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Comparative mapping in loblolly and radiata pine using RFLP and microsatellite markers

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Abstract Genetic linkage maps were constructed for loblolly pine (*Pinus taeda* L.) and radiata pine (*P. radiata* D. Don) using a common set of RFLP and microsatellite markers. The map for loblolly pine combined data from two full-sib families and consisted of 20 linkage groups covering 1281 cM. The map for radiata pine had 14 linkage groups and covered 1223 cM. All of the RFLP probes readily hybridise between loblolly and radiata pine often producing similar hybridisation patterns. There were in total 60 homologous RFLP loci mapped in both species which could be used for comparative purposes. A set of 20 microsatellite markers derived from radiata pine were also assayed; however, only 9 amplified and revealed polymorphic loci in both species. Single-locus RFLP and microsatellite markers were used to match up linkage groups and compare order between species. Twelve syntenic groups were obtained each consisting of from 3 to 9 homologous loci. The order of homologous loci was colinear in most cases, suggesting no major chromosomal rearrangements in the evolution of these species. Comparative mapping between loblolly and radiata pine should facilitate genetic research in both species and provide a framework for mapping in other pine species.

Key words Comparative mapping · RFLP · Microsatellite markers · Loblolly pine · Radiata pine

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Introduction

Loblolly pine (*Pinus taeda* L.) and radiata pine (*P. radiata* D. Don) are two of the most important pine species for plantation forestry. Loblolly pine is extensively planted in the south-eastern United States, and the largest producers of radiata pine are Australia, New Zealand and Chile. Taxonomically, *P. taeda* and *P. radiata* are in the same subgenus (*Pinus*) and section (*Pinus*), but different subsections (*Australes* and *Oocarpae*, respectively) (Little and Critchfield 1969). Both are diploid organisms with 2n = 24 chromosomes.

Loblolly or radiata pine restriction fragment length polymorphism (RFLP) probes readily hybridise to both species using the same stringency conditions. A common set of probes derived from loblolly pine cDNA were used to construct linkage maps for two loblolly pine full-sib families (Devey et al. 1994; Groover et al. 1994). A consensus map has also been constructed for these two families (Sewell et al. 1999). A subset of loblolly pine probes plus additional probes derived from radiata pine genomic DNA were used for map construction in a radiata pine full-sib family (Devey et al. 1996).

Microsatellite markers have also been used for map construction in radiata pine (Devey et al. 1996). They are highly polymorphic and usually reveal only a single locus. At least in animals, microsatellite markers are conserved among species, making it possible to use heterologous polymerase chain reaction (PCR) primers between related species (Moore et al. 1991). In plants, heterologous primers may not always amplify or reveal polymorphism depending on the degree of relationship between species (Byrne et al. 1996; Kijas et al. 1995).

Comparative mapping has provided a means for studying the chromosomal organisation of related species or genera. Genome maps using a common set of RFLP markers have been produced for a number of plant and animal species (Ahn and Tanksley 1993; Kowalski et al. 1994; Levan et al. 1991; Saghai Maroof et al. 1996; Tanksley et al. 1992; Van Deynze et al.; 1995). These maps show remarkable colinearity and provide insights

into genome structure and evolution of the species studied. There is also the possibility of identifying homologous genes or quantitataive trait loci (QTLs) for traits of economic importance (Lilley et al. 1996).

A similarity between loblolly and radiata pine genomes is expected based on their taxonomic relationship. In this report we present a comparison of the genetic maps for the two species which were constructed using a set of common RFLP and microsatellite markers.

Materials and methods

Mapping populations and genetic markers

Data from the published maps were obtained from existing databases. The two loblolly pine maps (Devey et al. 1994; Groover et al. 1994), referred to here as "base" and "qtl" maps, respectively, consisted primarily of RFLP loci revealed by cDNA probes derived from loblolly pine. The radiata pine map was constructed using two sources of probes: 93 of the loblolly probes and 76 probes derived from a radiata genomic DNA library (Devey et al. 1996). In the present study, 55 of the radiata probes which revealed a comparatively simple hybridisation pattern were used for mapping in the two loblolly families. Random amplified polymorphic DNA (RAPD) data were also available for the mapping families; however, as it would be very difficult to establish homology across species using RAPD markers, these data were not considered in the present study. The pedigrees and total number of RFLP loci analysed for each family are listed in Table 1.

DNA isolation and RFLP procedures were as described previously (Devey et al. 1996). For comparative purposes, the two radiata parents were included on loblolly progeny blots and two loblolly parents were included on radiata progeny blots.

PCR conditions for microsatellite primers were optimised for use in radiata pine (Smith and Devey 1994). A set of 20 markers were assayed in both species using the same conditions. DNA (50 ng) was amplified in a total volume of 10 µl containing 0.2 µ*M* of each primer, 20 m*M* TRIS-HCl (pH 8.4), 50 m*M* KCl, 1.8–3.0 m*M* $MgCl_2$, 100 µ*M* of each dNTP, 1 mg/ml BSA and 0.4 U Platinum™ *Taq* polymerase (GibcoBRL). Samples were processed through 30 cycles of 10 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final extension of 72°C for 1 h. One of three annealing temperatures (50°, 55° or 60°C) was used depending on the conditions determined for each primer pair. Primers were designed with different dye labels for *in situ* labelling of PCR products. Two-microliter aliquots of the PCR reaction were combined with 15 µl deionised formamide and an internal-lane size standard for analysis on an ABI PRISM™ 310 Genetic Analyzer.

Linkage analysis

Linkage analysis was performed using a method similar to that described by Sewell et al. (1999). Three mapping programmes were used: MAPMAKER Macintosh Ver 1.0 (Lander et al. 1987), MAPMANAGER Ver 2.6.5 (Manly 1993) and JOINMAP Ver 2.0 (Stam 1993). Separate sets of data were derived for those markers segregating on maternal and paternal sides of each pedigree. Progeny data were coded in a backcross configuration with respect to grandparental genotypes so that the markers could be analysed within a phase-known model. For those markers where grandparental data were missing or where both grandparents were heterozygous for the same pair of alleles, the data were "reciprocally" coded to represent both possible phase options (Nelson et al. 1993). Intercross markers (ab x ab) could not be analysed with MAPMAKER or MAPMANAGER.

Initial linkage analysis was done using JOINMAP. Chi-square values were determined by JMSLA and used to test markers for goodness-of-fit to expected Mendelian ratios. Those markers which showed highly significant segregation distortion ($P<0.001$) were excluded from further analysis. Marker loci from the two loblolly families were combined and grouped based on pairwise recombination at LOD >5.0. The radiata family, which had somewhat fewer markers, was grouped using a LOD >4.0. Single-locus RFLP and microsatellite markers were used to determine which groups were homologous between the two species, and corresponding groups were numbered accordingly.

Order within groups was determined with MAPMAKER. Separate male and female maps were constructed for each group using the "first order" command at LOD >3.0 and q <0.40 and verified by permutating all adjacent triplets using the "ripple" command. The "compare" command was used to calculate likelihoods for different orders if there were any discrepancies among families or between species. The data files were then imported into MAPMANAGER to visually inspect the orders and to check for excess double recombinants and possible genotyping errors.

The final maps for each species were then constructed with JOINMAP using the "fixed order" established by MAPMAKER. At this step, data from intercross markers could be placed relative to other markers in the fixed order. JOINMAP combined male and female maps from each species and was also used to merge the two loblolly mapping populations into a single family-averaged map.

Table 1 Loblolly and radiata pine mapping populations

Family	Female parent	Male parent	Number of offspring
Radiata	31053	31032	96
Loblolly base	20–1010	11–1060	95
Loblolly "qtl"	[a]	[a]	48

[a] Confidential

Results

Loblolly pine

Linkage analysis in loblolly pine was based on segregation of 158 RFLP and 11 microsatellite loci in the base pedigree, and 290 RFLP and 10 microsatellite loci in the "qtl" pedigree. Of these, 157 loci were common between both families. Initially, separate maternal and paternal linkage maps were constructed using MAPMAKER; marker order was colinear among the four maps with a few statistically non-significant exceptions (LOD <3.0 as determined from the "compare" command). JOINMAP was used to obtain a consensus map from combined data consisting of 20 linkage groups of 3 or more markers; unlinked markers and groups with only 2 loci are not shown (Fig. 1). Five of the smaller groups consisted only of markers segregating in the "qtl" family. Linkage groups consisted of from 3 to 27 loci and ranged in length from 9 to 127 cM. An average distance between markers of 4.3 cM was observed. A total distance of 1281 cM was covered. Of the 211 mapped RFLP loci, 52 were revealed by probes derived from radiata pine and 159 were revealed by loblolly probes (Table 2).

Although the present analysis included additional RFLP and microsatellite loci and did not include previously reported RAPD and isozyme loci, the correspondence with results obtained by Sewell et al. (1999) was

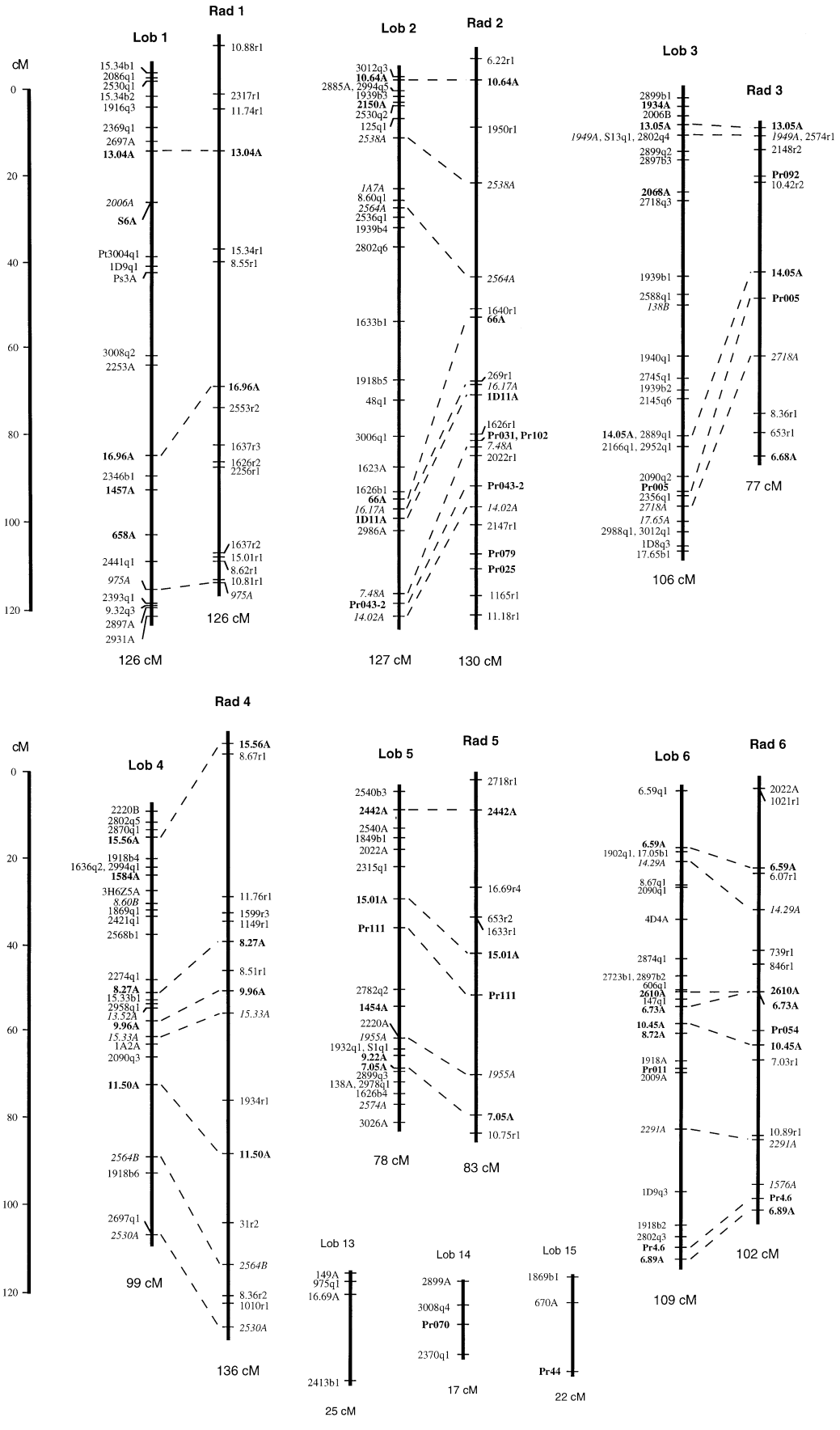

Fig. 1 RFLP and microsatellite linkage maps for loblolly and radiata pine. Loblolly linkage groups are on the *left*; radiata linkage groups are on the *right*; centi Morgan distances in Haldane (cM) are indicated on *scale to left*. Microsatellite loci are shown in *bold font* and begin with the letters *Pr*, e.g. **Pr9.3**. RFLP loci corresponding to cDNA probes derived from loblolly pine *do not have a decimal point*, e.g. *2564A*. Loci *1A7A* and *1D11A* were revealed by loblolly pine genomic DNA probes, and loci *S6A* and *S72q1* were revealed by a *P. sylvestris* Cab cDNA (Jansson and Gustafsson 1990). Those loci corresponding to genomic DNA probes derived from radiata pine *have a decimal point followed by two digits*, e.g. 15.01 A. Where an RFLP probe reveals only a single locus and is therefore likely to be homologous among species or families, it appears in *bold type*. Loci which were assumed to be homologous based on a comparison of size, number and pattern of hybridisation fragments and also confirmed by map position are lisited in *italics*. Other loci which were not used for hybridisations in both species or for which homology could not be clearly determined, end with *lowercase* b, *q* or *r* and *a number* depending on whether they came from loblolly base, qtl, or radiata families, respectively

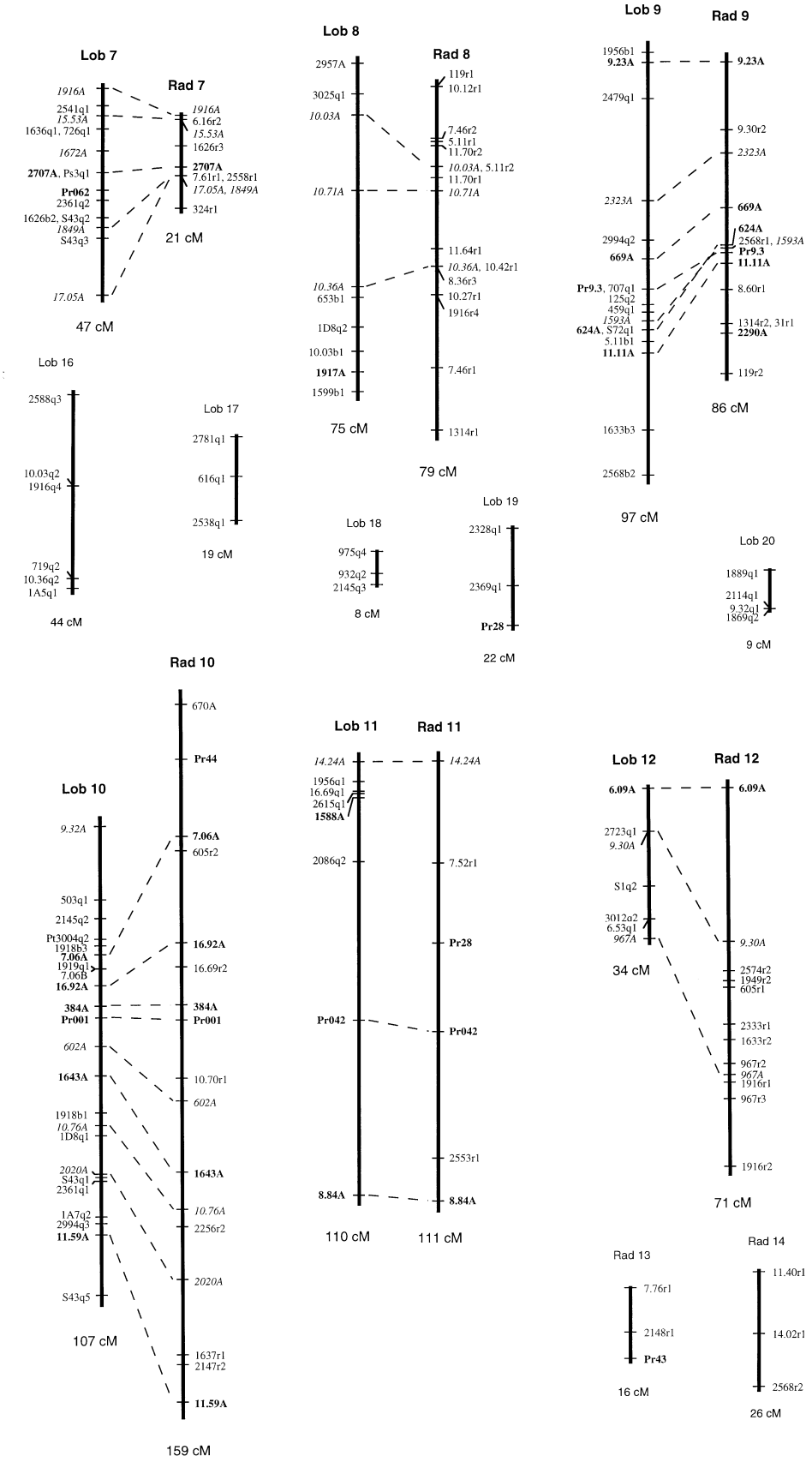
Lob 7
Rad 7
1916A
2541q1
15.53A
1636q1, 726q1
1672A
2707A, Ps3q1
Pr062
2361q2
1626b2, S43q2
1849A
S43q3
17.05A
1916A
6.16r2
15.53A
1626r3
2707A
7.61r1, 2558r1
17.05A, 1849A
324r1
21 cM
47 cM
Lob 8
Rad 8
2957A
3025q1
10.03A
10.71A
10.36A
653b1
1D8q2
10.03b1
1917A
1599b1
119r1
10.12r1
7.46r2
5.11r1
11.70r2
10.03A, 5.11r2
11.70r1
10.71A
11.64r1
10.36A, 10.42r1
8.36r3
10.27r1
1916r4
7.46r1
1314r1
75 cM
79 cM
Lob 9
Rad 9
1956b1
9.23A
2479q1
2323A
2994q2
669A
Pr9.3, 707q1
125q2
459q1
1593A
624A, S72q1
5.11b1
11.11A
1633b3
2568b2
9.23A
9.30r2
2323A
669A
624A
2568r1, 1593A
Pr9.3
11.11A
8.60r1
1314r2, 31r1
2290A
119r2
97 cM
86 cM
Lob 16
2588q3
10.03q2
1916q4
719q2
10.36q2
1A5q1
44 cM
Lob 17
2781q1
616q1
2538q1
19 cM
Lob 18
975q4
932q2
2145q3
8 cM
Lob 19
2328q1
2369q1
Pr28
22 cM
Lob 20
1889q1
2114q1
9.32q1
1869q2
9 cM
Lob 10
Rad 10
9.32A
503q1
2145q2
Pt3004q2
1918b3
7.06A
1919q1
7.06B
16.92A
384A
Pr001
602A
1643A
1918b1
10.76A
1D8q1
2020A
S43q1
2361q1
1A7q2
2994q3
11.59A
S43q5
670A
Pr44
7.06A
605r2
16.92A
16.69r2
384A
Pr001
10.70r1
602A
1643A
10.76A
2256r2
2020A
1637r1
2147r2
11.59A
107 cM
159 cM
Lob 11
Rad 11
14.24A
1956q1
16.69q1
2615q1
1588A
2086q2
Pr042
8.84A
14.24A
7.52r1
Pr28
Pr042
2553r1
8.84A
110 cM
111 cM
Lob 12
Rad 12
6.09A
2723q1
9.30A
S1q2
3012a2
6.53q1
967A
6.09A
9.30A
2574r2
1949r2
605r1
2333r1
1633r2
967r2
967A
1916r1
967r3
1916r2
34 cM
71 cM
Rad 13
7.76r1
2148r1
Pr43
16 cM
Rad 14
11.40r1
14.02r1
2568r2
26 cM

Table 2 Summary of RFLP loci mapped in loblolly, radiata, and common loci mapped in both species

	Source of probes			Homologous loci	
	Loblolly	Radiata	Total	Single-locus[a]	Others[b]
Loblolly	159	52	211	42	37
Radiata	80	77	157	33	30
Both	42	54	96	31	29

[a] Single-locus RFLP loci are listed in bold font in Fig. 1
[b] Others are loci assumed to be homolgous based on a comparison of size, number and pattern of restriction fragments and confirmed by map position. These are listed in italics in Fig. 1

very close. Linkage groups 1–11, 16, 17 and 19 are numbered to correspond with the previous study. The only differences are in linkage group 12, which was previously numbered 14, and in linkage groups 13, 15, 18 and 20, which did not seem to be present. Linkage groups 12 and 14 were renumbered because it was found that the previous group 14 was syntenic to radiata group 12. For the markers that were common to both studies, the order within groups was also very similar.

Radiata pine

A genetic linkage map for radiata pine was similarly constructed based on segregation of 190 RFLP and 20 microsatellite markers in one full-sib family. One hundred fifty-seven RFLP and 16 microsatellite markers were mapped, including 77 RFLP loci revealed by probes derived from radiata pine and 80 revealed by loblolly probes (Table 2). When grouped at a LOD >4.0, the loci which comprised group 3 were split into 2 smaller groups; however at a LOD of 3.0 and based on results obtained in loblolly pine (with an even higher LOD >5.0) these markers were combined to form a larger group. They were therefore combined in radiata. This resulted in 14 linkage groups of 3 or more markers and which covered 1223 cM (Fig. 1). The groups consisted of 3–22 markers and ranged in length from 16 to 159 cM, with an average distance of 7.0 cM between pairs of loci.

Comparison of loblolly and radiata pine

Determining homology of RFLP loci between species or even among families within the same species can be difficult in pines because the hybridisations often reveal complex gene families. There were 96 RFLP probes in common between loblolly and radiata pine. Among these, two classes of comparative loci were identified. The first class consisted of those in which the RFLP probe revealed only a single-locus – 2 fragments per individual or in some cases 3 or 4 cosegregating fragments, but still only a single locus detected. Single-locus probes are almost certainly homologous and were quite useful for comparisons between the species (Fig. 1, listed in bold font). An example of one of the single-locus RFLP probes, 14.05, is shown in Fig. 2. The number of these probes was limited, and unfortunately they were on average less polymorphic than other RFLP loci. Therefore only 31 revealed segregating loci and were mapped in both species. The second class of comparative loci were those assumed to be homologous based on a comparison of size, number and pattern of hybridisation fragments; there were 29 of these loci mapped in both species (Fig. 1, italics). If the locus was segregating in both species, homology could also be confirmed by map position. If a probe was not used for hybridisations in both species or if there was any uncertainty as to whether a particular locus was homologous, it was given a different locus designation depending on which mapping family the polymorphism was observed, e.g. *7.18b1*, *q1* or *r1* for a locus revealed by probe 7.18 in base, "qtl" or radiata mapping populations, respectively.

PCR conditions for microsatellite markers were optimised for radiata by varying the annealing temperature and magnesium concentration; the same conditions were used for amplification in loblolly pine. Of the 20 microsatellite loci assayed, 12 amplified and revealed polymorphic loci in one or both loblolly families. Of those markers revealing polymorphisms, null alleles were observed in at least one of the loblolly families for *Pr4.6*, *Pr9.3*, *Pr44* and *Pr111*. The presence of nulls made it difficult, or impossible in one case, to score the locus. Loblolly microsatellite loci were assumed to be homologous with the same loci in radiata pine and mapped to similar positions except for *Pr44* and *Pr28* which mapped to linkage groups 10 and 11, respectively, in radiata but appeared on smaller non-syntenic groups in loblolly pine.

Using homologous RFLP and microsatellite loci we were able to match up groups between loblolly and radiata pine and to compare orders. Twelve syntenic groups were obtained each of which had from 3 to 9 homologous loci. In most cases, the order was colinear. A slight discrepancy in order occurred on linkage group 9 for loci *624A* and *Pr9.3*; however, the differences in LOD were not significant, i.e. <3.0. The only significant discrepancy occurred on radiata linkage group 2. The order obtained from MAPMAKER and confirmed with MAPMANAGER was consistent with that obtained for loblolly group 2; however, JOINMAP would not place the markers in this order even when a "fixed order" was specified. The problem was attributed to marker *1A7*, which was subsequently dropped from the analysis in radiata. If this locus was included, JOINMAP would place it at either end of the linkage group (LOD >1). Its placement also significantly rearranged the order of other homologous loci. Without *1A7*, the other loci could be positioned colinearly with respect to loblolly and in agreement with MAPMAKER and MAPMANAGER.

A number of large linkage blocks spanning up to 9 homologous loci were conserved. Overall distances are nearly the same in both species (965 vs. 967 cM), al-

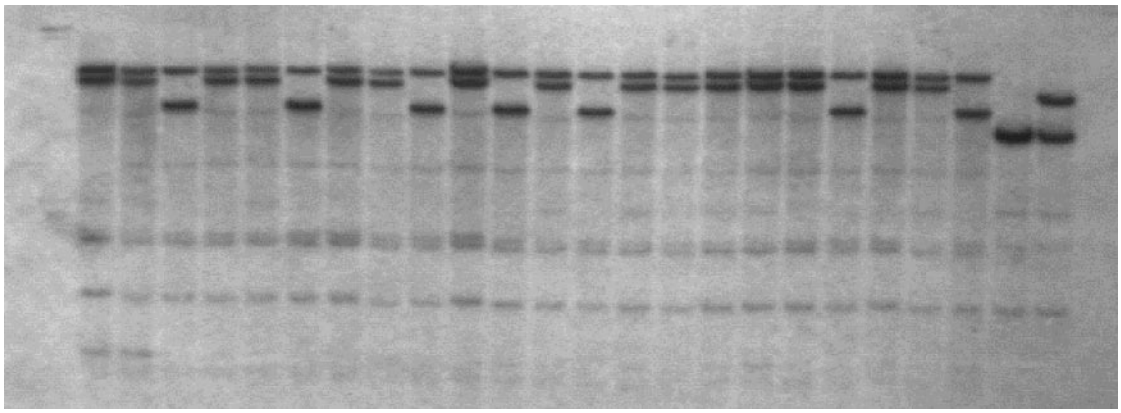

Fig. 2 Hybridisation of RFLP probe 14.05 to *Eco*RI-digested DNAs from 22 progeny of the loblolly pine base map family and radiata pine parents, 31053 and 31032 (right 2 lanes). Lambda/*Hind*III molecular weight standard is in the first lane

though considerable differences were observed in two regions on linkage groups 2 and 10. The difference observed on linkage group 2 is primarily due to the interval between *2564A* and *66A* which is 68 cM in loblolly pine and 9 cM in radiata. And on linkage group 10, the difference is primarily accounted for by the interval between *7.06A* and *602A*.

Discussion

Genetic linkage maps were constructed for loblolly and radiata pine using a common set of RFLP and microsatellite markers. A total of 60 comparative RFLP loci were identified and were useful for lining up the maps. There were also 9 microsatellite loci mapped in both species. Twelve syntenic linkage groups were obtained corresponding to the haploid number of 12 for pines. Each group consisted of from 3 to 9 homologous loci, and colinearity was observed in all groups except 2 and 9. The discrepancy on group 9 was not significant, and that which occurred on group 2 is probably due to some anomaly in the data or linkage programme rather than to a real genetic rearrangement. A colinear order for group 2 was obtained with MAPMAKER and confirmed with MAPMANAGER but could not be obtained with JOINMAP. The discrepancy appears to be attributed to locus *1A7A* on radiata group 2. This may have been a phase problem, since the locus could be placed at either end of the linkage group. JOINMAP determines phase from progeny segregation, whereas with MAPMAKER, phase was determined from grandparental data. It could not be determined which was correct, and this marker was subsequently dropped from the analysis. It is also possible that *1A7A* in radiata was not homologous with the same locus mapped in loblolly group 2, since there were 2 other loci revealed with this probe in these families.

Linkage groups in the present analysis were numbered to correspond with those obtained in Sewell et al. (1999). They had 14 consensus groups; however, there were no common markers between loblolly and radiata pine for groups 12 and 13, and it was found that loblolly group 14 was syntenic to radiata group 12. Loblolly linkage groups 12 and 14 were therefore renumbered in the present study.

Whereas all of the RFLP markers worked equally well in either species, there were some difficulties with the microsatellite markers. The microsatellite markers were derived from radiata pine, and a set of 20 were assayed in both species. All of these worked in radiata; however, only 12 amplified and revealed polymorphism in loblolly pine using the same PCR conditions that were used in radiata. Of the microsatellites that revealed polymorphisms in loblolly, there were 4 loci where null alleles were observed (or not observed). It has been reported that heterologous microsatellite primers do not amplify as well in distantly related species, as evidenced by a lack of amplification and null alleles (Byrne et al. 1996; Kijas et al. 1995). Loblolly and radiata pine are closely related; however, there is still evidence of some sequence divergence.

On average, linkage distances between common markers were conserved in loblolly and radiata pine, although there were some considerable differences. The biggest difference occurred on linkage group 2 between *2564A* and *66A*. Regions of unequal recombination between populations are common even within a species (Beavis and Grant 1991; Sewell et al. 1999) and are most likely due to sampling error. Double recombinants which occur as a result of scoring inaccuracies can also affect distances.

Genome length estimates for pines have varied considerably (Plomion et al. 1995; Nelson et al. 1993). Etch and Nelson (1997) compared three pines, *P. strobus*, P. *palustris* and *P. pinaster*, and found the average theoretical genome length to be close to 2000 Kosambi cM. The theoretical map distance was greater than the observed map coverage for all three species, ranging from 1204 to 1845 cM. Using the same methods, Sewell et al. (1999) estimated the average genome length of loblolly pine to be 1661 cM. If results obtained in the present study are comparable, a distance of about 1200 cM obtained for loblolly and radiata pine would represent about 75% of

the expected genome coverage (does not include linkage groups with only 2 markers). Additional informative and single-locus markers would be useful to join together smaller groups and increase map coverage.

The majority of pine RFLP probes reveal large gene families, and the size of these families can vary considerably among different species (Kinlaw and Neale 1997; Ahuja 1994). This was observed for 2 probes, 1457 and 658 on loblolly linkage group 1, both of which revealed single loci in loblolly pine; however, when used as probes in radiata pine they revealed complex hybridisation patterns with numerous restriction fragments. Interestingly, these 2 loci map to within 10 cM of each other. This would suggest an amplification of this region of the genome in radiata which has not occurred in loblolly pine.

The similarity of loblolly and radiata pine genomes should facilitate gene mapping in both species. Colinearity of marker loci is of particular interest for gene mapping since homologous loci linked to genes of importance in one species may predict the location of these genes in the other. Groover et al. (1994) identified a number of QTLs for wood density in loblolly pine. Some of these genes may also be segregating in radiata pine, and the markers identifying them in loblolly pine may be useful starting points for similar studies in radiata. Many of the RFLP probes used in this study have been shown to hybridise across a range of pine and other conifer species (Ahuja et al. 1994), suggesting the possibility for development of a "generic" pine map. The probes described here could provide a useful framework for such studies as well as information on the evolution of the pine genus.

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