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Genetic maps for Pinus elliottii var. elliottii and P. caribaea var. hondurensis using AFLP and microsatellite markers

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Abstract Genetic maps for individual *Pinus elliottii* var. *elliottii* and *P. caribaea* var. *hondurensis* trees were generated using a pseudo-testcross mapping strategy. A total of 329 amplified fragment length polymorphic (AFLP) and 12 microsatellite markers were found to segregate in a sample of 93 interspecfic F_1 progeny. The male *P. caribaea* var. *hondurensis* parent was more heterozygous than the female *P. elliottii* var. *elliottii* parent with 19% more markers segregating on the male side. Framework maps were constructed using a LOD 5 threshold for grouping and interval support threshold of LOD 2. The framework map length for the *P. elliottii* var. *elliottii* megagametophyte parent (1,170 cM Kosambi; 23 linkage groups) was notably smaller than the *P. caribaea* var. *hondurensis* pollen parent (1,658 cM Kosambi; 27 linkage groups). The difference in map lengths was assumed to be due to sex-related recombination variation, which has been previously reported for pines, as the difference in map lengths not be accounted for by the larger number of markers mapping to the *P. caribaea* var. *hondurensis* parent – 109 compared with 78 in *P. elliottii* var. *elliottii* parent. Based on estimated genome sizes for these species, the framework maps for *P. elliottii* var. *elliottii* and *P. caribaea* var. *hondurensis* covered 82% and 88% of their respective genomes. The pseudo-testcross strategy was extended to include AFLP and microsatellite markers in an intercross configuration. These comprehensive maps provided further genome coverage, 1,548 and 1,828 cM Kosambi for *P. elliottii* var. *elliottii* and *P. caribaea* var. *hondurensis*, respectively, and enabled homologous linkage groups to be identified in the two parental maps. Homologous linkage groups were identified for 11 out of 24 *P. elliottii* var. *elliottii* and 10 out of 25 *P. caribaea* var. *hondurensis* groups. A higher than expected level of segregation distortion was found for both AFLP and microsatellite markers. An explanation for this segregation distortion was not clear, but it may be at least in part due to genetic mechanisms for species isolation in this wide cross.

Keywords Marker-aided selection · Forest trees · Genetic markers · Linkage maps

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Introduction

Most attributes of interest to tree breeders require many years of growth before they can be reliably assessed (Zobel and Talbert 1984). Wood density, for example, an important indicator of timber strength in conifers, may only be reliably selected in *Pinus teada* after 6–11 years of growth (Zobel and Jett 1996). Temperate conifers require even longer, as long as 12–25 years for species such as Douglas fir. Marker-aided selection (MAS), the selection of individuals based on genetic markers linked with genes controlling traits, has been applied in agricultural crops to improve the efficiency of selection and accelerate returns from breeding [reviewed in Young (1999)]. The possibility of MAS for selection early in a tree's life, long before it expresses it genetic potential, is particularly advantageous in tree breeding, as the time savings are generally much larger than in agricultural crops (O'Malley and McKeand 1994; Tauer et al. 1992; Williams and Neale 1992). Consequently, genetic maps, generally a prerequisite for MAS, have been developed for trees belonging to most major forest tree genera, including *Eucalyptus, Pinus, Acacia* and *Populus* (eg. Bradshaw et al. 1994; Butcher and Moran 2000; Devey et al. 1994; Grattapaglia and Sederoff 1994).

Pinus elliottii var. *elliottii* Little and Dorman (PEE), *P. caribaea* Morlet var. *hondurensis* Barrett and Golfari

(PCH) and their hybrid are the principle forest tree plantation taxa in Queensland, Australia (Haines 2000). The hybrid is the taxon of choice for planting on around 70% of the 177,000-ha estate planted to *Pinus* spp. in Queensland, as it combines favourable characteristics from the parents, providing superior growth and form (Dieters 1996; Powell and Nikles 1996; Toon et al. 1996). Pine wood from these plantations is primarily used for structural timbers, veneer and plywood products (Nikles 1996). A shorter rotation and improved juvenile wood properties are viewed as key breeding objectives for the continued market success of hybrid pine products (Haines 2000). The breeding and deployment systems used with the hybrid pine, including its amenability to vegetative propagation, relatively short rotation age and a single breeding zone suggest that MAS will be more viable than for many temperate conifers (Johnson et al. 2000).

A number of strategies have been used to generate genetic maps in *Pinus* spp. and other outcrossing tree species [reviewed in Williams (1998)]. The pseudo-testcross (PTC) strategy is one approach for forest trees where inbreeding is usually undesirable and advanced generation crosses impractical due to long generation times (Grattapaglia and Sederoff 1994). In pines, however, their unusual reproductive biology provides an alternative approach for generating single-tree genetic maps as haploid mapping can be carried out on a population of megagametophytes from a single tree (Tulsieram et al. 1992). Other strategies for mapping and QTL detection are possible in the relatively rare situations where three generation pedigrees are available (Williams and Neale 1992). Although it has been used with pines (Kubisiak et al. 1995; Wilcox et al. 2001), the PTC mapping strategy has tended to be used most often in hardwood species such as eucalypts where megagametophyte maps are not feasible (Grattapaglia and Sederoff 1994; Marques et al. 1998; Shepherd et al. 1999). The PTC strategy may not be as efficient as single-tree genetic maps based on megagametophytes because not all heterozygous marker loci will segregate, nonetheless, a double-PTC where maps are generated for both parents may offset this lower efficiency (Grattapaglia and Sederoff 1994).

Until recently, genetic maps for pines have tended to be incomplete (i.e. not covering the total genome) and fragmented (more than the expected 12 linkage groups) (Remington et al. 1999). Using amplified fragment length polymorphism (AFLP) markers, Remington et al. (1999) constructed a complete genetic linkage map for *P. taeda*, a close relative of PEE and PCH. Genetic maps based on random amplified polymorphic DNA (RAPD) markers have been reported for *P. elliottii* (Kubisiak et al. 1995; Nelson et al. 1993) and a close relative longleaf pine (*Pinus palustris* Mill.) (Kubisiak et al. 1995; Nelson et al. 1994). A linkage map has also been generated for a PEE × PCH F_1 hybrid (Dale 1994), but as yet no linkage map has been reported for *P. caribaea* or PCH.

We report genetic maps for an individual of PEE and PCH. Maps were generated by linkage analysis of AFLP and microsatellite markers segregating in an interspecific F_1 hybrid family. Framework maps were generated using a PTC strategy to allow a comparison of map sizes with those of other pines and for future quantitative trait analysis. Comprehensive maps were generated using an PTC strategy that was extended to include markers in mating configuration other than a backcross (BC) to maximise genome coverage from markers available and allow alignment of homologous linkage groups between the two maps.

Materials and methods

Mapping population

The mapping population consisted of a family derived from two controlled crosses of a select *Pinus elliottii* var. *elliottii* (2PEE1–102) and a select *Pinus caribaea* var. *hondurensis* (1PCH1–63). Seedlings from the first pollination were planted in a field trial at two sites, Beerburrum and Toolara, Queensland. This pollination was carried out with a mix of dead 2PEE1–102 pollen and live 1PCH1–63 pollen in an attempt to enhance the fertilisation rate. Needles were collected from 89 three-year-old trees across the two sites in this experiment. From the second seedlot, five from a batch of 320 germinated seedlings were sampled to give a total of 94 progeny. These five individuals exhibited poor growth in the nursery and were unlikely to have survived in the field. Foliage material for the parents of this cross was collected from scions grafted in clonal seed orchards. A total of 93 individuals were used in the linkage analysis as one individual in our sample was found to be a product of the selfing of the maternal parent and was eliminated on the basis of microsatellite marker genotypes (data not shown).

DNA preparation

Needle samples were stored at –20 °C as soon as practical after harvest. Frozen foliage was used to prepare DNA as described in Graham et al. (1994). This method typically yielded 30–40 ug of DNA per 1 g of needle tissue by comparison with DNA weight standards on agarose gels.

Genetic markers

Microsatellite markers

A total of 50 microsatellite markers developed in *P. radiata, P. taeda* or *P. strobus* were tested for transfer to PCH, PEE and their hybrids (Echt et al. 1996, 1999; Elsik et al. 2000; Fisher et al. 1996; Smith and Devey 1994). The results of transfer of these loci, the conditions used for the polymerase chain reaction (PCR) and the methods used for detection and sizing of amplification products are reported in Shepherd et al. (2002).

AFLP markers

The method adopted for AFLP generation was based on Remington et al. (1999). This method largely follows Vos et al. 1995 but uses a 2-bp preselective amplification and 7-bp selective amplification strategy to adapt to the large genome size of conifers. The adaptor and primer sequences are given in Remington et al. (1999) and are listed with a corresponding primer combination (PC) number in Table 1. Adaptors and primers were synthesised by Genset (Lismore, NSW). Selective primers were synthesised with a 5' fluorescent dye, either HEX or TET, on the *Eco*RI primer.

Table 1 AFLP primer combinations[a] and band statistics

Primer combination	*Eco*RI primer extension[a]	*Mse*I primer extension[a]	Number of bands scored	Number A[b]	Number B	Number C	Minimum band size (bp)	Maximum band size (bp)	Average band size (bp)
1 PC1	ACA	CCAG	6	1	2	3	79	182	148
2 PC2	ACA	CCCG	14	7	5	2	75	431	264
2 PC3	ACA	CCGC	15	2	10	3	94	413	229
4 PC4	ACA	CCGG	14	4	8	2	82	458	253
5 PC5	ACA	CCTG	18	1	7	10	102	487	276
6 PC6	ACC	CCAG	7	3	2	2	123	309	203
7 PC7	ACG	CCAA	22	4	13	5	130	307	215
8 PC8	ACG	CCAC	16	5	8	3	74	219	148
9 PC9	ACG	CCAG	19	9	9	1	105	535	239
10 PC10	ACG	CCCA	15	0	12	3	77	264	177
11 PC11	ACG	CCGA	11	2	7	2	89	350	224
12 PC12	ACG	CCGC	8	0	5	3	107	523	313
13 PC13	ACG	CCTA	18	10	8	0	56	290	179
14 PC14	ACG	CCTC	22	0	16	6	54	348	184
15 PC15	ACG	CCTG	9	6	3	0	59	385	205
16 PC16	ACG	CCTT	26	13	11	2	122	432	253
17 PC17	ACT	CCAG	5	1	3	1	118	294	186
18 PC18	ACT	CCCG	5	0	2	3	81	343	188
19 PC19	ACT	CCGC	19	5	10	4	83	314	170
20 PC20	ACT	CCGG	22	12	9	1	114	324	199
21 PC21	ACT	CCTG	–[c]	–	–	–	–	–	–
22 PC22	ACC	CCCG	13	4	5	4	78	319	195
23 PC23	ACC	CCGC	15	1	5	9	92	350	198
24 PC24	ACG	CCCG	10	4	4	2	73	410	223

[a] Data from Remington et al. (1999)
[b] No. of bands in each quality rating (see Materials and methods)
[c] PC21 was not analysed due to gel failure

Restriction-ligation reactions were carried out in a total volume of 30 μl. Reactions contained 500 ng of genomic DNA, 0.125 U/μl *Eco*RI (New England Biolabs, Beverly, Mass), 0.125 U/μl *Mse*I (New England Biolabs), 0.02 U/μl T4 ligase, 1 × T4 ligase buffer (New England Biolabs), 50 ng/μl bovine serum albumin (BSA), 50 m*M* NaCl, 0.1 pmol/μl *Eco*RI adaptor (E) and 1 pmol/μl *Mse*I adaptor (M). Reactions were carried out at room temperature overnight. A 15-μl aliquot of this reaction was electrophoresed to verify digestion, the remaining 15 μl was diluted to 100 μl with $TE_{0.1}$ (20 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0).

Preselection reactions were carried out in a total volume of 20 μl and consisted of: 4 μl of the diluted R/L, 1 × PCR reaction buffer (Boehringer Mannheim, Indianapolois, Ind.), 0.4 U *Taq* polymerase (Boehringer Mannhiem), 0.8 m*M* total dNTPs with 30 ng of each preselective primer E + AC and M + CC per reaction. Cycling was carried out on a PE 9600 thermocycler (Perkin Elmer, Foster City, Calif.) with a 72 °C hold for 2 min followed by 20 cycles of 94 °C for 1 s, 56 °C for 30 s and 72 °C for 2 min, followed by a final hold at 60 °C for 30 min. A 10 μl aliquot of the reaction was electrophoresed on agarose to verify amplification; the remaining 10 μl was diluted to 200 ul with $TE_{0.1}$.

Selective amplifications were carried out in 10-μl total volumes consisting of 1 μl of diluted preselective template and using the same reaction conditions as for preselective amplification but with 30 ng of a *Mse*I primer and 5 ng of an *Eco*RI primer per reaction. Selective amplification cycling was performed on a PE 9600 with the following programme: an initial cycle of 94 °C for 2 min, 65 °C for 30 s, 72 °C for 2 min; then eight cycles of 94 °C for 1 s with an annealing temp starting at 64 °C for 30 s but decreasing by 1 °C each cycle, 72 °C for 2 min; finally, 23 cycles of 94 °C for 1 s, 56 °C for 30 s, 72 °C for 2 min, with a final hold at 60 °C for 30 min.

AFLP fingerprints were separated on 6% denaturing polyacrylamide gels using a Gel-Scan 2000 real-time gel electrophoresis system (Corbett Research P/L, Sydney, NSW) and the 18-cm (H) × 20-cm (W) × 0.25-mm (D) gel format. Gels were "pre-run" for 30 min at 40 °C and 1,400 V. A 1-μl aliquot of a 1:2 mix of PCR product and denaturing gel-loading dye for each sample was "pulse" loaded (20 s at 1,400 V) following denaturation of the samples by heating for 3 min at 94 °C, then rapid chilling on ice. Each gel had provision for 48 samples and three marker standards (GeneScan-500 TAMRA, Perkin Elmer, Foster City, Calif.). Gels were then electrophoresed at 40 °C and 1,400 V for 80 min, which allowed separation of bands up to 500 bp. Bands were scored by recording the presence of a band on a digital overlay of the gel images using GENE PROFILER version 3.45 software (Scanalytics, Fairfax, Va.). The software was then used to group bands into bins and report marker scores to a database programme (RFLPSCAN database, Scanalytics). A bin size of 1 bp was typically used, but bin sizes were adjusted manually if necessary. Data were exported to a spreadsheet for compilation with microsatellite data and testing for expected segregation ratios. On completion of scoring, each marker was reviewed and assigned a "quality" rating. High-quality bands had strong bands and were well separated (>2 bp) from other bands. Bands that were not as distinct were assigned to a second grade, whereas bands which were confounded with other bands or faint and difficult to score were assigned to a third grade.

Linkage analysis

Microsatellite and AFLP markers were coded as either BC or intercross (IC). Dominant AFLP of an IC mating type were identified by the genotypes of the parents (presence of a band in both parents) and approximate 3:1 (presence to absence) segregation in the progeny. AFLP markers coded as a BC were present in one parent but absent in the other and had approximate 1:1 segregation in the progeny. A separate data file was prepared for each parent with their respective sets of segregating AFLP and microsatellite markers.

Comprehensive maps

Comprehensive maps were constructed using MAPMANAGERQTX version b3 (MQTX) software (Manly et al. 2001) following the strategy outlined by Grattapaglia and Sederoff (1994) for a two-way PTC but extended to include AFLP markers, segregating in an IC configuration in the case of AFLP markers or IC or multiple intercross (MIC) configurations in the case of microsatellite markers.

Comprehensive maps were prepared separately for each parent, in three stages. An initial grouping and ordering analysis was carried out on a subset of markers for each parent that were in a BC configuration and fitted the expected segregation ratio of 1:1 at $P < 0.05$. Analysis was carried out with the following settings: "Arbitrary" cross type (allows for detection of markers linked in repulsion) and "Allow for mixed segregation types"; linkage evaluation threshold $P = 0.001$ and Kosambi (K) map function. The "Make Groups" was applied to form linkage groups and establish a best order by "rippling". Markers linked in repulsion were identified in the "Stats" window by the zero linkage distance reported. These markers were then re-coded using the "Flip Marker" command so that the program reported the correct linkage distance. Backcross markers, which did not comply with the expected segregation ratio test, were incorporated into the map in a second stage of analysis by "distributing" these markers over the existing linkage groups with the "Distribute" command. The "Make Groups" command was applied to the remaining unlinked markers. In the final stage, intercross markers were added to the map by repeating the "Distribute" and "Make Groups" process.

Detection of mis-typed AFLP and microsatellite phenotypes was carried out using the principle that double crossovers indicated likely mis-scored data points. Hence, once comprehensive maps were generated using unverified data, scores for markers that were linked but had one or more double crossovers in their best order were checked and corrected where necessary.

Framework maps

Framework maps were constructed using MAPMAKER experimental version 3 (MME) and were based on BC markers or multiple backcross (MBC) (Lander et al. 1987; Lincoln et al. 1993). Backcross markers that fitted a 3:1 ratio were excluded from analysis. The global grouping threshold was set at log of the odds (LOD) 5 and 50 cM (K). Best order maps for each linkage group were established first by using the ripple command. A maximum of eight markers per linkage group were rippled simultaneously. Those groups for which the best order was not greater than a LOD 2 than the next alternative order were retested after dropping one or more markers. Markers were dropped on the basis of their quality rating and whether they tended to swap order, until an order with an interval support greater than LOD 2 was achieved. The order was then retested by "rippling". With larger groups (i.e. more than eight markers), a subset of high-quality, well-spaced markers was chosen from the best order as an initial start group. Other markers were then added to the framework using the "build" command using a threshold LOD linkage of 2. The final order was retested rippling eight markers or less at a time.

Results

Microsatellite and AFLP markers for PEE and PCH

From the set of 50 microsatellite marker tested, 12 were found to segregate in the mapping cross and could be reliably scored (Table 2). Transpecific amplification of microsatellite markers is reported elsewhere (Shepherd et al. 2002). Two loci, RPTest1 and PtTX3018 showed

Table 2 Twelve microsatellite loci scored in the PEE × PCH F_1 family, their mating configuration, allele sizes and segregation ratios

Locus	Microsatellite description	Exp. size of PCR fragment[a]	Mating type[b]	PEE	PCH	Obs. no. 1[c]	Obs. no. 2	Obs. no. 3	Obs. no. 4	Expected seg. ratio[d]	*P* value[e]	Reference[f]
PtTX3018	na	155	MBC	138/138	135/141	54	34			1:1	0.03*	1
NZPR5	$(AG)_{29}$	114	BC_1	82/82	80/82	45	48			1:1	0.76	2
PtTX2123	$(AGC)_8$	202	BC_1	200/200	197/200	47	41			1:1	0.52	3
RPTest05	$(CAA)_6$ $(CAA)_5$ $(AAC)_4$	197	BC_1	193/196	196/196	51	43			1:1	0.41	4
RPTest11	$(CAT)_7$	213	BC_1	213/213	206/213	45	47			1:1	0.83	4
RPTest20	$(CAG)_5$	259	BC_1	252/230	252/252	44	49			1:1	0.71	4
PtTX3013	$(GTT)_{10}$	134	IC	131/141	131/141	15	52	27		1:2:1	0.13	3
RPTest01	$(ATA)_7$	125	IC	112/124	112/124	13	52	29		1:2:1	0.04*	4
NZPR1	$(AG)_{17}$	139	MIC3	121/139	121/137	18	21	25	22	1:1:1:1	0.61	2
NZPR7	$(AT)_{22}$ $(AG)_{27}$	149	MIC4	91/114	77/null	25	22	20	19	1:1:1:1	0.81	2
PtTX 2037	$(GTGA)_8$ $(GT)_{14}$	177	MIC4	146/148	162/null	19	28	16	27	1:1:1:1	0.20	3
PtTX 3034	$(GT)_{10}$ $(GA)_{13}$	207	MIC4	199/207	205/213	20	22	28	20	1:1:1:1	0.59	3

[a] Size of fragment in the individual from which the clone was derived

[b] Mating type, the mating type of the locus segregated in a mapping cross MBC, multiple backcross; BC, Backcross; IC, intercross; MIC 3 or 4, multiple intercross with three or four alleles segregating (see Haseman and Elston 1972). Markers with MBC or MIC were re-coded for linkage analysis based on a maximum of two segregating alleles

[c] Observed no. of progeny of genotype class 1

[d] Expected Mendelian segregation ratio of progeny genotype classes

[e] *P*-value for Chi-squared test. *$P \leq 0.05$

[f] References: 1, Shepherd et al. (2002); 2, Fisher et al. (1998); 3, Elsik et al. (2000); 4, Echt and Burns (1999)

Table 3 Polymorphic AFLP markers by quality classification and proportions which were linked in the framework map

Marker quality class[a]	Number (%)	Number mapped (%)[b]	Percentage of class which mapped
A	94 (29)	77 (31)	82
B	164 (50)	121 (49)	74
C	71 (21)	48 (20)	68
Total	329	246	

[a] A, High-quality marker; B, intermediate; C, low quality (See Materials and methods)
[b] The number or percentage of markers which were linked in a group of two or more markers in the comprehensive maps for both parents. This included framework and accessory markers. Markers that were 3:1 and appeared in both maps were only counted once

Table 4 Summary of framework genetic maps and genome coverage for PEE and PCH

Species	Number of groups	Number of markers	Total length of groups (cM) (K)	Average spacing[a]	Map length[b]	*E*(G)[c]	Percentage Genome coverage[d]
PEE	23	78	836	15.2	1170	1427	82
PCH	27	109	1283	15.7	1658	1881	88

[a] Average framework marker spacing = sum of length of all linkage groups upon the number of framework marker intervals minus the number of linkage groups (Remington et al. 1999)
[b] Map length determined as per Kubisiak et al. (1995), total length of groups adjusted for 24 true telomeric ends
[c] E(G), Estimated genome size using method 3 of Chakravarti et al. (1991)
[d] Genome coverage based on E(G)

slight distortion (*P* value for Chi-square test for goodness of fit = 0.04 and 0.03, respectively).

Twenty-three primer combinations (PC) were investigated for polymorphism of AFLP bands in the hybrid pine family (Table 1). Most markers occurred in a BC configuration, with 165 and 134 markers segregating in the PCH and PEE parent, respectively. Thirty markers that were present in both parents and segregated in an approximate 3:1 present-to-absent ratio in the progeny were also scored to give a total of 329 polymorphic bands, averaging 14.3 ± 5.6 per PC.

Markers were assigned a quality rating once all scoring was complete. Of the 329 polymorphic AFLP, 28% were in the highest quality class, i.e. class A; 50% were in class B and 22% were in class C (Table 3). The quality of markers appeared to be related to the ability to detect linkage with other markers, as markers that were scored with a higher confidence had a higher representation in the linkage maps. A high number of AFLP makers had segregation ratios that were distorted from expected Mendelian ratios (35% and 30% of markers from the PEE and PCH parents, respectively). Marker quality, however, was not significantly associated with segregation distortion (data not shown).

The framework map for PCH was larger than the PEE

The framework map for PEE consisted of 78 markers in 23 linkage groups and covered 1,170 cM (K) (Table 4, Fig. 1). The framework map for PCH was 50% larger, at 1,658 cM (K) and consisted of 109 markers in 27 linkage groups (Table 4, Fig. 2). Estimates of genome coverage for PEE and PCH were 82% and 88% respectively.

To investigate whether the larger number of markers in the PCH framework map contributed to the greater observed map size in PCH, we constructed a new framework map based on a similar number of markers to the PEE framework map. Thirty-one markers were randomly eliminated from the set of PCH framework markers (six other markers were eliminated because a number of groups, mainly pairs, no longer formed linkage groups) and a new framework map generated. This map consisted of 72 markers and covered a distance of 1,397 cM (K), which was still 19% longer [226 cM (K)] than the framework map of 1,170 cM (K) for PEE based on 78 markers. The average distance between markers in the PCH map was significantly larger than that in the PEE map (one-tailed *t*-test with unequal variances; $df = 97$; t-value = –2.17; P value = 0.016).

The distribution of markers exhibiting segregation distortion was examined. Both maps had similar numbers of distorted markers – 17 and 18 for the PCH and PEE maps, respectively. Some distorted markers tended to occur in clusters, and in some cases all markers in a group were distorted (eg. PCH group 21 and 14 PEE group 23), although other distorted markers were distributed over many linkage groups. The degree of distortion was found to increase along a linkage group in some cases (eg. Chi-square values increased from 4.5 to 11.9 for BC markers on PCH group 21). Intercross markers linked to these BC markers were also distorted from an expected 3:1 ratio.

Homology between PEE and PCH and maps of other pines

The comprehensive map for PEE consisted of 125 markers in 24 groups and covered 1,548 cM (K) (Table 5). The comprehensive map for PCH consisted of 155 markers in 25 groups and covered 1,823 cM (K).

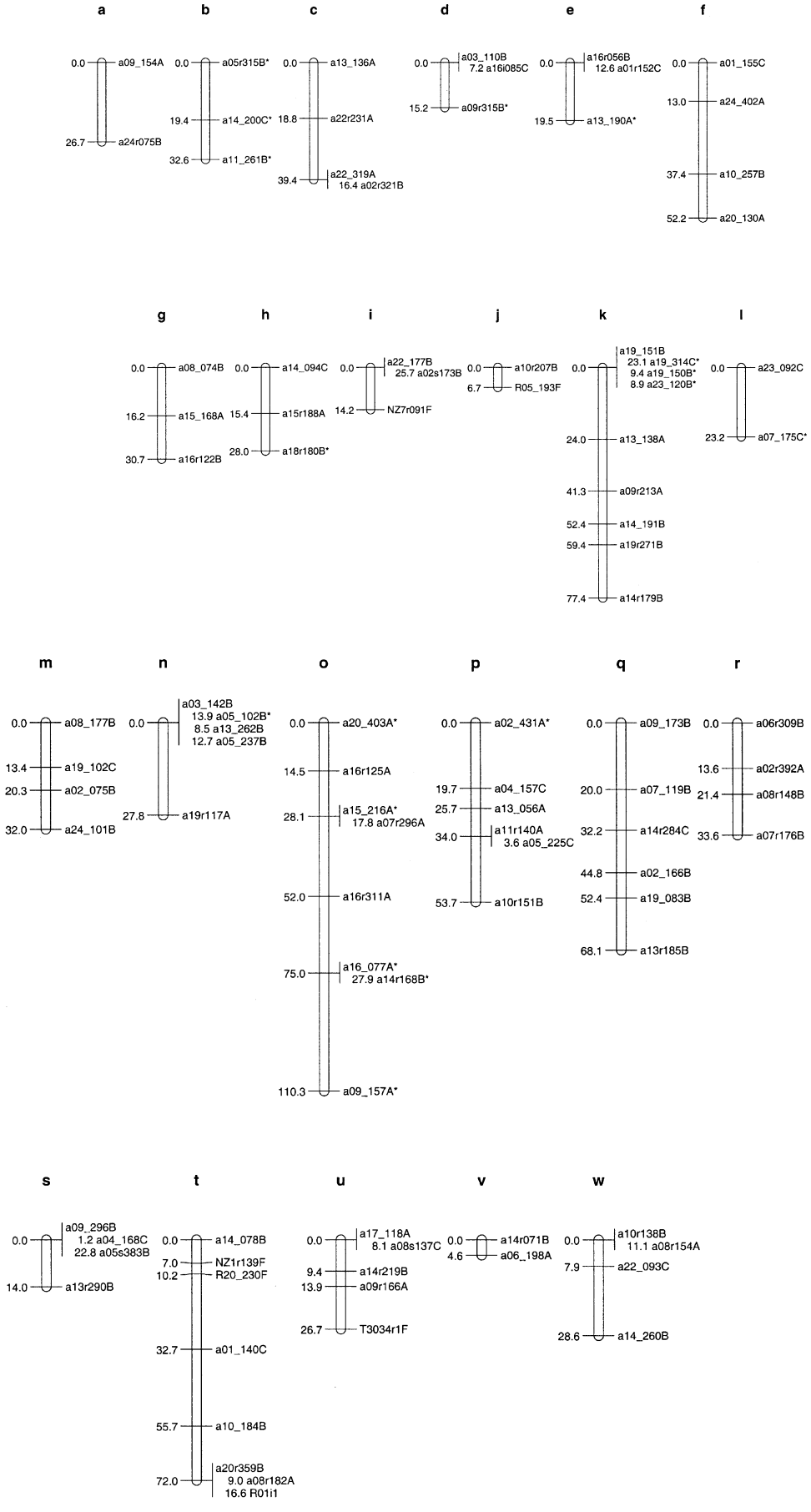

Fig. 1 Framework map for a *P. elliottii* var, *elliottii* (2PEE1–102). AFLP and microsatellite markers were mapped using a pseudo-testcross strategy using MAPMAKER v3 with the criteria of LOD 5 and 50 cM for grouping. Markers were ordered with an interval support minimum LOD of 2. Distances are in centiMorgans Kosambi. AFLP markers are labelled with an *a* prefix, followed by a *PC* number (see Table 1) and fragment size in base pairs interspersed with a *dash* or an *r*. A *dash* indicates the marker linked in the phase that it was scored, an "*r*" indicates it was re-coded. Microsatellite markers begin with a *capital letter*, and different alleles are either identified by the *size* in base pairs or a *sequential number* representing a different allele number. An * suffixed to a marker label indicates the marker had a distorted segregation ratio

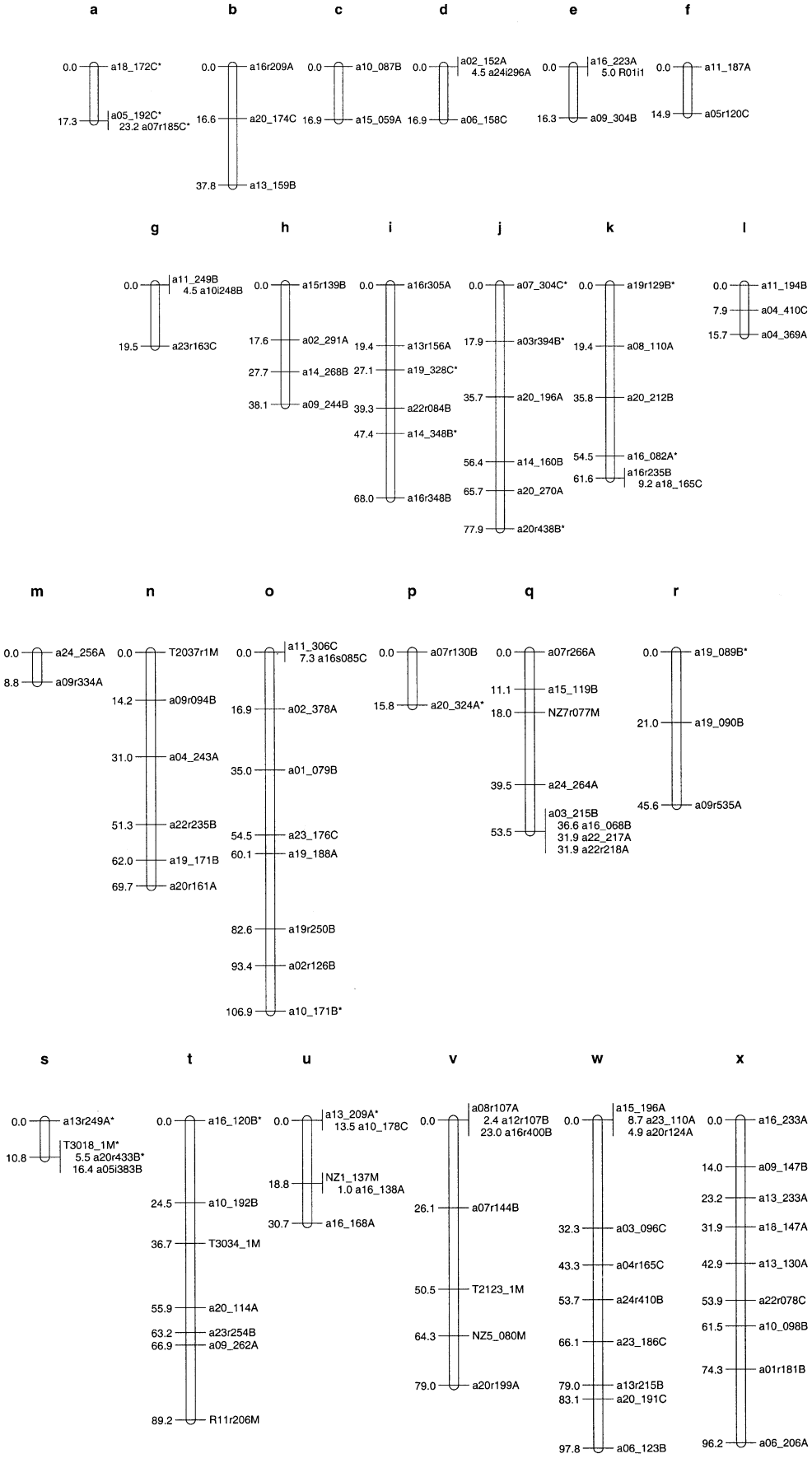

Fig. 2 Framework genetic map for a select *P. caribaea* var. *hondurensis* tree (1PCH1–63). See Fig. 1 for explanation

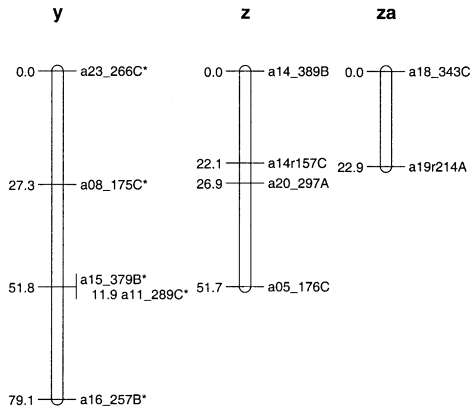

Fig. 2 continued

Twenty-seven AFLP or microsatellite markers in either an IC or MIC mating type were mapped onto one or both comprehensive maps. These markers were potential locus "bridges" that could allow homologous linkage groups to be identified in the two maps as well as enable a comparison of distances and map orders. Only 13 of these "bridge" markers occurred on both maps and therefore could be used as reference points (Table 6). By lowering the threshold for linkage to LOD 2, we found a further six "bridge" markers, giving a total of 19 reference points. These markers allowed homologous linkage groups to be established between 11 of the 24 PEE groups and 10 of the 25 PCH groups. There were three occurrences where markers from one group, from one parent, mapped to two or more groups in the other parent. There were only three instances where it was possible to compare linkage distances between the two maps. Two of these occurrences were based on tentative linkages, i.e. LOD <2.0. Nevertheless, similar distances were found in all three occurrences. Marker order was conserved in the

Table 5 Summary statistics of comprehensive genetic maps for PEE and PCH

Species	Number of groups	Number of core (accessory) markers	SSR	AFLP	Total length of groups cM (K)	Av. no. core marker spacing[a]	Number of 3:1 AFLP	Unlinked AFLP
PEE	24	113 (12)	8	105 (12)	1548	17.4	8	49
PCH	25	141 (14)	10	131 (14)	1823	15.7	19	47

[a] As per Table 4.

Table 6 Comparative mapping between the PEE and PCH comprehensive maps using "bridge" markers

Locus	Mating type[a]	PEE group[b]	PCH group[b]	Other tentative linkages
NZ1r139F	MIC3	1	2	
RO1i1	IC	1	17	
a05s383B	IC	2	7	
T3034r1F	MIC4	4	5	
a08s137c	IC	4	**17**	Linked to RO1i1 (group 17) at LOD 1.9
a07i269A	IC	5	21	
T2037r1F	MIC4	6	8	
a16s368B	IC	**6**	8	Linked to PtTx2037 (group 6) LOD 2
a24i296A	IC	**6**	18	Linked to T2037 (group 6) LOD 2
a07i124C	IC	6	–	No links to any bridge markers
a02s365A	IC	**11**	10	Linked to a16s432A (group 11) LOD 2
a16s432A	IC	11	21	
a02s173B	IC	13	**3**	Linked to NZ7_077M (Group 3) LOD 2.9
NZ7r091F	MIC4	13	3	
a10s148C	IC	13	**3**	Linked to a02i173B (group13) LOD 1.6
a08i097C	IC	18	8	
a16i085C	IC	20	8	
a24i073C	IC	21	1	
T3013i1	IC	21	1	
a07i147B	IC	23	8	
a07i274B	IC	–	4	Linked to a07i145B at LOD 1.7 which did not map in PEE
a07i238A	IC	–	8	
a05s317B	IC	–	11	
a11i089B	IC	–	12	
a10i248B	IC	–	15	
a07i141B	IC	–	23	
a07i145B	IC	–	24	

[a] For explanation of mating type designation see footnotes for Table 2[b] Group numbers in bold indicate markers not linked at threshold linkage criteria in the framework map, i.e. LOD < 5

one instance where three "bridge" markers were linked in both maps.

Linkage between microsatellite markers on the PEE and PCH maps was compared with previously reported linkage in other *Pinus* spp. For NZPR markers, no linkage was found in *P. radiata* between any of the NZPR markers we have studied (Fisher et al. 1998). The mapping of NZPR1 and NZPR7 to separate linkage groups on both the PEE and PCH maps was consistent with the observation of no linkage in *P. radiata*. Zhou et al. (submitted) reported that PtTX3018 and PtTX3034 map to separate linkage groups in *P. taeda*. Our results for these two markers in PEE and PCH were consistent with this observation for *P. taeda*. These two markers occurred in separate linkage groups in PCH; however, in PEE, the PtTX3018 marker was unlinked.

Discussion

Genetic maps for PEE and PCH using AFLP and microsatellite markers

Genetic maps based on AFLP and microsatellite markers are reported for individual PCH and PEE trees. Framework maps were generated using the PTC strategy by analysing markers segregating in an interspecific F_1 family. Framework maps covered an estimated 88% and 82% of the respective genomes. The PCH tree was apparently more heterozygous than the PEE tree because a larger number of markers were found to segregate from this parent. As a consequence, the PCH framework map was longer and, even after correction for the greater number of markers mapping to this parent, the PCH framework map was still 19% longer overall than the PEE framework map. Comprehensive maps were also generated that included markers segregating in an IC or MIC. The extension of the PTC strategy to include markers segregating in other configurations allowed regions of the genome not covered by markers in a BC configuration to be mapped as well as the alignment of homologous linkage groups between the two maps.

Genome size and map coverage

Based on chiasmata counts, the theoretical genetic distance for pines should be 1,500 cM (Plomion et al. 1995). Empirical estimates of genome size for *P. elliottii* based on partial genetic maps using the method of Hulbert et al. (1988) and modifications of Chakravarti et al. (1991) have varied because of the differences in methods and confidence limits applied during map generation (Echt and Nelson 1997). These investigators found, however, that when systematically compared, genome sizes for three species were similar [2,000 cM (K)], suggesting conservation of rates of recombinations amongst species of pines. Our estimates of genome sizes were lower but the estimate for PCH fell within their 95% confidence limit [1,828–2,242 cM (K)]. The lower estimates of genome size may be attributable to the use of framework maps for genome size estimates in this study. Other recent estimates of genome size for *P. taeda* by several methods were found to converge on 1,700 cM (K), which appears to be more consistent with theoretical expectations (Remington et al. 1999).

Sex-related recombination variation appears to be the likely explanation for the larger map for the PCH parent compared with the PEE parent. In two previous studies of recombination rates in pines, higher recombination rates were found for the pollen parents – an average of 26% in two crosses of *P. taeda*, and 28% for a pollen parent in *P. pinaster* – than for the respective megagametophyte parent (Groover et al. 1995; Plomion and O'Malley 1996). The 24% greater genome size for the PCH pollen parent was comparable with the higher recombination rates for pollen parents in previous studies of pines.

Segregation distortion in AFLP and microsatellite markers

A relatively high rate of distortion was found amongst AFLP and microsatellite markers in our study. Amongst those AFLP markers segregating in a BC configuration, 30% and 35% were distorted in PCH and PEE, respectively. Similarly, 2 out of 12 microsatellite loci showed distortion when no distorted loci were expected for a sample of this size at the tested probability level. Distortion of an AFLP locus was not related to its quality, and the number of distorted loci was similar on both maps. The rate of distorted markers in our study was high compared with the rates of distorted RAPD markers found in *P. elliottii* (10%) generated from a population of megagametophytes or from a *P. elliottii* × *P. palustris* hybrid (13%) (Kubisiak et al. 1995; Nelson et al. 1993) but lower than that found in a family from a self of a PEE × PCH F_1 individual (55%) (Dale 1994).

Hybrid dysgenesis and segregation distortion have been features of mapping experiments involving wide crosses in plants, and they tend to be the rule for studies of interspecific hybrids (Korol et al. 1994; Rieseberg and Linder 1999). Although there may be several methodological or biological explanations for segregation distortion, in our case hybrid incompatibility would appear to be a likely explanation, as there is some evidence for this from observations on germination rates and counts of "abnormals" in field tests (G. Nikles, personal communication). Hybrid incompatibility may be occurring in the F_1 hybrid due to pre- or post-zygotic barriers to interspecific gene flow. This may be manifest in germination rates for the hybrid which, although variable depending on the PEE mother (0.2–43.6%), on the whole are low compared with the parental taxa (G Nikles, personal communication). In addition, of those seedlings which survive to be planted in the field, the occurrence of seedling with aberrant morphology, is higher than the parental taxa and outcrossed second generation hybrids (Powell 2000).

There is evidence from studies in mammals and angiosperms to suggest that genomic imprinting (i.e. gene expression dependant upon parent-of-origin) may be the cause of the failure of interspecific crosses due to growth related defects (Kondoh and Higashi 2000). Methylation of DNA and other epigenetic phenomena may be contributing to poor seed set and differential survival in PEE × PCH F_1, however, the possibility of pollen lethals or other genic factors such as differential chromosomal loss have not been excluded (Bradshaw and Stettler 1994).

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References

Bradshaw HD, Stettler RF (1994) Molecular genetics of growth and development in *Populus* II. Segregation distortion due to genetic load. Theor Appl Genet 89:551–558

Bradshaw HDJ, Villar M, Watson BD, Otto KG, Stewart S, Stettler RF (1994) Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid popular composed of RFLP, STS and RAPD markers. Theor Appl Genet 89:167–178

Butcher P, Moran G (2000) Genetic linkage mapping in *Acacia mangium*. 2. Development of an integrated map from two outbred pedigrees using RFLP and microsatellite loci. Theor Appl Genet 101:594–605

Chakravarti A, Lasher K, Reefer J (1991) A maximum-likelihood method for estimating genome length using genetic linkage data. Genetics 128:175–182

Dale G (1994) Genetic mapping in an interspecific hybrid between *Pinus caribaea* and *Pinus elliottii*, Unpublished PhD thesis, University of Queensland, Brisbane

Devey ME, Fiddler TA, Liu BH, Knapp SJ, Neale DB (1994) An RFLP linkage map for loblolly pine based on a three-generation outbred pedigree. Theor Appl Genet 88:273–278

Dieters MJ (1996) Genetic parameters for slash pine (*Pinus elliottii*) grown in south-east Queensland, Australia: growth, stem straightness and crown defects. For Genet 3:27–36

Echt C, Burns R (1999) SSR derived from *Pinus taeda* ESTs. http://dendromeucdavisedu/Data/primerhtml

Echt CS, Nelson CD (1997) Linkage mapping and genome length in eastern white pine (*Pinus strobus* L.). Theor Appl Genet 94:1031–1037

Echt CS, May-Marquardt P, Hseih M, Zahorchak R (1996) Characterisation of microsatellite markers in eastern white pine. Genome 39:1102–1108

Echt CS, Vendramin GG, Nelson CD, May-Marquardt P (1999) Microsatellite DNA as shared genetic markers among conifer species. Can J For Res 29:365–371

Elsik CG, Minihan VT, Hall SE, Scarpa AM, Williams CG (2000) Low-copy microsatellite markers for *Pinus taeda* L. Genome 43:550–555

Fisher PJ, Gardner RC, Richardson TE (1996) Single locus microsatellites isolated using 5′-anchored PCR. Nucleic Acids Res 24:4369–4371

Fisher PJ, Richardson TE, Gardner RC (1998) Characteristics of single- and multi-copy microsatellites from *Pinus radiata*. Theor Appl Genet 96:969–979

Grattapaglia D, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137:1121–1137

Graham GC, Mayers P, Henry RJ (1994) A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. Biotechniques 16:48–50

Groover AT, Williams CG, Devey ME, Lee JM, Neale DB (1995) Sex-related differences in meiotic recombination frequency in *Pinus taeda*. J Hered 86:157–158

Haines RJ (2000) Clonal forestry in Queensland and implications for hybrid breeding strategies. In: Dungey HS, Dieters MJ, Nikles DG (compilers) Hybrid breeding and genetics of forest trees. Proc QFRI/CRC-SPF Symp. Department of Primary Industries, Brisbane, pp 386–389

Haseman JK, Elston RC (1972) The investigation of linkage between quantitative trait and a marker locus. Behav Genet 2:3–19

Hulbert S, Ilott T, Legg E, Lincoln S, Lander E, Michelmore R (1988) Genetic analysis of the fungus, *Bremia lactucae*, using restriction length polymorphism. Genetics 120:947–958

Johnson GR, Wheeler NC, Strauss SS (2000) Financial feasibility of marker-aided selection in Douglas-fir. Can J For Res 30:1942–1952

Kondoh M, Higashi M (2000) Reproductive isolation mechanism resulting from resolution of intragenomic conflict. Am Nat 156:511–518

Korol AB, Preygel IA, Preygel SI (1994) Recombination variation and evolution, 1st edn. Chapman and Hall, London

Kubisiak TL, Nelson CD, Nance WL, Stine M (1995) RAPD linkage mapping in a longleaf pine ×slash pine F_1 family. Theor Appl Genet 90:1119–1127

Lander E, Green P, Abrahamson J, Barlow A, Daley MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lincoln SE, Daly MJ, Lander ES (1993) Mapping genes controlling quantitative traits using MAPMAKER/QTL version 1.1: a tutorial and reference manual. whitehead Institute, Cambridge, Mass.

Manly KF, Cudmore RH, Meer JM (2001) MAP MANAGER QTX, cross platform software for genetic mapping. Mammal Genome 12:930–932

Marques CM, Araujo JA, Ferreira JG, Whetten R, O'Malley DM, Liu BH, Sederoff R (1998) AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*. Theor Appl Genet 96:727–737

Nelson CD, Nance WL, Doudrick RL (1993) A partial genetic linkage map of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) based on random amplified polymorphic DNAs. Theor Appl Genet 87:145–151

Nelson CD, Kubisiak TL, Stine M, Nance WL (1994) A genetic linkage map of longleaf pine (*Pinus palustris* Mill.) based on random amplified polymorphic DNAs. Hered 85:433–439

Nikles DG (1996) The first 50 years of the evolution of forest tree improvement in Queensland. In: Dieters MJ, Matherson AC, Nikles DG, Harwood CE, Walker SM (eds) Tree Improv Sustainable Trop For. QFRI-IUFRO Conf. Queensland Forestry Research Institute, Gympie, pp 51–64

O'Malley DM, McKeand SE (1994) Marker assisted selection for breeding value in forest trees. For Genet 1:207–218

Plomion C, O'Malley DM (1996) Recombination rate differences for pollen parents and seed parents in *Pinus pinaster*. Heredity 77:341–350

Plomion C, O'Malley DM, Durel CE (1995) Genomic analyses in maritime pine (*Pinus pinaster*) – comparison of 2 RAPD maps using selfed and open-pollinated seeds of the same individual. Theor Appl Genet 90:1028–1034

Powell M (2000) Genetic parameter estimation for hybrid pines in Queensland. Unpublished PhD thesis, University of Queensland, Brisbane

Powell MB, Nikles DG (1996) Performance of *Pinus elliottii* var. *elliottii* and *P. caribaea* var. *hondurensis* and their F_1, F_2 and backcross hybrids across a range of sites in Queensland. In: Dieters MJ, Matherson AC, Nikles DG, Harwood CE, Walker SM (eds) Tree Improv Sustainable Trop For. QFRI-IUFRO Conf. Queensland Forestry Research Institute, Gympie, pp 382–383

Remington DL, Whetten RW, Liu BH, O'Malley DM (1999) Construction of an AFLP genetic map with nearly complete genome coverage in *Pinus taeda*. Theor Appl Genet 98:1279–1292

Rieseberg LH, Linder CR (1999) Hybrid classification: insights from genetic map-based studies of experimental hybrids. Ecology 80:361–370

Shepherd M, Chaparro JX, Teasdale R (1999) Genetic mapping of monoterpene composition in an interspecific eucalypt hybrid. Theor Appl Genet 99:1207–1215

Shepherd M, Cross M, Maguire T, Dieters M, Williams C, Henry R (2002) Transpecific microsatellites for hard pines. Theor Appl Genet 104:819–827

Smith DN, Devey ME (1994) Occurrence and inheritance of microsatellites in *Pinus radiata*. Genome 37:977–983

Tauer CG, Hallgren SW, Martin B (1992) Using marker-aided selection to improve tree growth response to abiotic stress. Can J For Res 22:1018–1030

Toon PG, Dieters MJ, Nikles DG (1996) Components of a slash pine (*Pinus elliottii* var. *elliottii*) breeding strategy for the continued improvement of its hybrids. In: Dieters MJ, Matherson AC, Nikles DG, Harwood CE, Walker SM (eds) Tree Improv Sustainable Trop For. QFRI-IUFRO Conf. Queensland Forestry Research Institute, Gympie, pp 51–64

Tulsieram LK, Glaubitz JC, Kiss G, Carlson JE (1992) Single tree genetic linkage mapping in conifers using haploid DNA from megagametophytes. Biotechnology 10:686–690

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP – A new technique for DNA Fingerprinting. Nucleic Acids Res 23:4407–4414

Wilcox P, Richardson T, Corbett G, Ball R, Lee J, Djorovic A, Carson S (2001) Framework linkage maps of *Pinus radiata* D. DON based on pseudotestcross markers. For Genet 8:109–117

Williams CG (1998) QTL Mapping in Outbred Pedigrees. In: Paterson AH (ed) Molecular dissection of complex traits. CRC Press, Boca Raton, pp 81–94

Williams CG, Neale DB (1992) Conifer wood quality and marker-aided selection: a case study. Can J For Res 22:1009–1017

Young ND (1999) A cautiously optimistic vision for marker-assisted breeding. Mol Breed 6:505–510

Zobel BJ, Jett JB (1996) Genetics of wood production. Springer, Berlin Heidelberg New York

Zobel B, Talbert JT (1984) Applied forest tree improvement applied forest tree improvement. John Wiley & Sons, New York, pp 437–458