RAPD Genome Maps of Douglas-Fir

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We present linkage maps and estimate genome length for two hybrid individuals of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], a coniferous tree species of wide distribution in the western United States. The hybrids were produced by crosses between the coastal (var. *menziesii*) and interior (var. *glauca*) varieties. Haploid megagametophytes from 80 seeds of each individual were analyzed using 81 10-bp random amplified polymorphic DNA (RAPD) primers selected for polymorphism, fragment strength, and repeatability in preliminary segregation analysis. Most (82–90%) of the segregating fragments followed the expected 1:1 Mendelian segregation; however, 10–18% showed significant segregation distortion ($P < .05$) among megagametophytes of the two trees. In one tree, 201 of 221 segregating loci analyzed were combined into 16 major linkage groups of 4 or more loci (plus 1 group of 3 loci, 3 groups with pairs of loci, and 11 unlinked loci); in the other, 238 of 250 segregating loci were combined into 18 major groups (plus 2 groups of 3 loci each and 6 unlinked loci). Analyses of the distribution of markers indicated highly significant clustering in both trees ($P < .001$). Including flanking regions and unlinked loci, both trees had linkage maps of similar length, 2600 cM and 3000 cM; expected total map size ranged from 2800 to 3500 cM based on method-of-moments estimation. When a common RAPD protocol was used, more than one-third of the RAPD markers segregated in both hybrids. These maps of Douglas-fir are among the largest reported for conifers, a possible consequence of its 13 haploid chromosomes.

Gametic segregation and linkage in conifer trees can be directly analyzed using RAPD markers. The haploid megagametophyte of conifer seeds avoids problems of dominance of RAPD markers and allows any tree to be mapped without making controlled crosses (Adams 1983). The availability of hundreds of RAPD primers allows creation of dense linkage maps. RAPD markers have been used to produce genome maps for a number of trees [see Neale and Harry (1994) and Neale et al. (1994) for a review]. Inheritance of RFLP and RAPD markers have been established in Douglas-fir [*Pseudotsuga menziesii* Mirb. (Franco)] (Carlson et al. 1991; Jermstad et al. 1994), and both markers are being used in ongoing genome mapping studies (Carlson J, personal communication; Jermstad K, personal communication).

The goals of this study were to determine the genetic length of the Douglas-fir genome and to create a set of markers to use in mapping quantitative trait loci (QTL) in a related investigation of adaptive trait variation between the coastal and interior varieties (var. *menziesii* and var. *glauca,* respectively). We studied segregation in progeny of hybrids between the two varieties, whose high heterozygosity should facilitate linkage mapping.

Materials and Methods

Plant Materials

Two unrelated intervarietal Douglas-fir hybrid trees, 10D and 3B (Rehfeldt 1977), were crossed with one another and with pollen from single, unrelated coastal and interior males, the latter giving "varietal pseudobackcrosses." This mating structure (3 crosses per hybrid mother tree) was created to study how QTLs donated by maternal parents were influenced by varietal (paternal) genetic background. Crosses on 3B were made in a coastal test plantation near Corvallis, Oregon. Crosses on 10D were made in an interior test plantation near Moscow, Idaho. Seeds collected from these controlled crosses were used for both mapping and to establish an experimental seedling population for QTL analysis. Because the maps presented in this article are based solely on data from

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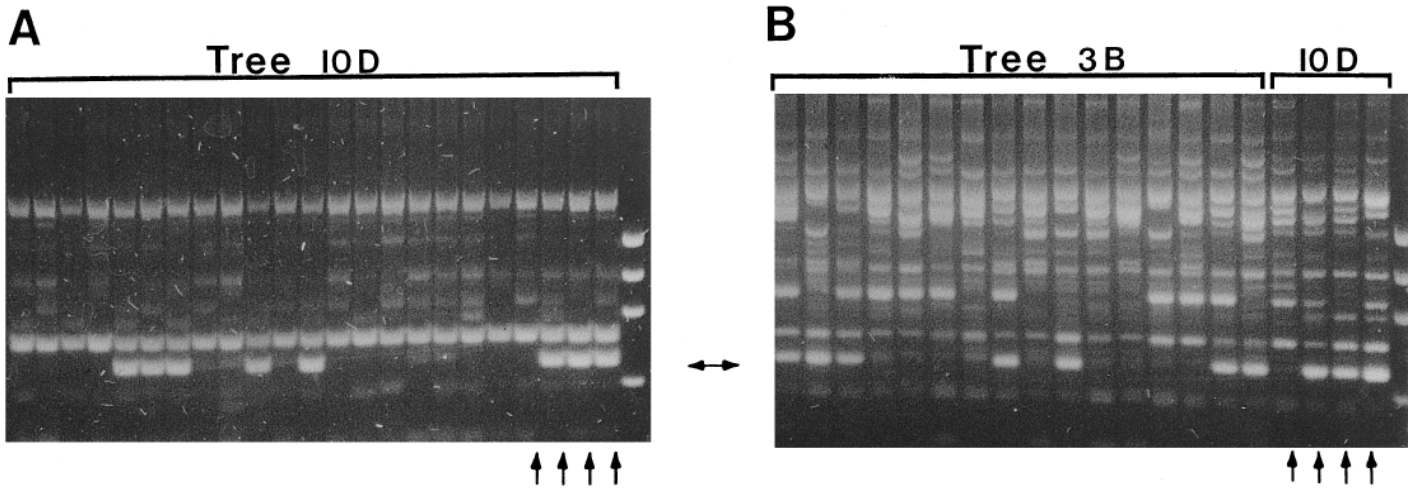

Figure 1. RAPD phenotypes generated by UBC primer no. 254 among megagametophytes of Douglas-fir hybrid trees 10D and 3B, using Promega **(A)** and Stratagene **(B)** *Taq* polymerases, respectively. Locus U254__380, common to both trees, is indicated by horizontal arrows (see also *Group 6* in Figure 2). Four samples of hybrid 10D amplified with both polymerases are indicated by vertical arrows. The rightmost lane contains a DNA size marker with fragment lengths of 1.3, 1.1, 0.9, and 0.6 kb (Ø174 DNA/*Hae*III digest).

megagametophytes, which derive from maternal tissues, cross-structure is unlikely to have had a significant influence on their content. Megagametophytes were carefully removed in nursery beds at an early stage of seedling development (stage 4: Krutovskii et al. 1997) and frozen until DNA extraction.

DNA Analysis

Total genomic DNA was isolated from individual megagametophytes following a modified CTAB procedure (Tsumura et al. 1996). DNA concentration was estimated by fluorometer Hoefer TKO 100. The extraction normally yielded 2.5 μg of DNA per megagametophyte (range 1.0–5.0 μg). We standardized working DNA preparations to 0.4 ng/μl in a TE buffer (10 mM Tris, 0.1 mM EDTA pH 8.0) and stored them at 4°C.

The screened random 10 bp primers were from Operon Technologies Inc. (primers OP-A8, -A9, -A16, -C4,-B5, -B8, -E12, -E17, -G9, -G10, -G12, -F7, -J1, and -Y17) and the Biotechnology Laboratory of the University of British Columbia (UBC #100–600). Polymerase chain reactions (PCR) were carried out in 96 well microtiter plates following standard procedures (Aagaard et al. 1995) in a volume of 25 μl with final concentrations of 10 mM Tris-HCl reaction buffer (Promega or Stratagene); 1.5 mM $MgCl_2$ (Stratagene) or 1.8 mM $MgCl_2$ (Promega); 100 μM each of dATP, dTTP, dCTP, and dGTP; 0.2 μM primer; 2 ng template DNA; and 1 unit of *Taq* (*Thermus aquaticus*) DNA polymerase from either Promega (hybrid 10D) or Stratagene (hybrid 3B) (catalog numbers M1864 and 600131, respectively). The change in *Taq* polymerase was necessitated by repeated failures of batches of enzyme after mapping of the first hybrid tree, 10D. The wells were overlaid with 50 μl of mineral oil, and amplification was performed in an MJR Research model PTC-100 thermocycler using settings of 3 min denaturation at 93°C followed by 44 cycles of 1 min of denaturation at 93°C, 1 min annealing at 37°C, 2 min extension at 72°C, and a final extension step of 10 min at 72°C. The reaction ended with an indefinite hold at 4°C.

Amplification products were electrophoresed using 32 cm × 20 cm, 2.0% agarose gels run for 4–5 h at 3.2 V/cm in TBE, pH 8.0 (Maniatis et al. 1982). Eighty megagametophytes were genotyped from each hybrid. Hybrid 10D seeds, which were analyzed first, were processed in two sets of 48 and 32 megagametophytes, respectively. To allow comparison of RAPD fragments between hybrids, four megagametophyte DNA samples of 10D that had been analyzed with Promega *Taq* were also included during analysis of 3B with Stratagene *Taq*. We ran 96 samples plus 6 standard ø174 DNAs (*Hae*III digest) on each gel (Figure 1). Gels were stained with 1 μg/ml ethidium bromide for 30 min in TBE, then destained in distilled water for 2 h and photographed.

Primer Screening

A total of 505 (491 UBC and 14 OPERON) primers were initially screened using four to eight megagametophytes in hybrid 10D (Table 1), of which 170 that gave some sharp, strongly amplified DNA fragments were selected for further testing. In a second screening with eight megagametophytes, a final selection of 96 primers was made based on the repeatability of fragments and the presence of at least one segregating fragment. This set was used for segregation analysis of the first set of 48 megagametophytes of hybrid 10D. The final 10D map we present was produced using 81 (76 UBC and 5 OPERON) primers that amplified successfully with both megagametophyte sets, giving 221 segregating loci; the 3B map was produced using 69 (64 UBC and 5 OPERON) primers and 250 segregating loci.

Segregation and Linkage Analysis

Segregation and linkage analyses were performed using the GMENDEL (Holloway and Knapp 1993) and MapMaker (Lander

Table 1. Screening of RAPD primers using megagametophytes of Douglas-fir hybrid trees

		Primers		Segregating fragments	
Stage	Tree	Screened	Selected	Screened	Selected
Primary screen with first set of 4–8 megagametophytes	10D	505	170	906	422
Secondary screen with second set of 8 megagametophytes	10D	170	96	616	247
Segregation and linkage analysis with 80 megagametophytes	10D	96	81	247	221 (2.7[a])
	3B	81	69	259	250 (3.6[a])

[a] Number of segregating fragments per selected primer.

Table 2. Efficiency of search for segregating RAPD loci in conifers

Species	Primers			Segregating loci				Reference
	Screened	Selected	%	Total	Per screened primer	Per selected primer	With segregation distortion[a] (%)	
Picea abies (L.) Karst.	328	96	29	186[b]	0.6	1.9	79[c]	Binelli and Bucci 1994; Bucci et al. 1995
P. glauca (Moech) Voss.	300	69	23	61[b]	0.2	0.8	31[d]	Tulsieram et al. 1992
Pinus brutia Ten.	95	15[e]	16[e]	42[b,e]	0.5[e]	2.8[e]	61[d,e]	Kaya and Neale 1995
P. elliottii Englm.	420	66	22	123	0.3	1.8[e]	10[d]	Nelson et al. 1993
	288[f]	91	32	127[e]	0.5[e]	1.4[e]	12[c]	Kubisiak et al. 1995
P. palustris Mill	576	128	16	188	0.3	1.5	8[c]	Nelson et al. 1994
	288[f]	113	39	157[e]	0.5[e]	1.4	13[c]	Kubisiak et al. 1995
P. pinaster Ait.	520	102	20	263	0.5	2.6	2[g]	Plomion et al. 1995b
P. strobus L.	288[f]	96	33	97	0.3	1.0	3[c]	Echt and Nelson 1997
P. sylvestris L.	220	104	47	298	1.4	2.9	5[c]	Yazdani et al. 1995
Taxus brevifolia Nutt.	345	67[e]	28	102[b]	0.3	1.5[e]	30[c]	Göçmen et al. 1996
Pseudotsuga menziesii (Mirb.) Franco	142	40	33	95	0.5	2.4	10[c]	Jermstad et al. 1994
	505	96	19	247	0.5	3.1[e]	18[c]	This study
Mean	345[h]	83	25[h]	153	0.5[h]	1.9	20[c]	

Sample sizes for screening when the number of megagametophytes sampled varied from 4 to 8.

[a] Percentage of segregating loci with segregation distortion.

[b] Loci showing segregation distortion were excluded.

[c] $P < .05$.

[d] Calculated using the number of loci showing segregation distortion provided in this article, although the probability level used to test segregation was not stated.

[e] Average of estimates provided in this study.

[f] Primers had been prescreened in previous experiments.

[g] $P < .01$.

[h] Mean excludes data from Kubisiak et al. (1995) and Echt and Nelson (1997) because primers were prescreened.

et al. 1987) programs. Segregating markers were scored for presence (1) or absence (0) of the amplified RAPD fragment, and (-) for missing data (i.e., failed or unscorable reactions). *G* statistics ($\alpha = 0.05$; Sokal and Rohlf 1981) were used to test the null hypothesis of 1:1 segregation of markers. All pairwise *r* and LOD values were computed for 221 and 250 loci in 10D and 3B, respectively. The *G* statistic was also computed to distinguish between independent segregation and genetic linkage under the null hypothesis that haplotypes segregate 1:1:1:1. Preliminary grouping was performed using likelihood odds (LOD) of 4.0–5.0 as a threshold for linkage. The probability of a type I statistic error for this test is $\alpha = 10\text{–}Y$, where *Y* is the critical value such that $LOD_r > Y$. A LOD threshold score of 4 means that we expected 2 out of 24,310 pairwise recombinations for 10D and 3 out of 31,125 pairwise recombinations for 3B to be statistically significant by chance alone.

Simulating annealing and multiple pairwise methods were used for locus ordering. Monte Carlo and bootstrap simulation with 100 iterations were applied to test the ordering and obtain confidence intervals for mapped positions of loci (Holloway and Knapp 1993). MapMaker functions, including NEAR and PLACE with relaxed LOD thresholds of 2.5–4.0, were used to map some of the unlinked loci and to merge some linkage groups. Map distances in centiMorgans were calculated using the Kosambi mapping function. Hypothetical expected map size was estimated using a method-of-moments estimator following Chakravarti et. al. (1991) and Hulbert et al. (1988). Two-point linkages at LOD values of 3.5 and 4.0 between distinct loci were determined and used to provide map size estimates. To study the distribution of markers over the map, the Kolmogorov-Smirnov and Lilliefors (on standardized data) one-sample test (Sokal and Rohlf 1981) was used to compare the shape and location of distribution of linkage intervals between adjacent loci with a normal distribution (SYSTAT 1992).

Results and Discussion

Primer Screening

Our selected set of 96 primers used for initial analysis of hybrid 10D gave 616 scorable fragments (6.4 per primer on average), nearly half of which segregated (Table 1). Our efficiency of searching for segregating loci was comparable to similar studies in other conifer species (approximately 0.5 loci per screened primer; Table 2). Of the 247 segregating loci scored for 10D, 45 (18%) showed statistically significant segregation distortion at the 5% probability level. Of the 261 segregating loci scored for 3B, 27 (~10%) showed statistically significant segregation distortion at the 5% probability level. Recombination estimators are still valid when distortion is observed at only one locus of a pair (Bailey 1961; Holloway and Knapp 1993; Ott 1991). However, segregation distortion for RAPD markers could result from comigration of fragments coded by multiple loci, competition among annealing sites for reaction components, or other nonbiological causes. Thus, of the loci showing significant segregation distortion, 18 in 10D and 16 in 3B showed only a relatively mild level of distortion ($.01 < P < .05$), and gave strong and reliable fragments which were retained in the linkage analyses (using the GMENDEL option to correct for segregation distortion). In sum, 81 primers and 221 segregating loci for 10D and 69 primers and 250 loci for 3B were included in the linkage maps.

Codominant Loci

Loci producing two cosegregating fragments of similar size in "repulsion phase" and amplified by the same primer were considered to be codominant, as demonstrated by Grattapaglia and Sederoff (1994) using Southern hybridization. Four such loci were found in 10D and seven in 3B (see underlined locus names marked by 'c' at the end in Figure 2). Thus, of all polymorphic loci we mapped, 2–3% (4 of 221 and 7 of 250) were putatively codom-

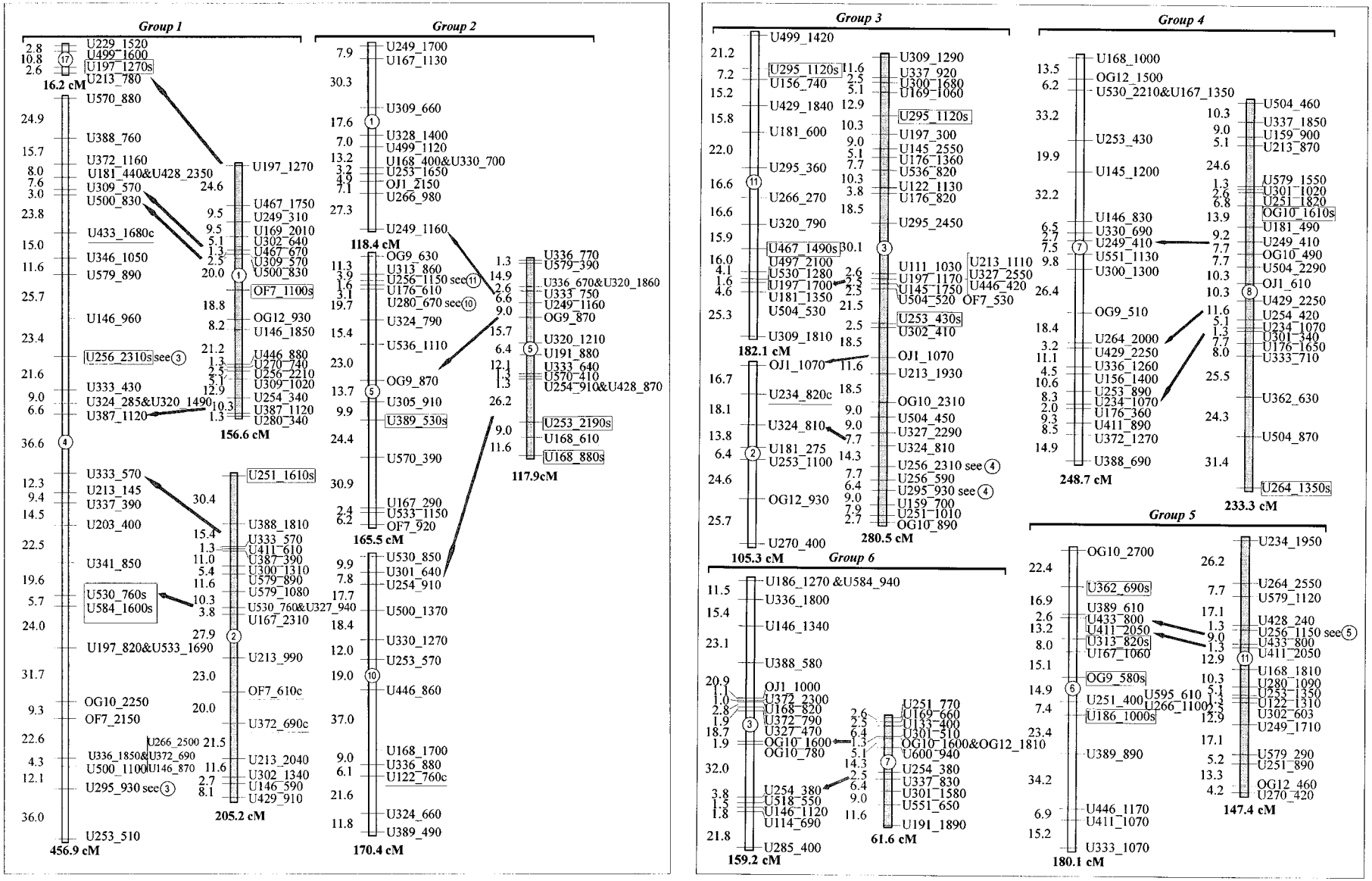

Figure 2. Synthetic genetic linkage groups of two Douglas-fir hybrid trees, 10D (white bars) and 3B (dark bars). Circled numbers refer to linkage group designations for each tree. Locus names are given on the right-hand side of the linkage groups, Kosambi distances (cM) are given on the left-hand side. The locus name starts with the capital letter of primer source (U stands for UBC and O for OPERON primers) followed by the serial number (numeric from 100 to 600 for UBC, and alphanumeric values of G9, G10, G12, F7, and J1 for OPERON primers), and the size of amplified DNA fragment (number of nucleotide base pairs). Loci showing segregation distortion are depicted within boxes and are followed by s; codominant loci are underlined and followed by c. Loci common to both hybrids are shown by arrows. Loci that mapped to different locations in the two hybrids are shown with their locus names followed by the circled number of the other linkage group.

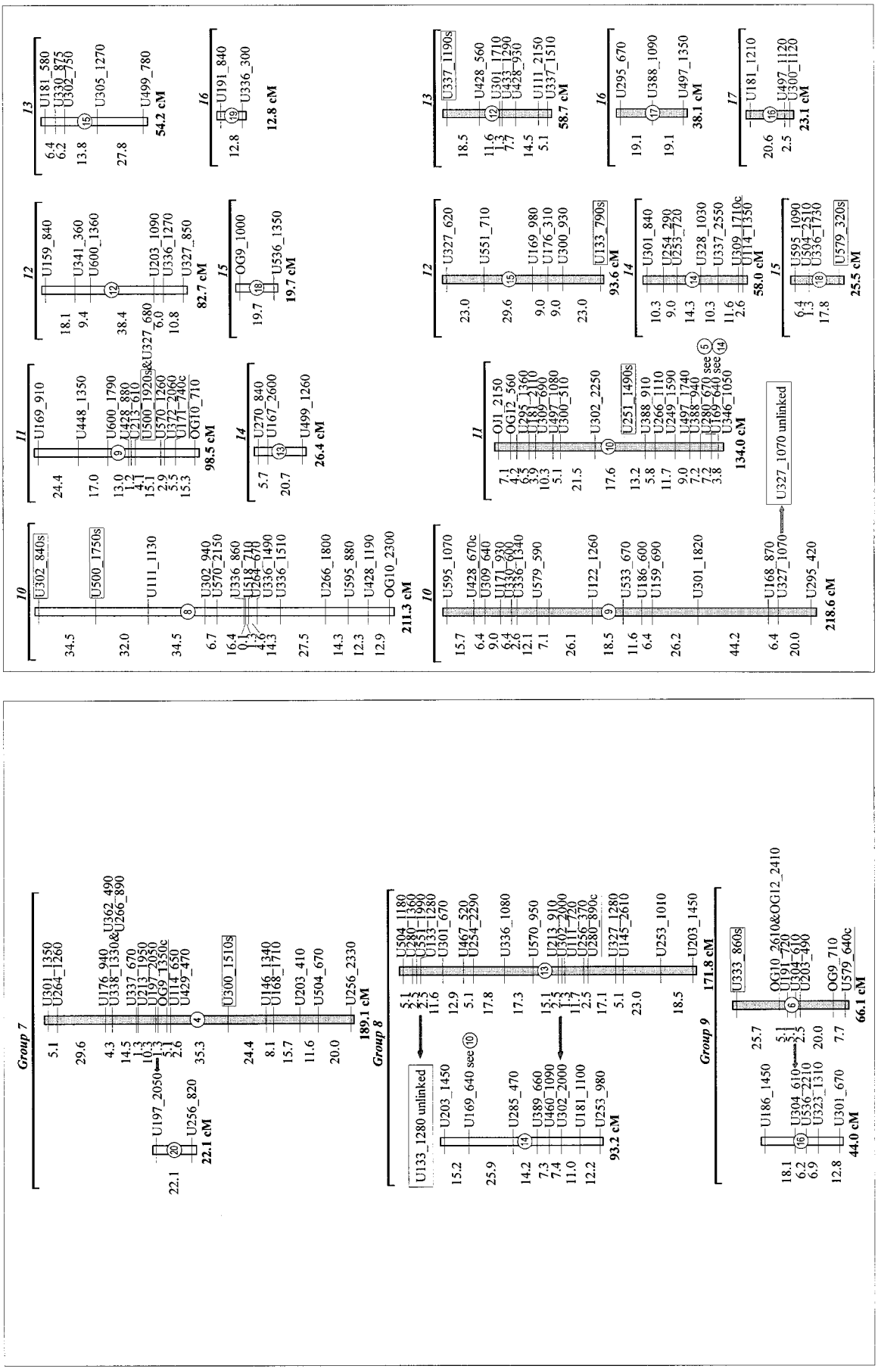

Figure 2. Continued.

inant and 97–98% dominant. Similar estimates have been made by others [e.g., 3% in longleaf pine (Nelson et al. 1994), 2% in eucalypts (Grattapaglia and Sederoff 1994), and 3% in maritime pine (Plomion et al. 1995b)].

Grouping and Ordering of RAPD Loci

Using LOD scores of 3.5, 4.0, or 5.0 as thresholds for grouping, most loci could be classified into 18 to 33 linkage groups of two or more loci. The number of linkage groups of three or more loci was only slightly different in the range of LOD scores of 3.0 to 4.0. Linkage groups and locus orders (see below) produced by GMENDEL and MapMaker were nearly identical.

We used exhaustive searches of all possible locus orders for groups with eight loci and less. For larger groups we applied GMENDEL kSAR, gSAR, and SAR functions to evaluate the position of each locus by examining various sets of its nearest neighbors. The simulated annealing method was used to improve ordering. The orders within groups obtained with a linkage criterion of LOD = 3.5 were nearly the same as those of the groups obtained with a linkage criterion of LOD = 4.0, differing only at some flanking loci that became unlinked under the higher LOD criteria. The only significant exception was that group 4 in tree 10D (Figure 2) was split into three groups and one unlinked locus under the LOD of 4.0. However, after analysis of all interorder estimates (*r, G,* and LOD), we found that this linkage group was valid after exclusion of a single problematic locus.

Segregation Distortion

Loci showing significant ($P < .05$) segregation distortion were widely distributed along the map (Figure 2). An exception was two tightly linked loci, U530_760s and U584_1600s in group 4 in 10D. Clustering of distorted markers has been commonly reported in mapping studies (e.g., Byrne et al. 1995; Cai et al. 1994; Jacobs et al. 1995; Mukai et al. 1995; Philipp et al. 1994; Uzunova et al. 1995). Segregation distortion in conifers, which usually maintain high genetic loads, has frequently been attributed to linkage between markers and detrimental or lethal genes (see Bucci and Menozzi 1993; Lanaud et al. 1995; Mukai et al. 1995; and Sorensen 1967 for references).

The number of RAPD loci that significantly deviate from Mendelian expectation has varied widely among mapping studies of trees (2–79%, mean of 20%: Table 2), but has often been well above that expected due to chance alone. The 18% of loci that we identified with significant distortion is far above the expected level of 5%, suggesting that biological (e.g., linkage to deleterious genes) or technical factors (e.g., low agarose-gel resolution, inconsistent PCR amplification) also influence ratios. Comigrating and overlapping polymorphic fragments can cause significant deviation from the expected 1:1 segregation of RAPDs in some cases (e.g., Plomion et al. 1995b).

Although there is no consensus among researchers as to whether markers showing segregation distortion should be used in linkage studies (see Kubisiak et al. 1995 for references), they have often not been included because of concerns over PCR-related problems. However, when linkage analyses included distorted markers many of them map to linkage groups. Bradshaw et al. (1994) observed that the proportion of distorted RAPD markers which failed to map to linkage groups (3 out of 16, or 19%) was comparable with the overall fraction of unlinked loci (19 out of 111, or 17%) in *Populus.* Nine of 20 and 8 of 14 distorted loci mapped in longleaf and slash pine trees, respectively (Kubisiak et al. 1995).

Although they can often be mapped, inclusion of distorted markers can weaken map structure. Kubisiak et al. (1995) reported that inclusion of distorted markers did not allow further convergence of either the longleaf or slash pine maps, and the number of linkage groups increased (e.g., from 19 to 22 in slash pine after distorted markers were included). We included 18 distorted markers in linkage analysis of hybrid 10D and 15 in the analysis of 3B. The percentage of unlinked loci among distorted markers, 22% in 10D and 7% in 3B, was higher than for normally segregating markers (3% and 2% for 10D and 3B, respectively). Moreover, when intervals with one or two distorted markers are considered separately, they had greater average map distances in both our maps. Mean linkage intervals were 16.4 for those involving distorted markers versus 12.5 cM for those with only nondistorted markers ($t = 1.84$, $P < .06$) in 10D, and 18.6 versus 9.2 cM ($t = 4.57$, $P < .01$) in 3B, respectively (Figure 3).

Comparison of Hybrid Maps

RAPD patterns revealed by the same primer were very different for 10D and 3B (Figure 1), a consequence of having to use *Taq* polymerases from different commercial sources (described in materials and methods). The Stratagene polymerase gave one-third more mappable fragments than did the Promega polymerase (3.6 versus 2.7 bands per primer; Table 1), and only 24 out of 221–250 loci (10–11%) were comparable for both hybrids (Figure 2). However, when a common amplification protocol was used, the level of conservation of RAPD loci was much higher. Between the 80 megagametophytes of 3B and the 4 control megagametophytes of 10B amplified using Stratagene *Taq,* 138 loci (~51%) were common, including 103 (~38%) segregating loci among the total 271 studied. This number of comapping loci is very likely to be an underestimate because many segregating loci in 10D were missed due to use of only four megagametophyte samples.

Comparison of the two maps indicated that some linkage groups were common (for instance, *Group 1* appears to include linkage groups 4 and 17 in 10D and groups 1 and 2 in 3B; see Figure 2). Thus, the total number of linkage groups of more than three loci can be reduced to 13–15 major groups, in good correspondence with the haploid set of 13 chromosomes in Douglas-fir. In some cases common loci were closely located, like pairs U309—570/U500—830 (3.0 cM in linkage group 4 in 10D and 2.5 cM in linkage group 1 in 3B; see Figure 2). However, there were several cases when closely linked loci in one hybrid were unlinked in another. For instance, loci U249—1160, OG9—870, and U254—910 were located in the same linkage group in 3B, but were located in linkage groups 1, 5, and 10 in 10D (Figure 2). The explanation may be that these loci are not the same, but rather contain similarly sized fragments. This may also account for several other mapping discrepancies (marked loci in Figure 2). Thus individual RAPD markers should be employed cautiously when comparing different genotypes.

Map and Genome Sizes

Trees 3B and 10D had 18 and 20 linkage groups, contained 244 and 210 mapped loci, covered 2279 and 2468 cM, and had average distances between adjacent loci of 10.1 and 12.9 cM, respectively (Table 3). Using a 30 cM map scale we can account for an additional 150 and 210 cM for flanking regions and 180 and 330 cM for unlinked loci, giving respective observed map sizes of approximately 2,600 and 3,000 cM and predicted map sizes of 2,810 and 3,540 cM. These estimates are in reasonable agreement with those of another RAPD analysis in Douglas-

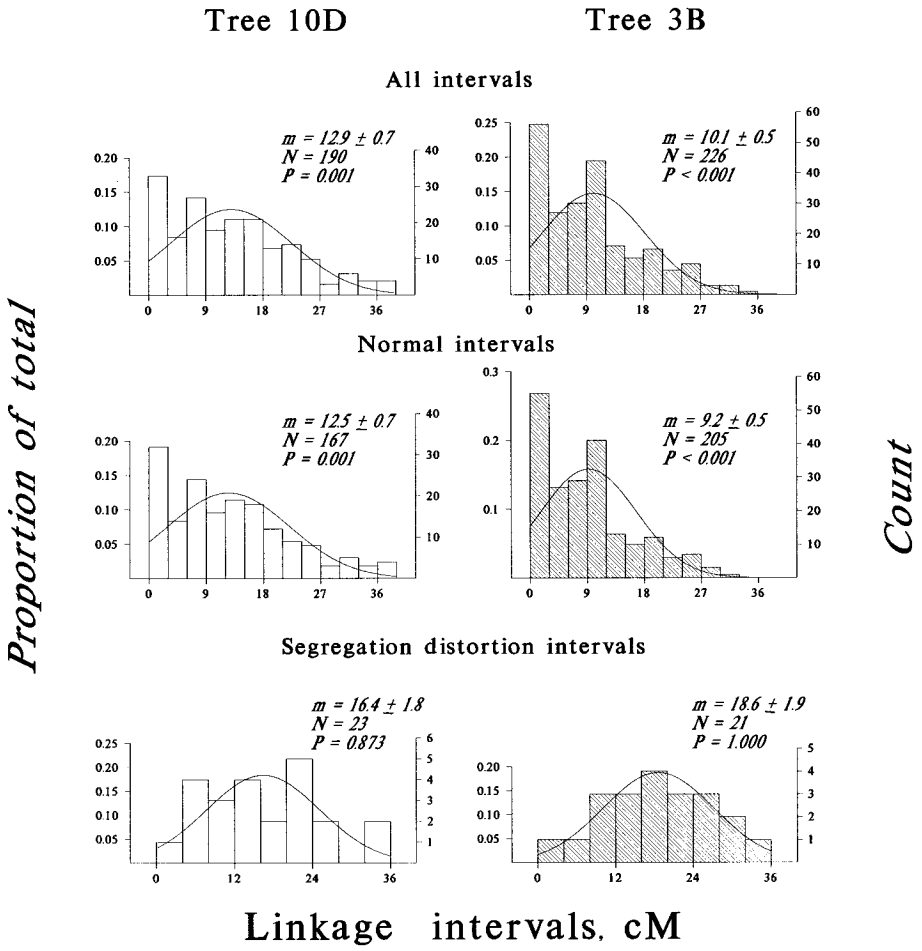

Figure 3. Distributions of linkage intervals for adjacent loci in genetic linkage maps of hybrids 10D and 3B. Normal intervals are those where both loci that define an interval do not show segregation distortion at the 5% level; segregation distortion intervals are those where at least one of the two loci that defines the interval show distortion. m = mean distance between adjacent loci ± standard error; N = number of intervals; P = the probability in Kolmogorov-Smirnov and Lilliefors (on standardized data) one-sample test comparing the distribution of linkage intervals between adjacent loci with that of a normal distribution.

Table 3. Summary of genome maps of two Douglas-fir hybrid trees

	Tree 10D (221 loci)	Tree 3B (250 loci)
Linkage criteria	r_{max} = 0.25–0.35, LOD_{min} = 3.0–4.0	r_{max} = 0.25, LOD_{min} = 4.0
Number of linkage groups	20: 16 > 3 loci, 1 = 3 loci, 3 = 2 loci	18: 16 > 3 loci, 2 = 3 loci
Number of unlinked loci	11	6
Average distance between adjacent loci (cM)	12.9 ± 0.7	10.1 ± 0.5
Total size of linkage groups (cM)	2,468	2,279
Total map distance of flanking regions (cM)[a]	210	150
Total map distance of unlinked loci (cM)[b]	330	180
Total observed map size (cM)[c]	3,008	2,609
Predicted map size (cM)[d]	3,540	2,810
Estimated genome coverage (%)[e]	85	93

[a] Assuming that 26 of the 40 ends of the 20 linkage groups for 10D and of the 36 ends of the 18 linkage groups for 3B trees cover 26 true telomeric regions (2 × 13 chromosome pairs in Douglas-fir), we can account for 210 cM for the other 14 of the 40 ends [(40 − 26) × 15 = 210 cM] for tree 10D, and 150 cM for the other 10 of the 36 ends [(36 − 26) × 15 = 150 cM] for 3B.

[b] Assuming 30 cM for each of 11 and 6 unlinked loci for 10D and 3B, respectively.

[c] Sum of total map distances for linkage groups, flanking regions, and unlinked loci.

[d] Estimated using a method-of-moments estimator following Hulbert et al. (1988) and Chakravarti et al. (1991).

[e] Ratio between total observed and predicted map sizes.

firir, where estimates for two trees varied from 2,100 to 2,300 cM (Carlson J, personal communication). However, they are significantly higher than estimates from an ongoing RFLP study of approximately 1,000 cM (Jermstad K, personal communication). The reasons for this difference are unclear, but may relate to the map locations of the cDNA-RFLP probes versus RAPD markers or the different quantitative analyses employed.

The Kolmogorov-Smirnov and Lilliefors one-sample test of normality of linkage intervals (on standardized data) revealed that the RAPD markers tended to cluster in both trees ($P \leq .001$; Figure 3). Clustering of RAPD and RFLP markers, though observed by several workers (e.g., *Eucalyptus grandis* and *E. urophylla,* Grattapaglia and Sederoff 1994; *Hordeum vulgare,* Giese et al. 1994; *Secale cereale,* Philipp et al. 1994), has only rarely been statistically tested, and has sometimes been found to conform to expectations under normality (Bucci et al. 1995; Byrne et al. 1995; Plomion et al. 1995b). Visual inspection of our maps (Figure 2) suggests that most clusters are medial, indicating that many may represent centromeres—areas where recombination is typically reduced (e.g., Chen and Gustafson 1995; Tanksley et al. 1992). Medial or submedial clusters of markers appear to be present in linkage groups 1, 2, 3, 4, 5, 6, and 8 (Figure 2). Medial clusters of markers were present in *P. sylvestris* (groups 1, 3, 7, 8, 9, 10, 13; Yazdani et al. 1995), *Eucalyptus nitens* (e.g., groups 1, 4, 9, 10; Byrne et al. 1995), and in several other published maps [e.g., *Citrus* (Cai et al. 1994), *P. pinaster* (Plomion et al. 1995a,b), *P. radiata* (Devey et al. 1996), *P. taeda* (Neale and Sederoff 1991), and *Eucalyptus* (Grattapaglia and Sederoff 1994)].

There were a number of gaps in our linkage maps. Map densities on the order of 15–20 cM have been recommended for QTL detection (e.g., Beckmann and Soller 1983; Darvasi et al. 1993). However, despite a low average distance between adjacent loci (9–12 cM) and the high percentage of genome coverage based on comparison of our observed and predicted total map sizes (85–93%, Table 3), there were many intervals larger than 20 cM. Assuming a random distribution of markers, a 3000 cM genome, and 95% probability that the maximum distance between loci is less than 20 cM, we would expect to achieve near saturation with 279 loci (Table 4), roughly 35 to 70 more loci than were placed on linkage groups for the two trees in this study. However, because of the tendency for clustering of markers, we

Table 4. Expected mapping efforts required to create a 3000 cM genetic linkage map

		Intervals exceeding maximum expected distance (%)				
		Observed				
P^a	c^a (cM)	10D	3B	Expected[b]	Ratio[c]	Expected number of markers required (n^a)
.90	20	20	12	10	1.6	214
	15	34	22	10	2.8	229
.95	20	20	12	5	3.2	279
	15	34	22	5	5.6	372

[a] Variables k (genome size in cM), P (probability of coverage), c (maximum distance between adjacent markers), and n (total number of markers) as used in the equation: $n = [log(1 - P)/log(1 - 2 \cdot c/k)] \cdot 1.25$, which assumes a random distribution of markers (following Beckman and Soller 1983; Lange and Boehnke 1982; Nelson et al. 1993).

[b] Assuming random distribution of markers for 3B and 10D, respectively.

[c] Ratio between observed (average for 3B and 10D) and expected number of intervals exceeding maximum expected distance.

observed 1.6- to 5.6-fold more intervals that exceeded the 15 or 20 cM thresholds than expected (Table 4), indicating that a substantially higher number of markers may be required for near saturation. Targeted saturation of poorly mapped areas, including unlinked markers and the ends of short linkage groups, is likely to be a more cost-effective strategy to achieve saturation than adding markers at random (e.g., Giovannoni et al. 1991).

Our maps and estimated genome sizes are among the largest published to date for *Pinaceae* (Table 5). Unlike most *Pinaceae* species and the other members of the genus *Pseudotsuga,* which have 12 haploid chromosomes (e.g., Price et al. 1974), Douglas-fir has an extra chromosome and thus a haploid number of 13. Of the 13 chromosomes, 5 have medial and 6 have subterminal centromeres, while 2 of the smaller chromosomes have terminal centromeres (Livingston 1971). In contrast, there are no terminal centromeres in the large majority of species in Pinaceae, and molecular phylogenetic evidence clearly demonstrates that this condition is a derived one within the genus *Pseudotsuga* (Strauss et al. 1990). The telocentrics have been suggested to originate from breakage of one of the ancient metacentric chromosomes, or possibly stimulated by a reciprocal translocation (Thomas and Ching 1968). If true, the genome length of Douglas-fir should be only modestly larger and its genome size nearly the same as for other species in the family. Data on genome length (Table 5) show that Douglas-fir is at the high end, but within the range of estimates for other species. Recent estimates of nuclear DNA content (O'Brien et al. 1996) show that Douglas-fir, at 38 pg (2C), is very close to the mean of 40 pg (range 11–88) for 41 *Pinus* species (data in O'Brien et al. 1996) and to the mean of 37 pg (range 24–63) for 74 *Pinaceae* species (Price et al. 1974).

Our results show that Douglas-fir has a large and complex genome, and that a large number of markers will be needed for comprehensive QTL and marker-aided selection projects. Cooperative efforts are currently under way to integrate genome maps and markers for the species (Neale D and Carlson J, personal communication).

Table 5. Estimates of genome size for *Pinaceae*

Species[a]	Markers	N^b	Linkage groups[c]	Markers mapped	Covered by linkage groups (Morgans)	Estimated total map length[d] (Morgans)	Predicted map size[e] (Morgans)	Expected coverage[f] (%)	Density per linked marker (cM)	Reference
Picea abies	RAPD	96	17–26	165	3.6	4.6[g]	—	—	22	Binelli and Bucci 1994; Bucci et al. 1997
Pinus elliottii	RAPD	66	13–22	73	0.8	2.2	2.9–3.4	64–75	10.7	Nelson et al. 1993
		91	13–19	91[h]	0.9	1.5	2.3–2.4	62–65	16.1	Kubisiak et al. 1995[i]
P. palustris	RAPD	102	16–22	133	1.6	2.3	2.6–2.7	85–87	14.7	Nelson et al. 1994
		113	18–21	122[h]	1.4	1.9	2.3–2.4	81–83	13.0	Kubisiak et al. 1995
P. pinaster	RAPD	102	13–18	263	1.4[j]	1.6[j]	1.2–1.4	90–100	9–10	Plomion et al. 1995b
	RAPD + proteins	142	12–13	436 + 27	1.9[k]	—	1.4–2.3	93–100	8.3	Gerber and Rodolphe 1994; Plomion et al. 1995a, 1996
P. radiata	RAPD + RFLP + SSR	25	14–22	195	1.4	—	—	—	7	Devey et al. 1996
P. sylvestris	RAPD	104	14	261[h]	2.6	3.3[g]	—	—	10.1	Yazdani et al. 1995
P. strobus	RAPD + SSR + STS	76	12–17	91 + 5	0.8–1.0[l]	1.2–1.6[l]	2.1–2.6[l]	58–60[l]	14–16[l]	Echt and Nelson 1997
P. taeda	RFLP		12	191	1.7	—	—	—	8.5	Grattapaglia et al. 1992
Mean		92	14–20	184	1.7	2.4	2.1–2.5	76–82	12.5	
Pseudotsuga menziesii	RAPD	81	16–20	210–244	2.3–2.5	2.8–3.2	2.8–3.5	91–98	10–13	This study

[a] The haploid number of chromosomes in all species except *Pseudotsuga menziesii* ($n = 13$) is 12.

[b] Number of RAPD primers.

[c] Major (>3 loci) and total number of groups.

[d] Including unlinked markers with 30 cM map scale, ends of linkage groups, and linked pairs.

[e] Estimated following Hulbert et al. (1988) and Chakravarti et al. (1991).

[f] Ratio between estimated (observed) and predicted map sizes.

[g] Estimated in this article as indicated (footnote d).

[h] Loci with significant segregation distortion were excluded from analysis.

[i] For 152 to 173 framework markers.

[j] Including 20 cM at both ends of five small linkage groups.

[k] For 244 framework markers.

[l] For 69 framework markers.

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