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An allele responsible for seedling death in *Pinus radiata* D. Don

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Abstract When inbred, most outcrossing species show high mortality, manifested at several life stages. The occurrence of homozygotes for deleterious or lethal alleles is believed to be responsible. Here, we report the identification of an allele responsible for the death of selfed *Pinus radiata* D. Don seedlings in their first month after germination. Among 291 S_1 seedlings of plus-tree 850.55, 76 died within 1 month of emergence. Their death appears to be caused by a single recessive lethal allele, SDPr (seedling death in *Pinus radiata*). SDPr is located in a linkage group with 28 RAPD markers, the closest of which is ai05800a. Of the 76 seedlings that died, megagametophytes of 73 could be genotyped. Of these, 71 had the null (no band) allele of ai05800a; only two had the band allele. Of the 190 surviving S_1 diploids that were genotyped, only two individuals were homozygous for the null allele of ai05800a. By two different methods, the map distance between SDPr and ai05800a was estimated to be between 1.0 and 2.7 cM respectively. The frequency of band and null alleles in the combined population of dead and surviving seedlings and in un-sown seeds shows no evidence of selection at this locus prior to germination.

Key words Lethal allele · Dominance
Inbreeding depression · Segregation distortion
Pinus radiata

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Introduction

Inbreeding depression is common and severe in many tree species, particularly conifers (Williams and Savolainen 1996). Slow seedling growth and abnormal morphology are two common manifestations of inbreeding depression. Reduced yield of filled seeds is one of the most severe and consistent results of inbreeding in conifers. High mortality of seedlings is also commonly observed (Pawsey 1964; Bingham 1973; Eriksson et al. 1973; Geburek 1986).

The severity of inbreeding depression varies among species, populations (Namkoong and Bishir 1987) and individuals (Koski 1971; Griffin and Lindgren 1985; Lundkvist et al. 1987). The reduction in viability upon inbreeding is well-studied, and the number of lethal equivalents has been estimated for many species (Williams and Savolainen 1996). The number of lethal equivalents is a close estimate of the number of genes involved only if the genes are completely lethal. Many more genes may be operating if their alleles are sublethal.

Lethal alleles can be purged from populations by simple recurrent inbreeding of survivors, provided the genes have high penetrance, their numbers are not too great, and the population is of sufficient size. If many sub-lethal genes are involved, however, such a practice may, instead, lead to the fixation of some of them (Hedrick 1994). In either case, purging of deleterious alleles will be more effective and efficient if the alleles can be associated with molecular markers so that marker-aided-selection (MAS) may be used to identify and eliminate those occurring in the heterozygous condition.

Both fully lethal and sublethal alleles can be identified using molecular markers. Following natural selection, these deleterious alleles should be present at lower frequencies than expected from Mendelian ratios. Tightly linked molecular markers should also show distorted Mendelian ratios, and provide information

on the location and gene action of the linked deleterious alleles. This approach has been used by Hedrick and Muona (1990) to identify a deleterious allele in Scots pine which was linked with fluorescent esterasase. A more sophisticated method, utilizing two markers, has recently been developed by Fu and Ritland (1994) and Cheng et al. (1996).

Pinus radiata has become one of most economically important tree species in the world. Breeding programs have been ongoing for almost half a century, and genetically improved trees are now used to establish all commercial stands. Unfortunately, like most tree species, *P. radiata* exhibits significant inbreeding depression which is expected to be increasingly manifested as recurrent selection proceeds. Selfing of eight *P. radiata* individuals resulted in seed production rates that were 15–99% less than those obtained from outcrossing (Griffin and Lindgren 1985), and survival rates of radiata pine selfs were found to be only 43% of the rate in outcrosses (Pawsey 1964).

Plus-tree 850.55 has been shown to be the best of all *P. radiata* selections in terms of growth rate. As a result, this tree and its progenies have been widely incorporated in breeding programs and their offspring extensively deployed in forest plantations. Nevertheless, plus-tree 850.55, like others of the species, exhibits significant inbreeding depression. Upon selfing, the number of filled seeds per cone was about half that of the outcrossing rate (Griffin and Lindgren 1985).

In this study, RAPD and microsatellite markers were used to study selfed progenies of *P. radiata* plus-tree 850.55. We report here on RAPD markers which appear to be associated with a lethal allele responsible for early seedling death in selfed progeny of plus-tree 850.55.

Materials and methods

Plant materials

Plus-tree 850.55 was selfed and 507 S_1 seeds were produced. Three hundred and seventy eight seeds were sown singly in containers and placed in a greenhouse. The remaining 129 seeds were saved for later use. Eighty seven of the sown seeds failed to germinate, leaving a total of 291 seeds that germinated successfully. Megagametophytes of the germinants were collected as soon as they were shed, and immediately stored in a $-20^{\circ}C$ freezer until DNA extraction. Seventy six of the 291 seedlings died within 1 month after germination. The megagametophytes of these 76 dead seedlings were used to investigate the genes responsible for their death. The remaining 215 seedlings survived for more than 1 year.

Molecular analysis

DNA was extracted from the haploid megagametophytes of the 129 un-sown seeds and the 291 seedlings that germinated. Megagametophytes from seeds that were sown but did not germinate were not available. DNA was isolated from needles of seedlings surviving past 1 month, but was not available from seedlings that died. DNA was extracted with a Fast Prep FP 120 machine (Savant) using a Bio 101 Kit H following the manufacturer's instructions.

RAPD PCR was performed as previously described (Richardson et al. 1995), using 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.4 μM primer (Operon or UBC primers), 15 ng genomic DNA, 0.8 unit of *Taq* DNA polymerase, 0.2 mM of each of dNTPs, and deionised water (up to 25 μl) with a 50-μl oil overlay. The PCR thermal cycle consisted of: one cycle of 94°C for 3 min/37°C for 1 min/72°C for 2 min, followed by 39 cycles of 94°C/37°C/72°C (1 min each), then one cycle at 72°C for 8 min. PCR was conducted in 96-well polycarbonate plates in a Techne PHC-3 thermal cycler. PCR products were separated by electrophoresis through 1.5% agarose gel in TBE buffer. Gels were stained in 0.25 μg/ml of ethidium bromide for 1 h, de-stained for 0.5 h, then photographed under UV light.

One hundred and twenty five RAPD primers were initially chosen based on previous work which showed segregating bands in plus-tree 850.55 (unpublished data). These primers were re-screened on DNA from needles and six megagametophytes of plus-tree 850.55 to confirm the presence of segregating markers. A total of 198 polymorphic markers were detected by 94 primers. Marker quality was classified as A, B or C, according to its reproducibility and band intensity. Four previously published microsatellite markers were also utilized (Smith and Devey 1994; Fisher et al. 1996), giving a total of 202 markers.

Genotyping and linkage analysis

The DNA of 198 megagametophytes from surviving seedlings was screened with all 202 markers. Because the mapping strategy using megagametophytes of conifers is identical to that for a backcross with the phase unknown, the data file was modified so that both coupling and repulsion phases could be identified (Nelson et al. 1993). A linkage map was constructed using the modified data file and Mapmaker with the Kosambi mapping function (Lander et al. 1987). Thresholds for LOD and recombination rate were 5.0 and 0.3 respectively.

Segregation distortion analysis

For each of the markers, segregation distortion in the megagametophytes of surviving seedlings, as measured by departure from the expected 1:1 ratio for presence (band allele) or absence (null allele) of a band, was tested by chi-square analysis. The 40 highest-quality markers, which included representatives from all regions identified as distorted in megagametophytes of surviving seedlings, were then used to test for segregation distortion in megagametophytes of the seedlings which died in the first month. Later, an additional five markers linked to a marker showing large distortion were examined to confirm the distortion. Segregation distortion favouring alternate alleles in the dead and surviving seedlings was taken as evidence of a linked gene affecting seedling survival.

Segregation distortion was also examined in the diploid surviving seedlings for 54 markers, representing all previously identified distorted regions. The RAPD diploid genotype was recorded as "+/?" if its megagametophyte produced a band; as "−/+" if its megagametophyte did not produce a band but the diploid did; and as "−/−" if neither the megagametophyte nor the diploid produced a band. Differences from the expected 2:1:1 ratio for the genotypes +/?, −/+, and −/− were tested by chi-square analysis. Segregation distortion in the diploids was used to determine the relative degree of selection against homozygotes and heterozygotes.

Finally, segregation distortion was tested in megagametophytes of un-sown seeds (65 markers) and in the combined population of megagametophytes from dead and surviving seedlings to determine if selection had operated prior to germination. To determine the

allele counts in the combined population, the band and null alleles from the megagametophytes of both dead and surviving seedlings were tallied. The genotypes of three megagametophytes from dead seedlings and 25 megagametophytes from surviving seedlings could not be scored. The frequencies of band and null alleles in the megagametophytes which could not be scored were assumed to be the same as observed in those which were scoreable for the dead and surviving classes. Altogether 139 megagametophytes had the band allele and 152 had the null allele.

Location of a putative lethal allele

Two methods were used to locate the position of a putative lethal gene on the linkage map. The first method used linkage analysis of megagametophyte genotypes from dead seedlings, assuming that all megagametophytes from the dead seedlings had the lethal gene. The second method used a maximum-likelihood estimate (MLE), derived from diploid genotypes of surviving seedlings (Cheng et al. 1996; Kuang, Richardson, Carson, Bongarten, in preparation).

Results

Linkage map

The data from 198 RAPDs and four microsatellites on 198 megagametophytes of surviving seedlings resulted in a linkage map with 19 linkage groups. This map is consistent with a previous map of plus-tree 850.55, which included 38 markers used in the present study (unpublished data). Of the 202 markers, 77 were significantly distorted from a 1 : 1 ratio ($P < 0.05$). The effects of distortion on linkage analysis were ignored because they are of minor consequence. (Lorieux et al. 1995; Kuang, Richardson, Carson, Wilcox, Bongarten, in preparation).

Segregation of markers and seedling death

Most of the 40 markers that were examined in megagametophytes of seedlings that died in the first month were either not distorted or else distorted in the same direction observed in the megagametophytes of surviving seedlings. Two markers were significantly distorted in the opposite direction in the dead and surviving seedlings. Distortion at the first marker, A11480B, was not great, and two closely linked markers were not distorted. Distortion at A11480B was therefore considered to be a statistical anomaly.

For the second marker, ai05800a, the difference between surviving and dead genotypes was very large, prompting further investigation (Tables 1, 2). For locus ai05800a, 71 of the 73 dead individuals genotyped had a null allele in their megagametophytes, whereas 121 out of 190 megagametophytes of the surviving seedlings had the band allele. This suggests that a gene with a deleterious allele is linked in coupling phase with the null allele of marker ai05800a.

Megagametophytes of dead seedlings were subsequently genotyped using five markers linked to ai05800a. The three markers most closely linked to ai05800a also showed significant segregation distortion, consistent with linkage to a deleterious allele (Fig. 1, Table 1). Markers f17115c and ad11120a were in the same linkage group as ai05800a, but so far away (more than 40 cM) from the lethal allele that they were not distorted.

In the surviving seedlings, a ratio of 2 : 1 : 1 for the genotypes $+/?$, $-/+$, and $-/-$ is expected if there is no segregation distortion. Genotypes at ai05800a and the closely linked U327600B of surviving seedlings departed significantly from the 2 : 1 : 1 ratio (Table 2). At ai05800a, only two individuals homozygous for the null allele survived. This is consistent with the hypothesis of a completely lethal (or nearly so) recessive allele not far from ai05800a.

Considering the live and dead seedlings together, no segregation distortion was detected for marker ai05800a ($x^2 = 0.58$, $P < 0.5$). This suggests that selection did

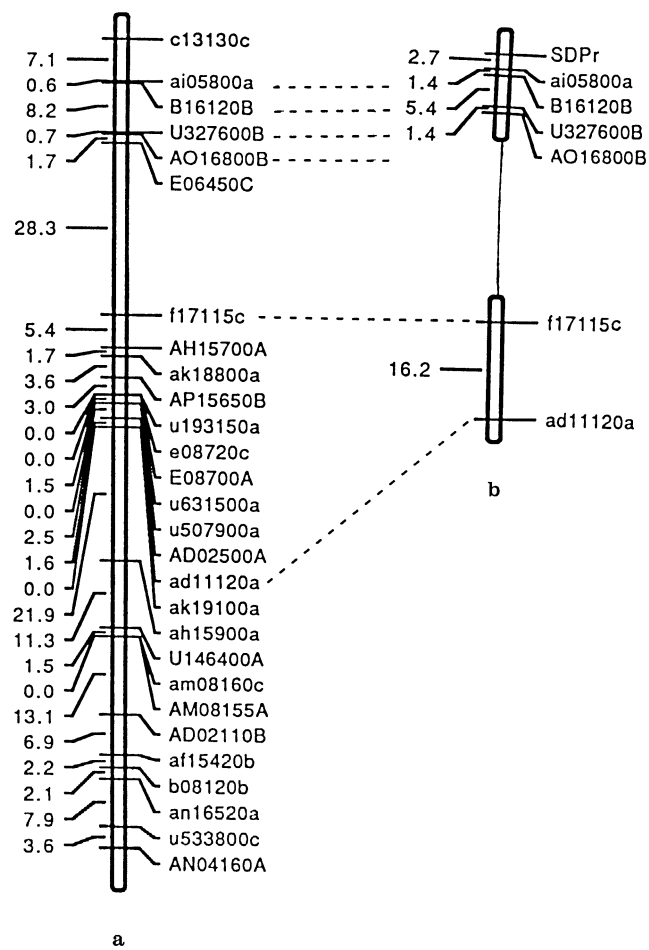

Fig. 1a, b Linkage group to which the lethal gene belongs: **a** constructed from megagametophytes of surviving seedlings; **b** constructed from megagametophytes of dead seedlings

not act against the putatively linked lethal allele prior to germination. In addition, allele frequencies in megagametophytes of the unsown seeds also indicates that selection against the putative lethal did not occur prior to germination. In the unsown seeds, 53 megagametophytes had the band allele of ai05800a, and 76 had the null allele. This represents a slight, but statistically significant, distortion ($x^2 = 4.1$, $P < 0.05$) in the opposite direction (i.e. favouring the allele associated with lethality after germination).

Location of the lethal allele

Assuming that all seedling death was caused by the lethal gene, and that the lethal allele is completely recessive, (i.e. all dead seedlings were homozygous for the lethal allele), the recombination rate and map distance between the linked molecular markers and the postulated lethal allele could be determined. Using data from the megagametophytes of the dead seedlings, the postulated lethal allele was located 2.7 cM from ai05800a on the side opposite to U327600B (Fig. 1 b). This is a maximum distance. If some seedling death was due to factors other than the lethal allele or the lethal allele was only partially recessive, the distance between ai05800a and the lethal allele would be less than 2.7 cM.

Using diploid genotypes of surviving seedlings, the MLE of the recombination rate between the lethal allele and marker ai05800a was calculated as 0.01, placing the lethal allele 1.0 cM from ai05800a, again on the side opposite to U327600B (Kuang, Richardson, Carson, Bongarten, in preparation). The viability of homozygotes for the lethal allele was estimated at 0.01 by the maximum-likelihood procedure (Kuang, Richardson, Carson, Bongarten, in preparation).

Table 1 Segregation of megagametophytes from dead seedlings

Marker[a]	Genotype[b]			Chi-square	P
	+	−	Unscored		
ai05800a	2	71	3	65.22	< 0.00001
B16120B	63	2	11	57.25	< 0.00001
U327600B	65	7	4	46.72	< 0.00001
AO16800B	63	8	5	42.61	< 0.00001
f17115c	29	38	9	1.21	
ad11110a	38	34	4	0.22	
Expected ratio	1	1	–		

[a] Nomenclature of marker name. When the RAPD marker is amplified by a UBC primer, its name begins with "U" and the first three digits indicate the series number of the UBC primer. When the marker is amplified by an Operon primer, its name begins with a letter other than "U" and the first letter(s) and the first two digits indicate the series number of the Operon primer. The last three digits in all RAPD marker names give the size of the PCR fragment if it is larger than 300, or 1/10 of the size of the PCR fragment if it is smaller than 300. The last letter in the marker name shows the marker quality, which was classified as A, B or C, according to its reproducibility and band intensity. The case of letters in the marker names shows their linkage phase

[b] "+" indicates band allele; "−" indicates null (no band) allele

Discussion

Our data suggest that *P. radiata* plus-tree 850.55 harbors a gene with a lethal allele that is closely linked with RAPD marker ai05800a. This is only the second simply inherited trait observed in radiata pine (the first gene, fastigiate, was published by Matheson in 1980). We propose to name this locus SDPr (seedling death in *P. radiata*). Four conclusions about the lethal allele at SDPr can be drawn from our results:

(1) The viability of SDPr in the homozygous recessive condition is near zero; only two surviving seedlings were homozygous-null for the linked marker, and recombination is the most likely explanation for their existence.
(2) The lethal allele appears to be completely recessive; there is no evidence of mortality in the heterozygotes.
(3) SDPr accounts for essentially all death of selfed progenies of plus-tree 850.55 in the first month after germination; only two dead seedlings had the marker associated with survival, and these are probably due to recombination.

Table 2 Segregation of markers in megagametophytes and surviving seedlings

Marker	Megagametophyte genotype[a]			Seedling genotype[b]			
	+	−	Chi-square (P)	+/?	−/+	−/−	Chi-square (P)
ai05800a	121	69	14.23 ($P < 0.0001$)	121	67	2	58.71 ($P < 0.00001$)
B16120B	58	106	14.05 ($P < 0.0001$)	–	–	–	
U327600B	70	126	16.00 ($P < 0.00001$)	70	61	65	17.16 ($P < 0.0001$)
AO16800B	60	105	12.27 ($P < 0.0001$)	–	–	–	
Expected ratio	1	1		2	1	1	

[a] For the meaning of genotype symbols, see Table 1

[b] For the meaning of genotype symbols, see text

(4) The wild-type allele of SDPr is not required in seed development or seed germination, but is indispensable in early seedling development. The germinant population as a whole showed no segregation distortion for the linked marker, and the slight distortion that was seen in the un-germinated seeds revealed excesses, not deficits, of the marker allele linked with the putative lethal allele. It is possible that the allele associated with survival after germination is selected against prior to germination, but it is more likely that sampling error accounts for the observed distortion since none was found in the germinant population.

Purging SDPr from the progenies of plus-tree 850.55 by simple recurrent selfing is a difficult task because SDPr appears to be recessive. Because of the dominant nature of RAPDs, one would have to use a linked RAPD marker in which the null allele was coupled with the wild-type allele to select for homozygotes of the wild-type allele. B16120B is the most closely linked marker having this property. By selecting for S_1 progenies that show no band for marker B16120B, the frequency of the lethal allele would be less than 5%. Recombination accounts for the failure to purge all lethals. Conversion of RAPD marker ai05800a to a co-dominant marker would make the selection more efficient in this case. Unfortunately, this type of selection can be used for only one generation because the marker will be fixed whereas the lethal allele will not. If a more closely linked marker were used, the recombination rate would be lower, but it would have to be very close to completely purge the lethal allele.

Another approach, backward selection without MAS, could purge the lethal allele completely in two selfed generations. S_1 individuals would be homozygous for the wild-type allele of SDPr if its S_2 progenies had no dead seedlings. However, this method requires producing S_2 progeny from all of the surviving S_1s, two-thirds of which will be heterozygotes. Marker B16120B could be used to identify most of the S_1 heterozygotes so that production of S_2s would be required for a much smaller number of S_1s.

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