtTX4001
PtTX_4003	-0.1489	3	93	0.559	0.440	0.391	NS	PtTX4003
PtTX_4011	-0.1568	2	46	0.543	0.400	0.317	ND	PtTX4011
PtTX_4030	-0.0688	9	312	0.913	0.802	0.773	***	PtTX4030
PtTX_4033	-0.0403	6	312	0.724	0.658	0.616	***	PtTX4033
PtTX_4056	-0.1279	2	57	0.456	0.355	0.290	ND	PtTX4056

Notes: FreNull: Frequency of null allele; K: Number of alleles per locus; N: Number of observation; HOBs: Observed heterozygoisty; HExp: Expected heterozygosity; PIC: Polymorphic information content; HW: Significance test of Hardy-Weinberg equilibrium; OffName: the official name used for the SSR markers in collaborative data base.

For H-W: NS = not significant, * = significant at the 5% level, ** = significant at the 1% level, *** = significant at the 0.1% level, ND = not done. These significance levels include a Bonferroni correction.

Table B.1 Continued

Locus	FreNull	k	N	HObs	HExp	PIC	HW	OffName
PtTX_4062	-0.1296	2	39	0.462	0.36	0.292	ND	PtTX4062
PtTX_4079	-0.0356	7	312	0.750	0.692	0.644	***	PtTX4079
PtTX_4092	-0.0712	3	312	0.734	0.625	0.554	***	PtTX4092
PtTX_4093	-0.2512	4	102	0.990	0.651	0.591	***	PtTX4093
PtTX_4114	-0.0774	3	312	0.708	0.601	0.527	***	PtTX4114
PtTX_4137	-0.0676	6	274	0.858	0.747	0.711	***	PtTX4137
PtTX_4181	-0.0363	8	312	0.776	0.720	0.677	***	PtTX4181
PtTX_4205	-0.0924	5	312	0.853	0.712	0.668	***	PtTX4205
PtTX_4221	-0.1519	2	312	0.529	0.390	0.313	***	PtTX4221
PtTX_4228	-0.1575	6	178	0.652	0.529	0.507	***	PtTX4228
ript0001	0.0143	3	312	0.622	0.630	0.553	*	PtRIP_0001
ript0006	-0.1295	5	95	0.989	0.773	0.731	**	PtRIP_0006
ript0011	-0.0546	3	95	0.705	0.616	0.543	NS	PtRIP_0011
ript0022	-0.1561	2	133	0.541	0.396	0.317	**	PtRIP_0022
ript0024	0.0958	6	178	0.624	0.739	0.698	***	PtRIP_0024
ript0031	-0.1041	2	312	0.490	0.399	0.319	**	PtRIP_0031
ript0032	-0.1227	5	227	0.476	0.396	0.368	***	PtRIP_0032
ript0033	-0.1465	2	39	0.513	0.386	0.309	ND	PtRIP_0033
ript0054	0.0270	2	95	0.474	0.503	0.375	NS	PtRIP_0054
ript0064	-0.1316	4	133	0.511	0.423	0.394	*	PtRIP_0064
ript0065	-0.1690	2	57	0.579	0.415	0.327	ND	PtRIP_0065
ript0066	-0.2025	2	46	0.674	0.452	0.347	NS	PtRIP_0066
ript0067	-0.0437	3	272	0.684	0.612	0.534	***	PtRIP_0067
ript0079	-0.1023	7	312	1.000	0.823	0.798	ND	PtRIP_0079
ript0101	-0.1382	2	41	0.488	0.373	0.301	ND	PtRIP_0101
ript0117	-0.0473	7	312	0.801	0.731	0.688	***	PtRIP_0117
ript0123	-0.2583	3	95	0.979	0.624	0.550	***	PtRIP_0123
ript0126	-0.0463	6	311	0.778	0.709	0.662	***	PtRIP_0126
ript0135	-0.0542	9	312	0.885	0.812	0.784	***	PtRIP_0135
ript0165	0.1516	5	157	0.510	0.704	0.661	***	PtRIP_0165
ript0211	-0.0511	6	312	0.776	0.704	0.653	***	PtRIP_0211
ript0255	-0.0213	3	312	0.510	0.476	0.391	NS	PtRIP_0255
ript0263	-0.1141	3	84	0.440	0.370	0.338	ND	PtRIP_0263
ript0287	-0.1304	3	312	0.785	0.606	0.523	***	PtRIP_0287
ript0293	-0.1404	3	119	0.529	0.422	0.378	*	PtRIP_0293
ript0305	-0.1617	2	95	0.558	0.404	0.321	NS	PtRIP_0305
ript0367	0.1253	4	229	0.472	0.597	0.547	***	PtRIP_0367
ript0369	-0.2546	3	55	0.982	0.631	0.555	***	PtRIP_0369
ript0388	0.0082	4	312	0.606	0.606	0.530	***	PtRIP_0388
ript0467	-0.0261	2	95	0.526	0.502	0.375	NS	PtRIP_0467

Table B.1 Continued

Locus	FreNull	k	N	HObs	HExp	PIC	HW	OffName
ript0767	0.1565	3	312	0.462	0.615	0.533	***	PtRIP_0767
ript0647	-0.1377	3	117	0.521	0.419	0.376	*	PtRIP_0647
ript0690	0.0042	5	256	0.711	0.711	0.659	***	PtRIP_0690
ript0791	-0.1347	4	312	0.49	0.374	0.306	***	PtRIP_0791
ript0792	-0.1303	4	124	0.508	0.422	0.394	*	PtRIP_0792
ript0814	0.1184	5	178	0.472	0.595	0.558	NS	PtRIP_0814
ript0852	-0.1296	2	312	0.462	0.356	0.292	***	PtRIP_0852
ript0947	-0.1204	3	312	0.462	0.381	0.348	***	PtRIP_0947
ript0968	-0.0693	6	312	0.913	0.798	0.768	***	PtRIP_0968
ript0984	-0.1243	3	175	0.469	0.381	0.340	*	PtRIP_0984
ript1027	-0.0909	8	312	0.974	0.823	0.799	ND	PtRIP_1027
ript1040	-0.0998	7	312	0.926	0.764	0.725	***	PtRIP_1040
ript1077	-0.1653	2	95	0.568	0.409	0.324	*	PtRIP_1077
ript9058	-0.1127	3	312	0.580	0.468	0.409	***	PtRIP_9058
ript9104	-0.1382	2	41	0.488	0.373	0.301	ND	PtRIP_9104
RPTest01	-0.1294	3	202	0.490	0.397	0.357	**	RPtest01
RPTest05	-0.1488	2	102	0.520	0.387	0.311	NS	RPtest05
RPTest06	-0.1653	2	95	0.568	0.409	0.324	*	RPtest06
RPTest09	-0.0658	4	312	0.554	0.474	0.417	NS	RPtest09
RPTest15	-0.1284	3	229	0.480	0.386	0.342	***	RPtest15
sifg1003	-0.1244	2	312	0.446	0.347	0.286	***	PtSIFG1003
sifg1004	-0.1328	2	312	0.471	0.361	0.295	***	PtSIFG1004
sifg1008	-0.0986	2	312	0.365	0.299	0.254	*	PtSIFG1008
sifg1018	0.0544	2	312	0.433	0.483	0.366	NS	PtSIFG1018
sifg1024	-0.1322	3	312	0.484	0.380	0.327	***	PtSIFG1024
sifg1035	-0.1423	2	312	0.500	0.376	0.305	***	PtSIFG1035
sifg1036	0.0403	3	312	0.548	0.549	0.485	***	PtSIFG1036
sifg1052	-0.1333	2	55	0.473	0.364	0.296	ND	PtSIFG1052
sifg1055	-0.0618	3	274	0.609	0.530	0.459	NS	PtSIFG1055
sifg1058	-0.1307	4	272	0.500	0.408	0.373	***	PtSIFG1058
sifg1060	-0.0166	7	312	0.667	0.645	0.580	NS	PtSIFG1060
sifg1061	-0.0318	7	312	0.728	0.677	0.632	***	PtSIFG1061
sifg1062	-0.2573	3	95	0.979	0.626	0.552	***	PtSIFG1062
sifg1064	0.0170	3	173	0.607	0.597	0.522	***	PtSIFG1064
sifg1069	-0.1066	2	41	0.390	0.318	0.265	ND	PtSIFG1069
SsPp_cn524	-0.1514	2	55	0.527	0.392	0.313	ND	SsPp_cn524
Ssrpt_ctg64	-0.1327	4	312	0.510	0.416	0.382	***	Ssrpt_ctg64
SsrPt_AW225917	-0.1545	2	41	0.537	0.397	0.316	ND	SsrPt_AW225917
SsrPt_AW981772	-0.1307	2	312	0.465	0.357	0.293	***	SsrPt_AW981772
SsrPt_Ctg1525	-0.1060	3	312	0.484	0.391	0.335	**	SsrPt_Ctg1525

Table B.1 Continued

Locus	FreNull	k	N	HObs	HExp	PIC	HW	OffName
Ssrpt_Ctg2300	-0.0543	3	312	0.577	0.500	0.44	NS	Ssrpt_Ctg2300
SSrPt_Ctg3021	-0.1452	4	41	1.000	0.757	0.700	ND	SSrPt_Ctg3021
Ssrpt_Ctg3754	0.0517	3	117	0.530	0.578	0.486	NS	Ssrpt_Ctg3754
Ssrpt_Ctg4698	-0.0162	4	312	0.548	0.477	0.43	NS	Ssrpt_Ctg4698
SsrPt_Ctg7024	-0.1575	2	55	0.545	0.400	0.318	ND	SsrPt_Ctg7024
SSrPt_Ctg7444	-0.1177	3	41	0.829	0.654	0.573	NS	SSrPt_Ctg7444
SSrPt_Ctg7731	-0.1223	2	41	0.439	0.347	0.284	ND	SSrPt_Ctg7731
SSrPt_Ctg865	-0.1545	2	41	0.537	0.397	0.316	ND	SSrPt_Ctg865

VITA

Lisha Wu was born in Beijing, People's Republic of China, in October, 1976. After graduation from Hepingmen high school in Beijing in 1994, she enrolled in China Agricultual University (CAU) and earned a Bachelor of Science degree in agronomy in July, 1998. Because of her outstanding performances during the undergraduate study, she was accepted as a master's student in department of agronomy without entrance examination for graduate school. Her research focused on establishing an efficient system for *Agrobacterium tumefaciens*-mediated gene transformation for sweet potato under the instruction of Dr. Qingchang Liu. Upon obtaining a master's degree in June, 2001, she worked in the genetic lab as research associate at the agronomy department. Her research was successful and her results have a patent with IPC Number: A01H 4/00; C12N 1/20; C12N 5/04.

In 2003, she came to Louisiana State University (LSU) and started her doctoral studies in the School of Renewable Natural Resources under the instruction of Dr. Michael Stine and Dr. Thomas Dean. She worked on the molecular breeding for inproving early height growth in longleaf pine, which is a project in cooperation with Southern Institute of Forest Genetics (SIFG), USDA Forest Service. She also enrolled in a dual degree program for experimental statistics at LSU, and received her 2nd master's degree of applied statistics from the Department of Experimental Statistics in May, 2008. Presently, she is a doctoral candidate at the school of renewable natural resources.