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RAPD markers linked to a gene for resistance to pine needle gall midge in Japanese black pine (*Pinus thunbergii*)

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Abstract Linkage of RAPD markers to a single dominant gene for resistance to pine needle gall midge was investigated in Japanese black pine (*Pinus thunbergii*). Three primers that generated linked markers were found after 1160 primers were screened by bulked segregant analysis. The distances between the resistance gene, *R*, and the marker genes *OPC06*$_{580}$, *OPD01*$_{700}$, and *OPAX19*$_{2100}$ were 5.1 cM, 6.7 cM and 13.6 cM, respectively. *OPC06*$_{580}$ was in coupling phase to *R*, whereas *OPD01*$_{700}$ and *OPAX19*$_{2100}$ were in repulsion phase to *R*. A linkage map for a resistant tree was constructed using 96 macrogametophytes. In linkage analysis, 98 out of 127 polymorphic markers were assigned to 17 linkage groups and six linked pairs. The total length of this map was 1469.8 cM, with an average marker density of 15.6 cM. The genome length was estimated to be 2138.3 cM, and the derived linkage map covered 67.5% of the genome. Although the linked markers *OPC06*$_{580}$, *OPAX19*$_{2100}$, and *OPD01*$_{700}$, belonged to the same linkage group, no precise positions were found for *OPC06*$_{580}$ or *OPD01*$_{700}$.

Key words *Pinus thunbergii* · Pine needle gall midge · RAPD · Bulked segregant analysis · Linkage

Introduction

Solving most of the major problems encountered in tree breeding is time-consuming because of the long generation times involved. Allogamy and the relative scarcity of genetic information are further limitations. However, it is anticipated that marker-assisted selection (MAS) will be developed as an important tool in breeding programs, with the capacity to curtail some of these problems. Although major genes are the most suitable for use as markers for MAS, only a limited number of these have been identified so far in forest trees. Devey et al. (1995) and Harkins et al. (1998) found random amplified polymorphic DNA (RAPD) markers linked to a resistance gene to white pine blister rust in sugar pine. Benet et al. (1995) identified three RAPD markers linked to a black leaf spot resistance gene in Chinese elm, while Lehner et al. (1995) found a RAPD marker linked to the pendula gene in Norway spruce. MAS has the potential to increase efficiency in breeding programs for all three of these characters, since they are all controlled by a single, dominant gene.

Pine needle gall midge (*Thecodiplosis japonensis*) is one of the major pest insects of Japanese black pine (*Pinus thunbergii*) and Japanese red pine (*P. densiflora*). It is distributed all over Japan and can significantly inhibit growth of the pines. Forty-two resistant Japanese black pine trees have been selected in breeding programs aimed to enhance resistance to this midge. Test-crosses have revealed that this resistance is controlled by a single dominant gene and that the resistant trees are heterozygotes for the resistance gene (Terada 1992).

We here report the identification of RAPD markers linked to pine needle gall midge resistance and the location of their linkage group in a linkage map derived using macrogametophytes in Japanese black pine.

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Materials and methods

Plant material

F_1 progeny were used from a cross between a resistant *Pinus thunbergii* tree, designated Taichu-touokuiku 17 (which is heterozygous, *Rr*, for resistance), and a susceptible tree, No. 6 (which is a homozygous recessive, *rr*). The progeny consisted of 35 resistant, and 27 susceptible individuals. They were planted in 1985 and surveyed for resistance to pine needle gall midge in 1988 and

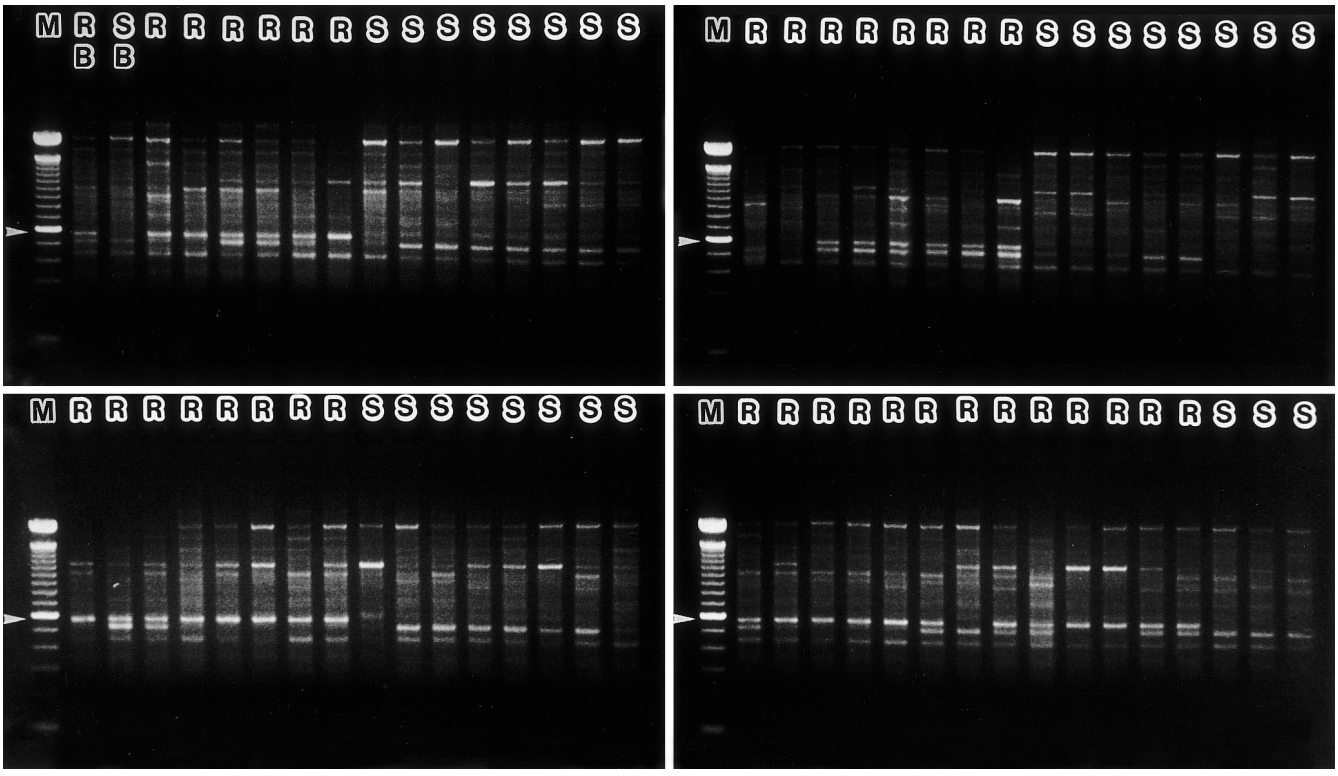


Fig. 1 A RAPD marker (580 bp, *arrowhead*) generated by primer OPC6 was detected in 32 out of 35 resistant individuals and absent in all of the susceptible individuals in progeny of the cross between the resistant tree (*Rr*) and the susceptible tree (*rr*), indicating it was linked to the resistance gene in coupling phase. *Lanes: M* 100-bp ladder markers, *RB* bulked sample from 3 resistant individuals, *SB* bulked sample from 3 susceptible individuals, *R* resistant individuals, *S* susceptible individuals

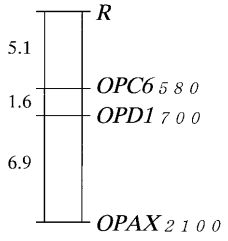


Fig. 2 Linkage map of the region around the resistance gene, *R*, to pine needle gall midge. Genetic distances shown in centiMorgans are to the *left* of the *vertical line*

1989. Leaves were collected for DNA extraction in July 1994 to locate markers linked to *R*. As RAPD markers are mainly dominant, it is generally impossible to distinguish the dominant homozygotes from heterozygotes. Therefore, when constructing linkage maps it is more practical to use macrogametophytes than crossed progeny. For this reason, open-pollinated seeds of Taichu-touokuiku 17 were also collected in October 1995 in order to develop a linkage map using macrogametophytes.

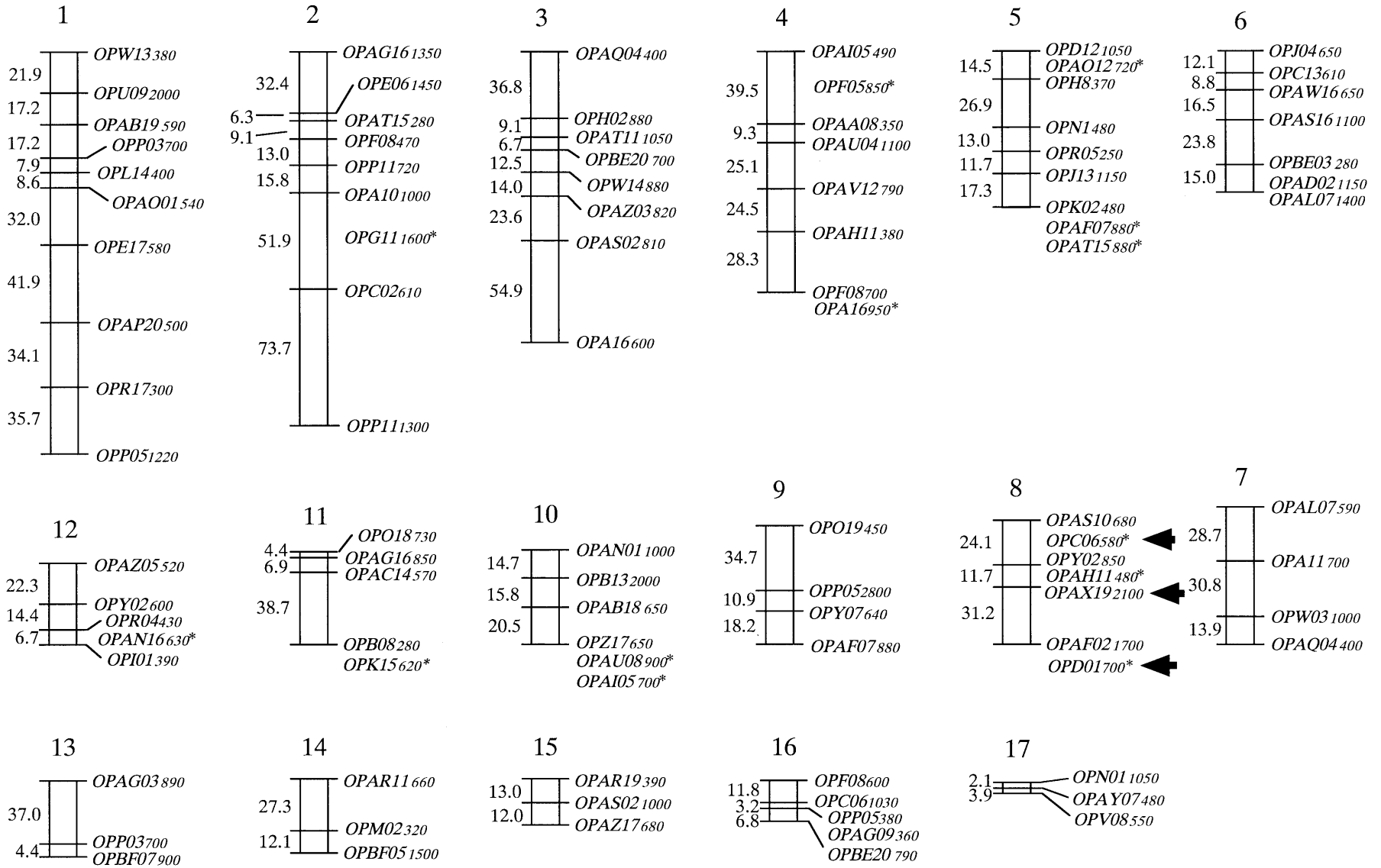
DNA isolation

Approximately 5 g of leaves was frozen in liquid nitrogen and ground with a grinder (Iwatani Co, IFM-150). The ground powder was suspended in 20 ml of ice-cold acetone and filtered through Toyo Rosi No. 2 filter paper using an aspirator. Total DNA was isolated from the residue according to the CTAB procedure (Murray and Thompson 1980). Using sodium dodecyl sulfate (SDS), (Tsumura et al. 1996), we also extracted DNA from macrogametophytes of the seed

RAPD analysis

RAPD analysis was carried out using 1160 different 10-mer primers (Operon Technologies). Each 25-µl aliquot of polymerase chain reaction (PCR) mixture contained 25 ng of leaf DNA or 10 ng of macrogametophyte DNA, 20 m*M* TRIS-HCl (pH 8.4), 50 m*M* KCl, 1.5 m*M* MgCl2, 100 µM each of dNTP, 0.2 m*M* of random primer, and 1 U of *Taq* DNA polymerase (Gibco BRL). Amplification was performed in a Astec PC-800 (Fukuoka, Japan) using the following protocal: a 3-min initial step, at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. This temperature regime was designated as PCR-1 and adopted for both leaf DNA and macrogametophyte DNA analysis. The amplification products were electrophoretically separated on 2% agarose gels, stained with ethidium bromide, and visualized on an UV transilluminator. Because there was only one linked marker, another temperature regime (designated as PCR-2) was also used to increase the number of linked markers. This protocol started with 1 min at 94°C, and continued with 35 cycles of 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C. The amplification products were separated electrophoretically on 2% agarose gels and polyacrylamide gels. As we hoped to find both tightly and loosely linked markers, relatively small numbers of samples were pooled. Thus, primers were screened with bulked samples (Michelmore et

Fig. 3 Linkage map of the Japanese black pine tree (Taichu-touokuiku 17) resistant to pine needle gall midge, derived using samples from macrogametophytes. *Arrows* indicate markers linked to the resistance gene in the crossed progeny. *Asterisks* indicate markers for which no precise positions were determined. Genetic distances, in centiMorgans, are shown to the *left* of the *vertical line* ▶


1
OPW13 380
21.9
OPU09 2000
17.2
OPAB19 590
17.2
OPP03 700
7.9
OPL14 400
8.6
OPAO01 540
32.0
OPE17 580
41.9
OPAP20 500
34.1
OPR17 300
35.7
OPP05 1220
2
OPAG16 1350
32.4
OPE06 1450
6.3
OPAT15 280
9.1
OPF08 470
13.0
OPP11 720
15.8
OPA10 1000
51.9
OPG11 1600*
OPC02 610
73.7
OPP11 1300
3
OPAQ04 400
36.8
OPH02 880
9.1
OPAT11 1050
6.7
OPBE20 700
12.5
OPW14 880
14.0
OPAZ03 820
23.6
OPAS02 810
54.9
OPA16 600
4
OPAI05 490
39.5
OPF05 850*
9.3
OPAA08 350
OPAU04 1100
25.1
OPAV12 790
24.5
OPAH11 380
28.3
OPF08 700
OPA16 950*
5
OPD12 1050
14.5
OPAO12 720*
OPH8 370
26.9
OPN1 480
13.0
OPR05 250
11.7
OPJ13 1150
17.3
OPK02 480
OPAF07 880*
OPAT15 880*
6
OPJ04 650
12.1
OPC13 610
8.8
OPAW16 650
16.5
OPAS16 1100
23.8
OPBE03 280
15.0
OPAD02 1150
OPAL07 1400
7
OPAL07 590
28.7
OPA11 700
30.8
OPW03 1000
13.9
OPAQ04 400
8
OPAS10 680
24.1
OPC06 580*
OPY02 850
11.7
OPAH11 480*
OPAX19 2100
31.2
OPAF02 1700
OPD01 700*
9
OPO19 450
34.7
OPP05 2800
10.9
OPY07 640
18.2
OPAF07 880
10
OPAN01 1000
14.7
OPB13 2000
15.8
OPAB18 650
20.5
OPZ17 650
OPAU08 900*
OPAI05 700*
11
OPO18 730
4.4
OPAG16 850
6.9
OPAC14 570
38.7
OPB08 280
OPK15 620*
12
OPAZ05 520
22.3
OPY02 600
14.4
OPR04 430
6.7
OPAN16 630*
OPI01 390
13
OPAG03 890
37.0
OPP03 700
4.4
OPBF07 900
14
OPAR11 660
27.3
OPM02 320
12.1
OPBF05 1500
15
OPAR19 390
13.0
OPAS02 1000
12.0
OPAZ17 680
16
OPF08 600
11.8
OPC06 1030
3.2
OPP05 380
6.8
OPAG09 360
OPBE20 790
17
OPN01 1050
2.1
OPAY07 480
3.9
OPV08 550

al. 1991) from 3 resistant and 3 susceptible individuals from the crossed progeny. After examining the selected primers using 4 resistant and 4 susceptible individuals, we surveyed putative linked markers using all F_1 individuals. To construct the linkage map, we first screened the capacity of 1160 primers to detect polymorphism using a test set of 8 macrogametophytes. Then, 96 macrogametopytes were examined using selected primers. Total genome length was estimated following the method of Hulbert et al. (1988) based on linkage data using macrogametophytes. Data were analyzed using MAPMAKER (Lander et al 1987) version 3.0 with parameters of a LOD score >4 and map distance <50 cM.

Results

RAPD markers linked to the pine needle gall midge resistance gene

In the PCR-1 temperature regime, 258 out of 1160 primers were selected after the bulked segregant analysis, from which 14 primers were chosen after testing 8 progeny. Only 1 of these primers, OPC-6, produced a marker linked to the resistance gene. This marker was present in 32 of the 35 resistant individuals sampled, but it was absent in all of the susceptible individuals (Fig. 1), indicating that it was linked to the resistance gene in coupling phase. In the PCR-2 temperature regime, 68 primers were selected after bulked segregant analysis, 7 of which were used in further studies after products from the 8 test individuals were analyzed on agarose gels. Although 2 primers produced markers linked to the resistance gene, 1 of them (OPC-6) produced the same marker found using the PCR-1 protocol. Another primer, OPAX-19, produced a marker present in 6 out of 35 resistant individuals and 25 out of 27 susceptible individuals, indicating it was linked to the resistance gene in repulsion phase. With polyacrylamide gels, only 1 primer, OPD-1, produced a detectable marker. This was present in 4 out of 35 resistant individuals and all the susceptible individuals, indicating that it was linked to the resistance gene in repulsion phase. These 3 markers were mapped in relation to the pine needle gall midge resistance gene, *R* (Fig. 2). *OPC06*$_{580}$ was closest to *R*, at a distance of 5.1 cM, *OPD01*$_{700}$ was also close (at 6.7 cM), and *OPAX19*$_{2100}$ was comparatively distant from *R* (at 13.6 cM).

Linkage map of the resistant tree

From the original 1160 primers, 208 were selected after samples from 8 macrogametophytes were analyzed. The potential of these primers to detect polymorphism was examined using 96 macrogametophytes; 92 were found to generate 125 polymorphic markers segregating in a 1:1 ratio. In addition to these markers, 2 linked markers, produced by OPD-1 and OPAX-19 using the PCR-2 protocol in crossed progeny, were also used. In linkage analysis, 115 out of 127 markers were definitively assigned to 17 linkage groups and six linked pairs, but the map positions of 17 markers remained ambiguous (Fig. 3). The total length of this map was 1469.8 cM with an average marker density of 15.0 cM. The genome length was estimated to be 2138.3 cM (LOD score 3.0), 2175.5 cM (LOD score 4.0), or 2101.2 cM (LOD score 5.0), with an average of 2138.3 cM. Thus, the linkage map covered 68.7% of the genome. Although the markers linked to *R*, (*OPC06*$_{580}$, *OPAX19*$_{2100}$, and *OPD01*$_{700}$) belonged to the same linkage group, no precise positions were determined for *OPC06*$_{580}$ and *OPD01*$_{700}$.

Discussion

It is desirable to use a marker linked tightly to the target character in MAS. Benet et al. (1995) found markers linked to a black leaf spot resistance gene in Chinese elm at a distance of 4.3 cM. Lehner et al. (1995) found a marker linked to the pendula gene in Norway spruce at a distance of 4.6 cM. We report here a marker linked to the resistance gene for pine needle gall midge, at a similar distance (5.1 cM). Devey et al. (1995) and Harkins et al. (1998) found markers linked, at a distance of less than 1 cM, to a resistance gene to white pine blister rust in sugar pine. To identify more tightly linked markers we would clearly need to use other markers in addition to RAPD markers.

In all, however, we found 3 linked markers: one in coupling phase to the resistance gene, and the other 2 in repulsion phase. As there are other resistant individuals, we should check whether or not they carry the linked markers in coupling phase to assess the full potential of these markers for use in MAS. Although we succeeded in constructing a linkage map in the region of the resistance gene using the crossed progeny, the order and positions of these markers were uncertain in the linkage map derived from macrogametophytes. To increase the resolution of this map, we would need to use other kinds of markers (such as AFLPs) in addition to the RAPD markers already identified.

The genome size estimates of several hard pine species have been already reported. Estimates have been obtained of approximately 2000 cM (Gerber and Rodolphe 1994) and 1860 cM (Plomion et al. 1995) for *P. pinaster*, and of 2880–3360 cM for *P. elliotii* var '*elliottii*' (Nelson et al. 1993) and 2612–2656 cM for *P. palustris* (Nelson et al. 1994). We estimated the genome size for Japanese black pine to be 2176 cM, which falls within the middle of the range found for other hard pine species.

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