APPLICATION OF GENETIC MARKERS TO TREE BREEDING

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Rapid advances have occurred concerning DNA markers in forest genetics: Two years ago, in June 1991, a workshop was organized by G.A. Tuskan and C.G. Williams, at Gatlinburg, Tennessee, on the potential use of genetic markers in tree breeding (Tuskan 1992). Few if any of the attendees would have predicted the rapid advances that have taken place in theory and methodology that can now be applied to genomic analysis of forest trees. The purpose of this paper is to reexamine some of the issues raised at the Gatlinburg meeting in view of this new technology and new theory.

High levels of genetic polymorphism in many forest tree species and the use of PCR based markers make possible moderate density genomic maps for individual trees. Map construction can be completed in weeks instead of years because PCR-based marker analysis has been automated (Nelson et al 1992a). The ability to map virtually any sexually mature tree (with sufficient heterozygosity) provides a basis for the reevaluation of the potential applications of genetic markers for tree breeding, as well as the potential application to studies of genetic diversity and selection in natural stands of forest trees.

Why were we skeptical two years ago? Widespread doubts were expressed about the utility of DNA markers because of both theoretical and technical limitations. Markers were considered to have limitations even in agronomic crops where genetic analysis was more advanced. Forest trees had high levels of linkage equilibrium, precluding predictable associations of markers and QTLs. No QTLs had been demonstrated in any forest tree. Finally, the technology required for mapping was expensive and relatively inaccessible to tree breeders. Strauss et al (1992) stated, in their contribution to the workshop, "we expect that the near term usefulness in most operational tree breeding programs will be limited". Furthermore, variation of quantitative trait effects depend upon genetic background, genotype by environment interaction, and variation over generations. Nonetheless, it was argued that marker aided selection had potential application for weakly heritable traits (Lande and Thompson 1990) where markers can "explain much of the additive variance within families".

Impact of PCR-based markers: Two factors have made it relatively easy to generate genetic maps of individual trees: the technology of PCR based markers, and the high levels of heterozygosity in most forest tree populations.

The first major obstacle to genomic mapping and marker analysis in forest trees was the problem of linkage equilibrium. In the absence of extended pedigrees, inbred lines or specific cultivars or other situations where linkage disequilibrium could be readily established, it was unlikely that specific associations of markers and important traits could be made. Several approaches have been taken to establish linkage disequilibrium for genetic mapping in forest trees.

Neale and coworkers (Neale and Williams 1991) have used a three generation pedigree of loblolly pine directed to the mapping and dissection of quantitative traits such as wood density (Williams and Neale 1992). Bradshaw has analyzed markers in an interspecies cross of Populus *trichocarpa* x *deltoides* (clone H11) (Bradshaw and Stettler 1992). Both Bradshaw and Neale used restriction fragment polymorphisms (RFLPs) as DNA based markers in pioneering studies of genomic mapping in trees. These markers provided a large number of polymorphic genetically neutral markers that made the earliest maps possible. In spite of these advances, most forest geneticists viewed RFLP marker analysis as laborious and limiting. Furthermore, extended pedigrees of adequate size were rare.

The high levels of heterozygosity in forest trees are an advantage for application of molecular markers. The third approach to achieve linkage disequilibrium is the use of individual trees for genetic analysis. This approach was pioneered years ago using isozymes (Conkle 1981) but was limited by the small number of enzymes for which assays were available. Isozyme markers have proved useful for the estimation of genetic variation, levels of heterozygosity, gene flow, genetic mapping and genetic relatedness. In contrast with agronomic crops, high levels of genetic polymorphism and heterozygosity were turned to advantage for genetic analysis of forest trees (e.g. Conkle, 1981; Wheeler et al 1983; Hamrick and Godt 1990; Millar et al 1988).

The advent of PCR based markers: In 1990, two research groups, Welsh and McClelland (1990) and Williams et al (1990) adapted the polymerase chain reaction technique to produce anonymous genetic markers without the requirement for prior sequence information. Williams et al. (1990) called their markers RAPDs for Random Amplified Polymorphic DNA, and Welsh and McClelland (1990) called their system AP-PCR for arbitrarily primed PCR. In both systems a short oligonucleotide primer is used to initiate synthesis at many sites in the genome. If two sites are adjacent and in opposite orientation, amplification of a band will occur, and the length of the band will be defined by the distance between the sites. Products of the amplification are readily detected by gel electrophoresis. Many bands show genetic polymorphism and therefore can be used as genetic markers. Markers can be highly heritable and reliable under well controlled conditions (Tingey et al 1992; Thormann and Osborn 1992; Weeden et al 1992; Caetano-Anollés et al. 1992; Skrotch et al. 1992; Kesseli et al. 1992; Grattapaglia et al. 1992; Gepts et al 1992; Smith and Chin 1992). Careful control of reaction parameters is essential because amplification reactions are extremely sensitive to initial conditions.

The major advantage of PCR based markers was that they did not depend on prior knowledge of DNA sequence and they required only nanogram amounts of DNA. The amount of DNA required for PCR based markers is about 1000 fold less than that required for RFLP analysis (10 micrograms per restriction digest). The low requirement for DNA makes possible analysis of the

haploid tissue in the megagametophyte. In concept, PCR based markers have many of the advantages of isozymes, are more technologically accessible than RFLPs because they do not require cloning or molecular hybridization, and they are potentially automatable. Although the methods are very similar, RAPD markers were used more widely than AP-PCR. Both types of markers were dominant, and differed from RFLP and isozyme markers that were typically codominant. The theory and methodology of RAPD markers has been extensively described, applied and reviewed (Williams et al. 1992, Tingey et al 1992; Thormann and Osborn 1992; Weeden et al 1992; Caetano-Anollé et al. 1992; Skrotch et al. 1992; Kesseli et al. 1992; Grattapaglia et al. 1992; Gepts et al 1992; Smith and Chin 1992).

The question of dominance: Most genetic analysis in plants and animals has been carried out with codominant markers. These types of markers are common in isozymes, RFLPs and many PCR based markers. RAPD markers are dominant and therefore are usually not informative when it is necessary to distinguish heterozygotes from homozygotes. This property has caused many geneticists to avoid RAPD markers and some consider dominance to be a "substantial practical impediment" (Strauss et al 1992). Our approach has been different. We have chosen to prescreen RAPD markers for those that are informative, as a result greatly increasing the genetic resolving power of the method. It is possible to convert certain RAPD markers to codominant markers either by restriction digestion after amplification or by the extension of the RAPD primers after sequencing the ends (Kesseli et al. 1992). So far we find it easier to screen large numbers of potential primers for informative dominant markers and to ignore the other markers than to convert RAPDs to codominant markers.

The PCR-based technology led to RAPD genomic maps in many conifers and hardwood forest trees. The advantages of RAPD markers made possible the construction of genomic maps from individual trees in a relatively short period of time (Tulsieram et al. 1992; Grattapaglia et al. 1991, 1992; Neale and Sederoff 1992; Hoey et al. 1992; Jermstad et al 1992; Song and Cullis 1992; Teasdale et al personal communication; Nance et al 1992a, van Buijtenen 1992; Kubisiak et al 1992; Nelson et al 1992b) The species included loblolly pine, slash pine, long leaf pine, Monterey pine, slash pine/caribaea hybrid, yellow poplar and several species of Eucalyptus. The methods allowed the integration of the concepts of isozyme analysis with the power of DNA technology, particularly in conifers where genetic analysis could be done on the haploid megagametophyte. Maps of moderate marker density (less than 10 cM average density) could be constructed with approximately 100 primers and 10,000 PCR reactions. Such linkage maps described the maternal genome only, but were relatively detailed, and contained inferred linkage phase. Perhaps the most important result was that maps could readily be made for individual trees directly from a small number of open pollinated seeds. The open pollinated mapping strategy is the same one used to order isozyme loci on the early conifer linkage maps (Conkle 1981).

The construction of linkage maps comes from the 1:1 segregation of heterozygous markers in a tree used as the maternal parent. Analysis of the genotype is made on DNA obtained from the haploid megagametophyte that is expected to segregate either the dominant allele (+) or the null allele (-). Cosegregation analysis of a large number of heterozygous loci provides assignment of loci to linkage groups either by a Chi square test or using

MAPMAKER (Lander et al 1987). MAPMAKER also provides the order of the loci within a linkage group. .

Mapping in diploid full sib crosses. The simplicity of the haploid mapping system in the conifer megagametophyte can be approached for mapping with RAPD markers in diploids. The strategy involves the selection of a subset of informative markers from a larger number of markers. In a cross between two highly heterozygous and genetically distant individuals, many dominant markers will be heterozygous in one parent and null in the other. In such a case, segregation will be 1:1 just as in mapping with the haploid megagametophyte. The mapping strategy now referred to as a double pseudotestcross and has been applied to different types of markers in several species (e.g. Carlson et al. 1991; Ritter et al. 1990, and Weeden et al. 1992). Screening for such RAPD markers is relatively easy using both parents and a small set of six to eight progeny. The frequency of such informative markers can be high.

In a cross between *Eucalyptus grandis x E.* urophylla, 305 primers were screened. Of these, 151 were used to amplify 571 polymorphic markers distributed in two maps. The major advantage of RAPD markers resides in the ability to screen a very large number of potential markers easily, and to select a subset that are informative. A double pseudotestcross strategy can potentially be applied to diploid conifers as well as hardwoods. Only one generation of parents and progeny are needed. The strategy makes detailed mapping possible for a large number of trees currently being studied in breeding programs.

Mapping in open pollinated diploids: A theoretical approach has been proposed for mapping in open pollinated diploids. If rare dominant alleles are present in a heterozygous state in a maternal parent, segregation in diploid progeny will be 1:1. RAPD marker screening makes it possible to find large numbers of rare alleles if the levels of heterozygosity are high. Simulations show that rare dominant alleles that have frequencies at least as high as 0.2 in the general pollen pool can still be useful as markers for mapping and for QTL analysis.

Dominant markers for targeted mapping (bulking). An important variation in mapping that involves pooled samples is particularly useful with dominant markers. This procedure called "bulked segregant analysis" (Arnheim et al 1985; Michelmore et al. 1991) allows the identification of markers flanking a defined locus without mapping of the entire genome. For example, an isozyme locus could be mapped by pooling 10 samples for each allele in a segregating population and preparing two DNA samples. The two samples are screened for primers that are present in one pool and absent in the other. In this way markers are detected that are linked to specific alleles. In a situation where a map already exists, bulking can quickly place new genes on a map or alternatively can be used to saturate a specific region for fine structure mapping. In a targeted mapping study in peach *(Prunus persica* L. Batsch) 15 markers were found linked to either the Gr (red leaf) locus or the Mdh 1 locus on linkage group 5 (Chaparro et al. 1993). Bulking is also applicable to saturation mapping for a specific chromosomal interval (Giovannoni et al 1991).

In theory, bulking can be used in systematics to find dominant markers that will serve to discriminate between species. O'Malley et al (1992) screened 200 primers and found 84 primers and 129 RAPD fragments that provided informative polymorphisms in a study of the relationships of Central American pines.

The main impediments to use of DNA markers for forest genetics that were perceived in 1991 have been lessened to a great extent. PCR based markers reduce direct costs, do not require clone banks, and eliminate the cumbersome technology of molecular hybridization. Theory and technology are now available to utilize the high levels of diversity and heterozygosity in forest trees for genomic mapping. Much of the genetic material in forest tree breeding programs is now accessible to genome mapping. Three kinds of mapping strategies are available, using haploid megagametophytes of open pollinated conifers, the pseudotestcross strategy for full sib crosses between diploids, and the "rare allele" strategy for mapping in an open pollinated diploid.

Mapping in any individual tree can be a powerful tool for quantitative trait dissection. If we now assume that it is relatively easy to map, how can mapping be applied to practical problems of tree breeding? The first level of application is the dissection of quantitative traits. For most traits of commercial importance in forest trees, the genetic structure underlying the phenotype is not understood. For decades, it has been assumed that most traits were controlled by many genes, being polygenic rather than oligogenic. Breeding strategies based on quantitative genetic theory of polygenic inheritance were highly successful in a wide variety of plants and animals. Genetic mapping now makes possible experimental testing of oligogenic versus polygenic inheritance in forest trees through genetic dissection of quantitative traits.

Bush and Smouse (1992) used allozyme polymorphisms to relate yield and fitness components in forest trees with a strategy similar to that used with DNA markers for QTL analysis, but did not find strong correlations. As Strauss et al (1992) argued, with increased genetic resolution, the architecture of quantitative traits affecting yield and fitness can be approached with unprecedented precision. However, the absence of adequate methods and theory led Strauss et al. (1992) to a negative view of the potential for quantitative trait dissection. Strauss et at 1992 argued that quantitative trait dissection in agronomic crops is of little relevance to forest tree populations, and that the near term usefulness of QTLs to tree breeding will be limited. Most studies of agronomic crops are based on inter or intra specific hybrids, and many of the species studied have been under domestication for hundreds or thousands of years.

It may be argued that domestication and inbreeding would make QTLs hard to find in agronomic crops. Nevertheless, quantitative trait loci abound in agronomic crops (Paterson et al 1990; 1991; Stuber et al 1987; Stuber 1992; Tanksley et al 1989; Kiem et al 1990). It can easily be argued that forest trees, as essentially undomesticated plants, would be rich in quantitative trait loci, particularly related to traits associated with growth and environmental adaptation. The immediate problem therefore, is the design of adequate tests to define QTLs in forest trees.

The design of adequate tests for QTLs should be different for forest trees than for crop species because of the different mapping strategies. QTLs are defined by statistically significant associations or correlations of specific markers (regions of linkage maps) with major components of a quantitative phenotype. A specific strategy, within family open pollinated analysis, can be used for QTL analysis in conifers using the haploid megagametophyte. For QTL analysis in full sib diploid crosses, traditional methods of maximum likelihood have been applied (Lander and Botstein 1989).

Quantitative trait dissection using within family analysis of open pollinated conifers.

The special genetic features of the haploid conifer megagametophyte makes possible tests for QTLs in open pollinated progeny from single trees. A single haploid megaspore gives rise to the maternal contribution to the embryo and to the megagametophyte. The genotype of the maternal nucleus before fertilization is identical to the megagametophyte. Therefore, megagametophytes rescued from germinating seeds can be used for genetic mapping, and the germinants can be scored for a quantitative phenotype. Correlation of maternal markers with the phenotype is equivalent to other kinds of QTL analysis, except only correlations of the maternal genotype with the trait is tested. Several such experiments are in progress.

An unusual feature of QTLs identified by within family analysis of open pollinated maternal trees, is that they would be detected in a mixed genetic background contributed by many different pollen parents, rather than a single parent as in a full sib cross. Such QTLs would not be cross specific, but would have general combining ability. QTL analysis in full sib crosses in diploids using the pseudotestcross is reduced to that of a standard backcross. Two sets of analysis are done, one for each parent, as it is a double pseudotestcross.

Adequate tests for QTLs, with open pollinated progeny may require large sample sizes and genotyping of many markers. Experiments in progress use sample sizes of approximately 1000, with the expectation that about 100 markers will be screened. Although sampling from the tails of the distribution would reduce the number of reactions required, it is necessary, at least in these early stages to expect to be able to process 100,000 reactions in a series of experiments. To do this it is necessary to do PCR reactions at a scale of one to two thousand per day. We have approached this problem by using a microtiter plate format (96 wells), multiple thermocyclers, a robotic pipetting station, and video still imaging for recording gel data, and data analysis, using MAPMAKER (Lander et al 1987), and GMENDEL (Liu and Knapp 1992a) on Macintosh computers and a Sparc II workstation. QTL analysis is done with MAPMAKER/QTL (Lincoln and Lander 1989) and QTLSTAT (Liu and Knapp 1992b). Similar automation of RAPD reactions has been set up by Nelson et al (1992).

Identification of QTLs provides predictive hypotheses to test in breeding.

QTLs are statistically defined and have the potential to be artifacts. What is needed to verify the biological reality of a QTL?. The detection of a QTL provides a predictive hypothesis to test in independent breeding experiments. Markers associated with QTLs in specific crosses or in open pollinated progeny should predict phenotype or performance. Identification of QTLs is only the first step in QTL analysis. Predictive tests will be the second stage of QTL experiments with forest trees. The best test of the putative QTLs will be in

actual breeding experiments, many of which are currently available for analysis. Verification will come from repetition, examination of related families, and retrospective analysis as well as prospective analysis.

What is really needed to use markers in breeding? The major limitation to tree breeding is the time required for one cycle of breeding and selection. The most valuable contribution that markers could make to breeding would be to save time by shortening the time before selection or to reduce the number of breeding cycles. This can be done in two ways, either by the early identification of superior progeny or by the identification of parents that will yield superior progeny. In contrast to annual crops where phenotypic selection can be carried out in weeks or months, woody plants may not show a mature phenotype for many years.

Identification of QTLs: Evidence is accumulating in several laboratories to identify QTLs for important traits in forest trees. Neale and coworkers have identified loci influencing wood specific gravity in loblolly pine (this conference) with a view toward marker aided selection (Williams and Neale 1992). Bradshaw and coworkers using hybrid poplar have identified loci for phenology and growth characteristics (Bradshaw, personal communication). In our group, QTLs for volume growth at rotation age have been identified in an open pollinated family of *Eucalyptus grandis.* In a full sib pseudotestcross of *E. grandis* and E. *urophylla,* QTLs for micropropagation ability (fresh weight of shoots in culture) have been identified. Even though QTL analysis is at an early stage, it would appear that association of markers with traits of significant biological effect or of commercial value can be readily detected.

Additional QTL experiments in progress: In loblolly pine, markers are being used to dissect the oligogenic nature of fusiform rust disease inprogeny from a series of crosses challenged with single aeciospore lines of fusiform rust. The genetic structure of resistance to rust is of interest as a "threshold" trait and because it provides a test case for marker aided selection. As argued by Lande and Thompson (1990), Strauss et al,. (1992), marker aided selection would be most efficient for a trait with relatively low between-family heritability. A general framework for the inheritance of resistance to rust in southern pines has been described (Kinloch and Walkinshaw (1991) and an approach to genetic analysis using advanced pedigrees for rust resistance has been presented (Nance et al. 1992b). Nance et al (1992) have also described strategies for use of marker aided selection for rust resistance in advanced pedigrees.

A series of experiments are in progress to analyze quantitative components of early height growth in loblolly pine using the within "half sib" approach. Cyclic shoot growth will be measured in open pollinated seedlings and correlated with maternal markers. QTL analysis will be carried out to identify regions of the genome that explain significant components of the early cyclic growth trait. Shoot elongation during first year is strongly correlated with field performance at 8 years (Bridgwater et al 1985; Williams 1987; Bridgwater 1990). The trait has high heritability and a strong juvenile mature correlation (Li et al 1991). A similar series of studies have been started to correlate variation in rooting ability within families with molecular markers by a similar analysis. Analysis of progeny from open pollinated trees is particularly appropriate and useful for coniferous forest trees. Half sib approaches to quantitative trait dissection using molecular markers have been

proposed for animal genetics (Haley 1991) where progeny genotypes are used to deduce the contribution of the common male parent.

The relationship of markers and breeding proceeds through three steps:
1, Construction of individual maps for important trees in a breeding program. Breeding applications will require many specific maps. The objectives for many breeding programs would be the construction of individual tree specific maps that had reasonably complete coverage at the 10 to 20 cM level. A single high density map for the species would be required to provide anchor points for the single tree, cross specific maps. Anchor points are esential to determine the relationship of QTLs localized in different trees.

2. Individual tree maps would be used in progeny tests to locate quantitative (QTLs) and qualitative genes of interest. Lethal or semilethal loci could be located and identified in specific trees. This information would provide the data base for the future generations of breeding and selection for each specific breeding population.

3. Use of specific markers in association with specific traits in genome map assisted plant breeding (GMAPB) (Liu and Hayes 1992). GMAPB would employ a new generation of computer software designed to use markers for marker aided selection, and in the design of breeding strategy. Selection of parents based on genomic mapping information would be particularly valuable for optimizing gain in selection experiments. Choosing parents with different QTLs contributing to the same phenotype would provide greater opportunity for increased gain. Breeding of trees with mapped lethal factors could lead to the establishment of inbred lines, and allow the development of new breeding strategies for forest trees.

Other aspects of genomic mapping: In addition to applications to breeding, genomic mapping in trees now has reached a level of technology so that it is possible to consider the targeting of important genes for map based cloning even for conifers. Fine structure maps based on RAPD markers are a first step to fine structure physical maps around important genes. It is not too early to begin such experiments. We are not at a stage where the identification of a specific gene in a well mapped interval is feasible, but this component of map based cloning should become an intense area of research, even in forest trees.

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