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Original article

Molecular dissection of the quantitative inheritance of wood property traits in loblolly pine

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Abstract – Significant progress has been made toward the molecular dissection of the quantitative inheritance of wood property traits in loblolly pine (*Pinus taeda* L.) and several other forest tree species. QTL mapping experiments have been used to reveal the approximate number of genes controlling traits such as wood specific gravity and microfibril angle and the individual effects of these genes on the total phenotypic variance for the trait. These analyses help to define the scope of the challenge to identify genes controlling complex traits. Verification experiments are needed to be certain of QTLs and to determine the effects of environmental variation and differences among genetic backgrounds. Genetic marker by QTL associations might be used for within family marker-aided breeding, although this application will have limited impact on wood quality improvement in pine. New technologies are being used to identify the genes underlying QTLs. Candidate genes can be identified by a variety of approaches such as functional studies, gene mapping and gene expression profiling. Once candidate genes are identified then it is possible to discover alleles of these genes that have direct effects on the phenotype. This will be accomplished by finding SNPs in linkage disequilibrium with the causative polymorphism. Discovery of such markers will enable marker-aided selection of favorable alleles and can be used for both family and within family breeding. DNA marker technologies will complement traditional breeding approaches to improve wood quality in parallel with growth and yield traits.

QTL / wood properties / SNP / marker-aided breeding / loblolly pine

Résumé – Décomposition au niveau moléculaire de l'hérédité quantitative des critères de qualité du bois de pin à l'encens (Loblolly pine, *Pinus taeda*). On a réalisé des progrès significatifs dans le domaine de la décomposition au niveau moléculaire de l'hérédité des critères de qualité du bois de *Pinus taeda* ainsi que de diverses espèces d'arbres forestiers. On a réalisé des essais de cartographie de QTL pour déterminer le nombre approximatif de gènes contrôlant des critères tels que la densité spécifique, l'angle des microfibrilles et pour estimer l'effet de ces gènes sur la variance phénotypique totale de ces critères. Ces analyses aident à définir le champ d'investigation permettant d'identifier les gènes contrôlant des critères complexes. Il convient de procéder à des expérimentations pour vérifier la validité des QTL, pour détecter les effets de variations des facteurs du milieu, et pour apprécier des différences éventuelles dues à la base génétique des populations en cause. La sélection intra-famille assistée par marqueur peut faire appel à des marqueurs génétiques associés aux QTL. Néanmoins cette voie n'ouvre que des perspectives limitées d'application pour l'amélioration de la qualité du bois chez les pins. On fait appel à des nouvelles technologies pour identifier les gènes qui sont à la base des QTL. Toute une série d'approches permettent d'identifier les gènes candidats telles que des études fonctionnelles, la cartographie génique, et le profilage d'expression des gènes. Une fois les gènes candidats identifiés, il est possible de trouver les allèles de ces gènes ayant un effet direct sur le phénotype. Cela sera fait en trouvant les SNP (polymorphisme d'un seul nucléotide) dans les déséquilibres de liaison avec le polymorphisme en cause. La détection de tels marqueurs va permettre la sélection d'allèles favorables pour la sélection de familles et la sélection intra-famille. Les technologies utilisant les marqueurs ADN constituent un appoint aux méthodes traditionnelles d'amélioration de la qualité du bois conduites en parallèle avec celle de la croissance et du rendement.

QTL / qualité du bois / SNP / amélioration assistée par marqueurs / *Pinus taeda*

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1. INTRODUCTION

The genetic improvement of wood property traits is a high-priority for nearly all forest tree-breeding programs. Rapid growth rates in plantation forests lead to higher proportions of lower quality juvenile wood; therefore, there is a critical need to improve wood quality as well as wood quantity. Target wood property traits can vary depending on whether wood is used for solid wood products or for pulp and paper. For example, increasing wood specific gravity and/or decreasing microfibril angle would have a positive effect on lumber strength, whereas decreasing lignin content might increase pulp yield.

A number of physical and chemical wood property traits are targets for genetic improvement, including wood specific gravity, microfibril angle, fiber length, cell wall diameter, cell wall thickness, pulp yield, modulus of elasticity, lignin content and cellulose content. Quantitative genetic inheritance is assumed for all wood property traits; there are no examples of wood quality traits under simple Mendelian control. Although studies are limited, heritabilities of wood property traits are generally quite high [35] suggesting that although genetic control is quantitative, these traits may be controlled by relatively few genes each. What these genes are is completely unknown.

The focus of our research is to identify the genes controlling wood property traits in loblolly pine (*Pinus taeda* L.), the most important timber species in the US. Our initial approach toward discovery of such genes was to use quantitative trait locus (QTL) mapping. Our QTL mapping experiments have provided estimates of the number of genes controlling some of these traits, the relative proportion of phenotypic variance controlled by each gene and the approximate position of these genes in the genome. QTLs, however, are only statistical entities; the genes coding for QTLs remain unknown. The second approach we have taken is to genetically map expressed sequenced tags (ESTs) for genes thought to effect wood property traits to the QTL maps and look for co-location of QTLs and ESTs on the genetic map. The ESTs chosen for mapping generally have a predicted function based on their protein-coding sequence. ESTs mapping near QTLs become "candidate genes" for the QTL. Finally, we are searching for single nucleotide polymorphisms (SNPs) within candidate genes so that SNPs can be associated with wood property phenotypes. Significant associations suggest, although do not prove, that the candidate gene does in fact partially control the quantitative trait. Continued application of these approaches should ultimately identify many of the most important genes controlling wood property traits in loblolly pine and other forest trees.

2. QTL MAPPING APPROACH IN LOBLOLLY PINE

There are four basic components common to any QTL mapping analysis: (1) a mapping population suitable for the experimental design of the study; (2) phenotypic data for the quantitative trait; (3) genetic segregation data from the markers used to monitor inheritance in the pedigree and (4) a statistical method of analysis used to correlate the phenotype with the inherited genotype. Each of these components, as they relate to QTL mapping for wood property traits in loblolly pine, is discussed below.

2.1. Mapping populations

A suitable mapping population must be identified to maximize the chances for detecting QTLs. A QTL can only be detected if it in fact segregates in the mapping population. Thus, at least one parent of the mapping population must be heterozygous for as many of the QTLs that control a trait as possible. Also, the phenotypic variation must be sufficiently large in the mapping population to enable the detection of a significant difference among the progeny classes.

An F_2 pedigree from a highly inbred crop species, such as corn or tomato [8, 24], is most amenable to mapping QTLs. Extreme phenotypes for a given trait can easily be selected from genetically divergent inbred lines that are most likely fixed for QTL alleles of opposite effect. The F_1 progeny generated from crosses among such divergent lines are therefore highly heterozygous for both genetic markers and QTLs.

The three-generation outbred population structure most closely approximates the structure of an inbred F_2 pedigree. Ideally, two crosses are made among four unrelated grandparents, where each mating pair is between individuals displaying divergent phenotypic values for the trait [10]. From each grandparental mating, a single phenotypically intermediate individual is chosen as a parent. Presumably, these intermediate parents are heterozygous for both marker and QTL alleles, and are potentially heterozygous for different allelic pairs that display a divergent phenotypic effect.

Four mapping populations from three-generation pedigrees are currently being used to map QTLs for wood properties in loblolly pine (*figure 1*). The original mapping

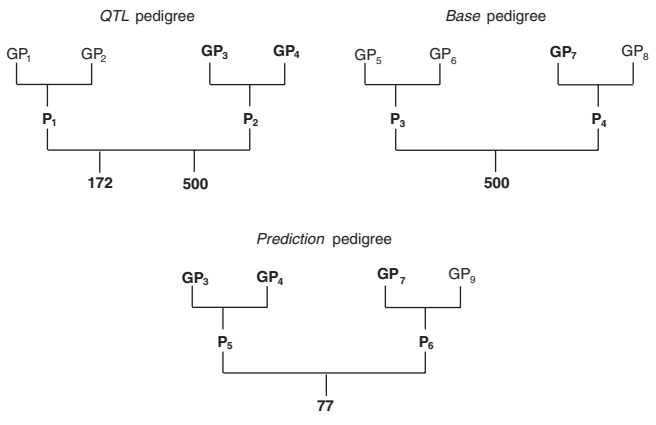


Figure 1. Diagram of the three-generation *P. taeda* pedigrees used in QTL mapping experiments.

population from the *qtl* pedigree (designated as IFGQTL) contains 172 progeny, and is grown at six different sites in North Carolina and Oklahoma [10]. Recently, larger mapping populations of ~500 progeny were generated for both the *qtl* and *base* pedigrees (IFGVEQ and IFGVEB, respectively), and are grown at a single site in North Carolina [4]. The *prediction* pedigree (IFGPRE) consists of 77 progeny, and is related to both the *qtl* and *base* pedigrees. The maternal grandparents of the *prediction* pedigree are the same as the paternal grandparents of the *qtl* pedigree. Therefore the *prediction* mother and the *qtl* father are full-sibs. Also, the paternal grandmother of the *prediction* pedigree is the same as that of the *base* pedigree. The prediction pedigree is grown at two different sites (Arkansas and Oklahoma). Each pedigree was constructed from first-generation selections of the North Carolina State University Industry Cooperative Tree Improvement Program and is maintained by Weyerhaeuser Company.

2.2. Physical and chemical wood property traits

Much of the success of a QTL detection experiment relies on the choice of the phenotypic trait under investigation. A trait controlled by a small number of genes each with a moderate to large effect, which exhibits only a minor influence from the environment (i.e., a highly heritable trait), has the highest chance of QTL detection. However, success in QTL detection does not necessarily equate with success in marker-aided breeding (MAB). Lande and Thompson [15] demonstrated that MAB is most efficient (relative to traditional phenotypic selection) with traits of low heritability. Therefore, for traits where QTL detection is most robust, phenotypic selection is equally effective. This dilemma can be overcome when selection for highly heritable traits is expensive or progress is slow relative to MAB [31]. Wood property traits are generally well suited for testing the efficacy of MAB because of their economic importance, high heritability, relative stability over ages and environments, late assessment of phenotypic value and high cost of phenotypic assay [34].

2.2.1. Wood specific gravity (wsg) and volume percentage of latewood (vol%)

Wood specific gravity is a measure of the total amount of cell wall substance in secondary xylem and is defined as the ratio of the density of oven-dry wood relative to the density of pure water at 4 °C [19]. The specific gravity of a given annual ring is a function of cell size and cell wall thickness. Both of these properties are heavily dependent upon whether the cells were differentiated during the development of earlywood or latewood. Earlywood is typically composed of large-diameter, thin-walled xylem cells, whereas latewood is typically composed of smaller, thicker-walled xylem cells. Therefore, the density of each individual annual ring is a direct combination of its three seasonal determinants: earlywood specific gravity, latewood specific gravity, and the relative percentage of each [19]. Wood specific gravity is the most reliable single index of wood quality because it is closely associated with many important wood properties [36, 37]. X-ray densitometry was used to estimate wood specific gravity and volume percentage of latewood from a radial wood core. Assays were made on a ring-by-ring basis for both earlywood and latewood [29].

2.2.2. Microfibril angle (mfa)

Microfibrils are long polysaccharide chains composed of a crystalline cellulose core surrounded by chains of hemicelluloses, which are encased by surrounding lignin and become rigid [23]. Microfibril angle refers to the mean helical angle that the microfibrils of the S_2 layer of the cell wall make with the longitudinal axis of the cell [20]. Lower fibril angles (closer alignment with the axis of the cell) have a positive influence on lumber strength, stiffness, and dimensional stability [19]. The thicker cell walls associated with latewood typically have lower fibril angles, although there is no constant relationship within a tree between specific gravity and fibril angle [19]. X-ray diffraction was used to estimate the average microfibril angle of both earlywood and latewood core sections from individual rings [20].

2.2.3. Cell wall chemistry (cwc)

The major chemical components of the cell wall are the polysaccharide fractions (holocellulose) and lignin. Holocellulose is composed of α-cellulose and a complex mixture of polymers formed from simple sugars known collectively as hemicellulose. The α-cellulose macromolecule is polymerized from thousands of glucose residues to form a highly stable, unbranched polysaccharide [23]. Lignin is derived from the polymerization of three different hydroxycinnamyl alcohols (monolignols): *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monolignols give rise to the *p*-hydroxyphenyl, guaiacyl, and syringyl units of the lignin polymer, respectively [1].

Pyrolysis molecular beam mass spectrometry (pyMBMS) was used to estimate the chemical content of α-cellulose, galactan and lignin from earlywood and latewood fractions [5]. PyMBMS is a high-throughput analytical method that combines a rapid spectroscopic technique with multivariate regression modeling to estimate the content of a particular cell wall constituent [22, 26, 35]. Using pyMBMS, the analysis of a single ground wood sample takes approximately two minutes, compared to traditional analytical methods that generally require several days.

In this study, chemical wood property traits were measured based on chemical content per unit weight rather than content per unit volume or per cell. Since wood is composed of approximately 97% lignin and holocellulose, an inverse relationship necessarily exists for lignin vs. holocellulose content, while the two components of holocellulose

(i.e., α-cellulose and hemicellulose) tend to vary directly [23]. Therefore an observed increase in lignin content could actually be the result of a decrease in holocellulose, or vice versa. As a result, the individual components of cell wall chemistry that were estimated by pyMBMS become an estimate of variation in overall cell wall chemistry, rather than an estimate of variation of the individual components.

2.3. Genetic markers and mapping

There are two important aspects to consider when choosing a genetic marker system for QTL mapping experiments: (1) the outbred nature of forest tree pedigrees and (2) the potential for comparative mapping. First, each parent of an outbred pedigree is typically a different, highly heterozygous individual, where the transmission of up to four different alleles must be followed from the parents to progeny. Therefore, multiallelic codominant markers are best suited to detect the maximum number of polymorphisms found in the heterozygous parents. Second, comparative mapping, both within species and among related taxa, is an important tool for relating results from different mapping experiments. Therefore a subset of the markers used in a mapping experiment should be orthologous across pedigrees and species [3].

The loblolly pine genetic maps used in QTL analyses have been constructed primarily from RFLP (restriction fragment length polymorphisms) markers [7, 10, 28]. Although an efficient method of mapping cDNAs, an RFLP analysis detects all members of multigene families, including pseudogenes [28]. By contrast, ESTP (expressed sequence tagged polymorphism) primers are designed from gene-coding regions and often amplify specific members of multigene families [32]. Because of this specificity, ESTPs are an excellent source of orthologous markers [3].

2.4. QTL analysis

The 4-allele model of an outbred pedigree complicates the analysis of QTLs in forest trees, where a significant difference in phenotypic variation must be detected among four genotypic progeny classes. The problem in implementing this outbred model is that both parents are not heterozygous at every locus. Therefore the four classes are not discernable at every position along a linkage group. However, it is possible to simultaneously utilize the linkage information from markers of all mating types to increase the informativeness at any given position on a linkage group [11]. Consequently, the four genotypic classes of an outbred pedigree can be identified at any given position in the genome, and the interval method can be used in a QTL analysis under an outbred model [14].

Traditional methods of estimating gene action under a two-allele model do not apply to an outbred pedigree. However, QTL results from an outbred analysis can be reported in terms of the individual parental effects and an interaction effect (*table I* [14]). For example, the maternal effect measures the difference in effect of each maternal allele against the background of the paternal alleles. The interaction effect measures the deviation from additivity, where a value of zero indicates complete additivity (although this measurement is only valid if both parents are heterozygous at that QTL).

Table I. Model used to test the effect of QTL alleles [14].

Parental cross	$Q_1Q_2 \times Q_3Q_4 \rightarrow Q_1Q_3$, Q_1Q_4 , Q_2Q_3 , Q_2Q_4
Maternal effect	$= (Q_1Q_3 + Q_1Q_4) - (Q_2Q_3 + Q_2Q_4)$
Paternal effect	$= (Q_1Q_3 + Q_2Q_3) - (Q_1Q_4 + Q_2Q_4)$
Interaction effect	$= (Q_1Q_3 + Q_2Q_4) - (Q_1Q_4 + Q_2Q_3)$; where Q*i* = QTL allele

3. PHYSICAL AND CHEMICAL WOOD PROPERTY QTLS IN LOBLOLLY PINE

Physical and chemical wood property traits have been analyzed for the presence of QTLs in the original *qtl* pedigree [29, 30]. Phenotypic data included rings 2–11 for *wsg* and *vol%*, rings 3, 5, and 7 for *mfa*, and ring 5 for *cwc*. Both earlywood and latewood were assayed for each trait. The outbred model for QTL analysis described in [14] was used to search the progeny population for significant associations among genetic markers and trait data. Each physical wood property trait (i.e., *wsg*, *vol%* and *mfa*) was analyzed as a composite trait (i.e., an average of individual-ring traits) and as an individual-ring trait. Composite traits were considered a more accurate measurement of phenotypic variation because they represented variation over a longer length of time.

3.1. Number and effect of QTLs associated with wood properties

Nine unique QTLs were detected from composite traits for *wsg*, five for *vol%*, and five for *mfa* (*figure 2*). Each of these composite trait QTLs were also supported by individual-ring QTLs, except for *vol%*_2.1, *vol%*_5.7 and *wsg*_14.1. Additional unique QTLs were also detected for individual-ring traits (*figure 2*). Eight unique *cwc* QTLs were identified from multiple chemical wood property traits (*figure 2*). The residual variance explained by each QTL ranged from 5.4 to 15.7% for *wsg*, 5.5 to 12.3% for *vol%*, 5.4 to 11.9% for *mfa* and 5.3 to 12.7% for *cwc*.

Fourteen of the 27 composite trait QTLs (two for *wsg*, four for *vol%*, three for *mfa*, and five for *cwc*) exhibited a strong non-zero interaction effect, which suggests some degree of non-additive expression (i.e., dominance or epistatis) for alleles at these QTLs. Of the remaining 13 composite trait QTLs, only one QTL for *wsg* and two for *cwc* exhibited a weak or zero interaction effect in conjunction with possible evidence that both parents are heterozygous. This combination

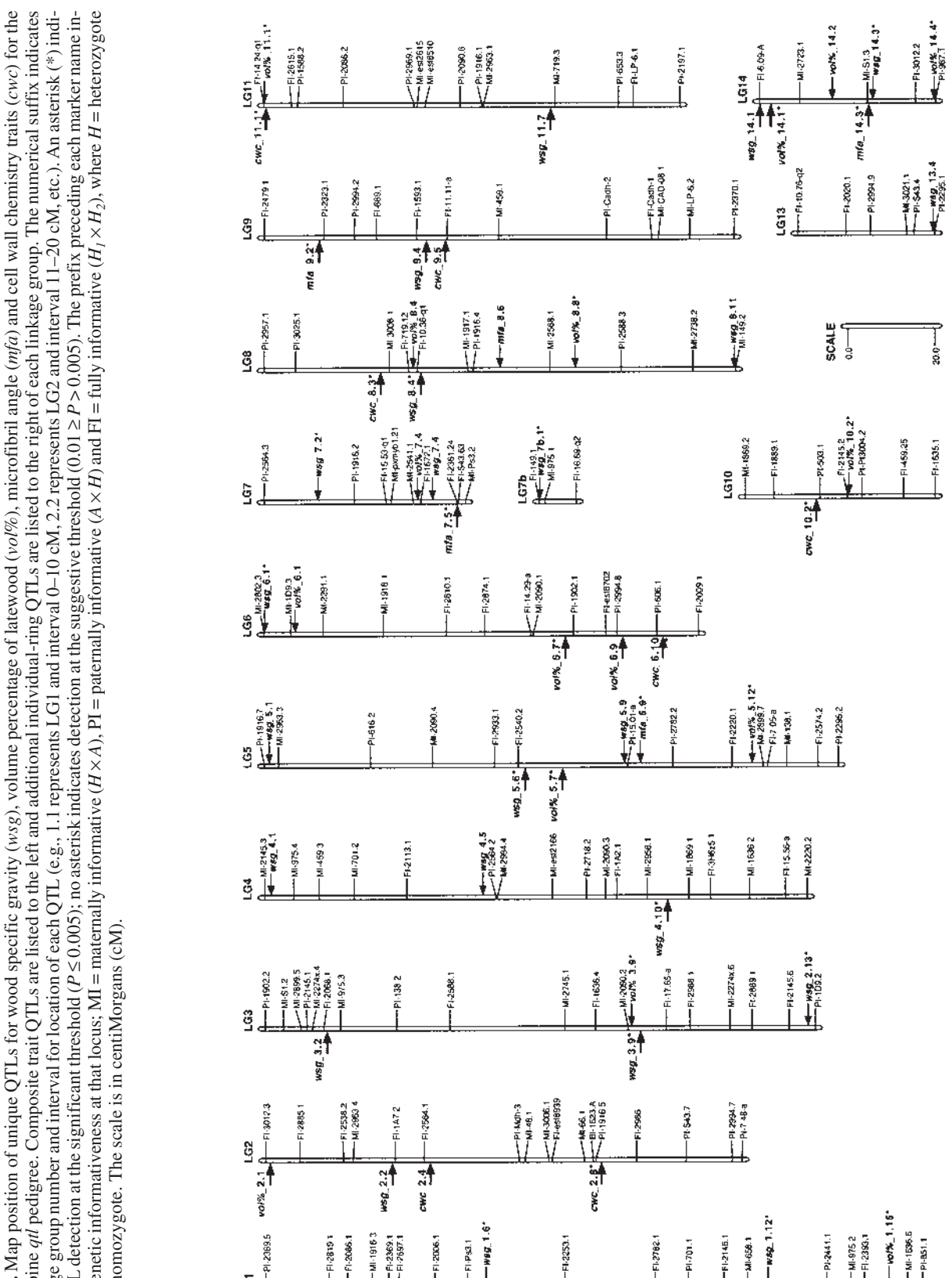


Figure 2. Map position of unique QTLs for wood specific gravity (*wsg*), volume percentage of latewood (*vol%*), microfibril angle (*mfa*) and cell wall chemistry traits (*cwc*) for the loblolly pine *qtl* pedigree. Composite trait QTLs are listed to the left and additional individual-ring QTLs are listed to the right of each linkage group. The numerical suffix indicates the linkage group number and interval for location of each QTL (e.g., 1.1 represents LG1 and interval 0–10 cM, 2.2 represents LG2 and interval 11–20 cM, etc.). An asterisk (*) indicates QTL detection at the significant threshold ($P \leq 0.005$); no asterisk indicates detection at the suggestive threshold ($0.01 \geq P > 0.005$). The prefix preceding each marker name indicates genetic informativeness at that locus; MI = maternally informative ($H \times A$), PI = paternally informative ($A \times H$) and FI = fully informative ($H_1 \times H_2$), where H = heterozygote and A = homozygote. The scale is in centiMorgans (cM).

provides potential evidence for additive expression at only these three QTLs. Therefore, the majority of the wood property QTLs exhibited some level of non-additive expression.

3.2. Temporal and environmental expression of QTLs associated with wood properties

Given the substantial genetic diversity within and among forest trees, and the variety of conditions in which they are grown, it is important to understand the stability of QTL expression over time and space. Even within a single site, genotype × environment (G × E) interactions will likely affect the temporal expression of QTLs. Long-lived trees also experience different developmental stages of growth (e.g., the change from juvenile to mature wood), which are likely controlled by different sets of regulatory factors.

A temporal dissection of QTL expression may provide insights as to how trees achieve their mature phenotype. For example, the physical wood property traits were analyzed over multiple growing seasons, and a subset of QTLs was consistently detected over that time. Other QTLs were detected only during a single year. For example, QTL *wsg*_4.10 appears to be consistently expressed over the duration of study, whereas QTL *wsg*_5.6 appears to be expressed only during the later stage of growth and is possibly associated with the onset of the development of mature wood.

In addition, significant differences in wood chemical contents were observed among the populations from North Carolina vs. Oklahoma. QTL × E analyses provide evidence that QTLs also interacted with environmental location. Four QTL × E interactions were detected for multiple cell wall chemistry components, two of which co-mapped with previously detected QTLs (*cwc*_6.10 and *cwc*_8.4).

3.3. Genomic distribution of QTLs associated with wood properties

A number of studies in forestry have used the same mapping population to identify and map QTLs for multiple traits. In several of these studies, QTLs for different traits have been mapped to the same genomic location [27]. For many of these QTL clusters, the traits exhibited a high degree of phenotypic correlation and similar allelic effects. This combined evidence suggests that pleiotrophy of a single QTL, rather than simple linkage among two QTLs, may likely explain these correlations [2].

Several chemical wood property QTLs co-mapped with QTLs for physical wood property traits. For example, *cwc*_1.5 and *mfa*_1.5 both mapped to approximately 45 cM on LG1. Even though both of these traits are associated with microfibrils, there is little phenotypic correlation ($-0.13 \leq r \leq 0.11$) and little congruence, either positive or negative, among the QTL effects for these traits. Similar observations are found among QTLs for *cwc* and *wsg* and *vol%*, supporting the hypothesis that different QTLs are represented in these QTL clusters.

4. QTL VERIFICATION

A large number of QTL mapping experiments in forest trees have been reported in recent years [27]. QTLs have been mapped for a variety of growth, yield, wood property, adaptive and disease resistance traits. In very few cases, however, have QTL verification tests been performed, making it almost impossible to assess the reliability of reported QTLs. The simple solution to such a dilemma is to add replication to all QTL mapping studies. Largely due to the significant costs associated with marker genotyping, cloning and phenotyping of some traits, replication is not part of most QTL experiments. Until replication becomes a standard aspect of QTL mapping, it is still possible to achieve some level of verification by comparing the non-replicated studies with one another. This assumes, however, that QTL maps among crosses or among species can be directly compared, which to date in forest trees is usually not possible. In this section, we briefly describe our efforts to develop comparative maps in conifers and how such maps can be used to verify QTLs.

4.1. Comparative mapping in conifers

Comparative maps among crosses and related tree species can be constructed by mapping orthologous genetic markers, such as RFLPs and ESTPs, to individual species maps. Comparative maps among crosses within *P. taeda* have been constructed [28]. An international collaboration, called the Conifer Comparative Genomics Project, has been formed to construct comparative maps among pines, spruces, firs and other conifers. Orthologous RFLP and SSR (simple sequence repeat) markers were used to construct comparative maps between *Pinus taeda* × *P. radiata* [6], whereas ESTP markers were used to create comparative maps between *P. taeda* and *P. elliottii* [3] and between *P. taeda* and *P. pinaster* (Chagné and Brown, unpublished). Comparative mapping in conifers has lead to identification of homologous linkage groups and soon it should be possible to associate linkage groups with individual chromosomes. Comparative genome analysis, including QTL verification, is now possible in conifers.

4.2. Comparative QTL mapping

Comparative mapping can be used to verify QTLs at many levels. Some comparisons are of basic biological interest whereas others have important consequences for the application of marker-aided breeding. QTL verification can be assessed in several ways: (1) among test environments; (2) among years; (3) within families; (4) among related families; (5) among unrelated families and (6) among species.

4.2.1. Among test environments and years

We discussed temporal and spatial variation in wood specific gravity QTL expression in *P. taeda* in an earlier section. Some QTLs were detected in nearly all rings (years), whereas some were detected only in one ring. Those expressed in all rings can be considered as verified QTLs but those expressed

in only one ring could easily be false positives. Likewise, not all QTLs were expressed in all environments, which could be due to lack of repeatability in detection or might be real QTL × E interactions. The effect of test site and year of measurement can be more precisely estimated if a clonal mapping population is used. We have conducted large QTL mapping experiments in *Pseudotsuga menziesii* for bud phenology and cold-hardiness traits using clonal mapping populations [12, 13]. Results of these studies show high repeatability of QTL expression among years within test environments but low repeatability among test environments. Although it is still difficult to generalize, it seems that QTL verification among years can be expected but will be difficult to establish among test environments.

4.2.2. Within families

Within family QTL verification can be accomplished using randomized and replicated field test designs in QTL mapping experiments. As noted previously, this is rarely done in forest tree experiments. An alternative is to compare QTL mapping results from the same mapping population where different progeny are tested at different test locations. Such a comparison confounds the effect of test site, but does provide some indication of within family verification. A comparison of results between the IFGQTL and the IFGVEQ experiments (*figure 1*) is one such test. Twenty-six percent (26%) of all QTLs detected were common to both experiments, whereas 48% were unique to the IFGQTL experiment and 26% were unique to the IFGVEQ experiment (*table II*). This is a surprisingly high percent of QTLs in common given our earlier conclusion regarding detecting the same QTLs in different environments. We expect that within family QTL repeatability would be nearly 100% if tested in the same environment. An example of some common QTLs were those for earlywood specific gravity at the top of linkage group 5 and volume percent latewood near the middle of the linkage group 5 (*figure 3*).

4.2.3. Among related families

We conducted an experiment to determine if the same QTLs could be detected in closely related families. The IFGQTL and IFGPRE experiments had two of four grandparents in common (*figure 1*). The paternal parent of IFGQTL and the maternal parent of IFGPRE were full-sibs. Even though IFGQTL and IFGPRE were planted at different test locations, 43% of the QTLs detected were common to both families (*table II*). QTLs for wood specific gravity and volume percent latewood on linkage group 5 are some of the QTLs common to both families (*figure 3*).

4.2.4. Among unrelated families

A concern often voiced by tree breeders is that QTLs detected in one family might not be found in other unrelated families. This concern can not be adequately addressed until QTL detection experiments are performed in large numbers of families in replicated tests (such as diallels), which is a very costly undertaking. In the interim, small comparisons can be made, such as results from the IFGVEQ and IFGVEB experiments. These families were planted at the same test site and phenotypic measurements were made simultaneously. Nevertheless, only 16% of the QTLs were common to both families (*table II*). One explanation for this could be that the

Table II. Percent of all wood property QTLs unique to individual experiments versus those common to pairs of experiments. See *figure 1* for pedigrees for each experiment.

IFGQTL	IFGPRE	IFGVEQ	IFGVEB	Common
48%	–	26%	–	26%
32%	25%	–	–	43%
–	–	68%	16%	16%

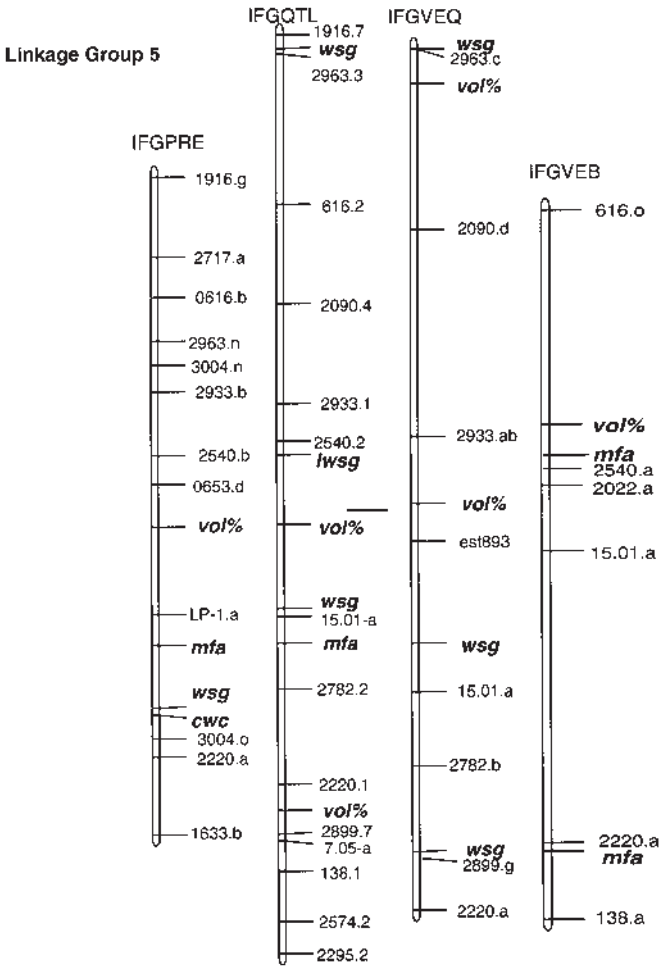


Figure 3. Comparative maps of linkage group 5 for four *Pinus taeda* experiments (IFGQTL, IFGPRE, IFGVEQ and IFGVEB). Wood property QTLs are shown in italics, e.g. wood specific gravity (*wsg*), percentage volume of latewood (*vol%*), microfibril angle (*mfa*), and cell wall chemistry (*cwc*).

IFGQTL family was selected because it was expected that wood specific gravity QTLs would segregate in this family [10], whereas no similar expectation was made about the IFGVEB family. These results suggest that QTLs segregating in multiple families may be less frequent.

4.2.5. Among species

Comparative maps between species will enable extending QTL verification to cross-species comparisons. Comparative maps between *P. taeda* with *P. elliottii*, *P. radiata*, *P. pinaster* and *P. sylvestris* are all under construction and these maps will have wood property QTLs. Detection of common QTLs across several species will provide another form of QTL verification.

5. CANDIDATE GENES, SNPS AND ASSOCIATION TESTS

Successful QTL detection and verification provides the opportunity for MAB. However, application will be limited to within family breeding in forestry due to linkage equilibrium between markers and QTLs in non-structured populations. In addition, within family MAB itself will be limited since QTL detection experiments require within family phenotypic evaluation of progeny, in which case, selection based on markers is no longer necessary. Therefore, MAB within families will only be useful when parent trees are remated, and early marker-selections are entered into a clonal propagation program (e.g., somatic embryogenesis).

If the genetic distance between a marker and a QTL were minimized (thereby increasing the opportunity for linkage disequilibrium), greater genetic gains would be realized through family selection using MAB. This will be achieved once the actual genes (or subset of such genes) controlling a quantitative trait are identified, and single nucleotide polymorphisms (SNPs) are discovered to detect alleles for these genes. Breeders can then apply selection directly at the allelic level, regardless of pedigree or family relationships.

One approach to identify such genes is a "candidate gene" analysis. Candidate genes (i.e., genes that putatively affect trait expression) can be identified when sufficient information is known about the regulatory or biochemical pathways associated with trait expression [16]. DNA sequences for candidate genes can be obtained from gene databases [25]. Alternatively, candidate genes can be identified from coincidental location with QTLs on well-characterized genetic maps (*figure 4*). The challenge is to identify DNA polymorphisms within candidate genes that will distinguish alleles and then associate alleles with differences among phenotypes. This can be accomplished through SNP discovery and association studies.

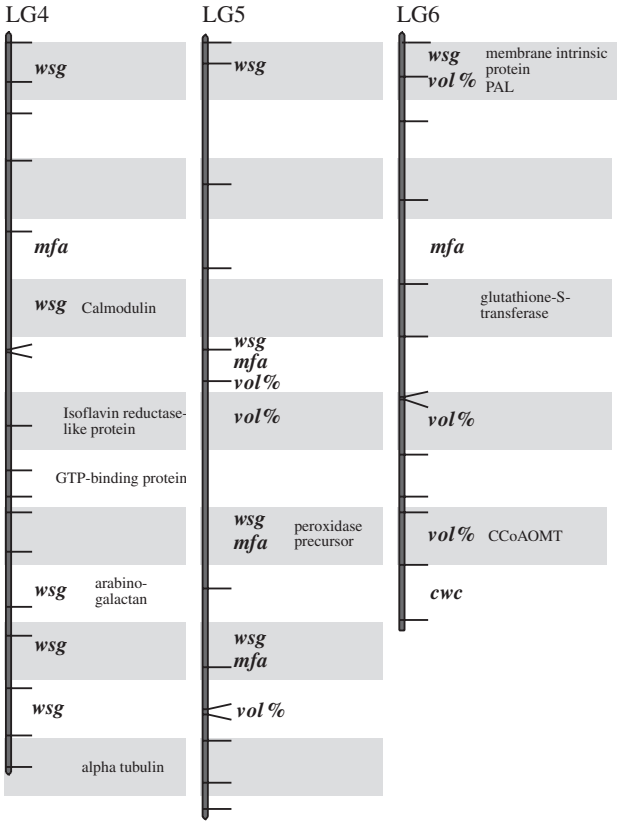


Figure 4. Three loblolly pine linkage groups with candidate genes and QTLs for wood specific gravity (*wsg*), percentage volume of latewood (*vol%*), microfibril angle (*mfa*), and cell wall chemistry (*cwc*).

Association studies are based on the existence of linkage disequilibrium in a natural population between a marker and a quantitative trait nucleotide (QTN) directly affecting the phenotypic value of the quantitative trait. Linkage disequilibrium (LD) is defined as the non-random association of alleles at linked loci and results from the two sites only rarely recombining from each other; it is an indirect estimate of how closely two loci are linked on the same chromosome. LD decays with time, and in older populations it is expected to extend over only short distances. For loblolly pine, it can be estimated that half of all locus pairs separated by physical distances on the order of 1.4 Mbp will show LD(1). Nonetheless, LD is expected to vary among genes and will have to be determined empirically.

(1) A perfect association between two linked loci decays with a "half-life" of $(1 - \theta)^t \cong 1/2$, where θ is the recombination rate and t is the number of generations (adapted from [16]). Approximately 200 generations have passed in the natural population of loblolly pine, based on an estimated 10 000 years since post-glacial recolonization and 50 years per generation. [Although loblolly pine can become reproductively mature before age 20 under open-grown conditions, substantial seed production does not occur under crowded, more typical, conditions until age 25–30. Furthermore, the species requires wind disturbance, such as a hurricane or tornado, for stand renewal – such an event is estimated to recur at any one site at 50 year intervals (Bongarten, pers. comm.)]. Therefore, $(1 - \theta)^{200} \cong 1/2$, and $\theta = 0.0035$ or 0.35 cM. The relationship between genetic and physical map distances in loblolly pine is unknown and is certain to vary both within and among chromosomes. For illustration purposes only, a value of 4Mbp/cM, hypothesized by Neale and Williams [21], was used. Thus 0.35 cM = 1.4 Mbp.

Our approach to conducting association studies in loblolly pine is to identify SNPs within regions of candidate genes implicated in the control of physical and chemical wood properties, to genotype a large number of individuals from the natural population at these SNPs, and to test for SNP by phenotype associations. The elements of each are discussed.

5.1. Association populations

An association population of approximately 500 individuals is sufficient to detect associations between a phenotype and a QTN responsible for 5% or more of the phenotypic variance [17]. Weyerhaeuser Company has provided a population of 475 unrelated first- and second-generation selections with 2 ramets/clone from the range of loblolly pine for this study. The clones are 16–25 years of age and planted at five different test sites in Georgia, Arkansas and Alabama. Increment cores and needle samples have been taken for wood property analysis and DNA extraction, respectively. The physical and chemical wood property traits being analyzed are the same as those described previously under QTL mapping approaches.

Population differentiation in loblolly pine follows the east-west division of the Mississippi River [9]. Admixture in the association population can lead to false positive associations since any wood property trait that is more frequent in one population will be positively associated with any allele that by chance is also more common to that group. Although the majority of genetic variation is found within populations, rather than between populations, the extent of random mating in the association population will also be evaluated.

5.2. Candidate gene identification

Candidate genes influencing wood property traits in loblolly pine are identified by three approaches (*table III*).

(1) Gene homology to identify genes with known roles inferred from functional studies in model species or pines.

(2) Gene linkage to QTL to provide tentative support for the role of a genetically mapped cDNA in determining the observed phenotype.

(3) Gene expression to identify genes that are induced or repressed in tissues and/or at differing times when key physiological events are occurring. Expression data is obtained from two sources: contig assemblies that are abundantly expressed in, or show differential expression between, normal wood and compression wood (http://web.ahc.umn.edu/biodata/nsfpine), and preliminary microarray experiments performed by our collaborators [33].

Table III. Candidate genes involved in wood formation.

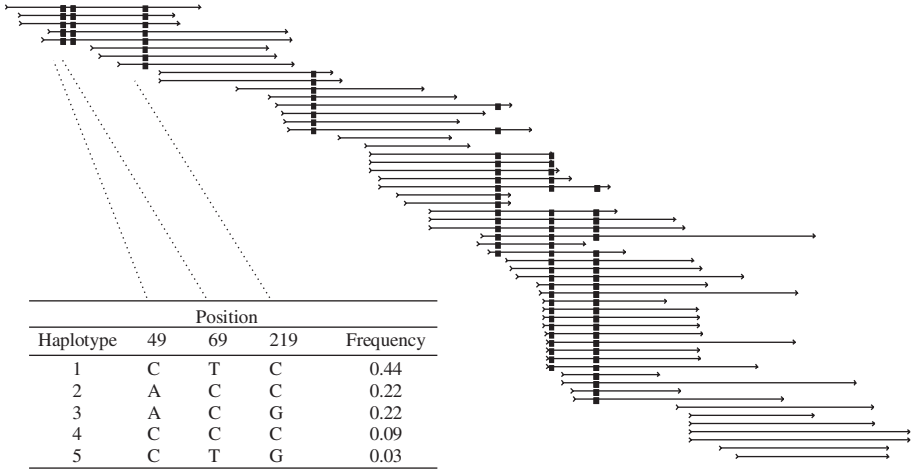
Phenylpropanoid pathway related	Genbank accession	Linkage group / cM
Cinnamoyl alcohol dehydrogenase	AA556583	9 / 88
Cinnamoyl-CoA reductase	AW754917	
CCoAOMT	AAD02050	6 / 92
Caffeic acid OMT	U39301	11 / 85
Diphenol oxidase	AI725182	1 / 84
4-coumarate CoA ligase	T09775	
Cinnamic acid 4-hydroxylase-1(2)	AA556362	3 / 70 (10 / 17)
SAM synthetase 2	AI725188	8 / 97
SAM synthetase	AI812759	3 / 116
SAM decarboxylase	AA556889	
S-adenosyl homocysteine hydrolase	O23255	11 / 3
Glycine hydroxymethyltransferase	AI812891	3 / 75
Isoflavone reductase-like	AA556842	4 / 65
PAL	AI813128	6 / 10
Phenylcoumarin reductase	AA556450	
Cell wall related		
Beta 1,3 glucanase	AA556234	8 / 55
Cellulase – cel2	AA557072	11 / 5
Cellulose homolog	AI812676	11 / 40
Cellulose synthase	AA556746	
Glucosyltransferase	AA556503	14 / 25
AGP6	AF101785	5 / 8
AGP-like14A9	U09556	3 / 78
AGP-like Pt3H6	U09555	4 / 95
Pectin methylesterase	AA557010	
Sucrose synthase	AA556396	
Xyloglucan endotransglycosylase	AA556947	

5.3. SNP discovery and genotyping

SNP allele discovery is conducted by a combination of in silico and de novo methods. The loblolly pine xylem EST databases include sequences from multiple genotypes and thus, inspection of contig assemblies provides a good indication of gene regions where SNPs occur (*figure 5*). In addition, the assemblies facilitate defining gene family members, thus allowing member-specific PCR primer selection. Primers are designed to amplify 500–600 bp from SNP-rich regions of the 5' and 3' ends of candidate genes. DNA samples from a panel of 32 megagametophytes of the association population are then sequenced in the forward and reverse direction for SNP validation.

To date, we have completed SNP discovery in the entire coding sequence of an arabinogalactan gene (AGP6) of loblolly pine, and for approximately 500 bp of 4-coumarate CoA-ligase, two members of the cinnamic acid 4-hydroxylase family, and an arabinogalactan-like gene. On first observation, the range of haplotypes for these five genes within the 32 gametes sampled is remarkable, varying from two for the arabinogalactan-like gene to 16 for AGP6.

We have optimized procedures for SNP genotyping of the entire association population on the Pyrosequencing SNP



Haplotype	Position 49	69	219	Frequency
1	C	T	C	0.44
2	A	C	C	0.22
3	A	C	G	0.22
4	C	C	C	0.09
5	C	T	G	0.03

Figure 5. In silico SNP detection and de novo sequence validation in the coding region of cinnamic acid 4-hydroxylase. The contig assembly detected seven SNPs (black squares). Amplification and sequencing of a 489 bp DNA fragment encompassing the 3 SNPs at the 5' end from 32 megagametophytes of unrelated trees revealed 5 haplotypes. No additional SNPs were found.

detection platform (http://www.pyrosequencing.com). Pyrosequencing is essentially high-throughput "sequencing-by-synthesis", and generates up to 20 nucleotides of DNA sequence around a SNP (*figure 6*).

5.4. Testing for SNP by phenotype associations

There is considerable debate over the power of single-locus versus haplotype analysis in identifying associations between markers and phenotype. Long and Langley [17] showed by simulation that single-marker-based permutation tests were more powerful than haplotype-based tests. However, in some cases, a multilocus/haplotype approach was shown to be more powerful [18].

A major advantage of single-marker based tests is that they do not require haplotypes to be inferred from diploid genotypic data. In its simplest form, a standard ANOVA can be used to determine if significant differences in quantitative trait values exist among SNP genotypic classes. Associations will also be tested for using the diploid marker permutation test [17].

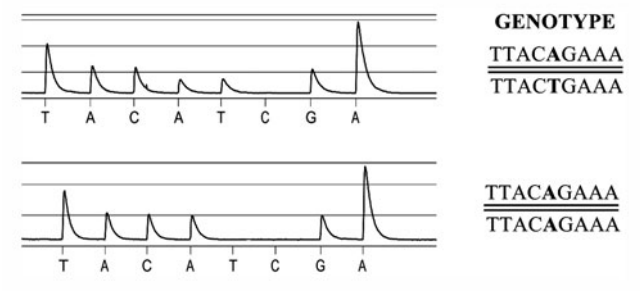


Figure 6. Pyrogram of a SNP in AGP6. Proportional signals are obtained for one, two, or three bases incorporations. Nucleotide addition is shown below the pyrogram and the genotypes of a heterozygote (top) and homozygote (bottom) are noted to the right.

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