Average effect of a mutation in lignin biosynthesis in loblolly pine

By: R. L. Wu, David L. Remington, J. J. MacKay, S. E. McKeand, and D. M. O'Malley

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Abstract:

Cinnamyl alcohol dehydrogenase (CAD, E.C. 1.1.1.195) is a monolignol biosynthetic enzyme that catalyzes the final step of lignin subunit biosynthesis in higher plants. Recently, a mutant allele of the *cad* gene, *cad-n1*, encoding for the CAD enzyme, was discovered in loblolly pine. By reducing the expression of the *cad* gene, this mutant has a decreased lignin content and major changes in the lignin composition in wood. In this study, we found that the substitution of a wild-type allele by *cad-n1* was associated with a significant effect on 2nd-year shoot elongation in a half-sib family of loblolly pine (designated family 7–1037). The average effect of *cad-n1*appeared to increase with tree growth and was greater for stem radial growth than height growth. An increase of 14.1% in de-barked volume in year 4 was associated with *cad-n1*. Co-segregation analysis indicated that the *cad* locus itself might represent a gene that governs stem growth in pine. The significance of the mutation *cad-n1* for tree growth and wood processing is discussed.

Keywords: Average effect | CAD | Lignin biosynthesis | Loblolly pine | Mutant | RAPDs

Article:

Introduction

Lignin is a complex phenolic polymer that reinforces the walls of certain cells in the vascular tissues of higher plants (Sederoff et al. 1994). Lignin plays an important role in mechanical support, water transport, and pathogen resistance (Vance et al. 1980; Bostock and Stermer 1989; Hawkins and Boudet 1994). In trees, high levels of lignin are synthesized in wood and account for 15–36% of the dry weight of wood (Whetten and Sederoff 1995). In the pulp and paper industry, lignin must be removed by harsh chemical treatments, which is a costly process both to the mill and the environment (Dean and Eriksson 1994). Genetic modification of the lignin content and composition of trees is thus receiving considerable attention in current forest biology

studies and wood production (Campbell and Sederoff 1996), although the effects of a modified lignin content on fitness and production are still unclear.

The final step in lignin precursor biosynthesis, the reduction of cinnamaldehydes to cinnamyl alcohols, is catalyzed by the monolignol biosynthetic enzyme cinnamyl alcohol dehydrogenase (CAD, E.C. 1.1.1.195). In herbaceous plants, the biological role of CAD had been studied using lignin mutants (Kuc and Nelson 1964; Muller et al. 1971). By analyzing inheritance patterns of CAD allozymes, MacKay et al. (1995) found that CAD appeared to be encoded by a single gene in loblolly pine. Recently, a mutated null allele, *cad-n1*, in the *cad* gene was discovered in loblolly pine by MacKay (1996). This mutation is associated with a large phenotypic effect on lignin composition (Ralph et al. 1997). The mutant pine seedlings grow normally in greenhouse conditions with little disruption of the essential functions of lignin (MacKay et al. 1997).

In the present paper, we present an analysis of the quantitative effects associated with the *cad-nl* allele based on megagametophytes in a half-sib family of loblolly pine. The pine megagametophyte is a haploid nutritive tissue (1n) surrounding the embryo in the mature seed. The megagametophyte develops from one of the four haploid megaspores that are the products of a single meiosis from a megaspore mother cell (Wilcox et al. 1996). The megagametophyte genotype is identical to the maternal gamete that forms the embryo. Thus, the haploid nature of the megagametophyte not only facilitates the molecular and genetic characterization of individual alleles, but also allows a direct estimate of the average effect of a gene of interest from half-sib progenies of a single heterozygous tree (O'Malley and McKeand 1994). This average effect has been used to study the genetic structure of a population and its evolution (Falconer and MacKay 1996). In this paper, it is used to describe the effect of the mutant allele, *cad-n1*, on stem growth in a half-sib pine family.

Materials and methods

The plant material used in this study was derived from open-pollinated progenies from an offspring (identified as selection 7–1037) of a cross between loblolly pine genotypes 7–56, an original tree from which the mutation in the *cad* gene was discovered, and 7–51. Selection 7–1037 has inherited the *cad-nl* allele from 7–56, based on the absence of CAD activity in approximately 50% of the megagametophytes from 7–1037 (MacKay 1996; MacKay et al. 1997). The pine seeds were collected from ramets of clone 7–1037 grown by the Federal Paper Board (currently the International Paper Company) in Lumberton, N.C., and were germinated and grown under greenhouse conditions. In 1993, 900 of these seedlings were transplanted to a field trial with nine square blocks in Lumberton, N.C. Within each block, 100 seedlings were planted in a 10×10 layout at a spacing of 0.6×0.9 m.

Height growth was measured for each tree in the plantation at the end of each of the first 2 years, from which 2nd-year shoot elongation was calculated. In 1995, three quarters of the trees were removed by harvesting every second row and column. In spring 1997, the remaining quarter of the trees was harvested within each of the first eight plots (one plot was dropped due to high levels of environmental heterogeneity), which generated a total of 158 destructively sampled trees. The traits measured on these trees included total 4-year height and diameter at breast height, of both outside bark and inside bark. These two diameters were used to calculate the stem

cross-sectional areas outside bark (AOB) and inside bark (AIB), respectively. The four-year stem volume outside bark (VOB) and inside bark (VIB) was calculated by multiplying height by the corresponding cross-sectional areas.

Megagametophytes were collected following seedling germination and stored at –80°C prior to DNA extraction. Genomic DNA was isolated using the Puregene DNA isolation kit (Gentra System Inc., Minneapolis, Minn.). A pair of 20-bp custom primers were designed from the DNA sequence of the region of the *cad* gene from genotype 7–56 to amplify genomic sequences upstream from the transcription unit of *cad*. The location of the *cad* locus was mapped using two flanking RAPD markers, according to the protocol of Williams et al. (1990). Primers producing polymorphic markers linked to the *cad* locus in other related loblolly pine families (see MacKay 1996; Wilcox et al. 1996) were screened to identify a pair of flanking markers at a genetic distance of approximately 20 cM on either side of the *cad* locus.

Since the association between the marker and the phenotype was based on haploid megagametophytes, the average effect for the *cad* gene could be directly calculated using the phenotypic difference between the two marker-allele types derived from megagametophytes of a heterozygous tree (O'Malley and McKeand 1994). The significance of the *cad*-associated average effect was tested using a linear regression model for the two-way analysis of variance:

$$y_{ijk} = \mathrm{m} + a_i + b_j + (a \times b)_{ij} + e_{ijk} \quad (1)$$

where y_{ijk} is the phenotypic value of the kth individual in the jth block from the ith allele type of megagametophyte, μ is the overall mean, α_i is the effect of the ith allele, b_i is the effect of the jth block, $(a \times b)_{ij}$ is the effect of interaction between the ith allele and jth block, and e_{ijk} is the residual term. In this model, the *cad* genotypes are assumed to be random and the block effect to be fixed. The variance components due to the *cad, cad*×block interaction, and residual effects were calculated from type-I sums of squares, PROC VARCOMP (SAS Institute 1988), and used to estimate the proportion of the phenotypic variance explained by the difference of the *cad* alleles.

The relationship between the *cad* gene and a real quantitative trait locus (QTL) that governs pine growth was inferred with the aid of the two RAPD markers flanking *cad*. A methodology for interval mapping based on an average effect was developed to determine if *cad* was consistent with the QTL (see Appendix).

Results

The two primers designed for PCR-amplification within the transcription unit of *cad* in family 7–1037 were 5′ TGA CTA GAC TCT GCC AAT CTC CTT AT 3′ (the sense primer) and 5′ GAT CGT CTG GCT CTT GTA CTG TAT T 3′ (the antisense primer). Amplification with these primers revealed length polymorphisms of PCR products in the megagametophytes of this family, the longer was associated with the mutant allele (null), *cad-nl*, inherited from 7–56, and the other, a wild-type allele, *Cad*, inherited from 7–51 (Fig. 1). The segregation ratio of 440:429 for these two alleles was not significantly different from a 1:1 ratio (χ^2=0.139), P=0.701), suggesting that genotype 7–1037 was heterozygous for the *cad* locus.

The average effect on shoot elongation in year 2 was 6 cm, or 3.6% relative to the population mean, which is significant ($P<0.001$) based on the result from the analysis of variance (Table 1). The favorable effect on growth was associated with the *cad-nl* allele. The *cad* locus accounted for a small but significant proportion of the observed variance (1.6%). The progeny seedlings of clone 7–1037 displayed significantly different height growth among blocks but, since there was no significant *cad*×block interaction effect (Table 1), similar trends in height response to blocks were detected between wild and *cad-nl* allelic types.

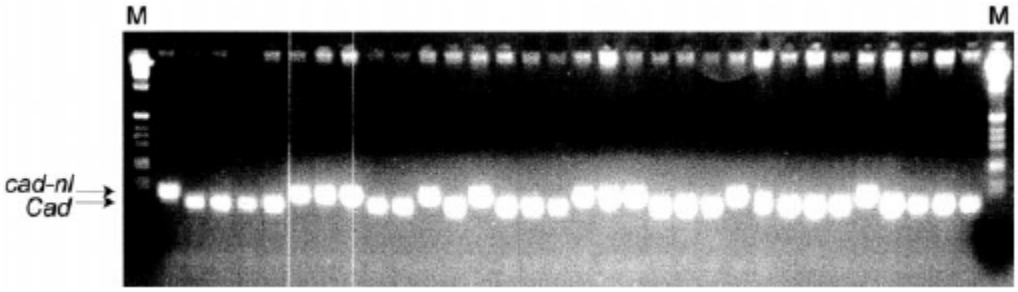

Figure 1. Segregation of a PCR-amplified length polymorphism of family 7–1037 based on a pair of primers designed from a targeted DNA sequence of the *cad* gene in family 7–56

Table 1. Results from the analysis of variance examining the influence of the *cad* gene on growth traits in family 7–1037 in Lumberton, N.C.

Source	*df*	MS	*F*-value	*P*	Average effect (%)	R^2 (%)[a]
Shoot elongation year 2						
cad	1	9741	13.38	0.0003	3.6	1.6
Block	8	18,893	103.76	0.0001		
cad×Block	8	184	0.25	0.9803		
Residual	868					
Stem height year 4						
cad	1	3536	1.49	0.224	2.7	–
Block	7	18,053	6.41	0.0085		
cad×Block	7	2816	1.19	0.313		
Residual	157	2373				
Cross-sec. area outside bark year 4						
cad	1	974	6.14	0.0142	9.7	2.8
Block	7	218	0.70	0.792		
cad×Block	7	312	1.97	0.0631		
Residual	156	159				
Cross-sec. area inside bark year 4						
cad	1	819	7.61	0.0065	11.5	4.1
Block	7	164	0.80	0.753		
cad×Block	7	206	1.91	0.0709		
Residual	156	108				
Volume outside bark year 4						
cad	1	360	5.72	0.0180	14.1	3.4
Block	7	155	1.47	0.543		
cad×Block	7	105	1.68	0.1187		
Residual	156	63				
Volume inside bark year 4						
cad	1	281	6.70	0.0106	14.1	3.4
Block	7	123	1.78	0.347		
cad×Block	7	69	1.64	0.1289		
Residual	156	42				

[a] R^2=the percentage of the total phenotypic variance explained by the *cad* gene

Fourth-year growth measurements were made on a subset of the sample which followed the 1:1 segregation ratio at the *cad* locus (χ^2=0.0058, *P*=0.942). This suggests that the sampling strategy used was random. Height in year 4 was not significantly different between the two *cad* allele groups (Table 1). As in year 2, height in year 4 displayed a significant block effect but a nonsignificant *cad*×block interaction effect. Significant average effects of allelic substitution at the *cad* locus were observed on stem radial and volume growth at age 4 years (Table 3); the mutant allele was associated with an increase in stem cross-sectional area inside bark and volume inside bark of 12% and 14.7%, respectively (Fig. 2). The proportion of the phenotypic variance for stem radial and volume growth in year 4 explained by the *cad* genotype was greater than that for 2nd-year shoot elongation. Unlike height, stem cross-sectional areas and volumes, both inside and outside bark, had no significant block effect. The effect due to *cad*×block interaction was nonsignificant for all growth traits.

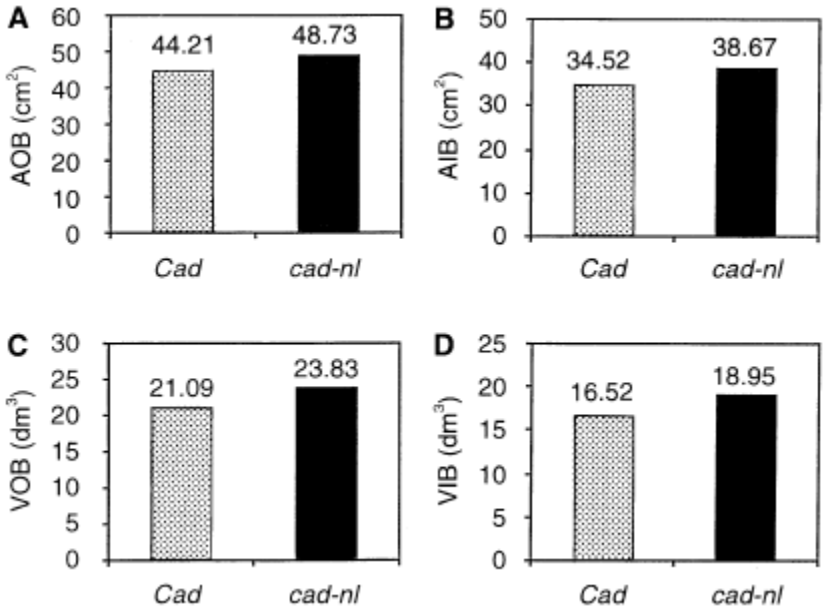

Figure 2. Comparisons of 4-year stem-growth traits between wild-type, *Cad*, and the mutant type, *cad-nl*, in open-pollinated progenies of family 7–1037. **A** Cross-sectional area outside bark (*AOB*), **B** cross-sectional area inside bark (*AIB*), **C** volume outside bark (*VOB*), and **D** volume inside bark (*VIB*)

The chromosomal location of the *cad* locus was previously mapped in three loblolly pine families using RAPD markers (MacKay 1996; Wilcox et al. 1996). Of the primers producing polymorphic markers linked to the *cad* locus in these families, primers F15 and H4 were found to generate a pair of flanking markers at a genetic distance of 15.8 and 23.9 cM on respective sides of the *cad* gene in family 7–1037 (Fig. 3). The presence of the fragment (+) for both RAPDs was linked in repulsion with the *cad-nl* allele (e.g., Fig. 4). The co-segregation analysis of the *cad* gene and the RAPD markers was used to infer whether the *cad* locus itself may be affecting growth. By assuming the putative QTL at any position between the two flanking RAPD markers, we tested the goodness-of-fit of the model derived from the average effects and genetic variances of maternal gametes for the two markers (see Appendix). When the assumed QTL position was around the *cad* locus, the model displayed the best adquacy for all growth traits, as

shown by the smallest *P* value (Fig. 3). Thus, the *cad* gene may be consistent with the QTL affecting stem growth in loblolly pine.

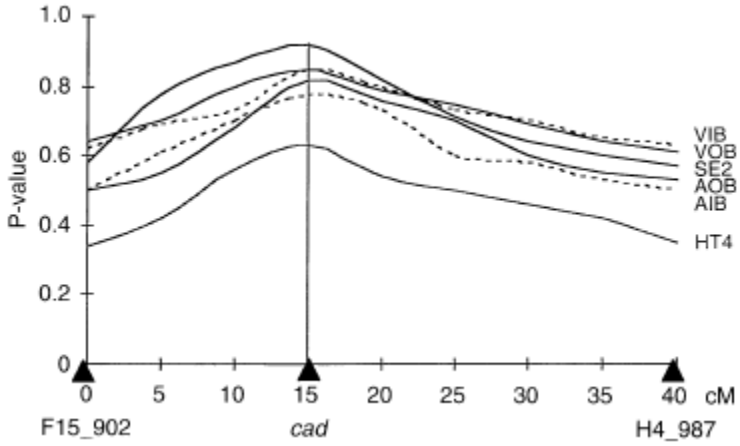

Figure 3. The profile of QTL position around the *cad* locus bracketed by two RAPD markers, F15_902 and H4_987, in family 7–1037. The map distance was calculated using the Haldane mapping function based on the recombination fraction estimated using a maximum-likelihood method. The adequacy of the model, expressed by the probability level (*P*), indicates the goodness-of-fit of an assumed QTL position to the system of nonlinear polynomial equations derived from average effects and genetic variances of megagametophyte genotypes. *SE2*=2nd-year shoot elongation, *HT4*=4-year total height, *AOB*=4-year stem cross-sectional area outside bark, *AIB*=4-year stem cross-sectional area inside bark, *VOB*=4-year stem volume outside bark, and VIB=4-year stem volume inside bark

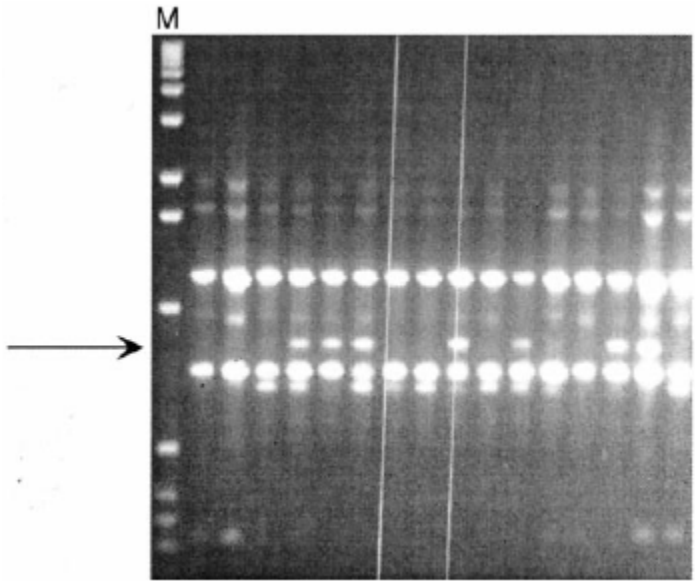

Figure 4. Agarose gel showing the segregation of RAPD marker F15_902 (*arrow*) in family 7–1037. The presence of the RAPD marker is associated with the wild-allele type of the *cad* gene

Discussion

A mutant allele of the *cad* gene affecting lignification was first discovered in a woody plant by MacKay (1996). This mutation, described as *cad-n1*, almost completely eliminates the expression of the *cad* gene and its product in megagametophytes, elongating shoot tips, and differentiating xylem. In the *cad-nl* homozygotes, the content of acid-insoluble lignin in dry weight was decreased by 9% relative to that in the *Cad* homozygotes (MacKay et al. 1997). It was also found that there were major differences in the chemical composition of lignin between the mutant and wild-type trees, resulting in a more-easily extracted lignin with potential benefits to the pulp and paper industry (MacKay et al. 1997).

By PCR-amplifying DNA polymorphisms within the *cad* promoter (MacKay 1996), we found that the wild-type allele and mutant allele of the *cad* gene followed a 1:1 segregation ratio in the open-pollinated progeny from a heterozygous pine 7–1037. The substitution of the wild-type allele by the mutant allele was associated with a significant average effect on stem-growth traits in this family.

The average effect of the mutant allele appeared to change with tree development. In year 2, the mutant allele produced 3.6% more shoot elongation than the wild-allele type and, in the progeny population, the *cad* gene accounted for a very small (1.6%), though significant, percentage of the phenotypic variance. Although the analysis of a subset of the sample showed no significant effect on stem-height growth in year 4, the *cad-nl* allele significantly increased radial (10.2–12%) and volume (12.9–14.7%) growth, as compared to the *Cad* allele. The proportions of the phenotypic variance explained by the *cad* locus ranged from 2.8 to 4.1% in the latter two traits. Two reasons may result in the observation of a nonsignificant average effect on 4-year height: (1) the effect of *cad-nl* on height growth may actually be small and cannot be detected unless an adequately large sample size is used (e.g., 800 trees in year 2), (2) the effect of *cad-nl* is confounded by the block effect which appeared to be significant for height. In addition, because the average effect is a function of additive and dominant genetic effects and allelic frequency (O'Malley and McKeand 1994; Falconer and MacKay 1996), the cancelling of these factors may blur the influence of the average effect. The mutant allele may display a greater influence on stem secondary increment than primary height growth, although the biochemical mechanisms for this relationship are unknown. A pleiotropic mechanism for the *cad-nl* allele may exist to decrease lignin content but increase volume growth. Perhaps the trees with *cad-nl* alleles invest fewer resources in the production of monolignols, which is an energy consuming process, providing additional resources for tree growth and stem-wood production. If so, the potential benefits of the *cad* mutant to the paper industry may extend beyond the altered lignin properties of the homozygous mutants.

The co-segregation analysis developed from the average-effect concept (see Appendix) suggests that the *cad* gene may well be a quantitative trait locus that affects stem growth in loblolly pine. Further mapping of the linkage group containing *cad*, extending beyond the flanking markers F15_902 and H4_987, as well as fine-structure mapping in the immediate vicinity of *cad*, will be important to further test this possibility. Loci with known mutations have been strongly implicated as functional (candidate) genes for quantitative variation in maize. Using QTL mapping with molecular markers, Doebley et al. (1995, 1997) suggested that *tb1-ref*, a recessive loss of function mutant, is apparently allelic to the alleles of a QTL that was detected to affect plant and inflorescence architecture.

Appendix: mapping the QTL affecting pine growth

In this appendix, we develop a genetic model for mapping QTLs based on the concept of average effect. Consider two flanking markers, *A* and *B*, between which a putative QTL is located. Two alleles at each of these three loci are denoted by A_1 and A_2, B_1 and B_2, and Q_1 and Q_2, respectively. The recombination fractions are denoted by *r* between the two markers, r_1 between marker *A* and the QTL, and r_2 between the QTL and marker *B*. For a heterozygous tree, maternal gamete types at the two markers are of four different kinds, A_1B_1, A_1B_2, A_2B_1 and A_2B_2, whose frequencies are $\frac{1}{2}(1-r), \frac{1}{2}r, \frac{1}{2}r$ and $\frac{1}{2}(1-r)$, respectively. Each of these gametes includes Q_1 and Q_2, and randomly mated with either paternal gametes from the same tree or paternal gametes from a natural population. Assume that the outcrossing rate of a maternal gamete is *t*. The population frequencies of A_1 and A_2 are denoted by p_A and q_A (=1–p_A), whereas those for the QTL and marker *B* are denoted by p_B and q_B (=1–p_B), and p_Q and q_Q (=1–p_Q), respectively. The gamete frequency for the three loci in the population is a function of the frequencies of individual alleles and their mutual gamete disequilibria. For example, the frequency of gamete $A_1Q_1B_1$ is expressed by:

$$p_{A_1Q_1B_1} = p_A p_Q p_B + p_A D_{QB} + p_B D_{AQ} + p_Q D_{AB} D_{ABC},$$

where D_{QB}, D_{AQ} and D_{AB} are the genetic disequilibria between alleles from two different loci and D is the gametic linkage disequilibrium among alleles from three different loci (Weir 1996). It is found that the average effect and genetic variance within each maternal-marker gamete derived from megagametophytes are independent of the allelic frequencies of markers and the linkage disequilibria between the loci. Following Falconer and Mackay (1996), we have obtained expressions for the average effects, $\alpha_{A_1B_1}, \alpha_{A_1B_2}, \alpha_{A_2B_1}$, and $\alpha_{A_2B_2}$, and genetic variances, $\sigma^2_{A_1B_1}, \sigma^2_{A_1B_2}, \sigma^2_{A_2B_1}$, and $\sigma^2_{A_2B_2}$, for the four maternal-marker gametes in terms of the QTL of interest (R.L. Wu, unpublished results). Five unknown genetic variables, *a* (The additive effect of the QTL), *d* (The dominant effect of the QTL), r_1 (or r_2), p_Q, and *t*, are used to describe this QTL. The genetic variance across the four maternal gametes is also derived, which is $\sigma^2_{AB} = \frac{1}{2}\left[(1-r)\alpha^2_{A_1B_1} + r\alpha^2_{A_1B_2} + r\alpha^2_{A_2B_1} + (1-r)\alpha^2_{A_2B_2}\right]$. In total, we can construct nine independent equations all of which are nonlinear polynomials with respect to the unknowns.

In an experimental population, the phenotypic value of a quantitative trait can be expressed by a linear regression model (see Materials and methods). In this model, the residual error is assumed to be normally distributed with a mean of zero and a variance of σ^2_e. Observed average effects and observed genetic variances from the real dataset can be used to solve the five variables describing the QTL and the residual variance, σ^2_e. Since the number of equations (9) is greater than the number of unknown varables to be estimated (5), a nonlinear optimization approach based on the weighted least squares analysis can be used to obtain the approximation estimate for each variable and its sampling error. The weights for the differences of across-gamete average effect and genetic variance are determined by the reciprocals of their variances multiplied by the corresponding degrees of freedom. The use of these weights results in the solution of the unknowns and their standard errors by the nonlinear optimization manipulation. These afford the means of calculating the weights for the second round of the iterative procedure. This step is

repeated until the final estimate converge to stable values. The adequacy of the model, i.e., the degree to which estimates of the unknowns fit the nine equations simultaneously, is tested based on the sum of squares of differences between the expected and observed values of the differences of average effects and genetic variances between a pair of maternal gametes and of across-gamete genetic variance (each square being multiplied by the weight) using χ^2-statistics. The degrees of freedom for the χ^2-test of goodness-of-fit would be the number of nonlinear equations used in the model minus the number of variables to be estimated. The adequacy of the model can be evaluated by the probability (P) level, which is the probability of getting a χ^2-value larger than the value actually obtained, given that the hypothesized position of the QTL is correct. The small values of P correspond to a poor fit and large values to a good fit.

In practice, the position of the QTL (r_1) between the flanking markers can be viewed as any value between 0 and r. Thus, for a particular value of r_1, one can obtain estimates for the unknown parameters and an adequacy test. A position at which the model displays the best adequacy is considered as the most likely location of the QTL.

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