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# Linkage of RAPD markers to NESTUR, a stem growth index in radiata pine seedlings

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**Abstract** Needle-to-stem unit rate (NESTUR) is a stem growth index of conifer seedlings that measures the efficiency of stemwood production per unit of foliage growth. The random amplified polymorphic DNA (RAPD) technique was applied to haploid DNA from the megagametophytes of a full-sib radiata pine cross to find markers linked to factors controlling the NESTUR trait. Using the bulked segregant analysis approach, 23 of 933 primers displayed putative linkage to factors controlling NESTUR. Based on the genotypic analysis of 174 individuals, two quantitative trait loci (QTLs) controlling NESTUR were identified at ANOVA *P*-levels of 0.01–0.001. The QTLs were identified by RAPD markers OPE-$06_{450}$ and OPA-$10_{1200}$, which were linked to each other ($r = 7\%$), and UBC-$333_{550}$, which was not linked to the other two. Linkage to components of NESTUR (increments in stem diameter and stem volume) was demonstrated for UBC-$333_{550}$, while the others were not linked to NESTUR components.

**Key words** Bulked segregant analysis · Quantitative trait locus · Epistasis · Stem growth efficiency

## Introduction

Needle-to-stem unit rate (NESTUR) is a stem growth index of conifer seedlings that measures the efficiency of stemwood production per unit of needle growth (Matheson et al., unpublished). This index, which is derived as a unit rate of growth in stem volume and needle volume, takes into account the influence of both photosynthetic capacity and carbon allocation on stemwood production. Its potential usefulness as an early selection trait in radiata pine (*Pinus radiata* D. Don) was suggested by Matheson et al. (1995), who observed correlations as high as 0.97 between stem : needle ratio measured on seedlings in the nursery and the family performance of 11-year-old trees growing on several sites in Australia. Although NESTUR shows high heritability between families of radiata pine (Matheson et al., unpublished data), the steps involved in its determination are too tedious and time-consuming to permit its use in practical selection programs, where thousands of individuals need to be assessed in a very short period of time.

Random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990) linked to factors controlling NESTUR could be used as indirect selection criteria. RAPDs are dominant markers and are therefore less informative at a locus than some other marker types. Heterozygous loci cannot be distinguished from homozygous dominant loci in diploid individuals. However, in conifers, this problem can be avoided by using DNA from haploid megagametophyte tissue. RAPD analysis of DNA from megagametophytes derived from a single tree show 1:1 segregation and are equivalent to a test-cross (Bucci and Menozzi 1993; Lu et al. 1995).

The identification of RAPD markers linked to quantitative trait loci requires a large number of individuals for statistical power (Darvasi et al. 1993), with a consequent relatively high cost for marker genotyping. An approach that has been proposed for reducing costs and time to identify linked markers is variously referred to as the *trait-based analysis* (Lebowitz et al. 1987), *selective DNA pooling* (Darvasi and Soller 1994) or *bulked segregant analysis* (Michelmore et al. 1991). The method is based on the principle of marker-allele

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frequency changes in the tails of a normally distributed phenotype as a result of linkage to the trait. In this procedure, two DNA pools are screened for marker-allele frequency changes, one pool representing individuals with a high phenotypic value for the trait of interest, and the other individuals with a low phenotypic value. Screening of the entire population is only required when polymorphisms for marker loci between the two bulks are detected, in which case there is a considerable saving in time and resources, particularly when used with RAPDs. This method is particularly useful when the analysis is aimed primarily at a single trait, and has been more widely applied to annual crops than to trees. In trees, the method has been used to detect markers linked to genes such as disease-resistance in sugar pine, apples and Chinese elm (Yuang and Kruger 1994; Benet et al. 1995; Devey et al. 1995), sex determination in *Pistacia* (Hormaza et al. 1994), the *pendula* gene in Norway spruce [*Picea abies* (L.) Karst. F. Pendula] (Lehner et al. 1995), and genes controlling agriculturally important traits in *Persea americana* (Mhameed et al. 1995).

The objective of the present research was to use bulked segregant analysis and haploid megagametophyte DNA to identify linkage between RAPD markers and QTLs influencing NESTUR in radiata pine.

## Materials and methods

### Plant material

*P. radiata* seeds from the full-sib cross 12038 × 10946 were obtained from CSIRO Forestry and Forest Products. The cross was selected based on parental variance for NESTUR values from previous evaluations. Open-pollinated (OP) seeds from the maternal parent (12038) had a high variance in NESTUR, while OP seeds from the paternal parent (10946) had a low variance. Approximately 300 seeds of the $F_1$ full-sibs were placed in a Petri dish lined with three layers of filter paper and stratified for 30 days at 4°C. Only 176 seeds germinated, and these were picked out on to 150 mm-diameter pots filled with a porous medium (3 parts by volume of river sand, 2 parts of medium-size vermiculite, and 1 part of perlite). Megagametophytes were collected following seedling germination and stored at −80°C for subsequent DNA extraction.

Seedling trees were initially grown in a glasshouse under ambient temperature and light conditions for 2 weeks, and then transferred to a controlled environment (Phytotron). The growth chamber was maintained at temperatures of 25°C (maximum or day) and 18°C (minimum or night), and a relative humidity of 65%. Nutrients were provided in the irrigation water through drippers at 12-h intervals.

### Measurements and determination of NESTUR

Seedlings were assessed for NESTUR at 4 months of age. Four separate measurements were made on each of two dates: (1) total height, measured from the pot rim to the growing point, (2) trunk height, from pot rim to the cotyledonary scar, (3) stem diameter, gauged with a dendrometer (0.001-mm resolution), and (4) needle volume. Needle volume was measured by a water-displacement method. First, the pots were clamped down firmly on a stand, and then carefully inverted. The seedling crowns were then positioned squarely above a cylinder of water resting on a top-loading balance (0.01-g resolution). Finally, the crowns were carefully lowered into the water to a known point (the cotyledonary scar) with caution taken to avoid contact of the foliage with the walls of the cylinder. To prevent trapping of air bubbles, a wetting agent was added to the water as a surfactant at a rate of 3 ml per 15 l. The measurements were repeated 2 weeks later. A detailed description of the measurements and calculation of NESTUR will be presented in a subsequent publication (Matheson et al., in preparation).

### DNA extraction and RAPD assays

Total genomic DNA was extracted according to Dellaporta et al. (1983) giving an average yield of 2.5 µg of DNA per megagametophyte. Reaction mixtures for RAPD assays consisted of 3 ng of DNA from each bulk, 2.0 mM of $MgCl_2$, 200 µM of each dNTP, 1 U of *Taq* polymerase, and 0.33 µM of primer in a reaction buffer containing 10 mM Tris-HCL (pH 8.3 at 25°C) and 50 mM KCL, and for a total reaction mixture of 15 µl. Amplifications were carried out in a Perkin Elmer Cetus 9600 DNA Thermal Cycler at three cycles of 94°C for 2 min, 37°C for 2 min, and 72°C for 2 min, followed by 40 cycles of 94°C for 45 s, 42°C for 45 s and 72°C for 120 s. Amplification products were separated by horizontal-gel electrophoresis in 1.5% agarose with 0.5 × TBE buffer, and detected by ethidium bromide staining.

### Bulked segregant analysis

The present study was based on haploid segregation analyses of the maternal parent of the full-sib family 12038 × 10946, although the phenotype was measured on the diploid offspring. Bulked samples consisted of 250 ng of DNA from the corresponding megagametophytes of nine High-NESTUR and nine Low-NESTUR individuals, representing 5% of the high and low tails of the normal distribution. The bulks were screened with a total of 933 random 10-mer oligonucleotide primers. We used 800 Operon primers supplied by Operon Technologies, Alameda, Calif., and 133 primers from the "conifers RAPD set" obtained from Dr. John Carlson, University of British Columbia. The presence of a RAPD band in one bulk and absence in the other provided evidence for a putatively linked marker. To exclude false positives, the RAPD assay was repeated for each polymorphism detected. Then, the 18 individuals that were used to construct the bulks of DNA were each tested for the putatively linked marker. Reliable markers were tested further in a two-stage approach to identify loci co-segregating with NESTUR QTLs. The markers were first tested on an additional 60 individuals divergently selected for extreme NESTUR values. Analysis of variance (ANOVA) was used to determine which loci appeared to be linked to the QTLs, using a Type-1 (false positives) error probability of $\leq 10\%$. In the second stage, all the remaining 174 progeny were genotyped for loci showing differences ($P \leq 0.10$). Linkage was considered significant when the probability levels associated with $F$-values were $\leq 0.01$.

### Linkage analysis

Goodness-of-fit to the expected 1 : 1 segregation for marker loci was tested by a chi-square analysis. Linkage among significant markers was calculated as $r = R/N$, where R equals the number of recombinants and N equals the total number of progeny. Epistatic interaction among significant loci was assessed by a factorial ANOVA model, using SPSS for Windows (SPSS, Inc.) computer software. Interaction terms were extracted from the following statistical

model: $Y_{ijk} = \mu + M_i + M_j + MM_{ij} + E_{ijk}$, where $Y_{ijk}$ is the observed phenotype, $\mu$ is the overall mean, $M_i$ and $M_j$ are the main effects of the $i_{th}$ and $j_{th}$ RAPD markers, and $E_{ijk}$ is the residual effect.

### Relationship of NESTUR to stem growth

Coincidence of QTLs for a complex trait with one or more of the trait's components can be interpreted as indirect biological validation for these QTLs. For two traits that are causally related, coincidence of at least some QTLs is likely (Lebreton et al. 1995). The relationship between NESTUR and growth in seedling height, stem diameter, stem volume and needle volume was assessed by standard Spearman correlations.

## Results

The two groups of nine extreme phenotypes had NESTUR values as low as 4.2 and as high as 20.4, with a mean of 8.6. The values ranged from −1.8 standard-deviation units below, and 4.9 units above, the mean, with a variation coefficient of 28.3% (Fig. 1). There were two 'runt' individuals with extremely low NESTUR values, and the data for these were discarded, reducing the sample size to 174.

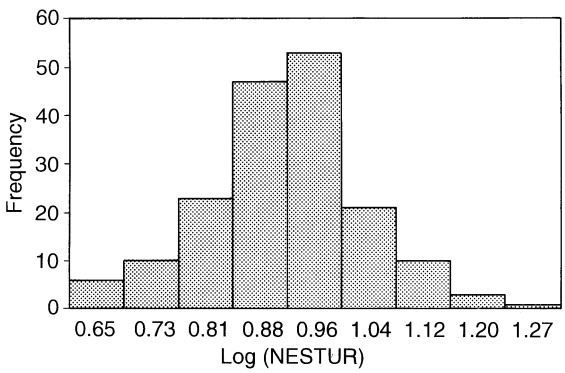


**Fig. 1** Frequency distribution of log10-transformed NESTUR values in 174 individuals of the full-sib cross of radiata pine (12038 × 10946)

The frequency distribution of phenotypes showed a skewness of 1.16, and a $\log_{10}$ transformation was applied to reduce skewness to 0.05. Analyses of variance (ANOVA) were carried out on the transformed data since ANOVA assumes the underlying variable to be normally distributed. However, results are presented for both transformed and untransformed data (Table 1).

### Identification and genetics of marker-linked QTLs

Of the 933 primers screened, 23 produced fragments that were polymorphic in the two bulks of DNA. However, only seven were found to be reliable when tested on the 18 individuals that were used to constitute the two bulks of DNA. These primers were then used to amplify DNA derived from an additional 60 individuals divergently selected from the family. All marker loci showed the expected 1:1 ratio of segregation (Table 1). Four RAPD markers were found to show differences ($P \leq 0.10$) among marker-genotype classes for NESTUR (Table 1). The three markers that did not show sufficient evidence of association with NESTUR QTLs were excluded from further analysis.

In the second stage of marker-trait analysis, RAPD genotypes were determined for the remaining 174 progeny using the four markers that showed differences in genotype class-means at $P \leq 0.10$. The full progeny data were then re-analysed for significant associations, using a threshold of $P \leq 0.01$ to declare linkage. Across the two stages of sampling, three RAPD markers were found to be linked significantly to NESTUR (Table 1). The differences among genotypic classes for these

**Table 1** Summary of RAPD loci in linkage with factors influencing NESTUR in radiata pine full-sib cross 12038 × 10946. Results are presented for log- transformed (Trans.) and untransformed (Untrans.) NESTUR values. $R^2$ values were calculated as the ratio of sum of squares (markers: total). Markers not considered further after the first stage of sampling are shown by (a), and after the second stage by (b)

| RAPD locus[a] | $\chi^2$ test for 1:1 ratio (*P*-value) | Stage I, extreme phenotype progeny ($n = 78$) | | Stage II, all progeny ($n = 174$) | | $R^2$ value (%) | Allele effect (SD units) |
|---|---|---|---|---|---|---|---|
| | | Trans. (*P*-value) | Untrans. (*P*-value) | Trans. (*P*-value) | Untrans. (*P*-value) | | |
| OPA-$10_{1200}$ | 0.100 | 0.004 | 0.005 | 0.001 | 0.001 | 10.56 | −0.50 |
| OPE-$06_{450}$ | 0.309 | 0.017 | 0.021 | 0.011 | 0.008 | 6.23 | −0.41 |
| UBC-$333_{550}$ | 0.346 | 0.005 | 0.007 | 0.002 | 0.003 | 9.23 | 0.46 |
| OPAM-$12_{600}$ | 0.938 | 0.056 | 0.109 | 0.028 | 0.046 | [b] | [b] |
| OPW-$01_{750}$ | 0.838 | 0.212 | 0.309 | [a] | [a] | [a] | [a] |
| OPO-$12_{650}$ | 0.221 | 0.216 | 0.123 | [a] | [a] | [a] | [a] |
| OPV-$17_{1100}$ | 0.307 | 0.350 | 0.319 | [a] | [a] | [a] | [a] |

[a] Primer name/fragment length in base pairs

markers were confirmed when all individuals in the family (174 individuals) were tested. An absence of RAPD-fragment markers generated by primers OPE-06 and OPA-10 was associated with low NESTUR values, while primer UBC-333 generated a 550 bp band that was associated with high NESTUR values.

Standardised allele-substitution effects of marker-linked QTLs were estimated as the b-value from simple linear regression. The marker loci OPA-$10_{1200}$ and OPE-$06_{450}$ had decreasing effects respectively, of 0.50 and $-0.41$ standard deviation units (SDU) from the family mean (Table 1), while the UBC-$333_{550}$ locus had an increasing effect of 0.46 SDU. The amount of variation attributable to the effects of these markers ranged from 6.2 to 10.6%, while a two-QTL model explained 20% of the phenotypic variation.

Pairwise comparisons among significant marker loci showed that the loci revealed by the Operon primers (OPE-$06_{450}$ and OPA-$10_{1200}$) were linked to each other at a recombination frequency of 7% ($SE_r = 0.02$). The locus UBC-$333_{550}$ was not linked to either OPE-$06_{450}$ or OPA-$10_{1200}$.

## QTL coincidence

The relationship of NESTUR with stem growth traits is shown by the correlation coefficients (Table 2). The most significant of the relationships were those with increments in stem diameter ($r = 0.82$) and stem volume ($r = 0.66$). The two linked RAPD markers (OPE-$06_{450}$ and OPA-$10_{1200}$) showed no significant ($P \leq 0.05$) linkage to any of these components, suggesting that this QTL is specific to NESTUR, and independent of its components (Table 2). However, the RAPD marker generated by the primer UBC-333 was associated with increments in stem diameter and stem volume, with effects similar to those observed for NESTUR (Table 2); that is, a 0.46 standard-deviation-units (SDU) increase in NESTUR was associated with an increase of 0.46 and 0.38 SDU in stem volume and diameter growths, respectively.

## Epistatic interactions

We sought to determine whether the action of one putative QTL might be dependent upon the allelic state at another locus. Using markers as treatments, we found that the QTL near markers OPA-$10_{1200}$ and OPE-$06_{450}$ appeared to influence phenotypic variation at the QTL linked to UBC-$333_{550}$ (Table 3). This is illustrated for NESTUR using all four possible two-locus marker genotypic class means for UBC-$333_{550}$ and OPA-$10_{1200}$ (Fig. 2). The presence of the UBC-$333_{550}$ marker is associated with increased values of NESTUR. However, this was found to be dependent on

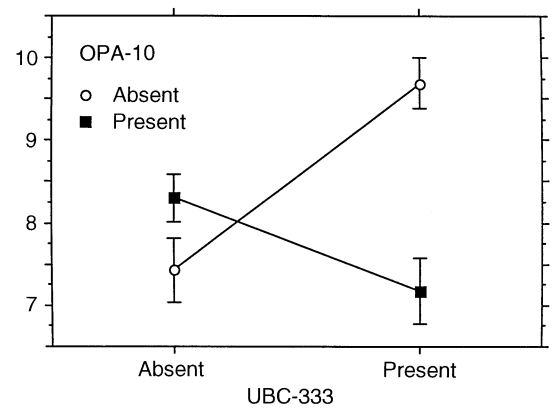


**Fig. 2** Illustration of epistasis between marker-linked QTLs influencing NESTUR, using four possible genotypic class means of markers UBC-$333_{550}$ and OPA-$10_{1200}$

**Table 2** Summaries of Spearman rank correlations of NESTUR with stem growth traits, and associations of NESTUR-linked RAPDs with stem growth traits

| Item | Associations with NESTUR ($r$) | Associations with RAPD locus | | |
|---|---|---|---|---|
| | | OPA-$10_{1200}$ ($P$-value) | OPE-$06_{450}$ ($P$-value) | UBC-$333_{550}$ ($P$-value) |
| Stem diameter growth | 0.819 | 0.12 | 0.43 | 0.01 |
| Stem volume growth | 0.662 | 0.09 | 0.34 | 0.004 |
| Height growth | 0.294 | 0.18 | 0.25 | 0.31 |
| Needle volume growth | $-0.122$ | 0.99 | 0.38 | 0.18 |

**Table 3** $P$-values from two-way analyses of digenic epistasis between putative QTLs, using markers as treatments. $P$-values are shown relative to NESTUR and stem growth traits

| Two-locus interaction | NESTUR | Stem diameter growth | Stem volume growth | Height growth | Needle volume growth |
|---|---|---|---|---|---|
| OPA-$10_{1200}$ × OPE-$06_{450}$ | 0.43 | 0.52 | 0.60 | 0.40 | 0.95 |
| OPA-$10_{1200}$ × UBC-$333_{550}$ | 0.0001 | 0.005 | 0.001 | 0.08 | 0.68 |
| OPE-$06_{450}$ × UBC-$333_{550}$ | 0.0001 | 0.002 | 0.001 | 0.12 | 0.70 |

the genotype at the OPA-$10_{1200}$ locus. The presence of the marker OPA-$10_{1200}$ appeared to influence phenotypic variation at the QTL linked to UBC-$333_{550}$ in an individual.

## Discussion

Many studies have demonstrated the power of bulked segregant analysis for tagging specific genes or QTLs (Hormaza et al. 1994; Yuang and Kruger 1994; Benet et al. 1995; Cheng and Roose 1995; Devey et al. 1995; Lehner et al. 1995; Mhameed et al. 1995). Most of these studies, however, considered simply inherited loci altering morphology or disease reaction, where the influence of non-genetic factors can be greatly minimised by more accurate phenotyping of the individuals. Using RAPD markers in combination with bulked segregant analysis, we were able to identify two QTLs significantly linked to factors controlling needle-to-stem unit growth rate in radiata pine, despite the lack of discrete variations. The QTLs identified explained 20% of the phenotypic variation, with standardised additive effects ranging from $-0.41$ to 0.50 SDU. Regression analysis, used in estimating standardised effects, and the amount of explained variation assumes no recombination between the marker and the QTLs. Violation of this assumption would cause the effect of the QTLs to be underestimated.

It is pertinent to note that, in the present study, only markers segregating from the maternal parent were assayed. Therefore, only some of the total number of QTLs will be potentially detectable, i.e. those segregating on the female side of the pedigree. The methods used, however, have served to establish marker-QTL linkage for NESTUR, as a first step in a program aimed at mapping QTLs with respect to flanking markers on the chromosome. The QTL for NESTUR identified with marker UBC-$333_{550}$ showed a significant association with stem-diameter growth and stem-volume growth (Table 3). The direction of additive gene effects of the QTLs on these traits, and the magnitude of phenotypic correlations, suggest a pleiotropic gene action. However, the independent effects of tightly linked loci cannot be ruled out at this stage since our data are insufficient to distinguish between the two possibilities.

The success of any QTL study is dependent on the influence of non-genetic factors. It is known that when a phenotype is controlled by multiple genetic loci, and the heritability is low, individuals can have extreme values due to non-genetic factors. Stem growth is the cumulative result of carbon assimilation and allocation over time. Since both assimilation and allocation are influenced by many biochemical pathways, physiological processes, as well as by environmental variables, it is reasonable to expect that the integration of stem growth with these variables will be genetically complex. Cross-contamination of DNA pools due to phenotyping errors will block the detection of polymorphism between pools (Wang and Paterson 1994), and thus reduce the power of bulked segregant analysis (Chaparro et al. 1994). The replication of phenotypic evaluations, e.g. by the use of clonal propagules, mitigates the effects of non-genetic factors by increasing the heritability of the trait (Bradshaw and Foster 1992); however, clonal materials were not available for the present study.

A common problem in bulked segregant analysis is the large number of false positives detected from pooled DNA samples. Lehner et al. (1995) identified 12 putatively linked loci among the two bulks of DNA constituted for the pendula gene, but only one was confirmed to be linked to the gene. For simply inherited traits, false positives can be screened out by genotyping individuals that were used to make up the bulks. For quantitative traits, only markers tightly linked to the QTLs will be detectable with this approach. We employed a sequential sampling procedure, testing all reliable markers on an additional 60 extreme-phenotype progeny. Markers that failed to show evidence of linkage ($P \leq 0.10$) at this stage were discarded. Subsequently, all progeny of the family were tested for markers that were retained, using a more stringent error rate of $\leq 0.01$. Potentially some QTL-linked markers, which would have been detected had all 174 progeny been genotyped, were missed by the use of bulked segregant analysis. By selecting individuals with the highest and lowest phenotypic values to form DNA pools, QTLs with small effects on the phenotype, which collectively explain a large portion of the genetic variation in a trait, probably escaped detection. However, it is unlikely that the sequential sampling we employed reduced the power of QTL detection since all markers identified from bulked DNA samples were given consideration. The sequential sampling procedure reduced costs by allowing an early decision to discontinue scoring markers in QTL-negative chromosomal regions.

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