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Linkage map of Japanese black pine based on AFLP and RAPD markers including markers linked to resistance against the pine needle gall midge

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Abstract Macrogametophytes derived from the seeds of a tree resistant to pine needle gall midge (PGM) were analyzed using amplified fragment length polymorphism (AFLP). A total of 244 segregating loci were detected among 71 macrogametophytes. Combining the AFLP results with previously reported segregation data for 127 random amplified polymorphic DNA (RAPD) markers, 157 AFLP and 50 RAPD markers with confirmed map positions were assigned to 20 linkage groups and three pairs covering 2085.5 cM with an average distance of 10.1 cM. The total map distance covers about 77.1–78.4% of the total genome, estimated to be approximately 2665–2719 cM in length. Thus, using AFLP markers, the previous RAPD map of this tree was improved in terms of the average distance between markers, the total map distance, and coverage of the genome. Three RAPD markers linked to a gene associated with resistance to PGM were also located on this map.

Keywords *Pinus thunbergii* · Macrogametophyte · Linkage map · AFLP · RAPD

Introduction

Recently, Kondo et al. (2000) developed a linkage map of Japanese black pine (*Pinus thunbergii*) using random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990) in a study of 96 macrogametophytes. They screened 1160 random primers, of which 92 were selected. The total map distance was 1469.8 cM, with 98 RAPD markers spaced 15.6 cM apart, on average, covering 67.5% of the genome. Although 1160 primers were used in the study, the total map distance and the density of markers were insufficient for some purposes. Additional types of DNA markers were used to extend the utility of the map.

It has been reported that amplified fragment length polymorphism (AFLP) markers (Vos et al. 1995) are more reliable and consistent, and show higher levels of polymorphism, than RAPD markers (Powell et al. 1996). Although AFLP markers were initially developed for plants with small genomes, they have been successfully used to analyze pine (which has a very large genome) after appropriate modifications (Travis et al. 1998; Remington et al. 1999; Costa et al. 2000). The linkage maps for Pine, developed using AFLP markers, each covered more than 85% of the genome.

By surveying AFLP markers as well as the RAPD markers analyzed in a previous study (Kondo et al. 2000), and using the same macrogametophytes assessed in the RAPD analysis, we aimed to reduce the average distance between markers, to extend the total map distance, and to integrate linkage groups. Further objectives were to compare key features of RAPD and AFLP markers, and to evaluate differences in the regions covered by the two types of markers, as discussed below. We also aimed to confirm the location of the markers linked to pine needle gall midge (PGM) resistance and, if possible, identify new AFLP markers linked to such resistance.

Materials and methods

Plant materials and AFLP procedure

Seventy one out of 96 macrogametophyte DNAs derived from openly pollinated seeds of the Japanese black pine (*P. thunbergii*) tree, designated Taichu-Touokuiku 17, mapped in a previous investigation (Kondo et al. 2000), were subjected to AFLP analysis in this study.

AFLP analysis was carried out according to the recommendations of the manufacturer (Perkin-Elmer) of the apparatus involved. Primers were complementary to the adapters *Eco*RI and *Mse*I, with two additional selective 3′ nucleotides (AC and CC, respectively; Remington et al. 1999) in the first amplification or ‘pre-amplifica-

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tion' cycle. Forty eight primer combinations, containing the same sequences as those used in the pre-amplification and one (for the *Eco* primer) or two (for the *Mse* primer) 3′ selective nucleotides, were used in the second amplification (or 'selective amplification') cycle. PCRs were carried out using a Perkin Elmer 9600 thermal cycler according to the procedure described by Remington et al. (1999). After selective amplification, AFLP fragments were detected on a model 373 DNA sequencer (Perkin-Elmer).

A large number of polymorphic fragments were detected, but each one used to score segregation had to meet the following criteria. First, the parent had to have the same size of fragment. Second, the peak intensity of the fragment had to be stable among the macrogametophytes. Third, there had to be no other fragments within 1 bp distance of the candidate fragment.

Linkage analysis

Mendelian segregation of loci was verified by chi-square tests of the expected 1:1 ratios. AFLP segregation data were combined with those obtained for the 127 RAPD markers used in our previous study (Kondo et al. 2000), and analyzed using the MAPMAKER/EXP 3.0 computer program (Lander et al. 1987; Lincoln et al. 1992). Marker phenotypes were reciprocally coded for each locus so that the program could calculate coupling- and repulsion- phase linkages from the arbitrary linkage-phase data (Nelson et al. 1993, 1994; Plomion et al. 1995a, b). To identify linkage groups, pairwise comparisons and grouping of markers were performed so that the LOD score was equal to, or greater than, 5.0. In order to establish the most-likely order of markers within each linkage group, we compared the best and second-best orders using a LOD score of 2.0 as an exclusion threshold, and the 'ripple' or 'compare' commands were employed to confirm the order of the markers. To evaluate the linkage map, we estimated the genome length, G, of Japanese black pine using the moment estimator method according to Hulbert et al. (1988), assuming threshold LOD values of 4.0, 5.0, and 6.0. The Haldane map function was used as in the previous paper.

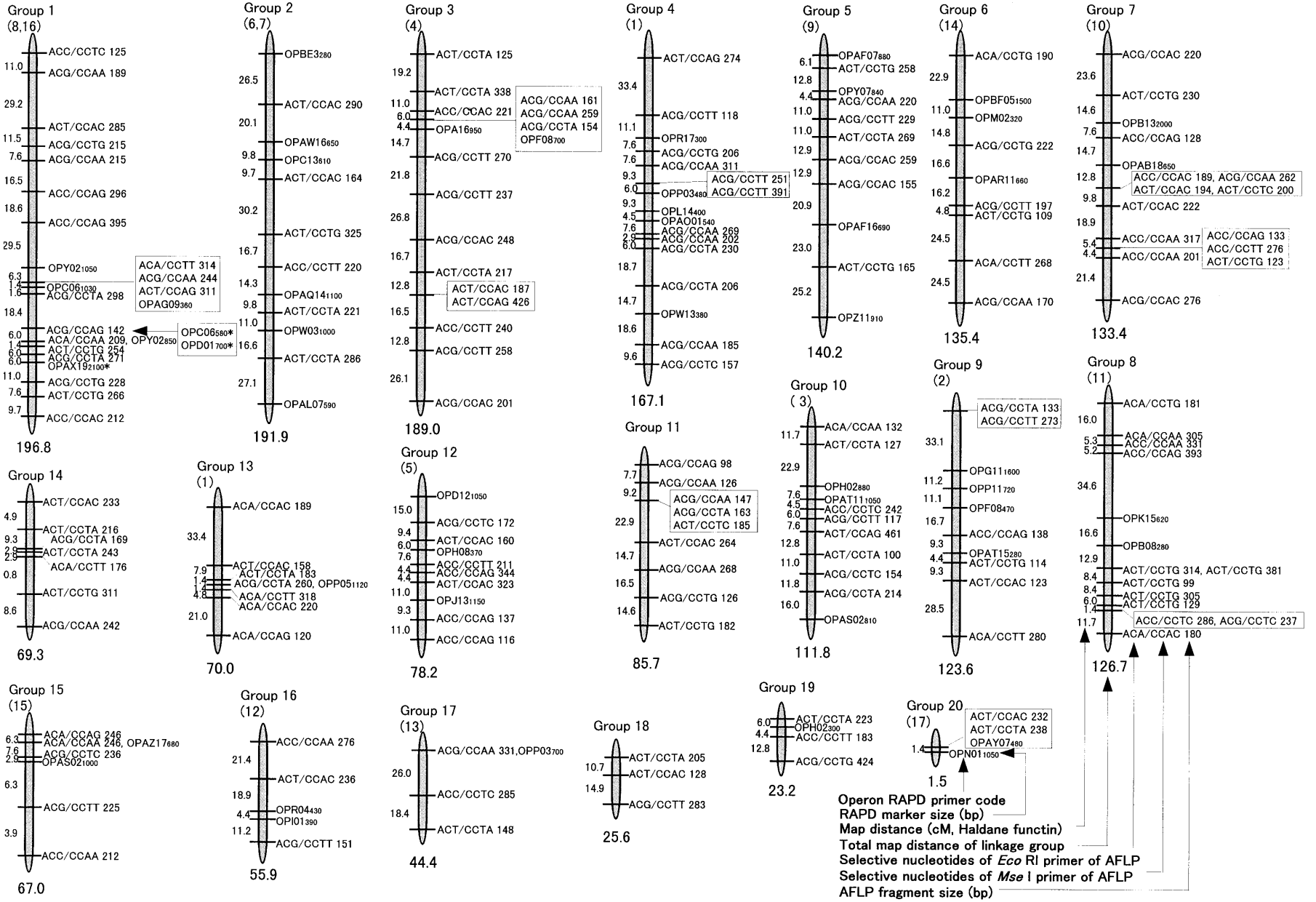
Results

Forty eight primer combinations were screened using eight DNA samples extracted from macrogametophytes derived from Taichu-Touokuiku 17, a tree of Japanese black pine (*P. thunbergii*) which is resistant to PGM. From these, 24 combinations were selected, which generated 244 polymorphic markers in 71 macrogametophytes (Table 1). As four of the 244 markers showed a significant deviation from a 1:1 ratio ($p<0.05$), they were excluded from linkage analysis. Segregation data for the 240 AFLP markers were combined with those obtained from 127 RAPD markers in previous work (Kondo et al. 2000), and analyzed using the MAPMAKER program. Of these 367 markers, 228 AFLP and 121 RAPD markers were assigned to 23 linkage groups. Of the 349 markers assigned, the map positions of 157 AFLP and 50 RAPD markers were confirmed, and assigned to 20 linkage groups and three pairs, covering 2088.1 cM with an average distance of 10.1 cM (Fig. 1). The other assigned markers (71 AFLP and 71 RAPD) were located as accessory markers at their

Fig. 1 Linkage map of Japanese black pine derived using 157 AFLP and 50 RAPD markers with confirmed map positions, assigned to 20 linkage groups, and three pairs (pairs are not shown). *Numbers in parentheses* show the linkage groups identified in a previous RAPD map (Kondo et al. 2000). Of the three markers linked to the PGM resistance previously reported (Kondo et al. 2000), only $OPAX19_{2100}$ was located with a confirmed map position. $OPC06_{580}$ and $OPD01_{700}$ were located as accessory markers at their most likely position (shown by an *arrow*). *Asterisks* indicate these three markers ▶

Table 1 Number of AFLP markers generated and mapped with confirmed positions. Primer combinations are represented in terms of the selective nucleotides used

Method	Primer combination		Number of scored fragments for mapping	Number of mapped fragments
	Eco+3	*Mse*+4		
AFLP	ACA	CCAA	10	4
	ACA	CCAC	6	3
	ACA	CCAG	5	2
	ACA	CCTT	5	5
	ACA	CCTG	5	2
	ACC	CCAA	5	5
	ACC	CCAC	5	3
	ACC	CCAG	15	9
	ACC	CCTC	4	4
	ACC	CCTT	9	5
	ACC	CCGA	8	2
	ACG	CCAA	20	17
	ACG	CCAC	11	6
	ACG	CCAG	2	2
	ACG	CCCA	3	0
	ACG	CCTA	11	12
	ACG	CCTC	7	4
	ACG	CCTT	17	13
	ACG	CCTG	11	6
	ACT	CCAC	19	15
	ACT	CCAG	9	4
	ACT	CCTA	24	16
	ACT	CCTC	5	2
	ACT	CCTG	24	16
Mean value for 24 primer combinations			10	6.5


Group 1
(8,16)
ACC/CCTC 125
11.0
ACG/CCAA 189
29.2
ACT/CCAC 285
11.5
ACG/CCTG 215
7.6
ACG/CCAA 215
16.5
ACC/CCAG 296
18.6
ACC/CCAG 395
29.5
OPY02$_{1050}$
6.3
1.4
OPC06$_{1030}$
1.6
ACG/CCTA 298
18.4
ACG/CCAG 142
6.0
1.4
ACA/CCAA 209, OPY02$_{850}$
6.0
ACT/CCTG 254
6.0
ACG/CCTA 271
OPAX19$_{2100}$*
11.0
ACG/CCTG 228
7.6
ACT/CCTG 266
9.7
ACC/CCAC 212
196.8
ACA/CCTT 314
ACG/CCAA 244
ACT/CCAG 311
OPAG09$_{360}$
OPC06$_{580}$*
OPD01$_{700}$*
Group 2
(6,7)
OPBE3$_{280}$
26.5
ACT/CCAC 290
20.1
OPAW16$_{650}$
9.8
OPC13$_{610}$
9.7
ACT/CCAC 164
30.2
ACT/CCTG 325
16.7
ACC/CCTT 220
14.3
OPAQ14$_{1100}$
9.8
ACT/CCTA 221
11.0
OPW03$_{1000}$
16.6
ACT/CCTA 286
27.1
OPAL07$_{590}$
191.9
Group 3
(4)
ACT/CCTA 125
19.2
ACT/CCTA 338
11.0
ACC/CCAC 221
6.0
4.4
OPA16$_{950}$
14.7
ACG/CCTT 270
21.8
ACG/CCTT 237
26.8
ACG/CCAC 248
16.7
ACT/CCTA 217
12.8
ACT/CCAC 187
ACT/CCAG 426
16.5
ACC/CCTT 240
12.8
ACG/CCTT 258
26.1
ACG/CCAC 201
189.0
ACG/CCAA 161
ACG/CCAA 259
ACG/CCTA 154
OPF08$_{700}$
Group 4
(1)
ACT/CCAG 274
33.4
ACG/CCTT 118
11.1
OPR17$_{300}$
7.6
ACG/CCTG 206
7.6
ACG/CCAA 311
9.3
6.0
OPP03$_{480}$
9.3
OPL14$_{400}$
4.5
OPAO01$_{540}$
7.6
ACG/CCAA 269
2.8
ACG/CCAA 202
6.0
ACG/CCTA 230
18.7
ACG/CCTA 206
14.7
OPW13$_{380}$
18.6
ACG/CCAA 185
9.6
ACG/CCTC 157
167.1
ACG/CCTT 251
ACG/CCTT 391
Group 5
(9)
OPAF07$_{880}$
6.1
ACT/CCTG 258
12.8
OPY07$_{840}$
4.4
ACG/CCAA 220
11.0
ACG/CCTT 229
11.0
ACT/CCTA 269
12.9
ACG/CCAC 259
12.9
ACG/CCAC 155
20.9
OPAF16$_{690}$
23.0
ACT/CCTG 165
25.2
OPZ11$_{910}$
140.2
Group 6
(14)
ACA/CCTG 190
22.9
OPBF05$_{1500}$
11.0
OPM02$_{320}$
14.8
ACG/CCTG 222
16.6
OPAR11$_{660}$
16.2
4.8
ACG/CCTT 197
ACT/CCTG 109
24.5
ACA/CCTT 268
24.5
ACG/CCAA 170
135.4
Group 7
(10)
ACG/CCAC 220
23.6
ACT/CCTG 230
14.6
OPB13$_{2000}$
7.6
ACC/CCAG 128
14.7
OPAB18$_{650}$
12.8
ACC/CCAC 189, ACG/CCAA 262
ACT/CCAC 194, ACT/CCTC 200
9.8
ACT/CCAC 222
18.9
5.4
ACC/CCAA 317
ACC/CCAG 133
4.4
ACC/CCAA 201
ACC/CCTT 276
ACT/CCTG 123
21.4
ACG/CCAC 276
133.4
Group 8
(11)
ACA/CCTG 181
16.0
5.3
ACA/CCAA 305
5.2
ACC/CCAA 331
ACC/CCAG 393
34.6
OPK15$_{620}$
16.6
OPB08$_{280}$
12.9
ACT/CCTG 314, ACT/CCTG 381
8.4
ACT/CCTG 99
8.4
ACT/CCTG 305
6.0
1.4
ACT/CCTG 129
11.7
ACC/CCTC 286, ACG/CCTC 237
ACA/CCAC 180
126.7
Group 9
(2)
ACG/CCTA 133
ACG/CCTT 273
33.1
OPG11$_{1600}$
11.2
OPP11$_{720}$
11.1
OPF08$_{470}$
16.7
ACC/CCAG 138
9.3
4.4
OPAT15$_{280}$
ACT/CCTG 114
9.3
ACT/CCAC 123
28.5
ACA/CCTT 280
123.6
Group 10
(3)
ACA/CCAA 132
11.7
ACT/CCTA 127
22.9
OPH02$_{880}$
7.6
4.5
OPAT11$_{1050}$
ACC/CCTC 242
6.0
ACG/CCTT 117
7.6
ACT/CCAG 461
12.8
ACT/CCTA 100
11.0
ACG/CCTC 154
11.8
ACG/CCTA 214
16.0
OPAS02$_{810}$
111.8
Group 11
ACG/CCAG 98
7.7
ACG/CCAA 126
9.2
ACG/CCAA 147
ACG/CCTA 163
ACT/CCTC 185
22.9
ACT/CCAC 264
14.7
ACG/CCAA 268
16.5
ACG/CCTG 126
14.6
ACT/CCTG 182
85.7
Group 12
(5)
OPD12$_{1050}$
15.0
ACG/CCTC 172
9.4
ACT/CCAC 160
6.0
OPH08$_{370}$
7.6
4.4
ACC/CCTT 211
4.4
ACC/CCAG 344
4.4
ACT/CCAC 323
11.0
OPJ13$_{1150}$
9.3
ACC/CCAG 137
11.0
ACC/CCAG 116
78.2
Group 13
(1)
ACA/CCAC 189
33.4
7.9
ACT/CCAC 158
ACT/CCTA 183
1.4
1.4
ACG/CCTA 260, OPP05$_{1120}$
4.8
ACA/CCTT 318
ACA/CCAC 220
21.0
ACA/CCAG 120
70.0
Group 14
ACT/CCAC 233
4.9
ACT/CCTA 216
9.3
ACG/CCTA 169
2.8
2.8
ACT/CCTA 243
ACA/CCTT 176
0.8
ACT/CCTG 311
8.6
ACG/CCAA 242
69.3
Group 15
(15)
ACA/CCAG 246
6.3
ACA/CCAA 246, OPAZ17$_{680}$
7.6
ACG/CCTC 236
2.9
OPAS02$_{1000}$
6.3
ACG/CCTT 225
3.9
ACC/CCAA 212
67.0
Group 16
(12)
ACC/CCAA 276
21.4
ACT/CCAC 236
18.9
4.4
OPR04$_{430}$
OPI01$_{390}$
11.2
ACG/CCTT 151
55.9
Group 17
(13)
ACG/CCAA 331,OPP03$_{700}$
26.0
ACC/CCTC 285
18.4
ACT/CCTA 148
44.4
Group 18
ACT/CCTA 205
10.7
ACT/CCAC 128
14.9
ACG/CCTT 283
25.6
Group 19
6.0
ACT/CCTA 223
4.4
OPH02$_{300}$
ACC/CCTT 183
12.8
ACG/CCTG 424
23.2
Group 20
(17)
ACT/CCAC 232
ACT/CCTA 238
OPAY07$_{480}$
1.4
OPN01$_{1050}$
1.5
Operon RAPD primer code
RAPD marker size (bp)
Map distance (cM, Haldane functin)
Total map distance of linkage group
Selective nucleotides of *Eco* RI primer of AFLP
Selective nucleotides of *Mse* I primer of AFLP
AFLP fragment size (bp)

most likely positions (not shown on the map in Fig. 1). Eighteen markers (4.9% of the total) remained unlinked. The estimates of the genome length (G) at various grouping LOD scores (Z) were 2718.5 cM at Z=4.0, 2665.0 cM at Z=5.0 and 2709.1 cM at Z=6.0. Two linkage groups in the previous RAPD map, 6 and 7, were combined together, and became Group 2 in the new map. Another two linkage groups, 8 and 16, were combined to form Group 1. In contrast to this integration of linkage groups, linkage group 1 was divided into two linkage groups, Groups 4 and 13. Therefore, the 17 linkage groups on the RAPD map derived in the previous study were assigned to 16 groups in this study. Of three markers linked to PGM resistance previously reported (Kondo et al. 2000), the map position of only one, $OPAX19_{2100}$, was confirmed. $OPC06_{580}$ and $OPD01_{700}$ were located as accessory markers (shown with an arrow). Asterisks in Fig. 1 indicate these three markers.

Discussion

Higher resolution from the AFLP analysis

Recently, Kondo et al. (2000) constructed a linkage map using the same materials surveyed in this study. Ninety eight RAPD markers, including three markers linked to PGM resistance, were assigned to 17 linkage groups, covering 1469.8 cM. The genome length was estimated to be 2138.3 cM, and the map covered 67.5% of the genome. Since the same macrogametophytes were used in the AFLP analysis as in the RAPD study, important features of RAPD and AFLP markers could be directly compared. In the AFLP analysis, the mean number of polymorphic markers detected per primer combination was ten (Table 1): 7.4-times more than in the RAPD assays of the previous study. The average distance between markers was reduced to 10.1 cM, from 15.6 cM for the RAPD map. Two pairs of linkage groups of the RAPD map were integrated into the new map. To identify linkage groups, a higher LOD score (>5.0) was used in this study than in the previous, purely RAPD analysis (LOD >3.0). Nevertheless, the number of unlinked RAPD markers decreased from twelve to 6. The genome length was estimated to be 2697.5 cM: 26% longer than the estimate obtained from the RAPD data. The total map distance was 2088.1 cM, covering 77% of the genome, and 42% longer than that of the RAPD map. Thus, the new map improved upon the previous RAPD map in terms of average distance, map distance and genome coverage. The increase in estimated genome length compared to the previous study might be due to the fact that the RAPD markers alone were only effectively sampling a portion of the genome. Six regions, spanning more than 50 cM with no RAPD markers, were mapped with AFLP markers in linkage groups 2, 5, 6, 7, 14 and 15.

Screening of polymorphic AFLP markers

Remington et al. (1999) and Costa et al. (2000) constructed linkage maps of loblolly pine and maritime pine, respectively. They used the same primer combinations (*Eco*RI-AC/*Mse*-CC in pre-amplification and *Eco*RI-ACX/*Mse*-CCXX, where X represents any selective nucleotide A, T, G or C, in selective amplification), as those employed in this study. Although many of the primer combinations we used were the same as those in the previous pine AFLP studies, the number of polymorphic markers per primer combination found in this study (ten) was less than in the other pines (24.8 in loblolly pine and 21.4 in maritime pine). However, of 235 AFLP markers surveyed in maritime pine, only 114 (49%) were assigned to confirmed map positions using 200 macrogametophytes and a LOD score >6.0. Similarly, in the loblolly pine study, only 184 markers out of 521 (35%) AFLP markers were assigned to confirmed positions. In comparison, the proportion of AFLP markers mapped in this study (65% of 240 markers) was much higher. From a large number of polymorphic fragments detected, we chose 244 markers based on the criteria described in Materials and methods. Some of the polymorphic fragment peaks had poor stability. Differences in the extent of amplification among DNA samples, or non-specific amplification of multiple fragments with the same molecular weight (Nikaido et al. 1999), might cause this instability. These errors would distort the segregation ratios and make it difficult to map fragments to unique positions. Some fragments detected in the macrogametophytes were sometimes absent in the parent DNA. This phenomenon might be due to the formation of artifacts during the preparation of template DNA or the PCR. Although fewer markers were used in this investigation, compared to the loblolly pine study (Remington et al. 1999), the higher proportion of markers with confirmed map positions might reflect the high quality of the markers selected according to the rigorous criteria employed.

Linked marker to R_{pgm}

Test crosses have revealed that a single dominant gene controls resistance to PGM (Terada 1992). Three RAPD markers linked to the resistance gene (R_{pgm}) were detected by bulked-segregant analysis (Kondo et al. 2000). Of these three markers, $OPAX19_{2100}$ was also located in the confirmed map position in the new map, and $OPC06_{580}$ and $OPD01_{700}$ were regarded as accessory markers at their most likely position. Four AFLP markers and one RAPD marker were found to be located within 15 cM of the two RAPD markers linked to R_{pgm} using macrogametophyte. Although we examined these markers in the segregating family described in the previous paper (Kondo et al. 2000), the segregation ratio of all the markers did not fit to 1:1. Therefore, it was assumed that both parents retained these markers heterozygously.

These markers might be useful in a different mapping population.

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