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QTL associations for density and diameter in *Pinus radiata* and the potential for marker-aided selection

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Abstract A large full-sib family of radiata pine (*Pinus radiata* Donn. ex D. Don) was used for quantitative trait locus (QTL) detection and independent verification. QTL detection experiments were carried out for juvenile wood density (JWD) and stem diameter at breast height (DBH) using selective genotyping. Evenly spaced RFLP and microsatellite markers were selected from an existing linkage map. QTLs were verified in an independent set of progeny from the same family. Based on map location, at least eight QTL positions for JWD and two for DBH were detected and verified. The percent variance accounted for by the markers ranged from 0.78% to 3.58%, suggesting a genomic architecture of many genes with small effect. Two unrelated "bridging" families were chosen as candidates for marker-aided selection (MAS), and six microsatellite markers showing an association with JWD or DBH were tested in these families. Of these, four markers showed a consistent association with JWD in one or both of the bridging families. Results from this study provide a basis for MAS in *P. radiata*.

Introduction

Quantitative trait analysis in radiata pine (*Pinus radiata*), as in most forest trees, has been challenging because the species is out-crossing and highly heterozygous. Linkage analysis in a pedigree obtained from a cross of two unrelated trees is complex, with segregation in the progeny resulting from separate meioses and crossovers in both parents. Up to four alleles may be segregating at each quantitative trait locus (QTL) and marker locus. For marker-aided selection (MAS), marker/QTL phase must be determined for each family from progeny means and segregation ratios.

There have been numerous reports of QTLs for various traits in conifers (Groover et al. 1994; Emebiri et al. 1997; Sewell et al. 2000; Jermstad et al. 2001); however, as yet, none of these have been confirmed or verified in an independent set of progeny. Of the few studies attempting independent verification, relatively few QTLs have actually been confirmed (Beavis 1995; Wilcox et al. 1997; Melchinger et al. 1998). This may be due to insufficient progeny numbers in the QTL detection and verification populations. The families used for this purpose in forestry have been largely determined by what was available at the time, as obtaining more appropriate pedigrees can take years. From simulated QTL detection experiments, it appears that the number of progeny required to detect and verify significant associations is far greater than what has been used in the past (Darvasi et al. 1993; Beavis 1998). For small effect QTLs, more than 1,000 progeny are recommended for detection and several hundred for verification populations (Carson et al. 2003a, 2003b). Fortunately, a number of large full-sib family block plantings of radiata pine were established in New Zealand in the early 1990s, when it first became apparent that larger numbers were needed (Wilcox et al. 1997).

Selective genotyping, using progeny from the extremes of the phenotypic distribution of a trait, has been suggested as a method to overcome the high costs of genotyping (Lander and Botstein 1989; Darvasi and Soller 1992; Muranty and Goffinet 1997). Simulations carried out by Carson et al. (2003b) suggested that selectively genotyping a small proportion of individuals in many circumstances would maintain most of the power of large population sizes and result in successful QTL detection.

Marker spacing does not appear to be critical, Darvasi and Soller (1994) reported that markers spaced up to 50 or

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60 cM along a framework map should be adequate for QTL detection. Also, Carson et al. (2003b) have suggested that at least in some circumstances, the loss from using more widely spaced markers is relatively small as compared to the gain which could be achieved with closely spaced markers. Further cost savings could therefore be obtained by using fewer markers spaced at regular intervals. The type of marker used can influence the outcome of QTL detection and verification experiments. Co-dominant markers, such as RFLPs or microsatellites, can be used to track multiple alleles at QTL loci and facilitate QTL mapping in an out-bred pedigree (Groover et al. 1994; Byrne et al. 1997a, 1997b).

Potential benefits from MAS were suggested for breeding programs for *Eucalyptus* and *Pinus radiata* (Kerr et al. 1996; Kumar and Garrick 2001); however, these did not include the costs of QTL detection. Johnson et al. (2000) evaluated the financial gains from MAS, including detection costs, for Douglas fir in the Pacific Northwest. Their study indicated that relatively large areas would need to be planted with MAS-improved germplasm to justify the initial investment. Wilcox et al. (2001), using simulated marker-based selection (MBS) in *P. radiata*, concluded that significant genetic and financial gains could be obtained even when selection is based on only a few loci each with relatively small effect.

Improvement of juvenile wood density (JWD) is an important objective for the radiata softwood industry in Australia and New Zealand. The emphasis on tree improvement since the 1950s has been on selection for growth and form, largely neglecting wood properties. The genetic correlation between JWD and stem diameter at breast height (DBH) is slightly negative. Individual tree heritability for JWD is relatively high, on average about 0.75, and that for DBH is low, about 0.25 (Cotterill and Dean 1990; Burdon and Low 1992). Selection for growth rate has reduced the rotation age and increased diameter, and as a consequence, the proportion of juvenile wood in the harvested tree has also increased. Juvenile wood has poorer grade recovery, lower density, lower strength, more distortion and surface checks and poorer finishing properties for structural timber (Zobel and Sprague 1998).

A collaborative project with CSIRO and Trees and Technology Ltd. was undertaken to determine if QTLs for JWD and DBH could be detected and verified given appropriate experimental design, and to compare results for high and low heritability traits. We also wanted to find out if the QTLs could be detected in other unrelated pedigrees leading to a commercial application of MAS.

Materials and methods

Populations for QTL detection and verification

A large full-sib family (approximately 10,000 trees) of *P. radiata* cross 268–405×268–345 was selected for QTL detection and verification. The trees were planted in 1994 in Kinleith Forest on the North Island of New Zealand.

Two individual QTL detection populations were derived by selective genotyping for JWD and DBH. The QTL detection population for JWD consisted of 1,379 trees, and from among these the 50 highest and 50 lowest density trees were utilised for marker genotyping. The QTL detection population for DBH consisted of 4,435 trees, and from among these the 100 highest and 100 lowest diameter trees were utilised for marker genotyping. Three trees which were substantially smaller than the others were thought to be outliers and were not included in the selectively genotyped population for DBH. Trees in the JWD population for the most part were also included among those utilised for DBH; however, only two trees were common between the selectively genotyped populations for JWD and DBH.

An independent set of 400 trees was randomly selected to be used for verification of marker-trait associations observed with initial QTL detection populations. The verification population was planted in the same block and the trees were intermixed with those from the detection populations, but none of the verification trees were included in either detection population. They were completely independent, but were measured and assessed for JWD and DBH at the same time as in the detection populations and in exactly the same way.

Two additional full-sib pedigrees each with more than 500 individuals were selected to evaluate QTL associations in unrelated families and as candidates for within family MAS. These families were referred to as the "bridging" populations and were unrelated to each other and also unrelated to the detection family; they were also grown on different sites and in different years than the detection family. The two populations were grown in Compartments 54 and 350 in Tarawara Forest, N.Z., referred to as Cpt. 54 and Cpt. 350. Each had about 400 progeny after eliminating 10–15% non-parental "rogue" progeny detected during routine marker genotyping.

Phenotypic measurement

Bark-to-bark wood cores were collected for the QTL detection and verification populations. The trees were 4 years old at the time of sampling for JWD. Basic density was determined on an oven dry weight, green volume basis, and the phenotypic values appeared to be normally distributed (Fig. 1A). JWD data was obtained similarly for the two bridging populations.

Diameter was measured when the trees were 5 years of age in September 1999. A height pole was used to ensure that the measurements were taken at 1.4 m. If branches occurred at this height, DBH was measured above and below the branches, and an average of the two measurements used to indicate DBH. Because the average diameter across the stand varied somewhat, rather than using raw data for selective genotyping and testing marker-trait associations, an analysis of variance was carried out using a defined blocking structure [block and plot(block)] to adjust the trait measurements. The two blocks represented somewhat different parts of the stand, and plots consisted of 25 adjacent trees. Deviations of each tree from the model (residuals) were calculated and used for QTL analyses. The phenotypic distribution for DBH is presented in Fig. 1B with actual values shown rather than residuals. DBH data was obtained similarly for the two bridging populations.

Marker genotyping

RFLP and microsatellite markers were selected at intervals of 10–20 cM based on a previously developed linkage map for *P. radiata* (Devey et al.1999). Seventy-four RFLP and 18 microsatellite markers were used for marker genotyping in the detection populations, covering about 1,200 cM. In selecting the markers, preference was given first to microsatellite markers and then to RFLP markers with simple, easy-to-score hybridisation patterns. Following initial collection of segregation data for RFLPs, six subsequently mapped microsatellite markers were used to substitute for RFLPs where two-point recombination values were less than 0.1.

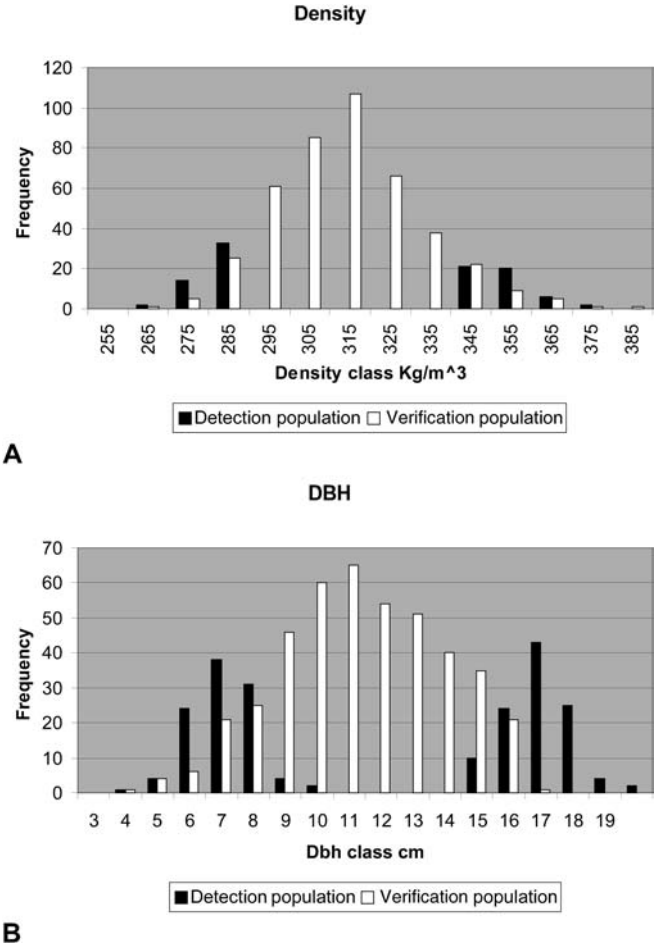

Fig. 1 Frequency distributions for juvenile wood density (**A**) and diameter at breast height (**B**) in the detection and verification populations

DNA isolation for the detection, verification, and Cpt. 54 populations were done using a scaled-down version of the CTAB method described in Devey et al. (1996). DNA isolations from the progeny from Cpt. 350 were done with a Qiagen MixerMill and Qiagen DNeasy 96 plant kit.

RFLP procedures were as described previously (Devey et al. 1996). About half of the RFLPs were derived from loblolly pine (*P. taeda*) cDNA clones, and the other half were derived *from P. radiata* genomic DNA clones (Devey et al. 1994, 1996). PCR conditions and primer sequences for microsatellite markers were reported in Devey et al. (2002).

QTL analyses of variance

Single-factor analyses of variance were carried out for each marker locus. The following linear model was fitted:

$$y_{ij} = \mu + m_i + e_{ij}$$

in which μ is the mean, m_i is the ith marker genotype and e_{ij} is the residual. Observations (y_{ij}) for DBH were adjusted for field layout effects as described above. JWD data were not adjusted. Analyses were carried out using the statistical package GenStat (Release 4.2, 5th edition, Lawes Agricultural Trust). Three separate analyses were carried out to detect marker/QTL associations for each marker locus: among alleles segregating in the male parent, among alleles segregating in the female parent and among all possible marker genotypes (maximum of four). "Percent variation accounted for" was calculated as:

100(1− residual m.s.)/(total m.s.) (Genstat 5 Committee 1993).

Results

Juvenile wood density

Basic density was determined for 1,379 full-sib progeny, and the 50 highest and 50 lowest individuals were selected for marker genotyping. Wood density of the high density trees ranged from 340.5 to 373 kg/m^3, and the low density trees ranged from 257.5 to 282.5 kg/m^3 (Fig. 1A). Analyses of variance indicated 27 markers had a significant (P<0.05) or highly significant (P<0.01) association with JWD in the detection population (Table 1). Two markers listed in Table 1 had a P-value between 0.05 and 0.10, and microsatellite marker *Pr233* was a substitution or second verification for the closely linked RFLP marker 602a. The percent variance accounted for by these markers is clearly overestimated in the selectively genotyped populations.

An independent verification population of 400 progeny from the same family was used to test all of the markers with P<0.10 in the selectively genotyped population. A slightly lower level of significance was used so as not to miss any markers that were potentially associated with JWD. Fourteen markers out of the 27 which were significant in the selectively genotyped population showed a significant association of P<0.05 with JWD in the independent verification population (Table 1). Marker *602a* was not significant in the verification population (P=0.151), but a closely linked marker, *Pr233*, was highly significant. The percent variation accounted for by each of the 14 markers ranged from 0.78% to 3.58%. Only one of the markers (7.05) was also detected in the population selectively genotyped for DBH and confirmed for DBH in the verification population ($P \leq 0.05$).

Linkage group, map order, and cM distances are listed in Table 1 according to Devey et al. (1999). Although there were 14 markers identifying QTLs for JWD, it was clear from their map position that a number of these were linked and possibly associated with the same QTL. There were seven linkage groups having one or more markers associated with JWD, corresponding to at least eight QTL positions as identified by markers *975, 1D11, 13.52a, 1955, 2442, 8.67b, 10.71a,* and *Pr233*. Assuming these eight QTLs are independent, the total percent variation accounted for is 14.1%.

With co-dominant markers it is possible to conduct separate maternal and paternal analyses of variance and determine which parent is contributing the QTL allele with the largest effect. Because the progeny number per class are greater for two genotypic classes than with four, it is possible to detect significance in one or the other parents when analysed individually, whereby when segregation data from both parents are analysed together the result may not be significant. For example, the maternal

Table 1 Detection and verification of quantitative trait loci (QTLs) for juvenile wood density, showing only markers with $P<0.10$ for the detection population (*d*) or $P<0.05$ for the verification population (*v*). The linkage groups and map order are based on Devey et al. (1999). Null alleles are indicated by (*9*). Analyses of variance were conducted using separate male, female and combined male/female marker segregation data

Linkage group	Map distance (cM)	Locus	Parental genotypes	Population	*F*-probability[c]			% Variation explained (Both)	Genotypic class means			
					Maternal	Paternal	Both					
									12[c]	22[c]		
1	80	2553a	*12×22*	d	0.000	–	0.000	12.96	298.9	326	–	–
				v	0.007	–	0.007	1.61	303.7	311.3	–	–
									13[c]	19[c]	23[c]	29[c]
1	126	975[b]	*12×39*	d	0.000	0.421	0.000	17.06	294.4	300.6	325.4	333.3
				v	0.003	0.089	0.001	3.58	308.1	296.8	311.1	311.7
									13[c]	23[c]	–	–
2	32	*Pr043–2* [a]	*33×12*	d	–	0.044	0.045	3.14	305.7	320.7	–	–
				v	–	0.864	0.864	0	306.7	307.2	–	–
									12[c]	13[c]	–	–
2	41	*Pr102* [a]	*32×11*	d	0.000	–	0.000	17.52	328.1	296.8	–	–
				v	0.004	–	0.004	1.93	311.2	303	–	–
									12[c]	13[c]	23[c]	33[c]
2	52	1D11[b]	*31×23*	d	0.000	0.558	0.000	15.52	320.9	334.2	304.2	293.3
				v	0.001	0.422	0.013	1.99	310.2	312.7	302.8	302.8
									12[c]	13[c]	22[c]	23[c]
2	71	66[b]	*23×12*	d	0.000	0.892	0.003	10.93	301.5	321.7	296.7	328.8
				v	0.036	0.633	0.103	0.90	306.9	309.3	301.6	311.2
									12[c]	14[c]	23[c]	34[c]
3	43	*Pr005* [a]	*31×42*	d	0.134	0.007	0.005	10.05	292.1	323.2	315.5	322.4
				v	0.090	0.061	0.060	1.22	303.4	305.9	305.5	314.0
									19[c]	99[c]	–	–
3	66	10.42b[b]	*99×19*	d	–	0.005	0.005	6.76	323.2	303.0	–	–
				v	–	0.070	0.070	0.59	309.6	304.5	–	–
									12[c]	22[c]	–	–
4	2	8.67d[b]	*22×21*	d	–	0.061	0.062	2.54	318.1	304.1	–	–
				v	–	0.047	0.047	0.78	310.2	304.5	–	–
									19[c]	29[c]	–	–
4	24	13.52a[b]	*99×12*	d	–	0.033	0.033	3.62	304.7	320.3	–	–
				v	–	0.049	0.049	0.82	305.1	310.3	–	–
									12[c]	13[c]	22[c]	23[c]
4	98	11.50[b]	*21×32*	d	0.540	0.036	0.047	5.11	302.0	331.0	309.2	312.2
				v	0.962	0.328	0.808	0	308.3	305.6	308.7	305.9
									11[c]	12[c]	–	–
5	4	7.05[b]	*11×12*	d	–	0.001	0.001	10.65	299.3	324.0	–	–
				v	–	0.029	0.029	0.97	303.9	310.0	–	–
									12[c]	13[c]	22[c]	23[c]
5	10	1955[b]	*23×12*	d	0.095	0.003	0.003	11.21	301.1	300.4	337.0	314.0
				v	0.097	0.031	0.038	1.45	308.0	299.7	310.6	309.3
									12[c]	13[c]	23[c]	33[c]
5	76	2442[b]	*13×32*	d	0.016	0.383	0.043	5.28	331.1	313.7	305.8	305.0
				v	0.003	0.063	0.004	2.81	312.5	309.8	306.6	297.7
									12[c]	22[c]	–	–
5	83	2718a[b]	*21×22*	d	0.084	–	0.085	2.03	307.4	320.3	–	–
				v	0.004	–	0.004	1.86	303.2	311.2	–	–
									19[c]	99[c]	–	–
6	32	8.67b[b]	*91×99*	d	0.003	–	0.003	8.04	323.2	301.3	–	–
				v	0.046	–	0.046	0.80	310.1	304.3	–	–
									12[c]	22[c]	–	–
6	49	2610[b]	*21×22*	d	0.005	–	0.005	6.84	323.3	302.9	–	–
				v	0.990	–	0.990	0	307.3	307.3	–	–
									13[c]	14[c]	23[c]	24[c]
6	62	10.45[b]	*12×34*	d	0.005	0.789	0.049	5.01	302.5	304.1	323.7	323.9
				v	0.528	0.300	0.527	0	309.8	303.6	308.5	307.9
									11[c]	12[c]	13[c]	23[c]
6	69	*Pr011* [a]	*31×12*	d	0.002	0.486	0.015	7.53	327.5	319.8	300.7	301.3
				v	0.052	0.287	0.088	0.96	309.0	310.6	308.5	301.5
									19[c]	99[c]	–	–
7	29	*Pr062* [a]	*99×19*	d	–	0.039	0.039	3.35	303.8	319.3	–	–
				v	–	0.993	0.993	0	307.2	307.2	–	–
									11[c]	12[c]	13[c]	23[c]
8	14	7.46[b]	*13×12*	d	0.005	0.753	0.047	5.27	324.2	321.0	300.3	302.5
				v	0.686	0.984	0.851	0	306.6	309.0	307.7	305.6
									11[c]	19[c]	–	–

Table 1 (continued)

Linkage group	Map distance (cM)	Locus	Parental genotypes	Population	*F*-probability[c]			% Variation explained (Both)	Genotypic class means			
					Maternal	Paternal	Both					
8	38	10.36a[b]	*91×11*	d	0.004	–	0.004	7.26	301.4	322.3	–	–
				v	0.059	–	0.059	0.67	304.5	309.9	–	–
									12[c]	19[c]	29[c]	99[c]
8	39	10.36b[b]	*91×29*	d	0.001	0.943	0.008	9.07	300.6	297.8	323.3	325.4
				v	0.071	0.572	0.243	0.31	305.2	304.3	308.0	311.8
									12[c]	13[c]	22[c]	23[c]
8	55	10.71a[b]	*23×21*	d	0.003	0.185	0.017	7.33	328.9	303.3	316.2	301.3
				v	0.012	0.680	0.070	1.15	310.3	305.2	311.7	301.7
									12[c]	13[c]	22[c]	23[c]
9	0	*Pr070* [a]	*23×12*	d	0.033	0.060	0.041	5.53	297.5	318.0	315.8	325.1
				v	0.137	0.305	0.093	0.89	308.4	309.4	310.6	301.5
									12[c]	13[c]	22[c]	23[c]
9	86	*Pr9.3* [a]	*23×12*	d	0.008	0.714	0.027	6.28	302.9	330.6	306.2	315.7
				v	0.178	0.765	0.392	0.00	306.0	312.4	307.2	308.4
									19[c]	99[c]	–	–
10	91	602a[b]	*99×19*	d	–	0.003	0.003	7.94	324.8	303.0	–	–
				v	–	0.151	0.151	0.28	309.3	305.2	–	–
									12[c]	13[c]	24[c]	34[c]
10	94	*Pr233* [a]	*23×14*	d	–	–	–	–	–	–	–	–
				v	0.569	0.004	0.033	1.50	311.2	310.9	301.2	304.5
									13[c]	23[c]	–	–
10	107	1643[b]	*21×33*	d	0.002	–	0.002	8.47	323.3	300.8	–	–
				v	0.709	–	0.709	0	306.6	307.6	–	–
									11[c]	12[c]	22[c]	–
13	0	*Pr43* [a]	*12×12*	d	0.034	0.034	0.034	4.84	327.6	310.4	301.6	–
				v	0.478	0.478	0.478	0	308.4	305.6	309.5	–

[a] Microsatellite locus
[b] RFLP locus
[c] Progeny genotypic classes derived from the mating in column 4

analyses for RFLP marker *66* had an *F*-probability of 0.036; however when both parents were analysed together the result was non-significant (P=0.103). In every case where the differences among allele classes segregating from a parent were significant in the verification population, the allele that expressed the highest value for the trait was the same in both the detection and verification populations. In addition, the parental allele with the highest value of the trait was always consistent among linked markers. For example, markers *1D11* and *66* on chromosome 2 are 19 cM apart, and the allelic effect for both markers is inherited through the maternal parent. This may indicate that both markers are detecting the same QTL and that marker *66* is associated with a real QTL effect.

Six additional markers were significant in the verification population at P<0.10 (*Pr005, 10.42b, Pr011, 10.36a, 10.36b, Pr070*), and may also represent "real" QTLs associated with very small effect genes.

Diameter at breast height

In parallel with the above objective, we used the same family to detect QTLs for DBH. Measurements were taken from 4,435 trees and the 100 largest and 100 smallest trees were selected for marker analysis using the same markers as above. DBH of the large trees ranged from 14.5 to 19.8 cm and from 3.5 cm to 8.7 cm for the small trees (Fig. 1B). The residual values from the analysis of variance ranged from 4.21 to 8.14 for the large trees, and from 4.85 to 9.14 for the small trees.

Analyses of variance indicated that 14 markers had a significant (P<0.05) or highly significant (P<0.01) association with DBH in the detection population (Table 2). The same 400 verification progeny as used above were used to test marker associations with DBH, and two markers, *7.05* and *7.46*, were verified and significantly associated with DBH (P<0.05). Two additional markers (*653a* and *602a*) that did not show an association with DBH in the detection population are also included in Table 2. They were tested because they showed an association with JWD in the detection population selectively genotyped for JWD. Interestingly, they did appear to be associated with DBH in the verification population (P<0.01). *Pr233* (also included in Table 2) was a substitution for closely linked RFLP marker *602a*, which had shown significance for JWD in the detection population and was also significantly associated with diameter in the verification population. *Pr233* was not tested in the detection population for JWD or DBH.

The two QTLs detected and verified for DBH were on separate linkage groups and could be assumed to be independent. The total percent variation accounted for by these markers is 2.20%. Of the two markers, only *7.05* also showed an association with density and the effect

Table 2 Detection and verification of QTLs for diameter at breast height, showing only markers with $P<0.10$ for the detection population (*d*) or $P<0.05$ for the verification population (*v*). Trait measurements for diameter at breast height were adjusted for deviations from a defined blocking structure [block and plot (block)]. Analyses of variance were conducted using separate male, female and combined male/female marker segregation data

Linkage group	Map distance (cM)	Locus	Parental genotypes	Population	*F*-probability			% Variation explained (Both)	Genotypic class means			
					Maternal	Paternal	Both					
									13[c]	19[c]	23[c]	29[c]
2	10	*Pr025* [a]	*93×12*	d	0.967	0.005	0.043	2.47	0.58	1.01	–1.16	–1.34
				v	0.557	0.548	0.140	0.63	0.14	0.52	0.49	-0.12
									13[c]	14[c]	23[c]	24[c]
3	6	653a[b]	*12×43*	d	0.795	0.396	0.753	0	0.04	–0.16	0.29	–0.83
				v	0.959	0.078	0.003	2.73	0.08	0.40	0.72	–0.45
									19[c]	99[c]	–	–
3	66	10.42b[b]	*99×19*	d	–	0.002	0.002	3.91	–1.46	0.76	–	–
				v	–	0.110	0.110	0.40	0.06	0.43	–	–
									12[c]	22[c]	–	–
4	3	8.67a[b]	*22×21*	d	–	0.075	0.075	1.04	–0.86	0.43	–	–
				v	–	0.649	0.649	0	0.16	0.27	–	–
									12[c]	13[c]	23[c]	33[c]
4	39	14.02a[b]	*31×23*	d	0.025	0.102	0.040	2.54	–0.46	1.29	–1.35	–0.88
				v	0.669	0.806	0.956	0	0.36	0.23	0.18	0.19
									12[c]	22[c]	–	–
4	140	2530[b]	*21×22*	d	0.052	–	0.052	1.33	–0.995	0.4207	–	–
				v	0.458	–	0.458	0	0.15	0.33	–	–
									11[c]	12[c]	–	–
5	4	7.05[b]	*11×12*	d	–	0.040	0.040	1.54	–1.00	0.49	–	–
				v	–	0.035	0.035	0.87	–0.03	0.45	–	–
									12[c]	13[c]	23[c]	33[c]
5	52	653b[b]	*31×23*	d	0.005	0.873	0.008	4.23	–1.92	–0.45	1.63	0.28
				v	0.870	0.975	0.551	0	0.02	0.36	0.40	0.08
									12[c]	22[c]	–	–
6	0	1021[b]	*12×22*	d	0.005	–	0.005	3.21	0.79	–1.22	–	–
				v	0.774	–	0.774	0	0.26	0.19	–	–
									12[c]	22[c]	–	–
6	19	6.59b[b]	*21×22*	d	0.029	–	0.029	1.85	–1.01	0.59	–	–
				v	0.517	–	0.517	0	0.29	0.15	–	–
									19[c]	99[c]	–	–
6	32	8.67b[b]	*91×99*	d	0.003	–	0.003	3.76	–1.24	0.92	–	–
				v	0.189	–	0.189	0.19	0.37	0.07	–	–
									12[c]	22[c]	–	–
6	49	2610[b]	*21×22*	d	0.004	–	0.004	3.38	–1.14	0.93	–	–
				v	0.259	–	0.259	0.07	0.36	0.10	–	–
									11[c]	12[c]	13[c]	23[c]
6	69	*Pr011* [a]	*31×12*	d	0.008	0.762	0.016	3.51	–1.61	–0.75	1.77	0.12
				v	0.052	0.142	0.128	0.71	0.54	0.40	0.31	–0.15
									11[c]	12[c]	–	–
6	99	*Pr4.6* [a]	*11×21*	d	–	0.029	0.029	1.84	–0.97	0.62	–	–
				v	–	0.437	0.437	0	0.12	0.29	–	–
									11[c]	12[c]	13[c]	23[c]
8	14	7.46[b]	*13×12*	d	0.036	0.829	0.122	1.36	–0.69	–1.29	0.07	1.13
				v	0.040	0.093	0.041	1.33	–0.11	0.07	0.17	0.77
									19[c]	99[c]	–	–
10	54	16.92[b]	*99×19*	d	–	0.003	0.003	3.73	0.83	–1.32	–	–
				v	–	0.686	0.686	0	0.28	0.19	–	–
									12[c]	13[c]	22[c]	23[c]
10	75	*Pr001* [a]	*12×23*	d	0.342	0.001	0.006	4.71	1.21	–0.85	0.92	–2.00
				v	0.065	0.696	0.311	0.15	0.48	0.44	0.11	–0.02
									19[c]	99[c]	–	–
10	91	602a[b]	*99×19*	d	–	0.620	0.620	0	–0.03	–0.40	–	–
				v	–	0.005	0.005	1.74	–0.09	0.55	–	–
									12[c]	13[c]	24[c]	34[c]
10	94	*Pr233* [a]	*23×14*	d	–	–	–	–	–	–	–	–
				v	0.952	0.007	0.012	2.04	–0.27	0.15	0.82	0.36

[a] Microsatellite locus
[b] RFLP locus
[c] Progeny genotypic classes derived from the mating in column 4

Table 3 ANOVA results for six microsatellite markers tested in detection, verification and bridging populations for associations with juvenile wood density (*JWD*) and diameter at breast height (*DBH*). Populations *d*, *v*, *c54* and *c350* refer to detection, verification, Cpt. 54 and Cpt. 350 populations, respectively

Lg	Locus	Parental genotypes	Population	JWD				DBH			
				F-Probability			% Variation explained Both	F-Probability			% Variation explained Both
				Maternal	Paternal	Both		Maternal	Paternal	Both	
2	*Pr102*	*23×11*	d	0.000	–	0.000	17.52	0.085	–	0.085	0.94
			v	0.004	–	0.004	1.93	0.852	–	0.852	-0.25
		12×11	c54	0.315	–	0.318	0.00	0.943	–	0.944	-0.25
		22×12	c350	–	0.687	0.687	0	–	0.266	0.266	0.06
3	*Pr005*	*13×24*	d	0.134	0.007	0.005	10.05	0.449	0.575	0.804	-0.98
			v	0.090	0.061	0.060	1.22	0.696	0.016	0.070	1.10
		12×23	c54	0.020	0.065	0.026	1.62	0.478	0.306	0.186	0.47
		12×12	c350	0.000	0.000	0.000	3.44	0.760	0.760	0.760	-0.36
6	*Pr011*	*31x12*	d	0.002	0.486	0.015	7.53	0.008	0.762	0.016	3.51
			v	0.052	0.287	0.088	0.96	0.052	0.142	0.128	0.71
		23×14	c54	0.096	0.811	0.293	0.19	0.395	0.281	0.585	-0.27
		23×12	c350	0.771	0.598	0.534	0	0.807	0.636	0.535	-0.21
8	*Pr114* [a]	*13×23*	d	–	–	–	–	–	–	–	–
			v	0.026	0.249	0.085	0.95	0.614	0.061	0.265	0.25
		11x12	c54	–	0.022	0.022	1.07	–	0.902	0.902	-0.25
		12×22	c350	0.178	–	0.178	0.21	0.422	–	0.422	-0.09
9	*Pr070*	*23×12*	d	0.033	0.060	0.041	5.53	0.470	0.839	0.722	-0.81
			v	0.137	0.305	0.093	0.89	0.729	0.828	0.893	-0.61
		12×11	c54	0.154	–	0.157	0.26	0.832	–	0.833	-0.25
		23×19	c350	0.002	0.228	0.010	2.17	0.293	0.373	0.300	0.18
10	*Pr233* [a]	*23×14*	d	–	–	–	–	–	–	–	–
			v	0.569	0.004	0.033	1.50	0.952	0.007	0.012	2.04
		13×24	c54	0.005	0.304	0.008	2.25	0.850	0.905	0.912	-0.64
		14×23	c350	0.791	0.255	0.570	0	0.324	0.385	0.609	-0.30

[a] Pr114 and Pr233 were substitutions for closely linked RFLPs and were not tested in the detection populations

was positive for both traits. For the putative association of DBH and marker *Pr233*, the marker allele showing an increasing effect for density showed a decreasing effect for diameter at *Pr233*.

Bridging populations

Some of the marker-trait associations detected in the cross 268–405×268–345 were tested in two unrelated "bridging" families selected as candidates for MAS. Only microsatellite markers were used for this part of the study, since using RFLPs to genotype the number of progeny needed for MAS would be impractical. Therefore, several important regions were not tested due to lack of linked microsatellites. Again, using a slightly lower level of significance ($P<0.10$) so as not to miss any real QTLs, six microsatellite markers were tested which showed an association with JWD or DBH in the verification population. A summary of results for these markers in the detection, verification, and bridging populations is presented in Table 3.

Analyses of variance in the two bridging families indicated that four of the six markers showed an association ($P<0.05$) with JWD in one or both families. *Pr005* was associated with JWD in both families. *Pr114* and *Pr233* were each associated with JWD in Cpt. 54, but not in Cpt. 350; and *Pr070* was associated with JWD in Cpt. 350, but not in Cpt. 54. Of the two markers that had shown an association with DBH in the verification population, neither was associated with this trait in the bridging families.

Discussion

The most important outcome from this research is that it demonstrates that small effect QTLs can be detected and independently verified in *P. radiata*, thereby providing a basis for MAS. At least eight QTLs explaining more than 14% of the variation for juvenile wood, and two QTLs for DBH explaining 2.2% of the variation were detected and verified. In two unrelated bridging populations, four of the six microsatellite markers tested showed a consistent relationship with density. Additional investigation is needed to determine whether the technology is commercially viable. This would depend, for example, on such things as clonal deployment and comparisons of the extra gain from MAS relative to conventional selection. Wilcox et al. (1997) suggested that genetic and economic gains from marker-based selection for density and diameter can be achieved even using only a few QTLs of small effect.

Marker-trait associations were identified using a straightforward and robust experimental approach for QTL detection and verification. A very large population was phenotyped; a small number of individuals from each phenotypic extreme were genotyped using markers spaced at regular intervals along a framework map. QTLs

were then verified in an independent set of progeny. The most important difference between the approach used here and that used previously in conifer QTL studies is the population sizes for detection and verification. The experimental design used here was based on simulation studies which suggested that larger numbers were needed and that selective genotyping would be a cost effective approach (Carson et al. 2003a, 2003b).

The experimental designs were more efficient in identifying QTLs for JWD than for DBH. Of the 27 markers showing an association with JWD, 14 were verified in an independent set of progeny; whereas of the 14 markers showing an association with DBH, only two were verified. QTLs for JWD were distributed on seven linkage groups, and the percentages of variation accounted for by markers in the verification population ranged from 0.78 to 3.58, suggesting that there are numerous genes of relatively small effect controlling this trait. In contrast, only two QTLs were verified for DBH, each on a different linkage group with the percent of the variation explained being 0.87 and 1.33. It is important to note that the proportion of variance explained by a QTL and the relative difference in means are over-estimated in selectively genotyped populations as compared to large randomly selected populations (Davarsi and Soller 1992).

It is not known why fewer QTLs were detected and verified for DBH than for JWD. It may be that DBH is controlled by smaller effect genes than JWD, although the lower heritability of DBH (0.25 vs 0.75) is probably also contributing to the lower power of the experimental approach for detecting QTLs for DBH. An increased number of progeny, both phenotyped and genotyped for DBH, were intended to compensate for the lower heritability; however, this compensation may not have been enough. It has been suggested that selective genotyping of low heritability traits using a small proportion of the total population may reduce power to detect QTLs, as a disproportionately greater number of individuals may be included in the tails whose phenotypes are a result of non-genetic factors (Wilcox et al. 2001).

Various publications have presented and compared methods for QTL detection (Lander and Botstein 1989; Darvasi et al. 1993; Zeng 1993,1994; Rebai et al. 1995, Carson et al. 2003a, 2003b). Rebai et al. (1995) concluded that the advantages for interval mapping over single factor ANOVAs decreased as distance between markers decreased. For intervals of 20 cM, interval mapping was only about 5% more powerful than ANOVA. Carson et al. (2003a) compared four methods for choosing markers for MAS; choosing markers at a peak for single factor ANOVAs appeared to be more efficient than interval mapping or composite interval mapping. Choosing markers for MAS using single marker analyses with large sample sizes resulted in almost as much gain from MAS, but required much fewer markers. With large sample sizes, these markers contributed little additional gain in the simulations, but would increase the cost of MAS substantially.

Clonal replication and testing may be an alternative to large progeny numbers in some species to improve the accuracy of phenotypic assessment of low heritability traits (Bradshaw and Forster1992). Clonal replication has the effect of increasing the heritability of individual genotypes, and in combination with relatively large population sizes might be more successful in identifying very small effect QTL, or QTL controlling traits with low heritability. In addition, it may be that including additional markers suggested as being associated with DBH by the JWD QTL detection and verification process would increase the gains from MAS in this trait. The correlated response of DBH with JWD might provide a better detection approach than looking at DBH alone.

With a negative genetic correlation between density and diameter, we would expect markers for density QTLs to also show an association with diameter QTLs, and that alleles expressing an increased effect for one trait would have a decreasing effect on the other. Actually, many of the markers for density had no detectable association with diameter. Only two markers appeared to have an association with both JWD and DBH, and one of these, *7.05*, had an allelic effect which was positive for both traits. There are likely to be other negative associations present for these two traits, but the associations for DBH were not statistically significant. It is worth noting however, that with appropriate marker selection, it may be possible to improve density without adversely affecting diameter.

In outbred conifer populations, both the marker locus and the QTL are in linkage equilibrium, and the marker and/or QTL may not segregate in different families. As a result the phase relationships can change, and a marker-QTL association in one pedigree may not necessarily be detected in another pedigree (Strauss et al.1992). Therefore, we did not necessarily expect that markers associated with QTLs in the detection/verification family would also be associated with these traits in the unrelated bridging families. With co-dominant markers the inheritance of QTL effects can be compared in different families. For example, with *Pr233* the QTL appears to be segregating in the paternal parent in the verification population, but in the maternal parent for Cpt. 54.

For commercial application of MAS, it is important that the marker assays are completed as quickly and efficiently as possible. RFLP markers significantly associated with density or diameter were not tested in the bridging populations due to time and resource limitations, and also because they would not be suitable markers for MAS. Since the completion of this study, additional microsatellite markers have been mapped, and there are now one or more microsatellites closely linked to each of the RFLPs associated with density or diameter QTLs. These markers could be substituted for the RFLPs; however, additional testing is needed to determine if the microsatellites are also associated with the QTLs. MAS using microsatellite markers is feasible, but the number of assays required is still daunting. Access to a commercial genotyping facility with a 96- or 384-well capillary machine for microsatellite assays would be desirable.

The results of this study show consistent marker-QTL relationships among three unrelated families and indicate that MAS should be possible in radiata pine. Application of this technology to conifer tree improvement appears promising, but will be challenging.

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