A Chromosomal Region Promoting Outcrossing in a Conifer

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ABSTRACT

Prefertilization mechanisms influencing selfing rates are thought to be absent in conifers. Outcrossing in conifers is promoted via an embryo-lethal system, but the genetic mechanism is poorly understood. This study is the first experimental profile of the genetic mechanism promoting outcrossing in conifers. Molecular dissection of a *Pinus taeda* L. selfed pedigree detected a chromosomal region identified as *PtTX3020-RPtest9*. Within this region, a semilethal factor was tightly linked ($r = 0.0076$) to a polymorphic expressed sequence tag (EST). The linkage group flanking the lethal factor showed strong heterozygote advantage. Using genotypic frequencies for the linkage group, three hypotheses about the semilethal factor could be tested: (1) the presence of a balanced lethal system, *i.e.*, a lethal factor present in each of the two marker intervals; (2) gametic selection operative prior to fertilization; and (3) a stage-specific lethal factor. Selection acted via the embryo-lethal system. No support for a genetic mechanism operating prior to fertilization was found. The semilethal factor exerted no effect after embryo maturity. The genetic mechanism promoting outcrossing in *P. taeda* L. appears to have a balancing selection system due to either pseudo-overdominance or true overdominance.

I T is well established that selfing is typically low in conifers but the genetic mechanism promoting outcrossing is poorly understood. Prefertilization mechanisms such as the self-incompatibility (SI) systems in flowering plants are considered absent in conifers (Hagman 1975) although definitive experimental results have not been reported. Koski (1971) observed in his pioneering work that embryo death occurred immediately after fertilization in the primary proembryo stage and that "the embryonic lethals of *Pinus sylvestris* eliminate 95 per cent of the self-fertilized zygotes." The embryo-lethal system is hypothesized as the selective mechanism promoting outcrossing in conifers (Bramlett and Popham 1971; Koski 1971). To date, the embryo-lethal system in conifers has been studied using biometrical genetics because of its importance to evolution, conservation biology, and plant breeding. In this respect, conifers are a model because they have the highest embryo genetic loads reported for plants (see review in Williams and Savolainen 1996). Embryo-lethal equivalent values tend to range from 8 to 10 for many conifer species but it is unclear whether these phenotypic values represent lethal alleles or mildly deleterious defects at independent viability loci.

Embryo genetic loads and gametic selection: Inbreeding effects should be greatest in early development (Haldane 1957). This prediction is supported by a meta-analysis of inbreeding studies in plants; defective alleles in viability loci expressed early in seed development are often fully lethal (Husband and Schemske 1996). This prediction is difficult to test because prefertilization events such as gametic selection can upwardly bias the magnitude of inbreeding effects by mimicking zygotic selection (Husband and Schemske 1996; Vogl and Xu 2000). Estimating dominance for viability loci requires the removal of gametic selection bias. For example, opposing gametic selection occurs when one allele is selected in the maternal gamete and the alternate allele is selected in the paternal gamete. Opposing gametic selection prior to fertilization results in an excess of heterozygotes without causing postzygotic mortality. Selection acts on haplotypes prior to fertilization so that homozygotes are not created. In conifers, pre- and postfertilization events can be separated by haplotyping haploid megagametophyte tissue.

High embryo genetic loads in conifers can be maintained by (1) mutation-selection balance accompanied by high mutation rates and partial dominance or (2) overdominance (Namkoong and Bishir 1987). The partial dominance hypothesis attributes inbreeding depression to increased homozygosity of alleles that are both deleterious and at least partially recessive. The overdominance hypothesis is based on a higher fitness of a heterozygote over either homozygote. Inbreeding depression arises from a loss of heterozygosity. The level of dominance, h, measures the degree to which the mutant allele is expressed in the heterozygote. Negative values of h represent overdominance.

Few cases of true overdominance have been reported for viability loci in plants (Mitchell-Olds 1995; Kärk-

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KÄINEN *et al.* 1999) but these loci can exert a profound effect. A few overdominant loci, constituting the balanced or heterotic load (WRIGHT 1977, pp. 479–480; CROW 1993), can dominate the dynamics of a mating system. Inbreeding depression estimates can exceed 50%, given a selfing rate of 10%, 10 viability loci, and a wide range of selection coefficients $0.27 \leq s \leq 0.91$ (ZIEHE and ROBERDS 1989). True overdominant loci are difficult to separate from those exhibiting pseudo-overdominance, a case where heterozygote excess is caused by two loci with deleterious recessive alleles linked in repulsion phase (STUBER *et al.* 1992).

Molecular dissection of embryo-lethal system: The relative importance of overdominance and dominance can be addressed in principle using molecular dissection (*e.g.*, HEDRICK and MUONA 1990; FU and RITLAND 1994). The mapping of the embryo-lethal system is based on a binomial analysis of marker genotype frequencies rather than quantitative trait means. Marker loci tightly linked to loci undergoing viability selection are expected to exhibit transmission ratio distortion (TRD) with certain marker genotypes underrepresented relative to Mendelian segregation ratios of 1:2:1. Unlike segregation distortion, TRD represents a statistically significant departure from expected Mendelian inheritance regardless of the genetic mechanism (CROW 1991; Montagutelli *et al.* 1996). Using interval mapping to dissect genetic load, KUANG *et al.* (1999) and REMINGTON and O'MALLEY (2000) showed a prevalence of semilethal rather than lethal factors in two conifers. These studies, based on amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) markers, support dominance as the prevalent model of gene action. Two drawbacks to using dominant RAPD and AFLP marker systems to study outcrossing mechanisms are (1) the difficulty in testing for gametic selection and (2) the weak correspondence between genetic and physical maps in large plant genomes. Whatever molecular marker system is used, moving from a lethal factor within a marker interval to positional cloning can be problematic in *P. taeda* because it has a large genome (20,000 Mb per haploid nucleus) composed of ∼86% highly repetitive DNA (ELSIK and WILLIAMS 2000).

Reproductive biology of conifers: In conifers, meiosis in the megaspore mother cell yields four meiotic products, one of which undergoes mitosis and becomes a multicellular haploid megagametophyte. The haploid megagametophyte, in turn, gives rise to one or more egg cells, each within its own archegonium (GIFFORD and FOSTER 1989, pp. 432–443). The megagametophyte can thus be used to deduce the egg cell's haplotype. There are an average of 2.3 fertilized archegonia in a *P. radiata* megagametophyte (LILL 1974) although archegonia numbers are highly variable within a single tree. Both simple and cleavage polyembrony occur in pines (SORENSEN 1982) but polyembrony does not change

the proportion of genotypes at the marker locus (HEDRICK and MUONA 1990).

There is a developmental basis for prefertilization (gametic) selection in conifers. In *P. taeda*, pollination and fertilization are separated by 12 months (GIFFORD and FOSTER 1989, pp. 432–443). The pollen tube slowly grows through the nucellus prior to egg maturity. Ovular secretions initiate pollen tube development about 1 week before fertilization (TAKASO *et al.* 1996). The prefertilization fluid stimulates pollen tube growth and even distorts pollen tube morphology, which may be related to prezygotic selection (TAKASO *et al.* 1996).

Most deaths from selfed matings occur soon after fertilization. Deaths are observed at the start of embryo development but not at the earlier zygotic stages (KOSKI 1971). This opens the question of whether self-fertilization prompts death at two stages: (1) death of the egg cell prior to fertilization as a consequence of pollen-egg signaling and (2) death of early embryos. At embryo maturity, X-ray analysis is used to distinguish filled seeds from dead, empty seeds.

The purpose of this study is to develop an experimental system for testing hypotheses about the genetic mechanisms underlying the *P. taeda* embryo-lethal system. Using a chromosomal region with a putative lethal factor adjacent to an expressed gene, we were able to test for (1) the presence of a balanced lethal system, *i.e.*, where a lethal factor is present in each of the two marker intervals; (2) gametic selection prior to fertilization; and (3) stage-specific lethal expression.

MATERIALS AND METHODS

Genetic load estimation and branch replicate design: Genetic load was estimated from 1805 selfed seeds and 359 outcrossed seeds from *P. taeda* parent 7-1037. Five to seven self-pollinations of one to six cones per pollination bag were made in each of three separate parts of the crown. Three branches each served as a replicate for estimating embryo genetic load. Three outcross pollinations with one to three cones per bag on a fourth branch used harvested wind-borne pollen. Developing strobili were caged until harvest to prevent insect predation. Lethal equivalents were estimated using the combinatorial method (BRAMLETT and POPHAM 1971; KOSKI 1971), which is based on proportions of empty seeds from self-pollinations (Es) and biparental outcrosses (Ec) from the same parent with polyembryony, assuming independent gene action (BISHIR and NAMKOONG 1987). The combinatorial model assumes that a seed produced by self-fertilization of a parent carrying lethal factors is empty due to homozygous recessive lethal alleles at a locus after adjustment for extraneous mortality (SAVOLAINEN *et al.* 1992).

Experimental design for marker analysis: A random sample of polymorphic triplet-repeat microsatellites was assayed for a molecular dissection study. As part of this study, three cohorts of selfed seeds from *P. taeda* parent 7-1037 were assayed for three linked microsatellite markers, *PtTX3020*, *PtTX2082*, and *RPtest9* (Table 1). First, embryos from 210 filled seeds were destructively sampled for molecular dissection. Second, haploid megagametophytes and embryos from 157 selfed seeds were destructively sampled to test for opposing gametic

Marker	GenBank accession no.	Primer	Repeat motif
PtTX2082	AF277841	F: 5′-AAATGTTTAATAYGAAGTTGAG-3′	$(GT)_{14}(GAGT)_7(GA)_{13}$
		R: 5′-GATGGATCTATGTTGGTTT-3′	
PtTX3020	AF143969	F: 5′-GTCGGGGAAGTGAAAGTA-3′	$A_{16}(CAA)_9$
		R: 5′-CTAGGTGCAAGAAAAGAGTAT-3′	
RPtest9	AA556811	F: 5′-CCAGACAACCCAAATGAAGG-3′	$A_5\ldots(TG)_4\ldots(GAG)_5\ldots$
		R: 5′-GCCTGCTATCGAATCCAGAA-3′	$(CAG)_8CGG(CAG)_7CGG(CAG)_4$

selection. Marker *RPtest9* was assayed in megagametophytes of heterozygous embryos to deduce gametic contributions. Gametic selection prior to fertilization was tested by comparing ratios of the two heterozygote classes C_1C_2 and C_2C_1. In the third cohort, a total of 145 seeds were cold stratified for 30 days and then checked for germination on filter paper in a petri dish. Of the 145 filled seeds, only 129 (89%) germinated. Sixteen were filled seeds that did not germinate and 6 were dead germinants at the cotyledon stage. Markers could be assayed for all surviving seedlings, 13 of the 16 dead filled seeds, and 3 of the 6 dead germinants. Mortality was scored at stage 1 (germination), stage 2 (cotyledons emerging from seeds), and stage 3 (seedlings with emerging primary needles). Genomic DNA was extracted from embryo tissue, needles, and megagametophytes using a modified Doyle and Doyle (1987) protocol.

Microsatellite and expressed sequence tag markers: Enriched-copy microsatellite libraries were the source of all markers including microsatellite *PtTX2146*, which was the same sequence as *RPtest9*, an expressed sequence tag (EST) with polymorphic repeat motifs (Elsik *et al.* 2000; Table 1).

During marker development, Mendelian inheritance was tested on all microsatellites using parents and grandparents in an outbred pedigree (Elsik *et al.* 2000). No transmission ratio distortion was observed in a sample of 118 outbred offspring. In the standard PAGE gel, PCR products were separated on 6% polyacrylamide denaturing gels. Gels were run at a constant temperature of 55° for 1.5–3.5 hr, followed by silver staining (Echt *et al.* 1996).

Linkage mapping: Microsatellites were in fully informative intercross mating-type configuration so the selfed offspring data were analyzed using the F_2 intercross option in MAPMAKER version 3.0 (Lander *et al.* 1987). Markers were assigned to linkage groups from a two-point analysis on the basis of LOD ≥ 3.0 and $r < 0.4$ as thresholds. Markers in each linkage group were ordered using the "order" command and tested in three-point analysis using command "ripple" (LOD threshold of 3.0). Deviation from the expected 1:2:1 ratios was tested with a χ^2 analysis for each marker locus. The Bonferroni experimentwise error rate (α') was used to adjust the significance threshold upwardly for each individual χ^2 test for distorted ratios ($\alpha = 0.005$; $\alpha' = 0.113$).

Mapping a lethal factor: Genotypic frequencies for each set of flanking markers were combined with a maximum likelihood approach, the expectation/conditional maximization (ECM) algorithm, to infer the following: lethal factor position, degree of dominance, and selection coefficient(s) for lethal factors (Cheng *et al.* 1996; Kuang *et al.* 1999). The ECM algorithm is an extension of the EM algorithm that decomposes the M step into a series of conditional M steps. In the overdominant case, one cycle of iteration of ECM consisted of one E step to estimate a_{ij} and two CM steps to estimate r_1, r_2, s_1, and s_2 (Meng and Rubin 1993; Cheng *et al.* 1996; Kuang *et al.* 1999). When the differences between the value of an estimate in the previous CM step and the current one was <0.001, then the iteration was stopped and the resulting estimates of recombination fractions (r_1, r_2) and selection coefficients (s_1, s_2) were reported for the overdominant case. In the case of dominance, degree of dominance (h) and a selection coefficient were estimated. A LOD significance threshold of 2.5 was suited to a single pedigree at 10-cM spacing (Lander and Botstein 1989).

Consider two linked markers A and B in coupling (AB/ab) linked to a putative lethal locus in locus order A-L-B. Recombination distances between A-L and A-B were defined as r_1 and r_2. Differential zygotic selection at the lethal locus is expressed as relative fitness of 1.0 for Ll, $1 - s_1$ for LL, and $1 - s_2$ for ll for the overdominant-type selection model. Observed marker genotypes are defined as a_i, where $i = 1, 2, \ldots, 9$. The LL, Ll, and ll genotypes must be inferred from these nine genotypic (a_i) classes for flanking marker genotypes. The direct counts of the observed nine marker genotype classes (a_i) can be used to obtain the expected counts. Expected counts are defined by a_{ij}, where $i = 1, 2, \ldots, 9$ and $j = 1, 2, 3$ for each of the 27 A-L-B genotypes (Cheng *et al.* 1996; Kuang *et al.* 1999). Expected counts are calculated from the selection model (G_j, where $j = 1, 2, 3$) and the 27 expected genotypic frequencies (F_{ij}) for a given locus order (Cheng *et al.* 1996). In our study, the G matrix was represented as $g_1 = 4/(4 - 2hs - s)$ for LL genotypes; $g_2 = 4(1 - hs)/4(4 - 2hs - s)$ for Ll genotypes, and $g_3 = 4(1 - s)/(4 - 2hs - s)$ for ll genotypes. Detailed ECM procedure for estimating recombination fractions and selection coefficients for the overdominant case are given in Kuang *et al.* (1999). Log-likelihood models for overdominant gene action and zygotic selection were compared for two gene orders (A-L-B *vs.* A-B-L). In all cases, the locus-order model with the lowest χ^2 value was considered the best fit. Variances of the final estimates were determined using Fisher's information matrix (Bailey 1961; Cheng *et al.* 1996).

RESULTS

Genetic load estimation: Upon selfing, parent 7-1037 averaged 19.8% filled seeds per cone compared to 82.7% filled seeds per cone for outcrossing. Embryo genetic load was 7.3 lethal equivalents per zygote for *P. taeda* parent 7-1037 assuming two archegonia. Genetic load adjustment for extraneous mortality bias was slight. Genetic load estimates varied slightly by branch replicate (Table 2).

A chromosomal region for a lethal factor: Microsatel-

TABLE 2

Estimation of embryo genetic load using the branch-replicate design

Branch ID	No. of seeds	Filled seeds (%)	ADJ-COMB $k = 1$	ADJ-COMB $k = 2$	ADJ-COMB $k = 3$
159	538	22.12	4.3	6.2	7.7
163	549	16.21	5.9	7.8	8.9
169	718	19.64	5.0	6.9	8.0
Total	1805	19.76	5.0	6.9	8.0

The combinatorial estimates of genetic load were estimated given variable archegonial number (k) within a strobilus on a single tree and adjusted for extraneous mortality (ADJ-COMB). Outcross had a mean 82.7% filled seed per cone.

lites *PtTX3020-PtTX2082-RPtest9* (denoted as *A-B-C* in locus-order models) were linked within a 25.9-cM interval (Table 3). The linkage group showed one or more lethal factors with a strong semilethal effect (Table 4). One marker in the linkage group, *RPtest9*, was part of a coding region or EST (GenBank accession no. AA556811). Direct counts showed that homozygotes for *RPtest9* alleles 1 and 2 had 25.7 and 37.14% lower viability, respectively, than heterozygotes. Three hypotheses could be tested to account for these findings: (1) a lethal factor is present in each of the two marker intervals within the linkage group; (2) lower viability is due in part to gametic selection; and (3) the lethal factor is stage specific.

Balanced lethal factors *vs.* a single lethal factor: Locus-order models were tested using maximum-likelihood methods to infer the position of lethal factors. The best fit for locus order depended upon estimates of recombination fraction, degree of dominance, and selection coefficients for viability loci. The first set of locus-order models *B-L-C* and *B-C-L* assumed dominance, but the estimates of *h* were negative and thus an overdominant model was more appropriate. For example, the locus-order model *B-C-L* had a degree of dominance (*h*) value of −0.455 and its χ^2 value was 53.48. The second locus-order *B-L-C* model for dominance was better supported with an estimate of $h = −0.485$ and its χ^2 value was reduced to 2.02.

All remaining locus-order models were based on overdominance. The first hypothesis was tested for balanced lethal factors in repulsion (pseudo-overdominance).

TABLE 3

Recombination fraction (r) and map units (Haldane centimorgans) for two linkage groups in a selfed *P. taeda* parent

Locus 1	Locus 2	Embryo r	Embryo cM (H)	Germinant cM (H)
PtTX3020	*PtTX2082*	0.137	16.0	17.0
PtTX2082	*RPtest9*	0.090	9.9	15.2

Balanced lethal factors can be detected with two-point locus order models by searching for one lethal factor in each marker interval. The combined locus order for the balanced lethal hypothesis would be $A-L_1-B-L_2-C$ and thus $A-L_1-B$ and $B-L_2-C$ locus-order models would be the best fit. The alternative single-factor model was *A-B-L-C*. The single-factor model was supported by two-point analyses; no lethal factor was detected in the *A-B* interval and a lethal factor was found in the *B-C* marker interval. If the lethal factor is in or close to the EST coding region, then the recombination fraction between the lethal factor and the *RPtest9* marker should approach zero.

The single-factor hypothesis was accepted because locus-order models *A-B-L* and *B-L-C* had the best fit: locus-order model *A-B-L* had a χ^2 value of 2.1 compared to the higher χ^2 value of 26.0 for locus-order model *A-L-B*. For the *A-B-L* model, the selection coefficients for the lethal factor were asymmetric and similar to selection coefficients for the lethal factor estimated from locus-order *B-L-C* (Table 5).

Locus-order *B-L-C* also had the best fit, supporting a single lethal factor in the marker interval *PtTX2082-RPtest9*. The LOD value exceeded 3.0, supporting the presence of a viability locus in the *PtTX2082-RPtest9* interval (Table 5). This lethal factor was tightly linked to *RPtest9* ($r_2 = 0.0076$) and its effects were semilethal and asymmetric ($s_1 = 0.335$, $s_2 = 0.665$; Table 5). The hypothesis of balanced lethal factors located in two different marker intervals was rejected in favor of the single-factor hypothesis.

Opposing gametic selection prior to fertilization: Opposing gametic selection was tested as a contributor to the observed heterozygote advantage. The maternal and paternal allelic contribution to each embryo was determined by haplotyping the megagametophyte for each heterozygous embryo. Genotypic ratios for *RPtest9* were as follows: C_1C_1 18 : C_1C_2 55 : C_2C_2 70 : C_2C_2 14. The two heterozygote frequencies $C_1C_2 : C_2C_1$ were not statistically different, and thus no gametic or prefertilization selection was detected.

The haplotyping data also validated the presence of a

TABLE 4

Genotypic ratios and transmission ratio distortion at embryo maturity during germination (stage 1) and seedling development (stages 2–3)

Marker	Embryo maturity	χ^2	Stage 1	χ^2	Stages 2–3	χ^2
PtTX3020	41:126:31	20.56**	19:84:35	10.23**	32:65:15	8.05*
PtTX2082	45:123:25	18.70**	21:75:42	7.44*	28:66:17	6.15*
RPtest9	41:126:31	20.56**	23:71:40	4.79	34:58:18	4.98

* Suggestive significance level ($\alpha = 0.05$). ** Threshold significance ($\alpha = 0.005$).

viability locus near marker *RPtest9*. Pronounced marker genotype distortion occurred at embryo maturity between homozygote and heterozygote classes in this second cohort of selfed 7-1037 offspring; the distorted ratio was 18:125:14.

Stage-specific selection: Stage-specific selection was tested at germination and seedling development. If the *RPtest9* lethal factor exerts stage-specific selection, then the pattern of marker distortion present at embryo maturity should shift or even dissipate by seedling emergence. The lethal factor linked to *RPtest9* exerted an overdominant effect at embryo maturity, which was later absent at germination and seedling development (Table 5).

Most notably, one of the two selection coefficients (s_1) decreased at germination, increasing the asymmetry of overdominant selection (Table 5). This was detected as a change in the genotypic ratios (Table 4) and as a shift in the direction of selection after embryo maturity (Table 5). This change was not statistically detectable given the smaller sample size of the germinants. It is notable that the detection of the lethal factor linked to marker *RPtest9* coincided with the separation of the embryo from its megagametophyte.

Mortality after embryo maturity was low compared to mortality during embryo development (15.2% *vs.* 80.2%). Two of the three linked markers, *PtTX3020* and *PtTX2082*, exerted a clear phenotypic effect at germination and seedling development (Table 4). Marker *PtTX2082* had the most mortality with its homozygote B_1B_1 marker genotypes showing 9 out of 12 deaths at germination. Other marker genotypes among the dead seedlings were randomly distributed across all stages.

DISCUSSION

Genetic load for parent 7-1037 was representative for *P. taeda* and other conifer species. A single semilethal factor was tightly linked to a polymorphic EST marker, providing an experimental system for testing the genetic mechanism that promotes outcrossing in *P. taeda*.

A stage-dependent transition from overdominance to dominance: Our results suggest that overdominance may prevail during early embryo development, possibly giving way to dominance as offspring assume the adult growth form and onset of reproduction. If so, a stage-dependent transition from overdominance to dominance may explain previous studies. The previous studies were conducted on young trees at 1–2 years of age (KUANG *et al.* 1999; REMINGTON and O'MALLEY 2000). At this late stage of seedling development, these studies would be expected to show dominance, rather than overdominance, as the prevalent genetic model. This explanation must be tested by using more markers to scan the genome at embryo maturity to check the prevalence of overdominance *vs.* dominance. Another explanation is that this overdominant chromosomal region is not representative of the entire embryo-lethal system and thus is the same region as the overdominant viability factor reported in the two previous genome scans using large numbers of RAPD and AFLP markers.

Each of these studies was conducted on a single selfed family. Additional heterozygous parents are needed to discern between global and pairwise heterosis, to determine allelic interactions for a range of multiple alleles, and to determine the importance of epistatic interactions for embryo viability.

TABLE 5

Maximum likelihood estimates for r_1, r_2, and s_1, s_2 from the expectation-conditional maximization method for the stages of embryo maturity and germination

Stage	Best locus order	Model	r_1 ($\pm$SE)	r_2 ($\pm$SE)	s_1 ($\pm$SE)	s_2 ($\pm$SE)	LOD
Embryo	*A-B-L*	OV	0.0968 (0.0860)	0.0151 (0.0251)	0.3050 (0.8077)	0.6730 (0.4007)	4.1
	B-L-C	OV	0.0822 (0.1044)	0.0076 (0.0359)	0.3350 (0.7720)	0.6650 (0.4055)	3.1
Germinant	*A-B-L*	OV	0.0142 (0.0910)	0.0153 (0.0279)	0.0050 (1.1651)	0.5520 (0.5898)	1.1
	B-L-C	OV	0.0860 (0.0925)	0.0062 (0.0343)	0.0185 (1.1568)	0.4775 (0.6759)	0.7

L, putative lethal factor; *A*, *PtTX3020*; *B*, *PtTX2082*; *C*, *RPtest9*; OV, overdominance.

The detection of the lethal factor linked to marker *RPtest9* coincided with the separation of the embryo from its megagametophyte. This, coupled with the importance of overdominance during embryo maturation, raises the possibility that there is an early self-recognition mechanism for conifers operating in addition to the embryo-lethal system. The mechanism may be an interaction between the megagametophyte and its developing embryo, operative soon after fertilization. If so, the early self-recognition system would cease to exert selection pressure once the embryo is separated from its megagametophyte, if not earlier.

Evolutionary implications for outcrossing in conifers: This is the first experimental system for a genetic mechanism promoting outcrossing in conifers. Our results supported overdominant selection on viability loci and were consistent with the embryo-lethal system as proposed by Koski (1971). There was no evidence for selection prior to fertilization. Pseudo-overdominance and true overdominance could not be distinguished because selection coefficients were asymmetric. To test for pseudo-overdominance, an intervening marker locus is needed within the *PtTX2082-RPtest9* interval to separately detect each lethal factor. Otherwise, a balanced lethal system and pseudo-overdominance cannot be ruled out.

Two models can account for these results. The first is a model based on directional selection coupled with antagonistic pleiotropy. If so, the two homozygotes at a viability locus would be under selection in opposing directions at different points in the life cycle or for different traits. One of two opposing selective forces would dissipate early, leaving unidirectional selection at later stages (Charlesworth and Charlesworth 1999). This model seems unlikely for the factor tightly linked to *RPtest9* because the deleterious effect should have been detected for both homozygote classes at embryo maturity and then shifted selection against only one of its homozygotes at later stages. Instead, there was no detectable selection against marker *RPtest9* after embryo maturity.

The second and more likely model is that multiple mutations exist within the same "superallele" at a viability locus or even a co-adapted viability complex. A viability complex is indicated because a balanced lethal system could be operative *within* the *B-C* marker interval (pseudo-overdominance) rather than a single lethal factor. If so, the true locus order might actually be $A-B-L_1-L_n-C$, where n is the number of lethal factors altering the viability of the phenotype. In addition, there may be a loosely linked viability locus outside the linkage group that would account for the extreme TRD throughout the entire 25-cM linkage group. Additional flanking markers will be needed to extend the search along the chromosome.

There is precedent for the superallele model. Multiple defects within the same allele for a viability locus or a co-adapted viability complex have been reported for

Drosophila. Noncoding and coding regions within a candidate gene region can interact to form superalleles (Stam and Laurie 1996; Long *et al.* 1998). A superallele undergoes multiple changes within a locus or small chromosomal region, forming an aggregate of deleterious mutations that affect transcription rates. Epistatic control within a superallele sequence can account for the transient effects of the *RPtest9* factor.

If the chromosomal region has true overdominance, then its viability locus or loci should have long persistence times on an evolutionary time scale and its balanced polymorphism system may be more ancient than speciation events. Persistent overdominant loci would maintain outcrossing even with sharp population size contractions, thus explaining the high genetic load for pines observed with small population sizes or even founder events (Williams *et al.* 1999). If so, purging conifer populations will be an ineffective way to reduce the embryo genetic load. Such a balancing selection system for outcrossing in pines would present a clear parallel to the overdominant systems that have been well characterized for SI genes in flowering plants and major histocompatibility genes in animals.

LITERATURE CITED

Bailey, N.T.J., 1961 *Mathematical Theory of Genetic Linkage*. Oxford University Press, London.

Bishir, J., and G. Namkoong, 1987 Unsound seed in conifers: estimation of numbers of lethal alleles and magnitude of effect associated with the maternal parent. Silvae Genet. **36:** 180–185.

Bramlett, D. L., and T. W. Popham, 1971 Model relating unsound seed and embryonic lethals in self-pollinated pines. Silvae Genet. **20:** 192–193.

Charlesworth, B., and D. Charlesworth, 1999 The genetic basis of inbreeding depression. Genet. Res. **74:** 329–340.

Cheng, R., A. Saito, Y. Takano and Y. Ukai, 1996 Estimation of the position and effect of a lethal factor locus on a molecular marker linkage map. Theor. Appl. Genet. **93:** 494–502.

Crow, J. F., 1991 Why is Mendelian segregation so exact? Bioessays **13:** 305–312.

Crow, J. F., 1993 Mutation, mean fitness and genetic load, pp. 3–42 in *Oxford Surveys in Evolutionary Biology*, Vol. 9, edited by D. Futuyma and J. Antonovics. Oxford University Press, Oxford.

Doyle, J. J., and J. L. Doyle, 1987 A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19:** 11–15.

Echt, C. S., P. May-Marquardt, M. Hseih and R. Zahorchak, 1996 Characterization of microsatellite markers in eastern white pine. Genome **3:** 1102–1108.

Elsik, C. G., and C. G. Williams, 2000 Retroelements contribute to excess low-copy number DNA in pine. Mol. Gen. Genet. **264:** 47–55.

Elsik, C. G., V. T. Minihan, S. E. Hall, A. M. Scarpa and C. G. Williams, 2000 Low-copy microsatellite markers for *Pinus taeda* L. Genome **43:** 550–555.

Fu, Y-B., and K. Ritland, 1994 Evidence for the partial dominance of viability of viability genes contributing to inbreeding depression in *Mimulus guttatus*. Genetics **136:** 323–331.

GIFFORD, E. M., and A. S. FOSTER, 1989 *Morphology and Evolution of Vascular Plants*. W. H. Freeman, New York.

HAGMAN, M., 1975 Incompatibility in forest trees. Proc. R. Soc. Lond. Ser. B **188:** 313–326.

HALDANE, J. B. S., 1957 The cost of natural selection. J. Genet. **55:** 511–524.

HEDRICK, P. W., and O. MUONA, 1990 Linkage of viability genes to marker loci in selfing organisms. Heredity **64:** 67–72.

HUSBAND, B. C., and D. W. SCHEMSKE, 1996 Evolution and timing of inbreeding depression in plants. Evolution **50:** 54–70.

KÄRKKÄINEN, K., H. KUITTINEN, R. VAN TREUREN, C. VOGL, S. OIKARINEN *et al.*, 1999 Genetic basis of inbreeding depression in *Arabis petrea*. Evolution **53**(5): 1354–1365.

KOSKI, V., 1971 Embryonic lethals of *Picea abies* and *Pinus sylvestris*. Commun. Instit. Forestalia Fennica. **75:** 1–30.

KUANG, H., T. E. RICHARDSON, S. D. CARSON and B. C. BONGARTEN, 1999 Genetic analysis of inbreeding depression in plus tree 850.55 of *Pinus radiata* D. Don. II. Genetics of viability genes. Theor. Appl. Genet. **99:** 140–146.

LANDER, E. S., and D. BOTSTEIN, 1989 Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics **121:** 185–199.

LANDER, E. S., P. GREEN, J. ABRAHAMSON, A. BARLOW, M. DALY *et al.*, 1987 MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1:** 174–181.

LILL, B. S., 1974 Development of the female cone of *Pinus radiata* D. Don. Ph.D. Thesis, University of Canterbury, New Zealand.

LONG, A. D., R. F. LYMAN, C. H. LANGLEY and T. F. C. MACKAY, 1998 Two sites in the *Delta* gene region contribute to naturally occurring variation in bristle number in *Drosophila melanogaster*. Genetics **149:** 999–1017.

MENG, X-L., and D. B. RUBIN, 1993 Maximum likelihood estimation via the ECM algorithm: a general framework. Biometrika **80:** 267–278.

MITCHELL-OLDS, T., 1995 Interval mapping of viability loci causing heterosis in *Arabidopsis*. Genetics **140:** 1105–1109.

MONTAGUTELLI, X., R. TURNER and J. H. NADEAU, 1996 Epistatic control of non-Mendelian inheritance in mouse interspecific crosses. Genetics **143:** 1739–1752.

NAMKOONG, G., and J. BISHIR, 1987 Frequency of lethal alleles in forest tree populations. Evolution **41:** 1123–1127.

REMINGTON, D., and D. M. O'MALLEY, 2000 Whole-genome characterization of embryonic stage inbreeding depression in a selfed loblolly pine family. Genetics **155:** 337–348.

SAVOLAINEN, O., K. KÄRKKÄINEN and H. KUITTINEN, 1992 Estimating numbers of embryonic lethals in conifers. Heredity **69:** 308–314.

SORENSEN, F., 1982 The roles of polyembrony and embryo viability in the genetic system of conifers. Evolution **36:** 725–733.

STAM, L. F., and C. C. LAURIE, 1996 Molecular dissection of a major effect on a quantitative trait: the level of alcohol dehydrogenase expression in *Drosophila melanogaster*. Genetics **144:** 1559–1564.

STUBER, C. W., S. E. LINCOLN, D. W. WOLFF, T. HELENTJARIS and E. S. LANDER, 1992 Identification of genetic factors contributing to heterosis in a hybrid from two elite maize inbred lines using molecular markers. Genetics **132:** 823–839.

TAKASO, T., P. VON ADERKAS and J. N. OWENS, 1996 Prefertilization events in ovules of *Pseudotsuga*: ovular secretion and its influence on pollen tubes. Can. J. Bot. **74:** 1214–1219.

VOGL, C., and S. XU, 2000 Multiple point mapping of viability and segregation distorting loci using molecular markers. Genetics **155:** 1439–1447.

WILLIAMS, C. G., and O. SAVOLAINEN, 1996 Inbreeding depression in conifers: implications for breeding strategy. Forest Sci. **42:** 102–117.

WILLIAMS, C. G., R. B. BARNES and I. NYOKA, 1999 Embryonic lethal load for a neotropical conifer, *Pinus patula* Schiede and Deppe. J. Hered. **90:** 394–398.

WRIGHT, S., 1977 *Evolution and the Genetics of Populations: Experimental Results and Evolutionary Deductions*, Vol. 3. University of Chicago Press, Chicago.

ZIEHE, M., and J. H. ROBERDS, 1989 Inbreeding depression due to overdominance in partially self-fertilizing plant populations. Genetics **121:** 861–868.

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