THE UNIVERSITY OF ALBERTA

POLLEN CONTAMINATION AND MATING SYSTEM IN A CLONAL DOUGLAS-FIR SEED ORCHARD

by

WILLI FAST

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DATED 17 December 1985

.....Die Hybriden derselben müssen während der Blüthezeit vorder Einwirkung jedes fremdartigen Pollens geschützt sein oder leicht geschützt werden können. Fälschungen durch fremden pollen, wenn solche im Verlaufe des Versuches vorkämen, müssen zu ganz irrigen Ansichten führen.

Gregor Mendel
(aus Versuche über Pflanzenhybriden)

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled POLLEN CONTAMINATION AND MATING SYSTEM IN A CLONAL DOUGLAS-FIR SEED ORCHARD submitted by WILLI FAST in partial fulfilment of the requirements for the degree of MASTER of SCIENCE.

Bruce P. Dancik
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Supervisor

James Beck
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Curtis Strobeck
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Date: 17 December 1985

This is for Janet..... my inspiration.

ABSTRACT

Pollen contamination and mating system of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) clone banks near Nanaimo, B.C. were estimated. Pollen contamination (m) from surrounding clone banks and natural stands ranged from 0.44 to 0.89. Overall contamination over two years was estimated to be 0.65. Pollen contamination varied significantly over two pollination years. Multilocus outcrossing rates (t) of four clone banks over two years ranged from 0.51 to 1.09, with an unweighted mean outcrossing estimate of 0.72. Outcrossing varied significantly among clone banks and pollination years. Excess homozygotes suggest that this seed may be inbred.

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I. Introduction

Traditional forest tree breeding programs typically rely on wind-pollinated seed orchards for the production of improved seed. Genetic gain calculations are based on the ideal wind-pollinated seed orchard operating with complete panmixia and no pollination from unselected trees. For the wind-pollinated general combining-ability orchard, this is possible only when (1) orchard ramets are completely isolated from surrounding unselected trees; (2) ramets are equally productive of male and female flowers, (3) pollen anthesis and female flower receptivity coincide, (4) all clonal crosses are equally compatible, and (5) no progeny result from self-fertilization or related matings (Woessner and Franklin, 1973). Violation of one or more of these conditions can result from problems related to pollination. Inbreeding and pollen contamination from background pollen sources have been cited as potential problems in seed orchards (Koski, 1975).

Pollen Contamination

Pollen contamination may be the single most important factor reducing genetic efficiency of seed orchards (Friedman and Adams, 1982). Genetic gain of clonal orchard seed pollinated by unselected trees would be half that expected with no contamination (Franklin, 1971; Squillace and Long, 1981; Woessner and Franklin, 1973). This seed would be genetically equivalent to open-pollinated seed from

selected parents in natural stands.

Heavy cone crop years can produce background levels of pollen sufficiently high to make pollen contamination in seed orchards a problem (Silen, 1962). This is true even when large pollen dilution or isolation zones surround seed orchards, (Squillace, 1967), and when temporal isolation is attempted by cooling orchard trees with sprinklers (Fashler and Devitt, 1980; Silen and Keane, 1969), or by moving orchard ramets to other ecological zones (Silen, 1963).

A number of attempts have been made to estimate directly pollen contamination in coniferous seed orchards (Friedman and Adams, 1982; Smith and Adams, 1983; Squillace, 1967; Squillace and Long, 1981). Allozyme analysis provides advantages over other techniques because of the potentially large number of variable single locus genetic markers (Smith and Adams, 1983). Conifers are particularly suitable trees to work with since the megagametophyte tissue is haploid, reflecting the maternal genetic contribution to the embryo. Electrophoresis of both the megagametophyte and embryo permits the direct determination of the genotypes of maternal and paternal contributions to an embryo.

Inbreeding

Inbreeding is a major concern in seed orchards because it often leads to reduced seed yields and less vigorous seedlings (Franklin, 1970; Orr-Ewing, 1954, 1957, 1965; Rudin *et al.*, 1977; Sorensen, 1971, 1982).

Self-fertilization is the most serious form of inbreeding in seed orchards (Hadders and Koski, 1975), and could be exaggerated by multiple ramets of the same clones. One of the most effective ways to reduce selfing in a clonal seed orchard is to increase the number of clones and reduce the number of ramets per clone (Müller-Starck, 1982), but this will not reduce the occurrence of selfing within the crowns of individual ramets (Franklin, 1971).

A number of studies have estimated the proportion of progeny due to outcrossing (t, the outcrossing rate) in seed orchards and natural forest tree populations, generally finding a very low amount of selfing (El-Kassaby *et al.*, 1981; King *et al.*, 1984; Neale and Adams, 1985; Moran *et al.*, 1980; Shaw and Allard, 1982). These studies usually have employed allozyme genetic markers and various statistical estimators of t (Brown and Allard, 1970; Brown *et al.*, 1975; Cheliak *et al.*, 1983; Green *et al.*, 1980; Shaw *et al.*, 1981).

Objectives

The mating system in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) clone banks was examined. Objectives were (1) to estimate the level of pollen contamination, (2) to estimate the outcrossing rate, and (3) to test if either of these factors varied by year of pollination.

II. Methods

Seed Collection

All cones were collected in 1982 and 1983 from all seed-cone producing ramets in the Douglas-fir clone banks of the MacMillan Bloedel Harmac Tree Improvement Center near Nanaimo, British Columbia (Figure 1). Seed was extracted and kept separate by clones within clone bank, and by year of collection. For some clones only bulked seed was available. Cones and seed also were collected from ninety dominant or codominant cone-bearing Douglas-fir trees from three natural stands surrounding the Tree Improvement Center, and from a Douglas-fir arboretum next to the clone banks. All seed was stored at 2° C.

Electrophoretic Methods

Seeds were dissected, and megagametophyte and embryo tissues were homogenized separately in an extraction buffer (Yeh and O'Malley, 1980). Resulting homogenates were assayed for eight polymorphic allozyme loci (Table 1) using starch gel electrophoresis (12.5% Connaught Hydrolysed Starch). Loci were chosen on the basis of clear resolution in both megagametophyte (haploid) and embryo (diploid) tissue. Inheritance of these loci was verified by El-Kassaby *et al.*, (1982). Multilocus allozyme genotypes of all sampled trees were derived from a sample of eight megagametophytes per tree. The probability (p) of misclassifying a heterozygous

Legend for map of Harmac Tree Improvement Center near Nanaimo, British Columbia.

Map Number	Description
	CLONE BANKS
1	Douglas-fir #1 - low elevation, dry
2	Douglas-fir #2 - mid elevation, dry
3	Douglas-fir #3 - mid elevation, wet
4	Douglas-fir #4 - high elevation
	OTHER
5	Other Douglas-fir clone banks
6	Douglas-fir seed orchards
7	Clone banks, other species
8	Arboretum
9	Isolated natural Douglas-fir stand
10	Continuous natural Douglas-fir stand
11	Continuous natural Douglas-fir stand

Figure 1. Map of Harmac Tree Improvement Center near Nanaimo, British Columbia.

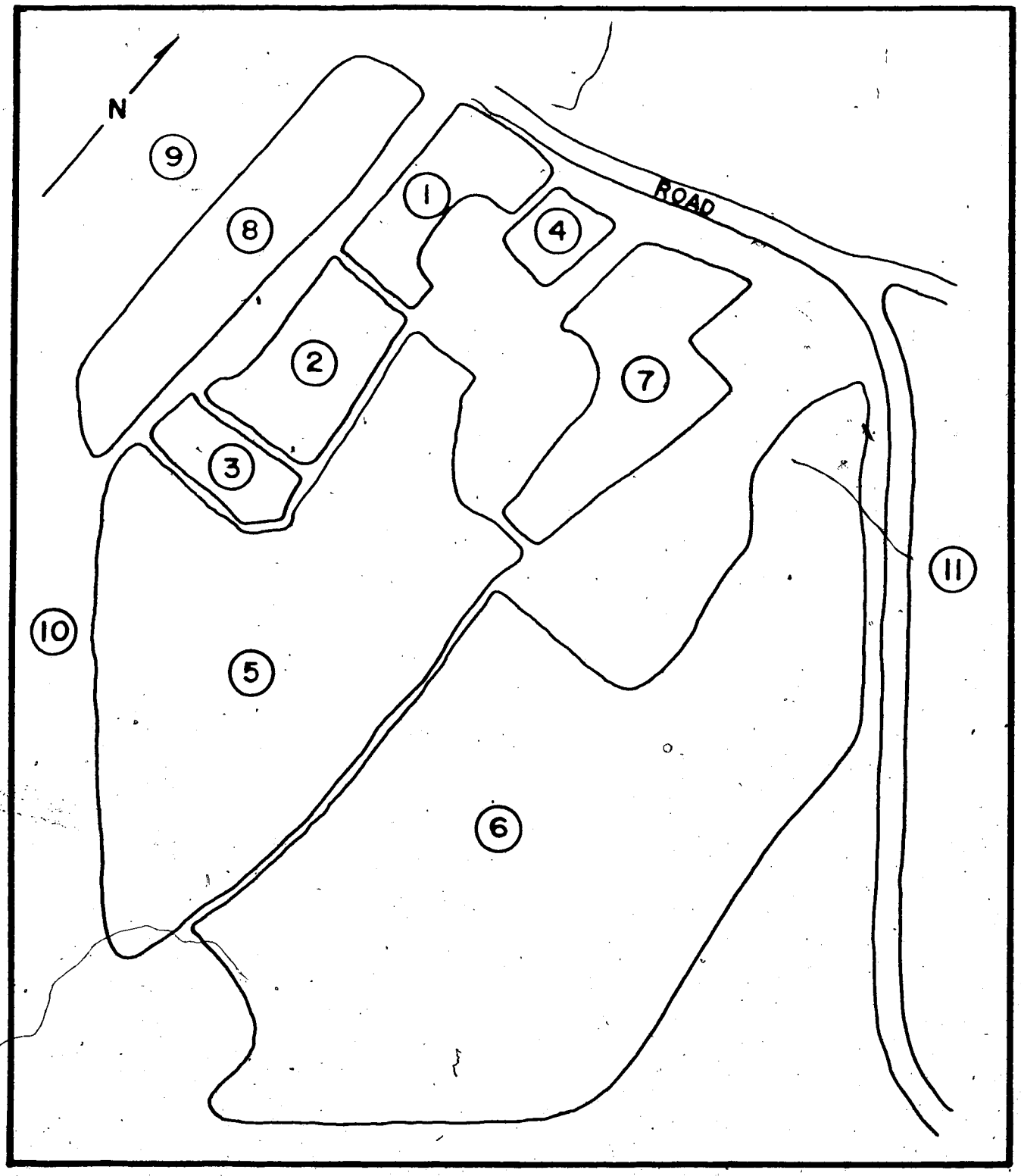

TABLE I. Enzymes assayed and buffer systems and staining procedures used in electrophoretic analysis of Harmac Douglas-fir clone bank seed.

Enzyme	Locus Abbreviation	E.C. Number	Buffer System	Stain Reference
leucine aminopeptidase	(LAP)	3.4.11.1.	A	Shaw and Prasad (1970)
malic enzyme	(ME)	1.1.1.40.	A	Yeh and O'Malley (1980)
phosphoglucose isomerase	(PGI)	5.3.1.9.	A	Yeh and O'Malley (1980)
malate dehydrogenase	(MDH-3)	1.1.1.37.	B	Yeh and O'Malley (1980)
isocitrate dehydrogenase	(IDH)	1.1.1.42.	B	Yeh and O'Malley (1980)
phosphoglucomutase	(PGM)	2.7.5.1.	B	Yeh and O'Malley (1980)
glucose-6-phosphate dehydrogenase	(G6P)	1.1.1.49.	C	Yeh and Layton (1979)
6-phosphogluconic dehydrogenase	(6PG-1)	1.1.1.44.	C	Yeh and O'Malley (1980)

A) Electrode buffer: lithium borate pH 8.1
Gel buffer: tris-citrate pH 8.5; Power: 300 volts
(Ridgeway, *et al.*, 1970)

B.) Electrode buffer: tris-citrate pH 6.3
Gel buffer: tris-citrate pH7.0; Power: 200 volts
(Selander, *et al.*, 1971)

C.) Electrode buffer: tris-citrate pH 7.0
Gel buffer: L-histidine-HCl pH 7.0; Power: 200 volts
(Namkoong, *et al.*, 1979)

Two alleles scored for all loci except ME, MDH-3, and IDH, for which three alleles were scored.

maternal genotype at any locus was less than 0.0078 when eight megagametophytes were sampled ($p=(1/2)^{n-1}$, where n is the number of megagametophytes sampled from each tree). By comparing allozyme segregation in embryo tissue with that in the corresponding megagametophyte, the genotypes of pollen contributions to clone bank seeds were deduced. This was done for eight embryos chosen at random from each fruitful ramet in each of clone banks one through four.

Statistical Methods

Pollen contamination analysis requires the knowledge of the genotype of all clones in the clone bank of interest. Since clone bank number one was the only clone bank for which this information was known, this was the only clone bank for which pollen contamination was estimated. Pollen contamination (m) in clone bank number one from surrounding Douglas-fir clone banks and nearby natural stands was estimated by the multilocus technique of Smith and Adams (1983). This technique does not require significant gene frequency differences between the studied clones and background pollen sources as do single-locus pollen contamination estimates (Rudin and Lindgren, 1977; Smith and Adams, 1983). Since a large proportion of contaminants was observed directly, the multilocus pollen contamination estimator was much more efficient than single-locus estimates (Smith and Adams, 1983). The multilocus model assumed that: (1) genes in the analysis were in linkage

equilibrium in the nearby stand, and (2) pollen production was independent of multilocus gametic genotype (Smith and Adams, 1983). These assumptions were reported to be reasonable when a large number of variable loci were sampled from large outcrossing populations of forest trees (Smith and Adams, 1983).

The model for estimating pollen contamination compared the multilocus pollen genotype of each sampled embryo with the array of all possible multilocus pollen genotypes in the clone bank. That proportion of pollen gametes with genotypes not matching those produced in the clone bank is the minimum observed contamination, b. The actual pollen contamination rate, m, is estimated by dividing the proportion of observed contaminants, b, by the probability that a background pollen grain carries a distinguishable multilocus marker, d (m=b/d) (Smith and Adams, 1983).

The normal approximation to the binomial distribution was used to construct confidence intervals on b and m, and to test the null hypotheses: H_0: b=0, and H_0: m=0. For a binomial variable (i.e. b, m) with frequencies p and q, this approximation is valid in the large sample (n) case when the value of $p \pm 2((pq/n)^{1/2})$ lies in the interval (0,1) (Mendenhall *et al.*, 1981). A chi-square homogeneity test (Rao, 1973) was used to test for differences between years in pollen contamination.

Single-locus outcrossing estimates have been shown to vary significantly among loci (Brown and Allard, 1970; Brown

et al., 1975); therefore, outcrossing rates of clone banks one through four were estimated using the multilocus procedures of Neale and Adams (1985). These are a modification of the maximum likelihood method of Green *et al.* (1980). Although several single-locus outcrossing rate estimators have been developed, some of which can handle multiple alleles per locus, they generally give comparable results (Cheliak, *et al.*, 1983). In this study, a maximum of three alleles per locus were scored. The outcross pollen pool allele frequencies were estimated by the single-locus method of Shaw and Allard (1982) modified by Neale and Adams (1985) to accomodate three alleles.

The multilocus outcrossing estimator was based on the mixed mating model (Shaw and Allard, 1982), with the assumptions that: (1) observed progeny result from either a random outcross (with probability t), or self-fertilization (with probability 1-t=s), (2) there is no selection between germination and analysis of progeny, (3) the probability of an outcross is independent of maternal genotype, (4) allele frequencies in the outcross pollen pool are homogeneous among maternal parents, and (5) all loci in the outcross pollen pool are independent (Shaw *et al.*, 1981). El-Kassaby *et al.* (1982) found no significant linkage in Douglas-fir among the allozyme loci used here. Sample sizes in this study were not large enough to test for spatial homogeneity of the outcross pollen pool. Although it is uncertain if all assumptions of the mixed mating model are fully satisfied,

it is clear that multilocus estimation of outcrossing rate is less sensitive to such violations than single-locus estimation (Shaw *et al.*, 1981).

A liklihood ratio test (Mendenhall *et al.*, 1981) was used to test the null hypothesis: H_0: t=1. A chi-square homogeneity test (Rao, 1973) was used to test for clone-bank and year differences in outcrossing rate. Wright's fixation index (Wright, 1951), measuring the deviation of observed heterozygotes from the proportion expected under Hardy-Weinberg equilibrium, was calculated as $F = 1 - H/h$, where H is the observed proportion of heterozygotes, and h is the expected proportion of heterozygotes under Hardy-Weinberg equilibrium. A chi-square goodness-of-fit test was used to test for deviations from Hardy-Weinberg equilibrium in the clone bank seeds (Mendenhall, *et al.*, 1981). All tests were done at the $p \leq 0.05$ significance level.

III. Results and Discussion

The proportion of observed contaminants (b) in clone bank number one was significant in both years (Table 2). Estimated pollen contamination (m) of clone bank number one was significant in both years, and varied significantly between years (Table 2).

Pollen contamination rates were within the range of those reported in other studies (Smith and Adams, 1983; Friedman and Adams, 1985). Although such contamination can increase genetic variability in a seed crop, it also decreases the genetic gain obtained from seed in the orchard (Franklin, 1971). Several factors could contribute to these high pollen contamination rates. Material in the clone banks is relatively young (oldest ramets are now eleven years old) and pollen production is low relative to both seed and pollen production of nearby natural stands. Local Douglas-fir stands are composed of open-grown trees with large crowns that produce heavy pollen crops. The fact that these stands are very close, and often upwind of the clone banks, virtually assures that some pollen contamination will occur.

The clones in clone bank number one are from the same general elevation and moisture regime as the local Douglas-fir (low elevation and dry moisture regime). As would be expected, female flower receptivity in this clone bank has been observed to reach its maximum at the peak of the local pollen flight (Hooper, 1979). This may predispose

Table 2. Proportion of observed contaminants (b), probability that a background pollen grain carries a distinguishable multilocus marker (d), and pollen contamination estimates (m=b/d) for Douglas-fir clone bank number one at Harmac. 95% confidence intervals given in parentheses.

Year	b	d	m
1982	0.16+ (0.10, 0.23)	0.38	0.44* (0.29, 0.59)
1983	0.34+ (0.25, 0.42)	0.38	0.89* (0.68, 1.11)
Total	0.24+ (0.19, 0.30)	0.38	0.65* (0.52, 0.78)

+ Denotes significant ($p \leq 0.05$) departure from H_o: b=0.

* Denotes significant ($p \leq 0.05$) departure from H_o: m=0.

this particular clone bank to higher levels of pollen contamination from local stands than other clone banks that contain material from elevations and moisture regimes different from those of native stands (Silen, 1963). Similar pollen contamination rates in those clone banks could have more serious consequences, since local stands may be adapted to different site conditions than sites for which those clone banks' seeds are intended (Heslop-Harrison, 1964; Herman and Lavender, 1968; Sorensen, 1983). Similarly, contamination of clone bank one by pollen from other clone banks may be more undesirable than contamination from local, presumably similarly adapted stands. In this study, it is not clear what proportion of pollen contamination comes from local stands versus surrounding clone banks.

As in previous reports (Friedman and Adams, 1985), pollen contamination in clone bank number one was found to vary significantly from year to year. This variation may be due to annual fluctuation in phenology of clone bank trees relative to that of nearby stands. Fluctuations in both pollen production and female receptivity could be involved. Annual differences in pollen contamination may also be a function of annual weather variation (i.e., temperature, moisture, wind). If the clone bank trees produce greater pollen crops as they mature, pollen contamination may become less significant (Sorensen, 1972). This may necessitate continual testing of contamination, to determine when, if ever, clone bank seed can be safely used operationally.

Because of the amount of natural Douglas-fir in the area, isolation strips around the clone banks probably would not be effective in reducing pollen contamination. Temporal isolation by cooling seed orchard clones with sprinklers, however, may reduce pollen contamination (Fashler and Devitt, 1980; Silen and Keane, 1969). Supplemental mass pollination may also be effective (Woessner and Franklin, 1973).

Multilocus outcrossing estimates (t) of clone banks one through four for two years ranged from 0.511 to 1.093 (Table 3). All but three of the estimates were significantly different from one. Outcrossing varied significantly among clone banks in 1982 and 1983. The estimates of t for all four clone banks combined varied significantly over the two years. The unweighted mean estimate of outcrossing for all four clone banks over both years was 0.72.

The outcrossing rates reported here were within the range reported for Douglas-fir (El-Kassaby, *et al.*, 1981; Omi and Adams, 1986; Shaw and Allard, 1982), although the overall rate of outcrossing was significantly lower than those observed in these studies. In a Douglas-fir clonal seed orchard, Omi and Adams (1986) reported an overall outcrossing rate greater than 0.9. Shaw and Allard (1982) reported similar results in a Douglas-fir seed orchard.

The extremely low outcrossing rates found in the clone banks at Harmac may be due to the layout of the ramets. Production seed orchards are designed to minimize

Table 3. Multilocus outcrossing rate (t) estimates for Douglas-fir clone banks 1-4 at Harmac with standard errors in parentheses.

Clone Bank Number	1982	1983
One	0.63* (0.035)	0.56* (0.043)
Two	0.51* (0.076)	0.65* (0.046)
Three	0.82 (0.096)	0.95 (0.088)
Four	0.56* (0.06)	1.09 (0.110)
Total	0.59* (0.018)	0.69* (0.018)

* Denotes significant ($p \leq 0.05$) departure from H_o: $t=1$.

self-fertilization by maximizing distances between ramets of the same clone (Giertych, 1975). The Douglas-fir clone banks at Harmac typically are laid out in clonal rows, with up to six adjacent ramets of the same clone in the same row. Crosses between ramets of the same clone are, in effect, self-fertilization.

Since clonal layout varies among clone banks, it is not surprising to find significant variation in outcrossing among clone banks. It is conceivable that clone banks may have either characteristically high or low outcrossing rates, depending on their layout. Because of the variability in other factors affecting the outcrossing rate (i.e., wind, weather, phenology), it seems reasonable to find significant annual variation in outcrossing rate.

A significant deficiency of heterozygotes was found for all loci but 6PG-1 in clone banks one through four. Wright's Fixation Index (F) ranged from -0.274 to 0.945 (Table 4).

The excess of homozygotes found in the clone bank seed suggests that this seed may be inbred, a reasonable conclusion considering the high levels of self-fertilization observed. Outcrossing between related individuals is also a part of inbreeding. However, since material in these clone banks originates from single-tree collections in many stands over a large geographic range, the effect of consanguineous matings is probably negligible. Inbreeding may have serious implications for any potential operational use of this seed. Further, the low outcrossing rates found in these clone

Table 4. Wright's Fixation Index (F) for combined (1982,1983) seed from Douglas-fir clone banks 1-4 at Harmac.

Clone Bank No.	Locus							
	G6P	6PG-1	IDH	LAP	MDH3	ME	PGI	PGM
1	0.364*	0.228	0.539*	0.801*	0.545*	0.928*	0.131*	0.709*
2	0.044	-0.090	0.129*	0.745*	0.190*	0.915*	0.650*	0.725*
3	0.282*	-0.078	0.293*	0.741*	0.894*	0.945*	0.692*	0.427*
4	0.122	-0.274*	0.229*	0.069	0.067	0.579*	0.431*	0.376*

* Denotes significant ($p \leq 0.05$) departure from Hardy-Weinberg expectations.

banks indicate the importance of proper seed orchard design to minimize inbreeding through self-fertilization.

The sum of the proportion of seeds arising from self-fertilization and pollen contamination indicates that proportion of seeds which may be undesirable for operational use. In 1982, as much as 81% of clone bank number one seed resulted from either self-fertilizion or pollination by trees outside the clone bank. In 1983, that estimate rose to over one. This discrepancy emphasizes that both self-fertilization and pollen contamination rates are estimated with some error. More precise estimates may be possible with joint estimation of self-fertilization and pollen contamination.

The magnitude of the pollen contamination estimate is highly sensitive to the magnitude of d, which is based on a random sample of genotypes from surrounding stands. An underestimate of d will result in an overestimate of pollen contamination (m=b/d). Smith and Adams (1983) suggested that 50 to 100 genotypes from potential background sources would be sufficient to reliably estimate d. If the sample of background genotypes used in this study does not accurately reflect the allele frequencies of all outside pollen sources combined, then d will not have been correctly estimated. In that case, a larger sample of genotypes from surrounding stands and clone banks may yield a more reliable estimate of d.

Although the observed proportion of contaminants (b) is adjusted by d, the model does not fully consider the probability that an indistinguishable pollen gamete is a contaminant. This probability may be influenced both by genotype locations in the clone bank, and by pollen production of indistinguishable genotypes in the clone bank relative to outside sources. Therefore, the pollen contamination estimate must be considered a minimum, given that d has been accurately estimated. Further, the confidence intervals around the pollen contamination estimates must be viewed as minimum confidence limits, since the variance used in their construction is a minimum estimate. This variance does not account for the sampling variance involved in estimating d (Smith and Adams, 1983). Nevertheless, the minimum observed pollen contamination (b) was significant in both years (Table 2).

Outcross pollen pool allele frequency estimates are dependent on the estimate of the outcrossing rate. Single-locus outcross pollen pool allele frequencies were used in the multilocus estimation of outcrossing, but it is unclear what effect they would have had on the multilocus outcrossing estimate. Although it probably would have been better to use a multilocus estimator of outcross pollen pool allele frequencies, none were available. Despite the error associated with m and t estimates, there is little doubt that pollen contamination and self-fertilization are serious problems at the Harmac clone banks. Due to the magnitude of

these problems, caution is advised in the operational use of this seed. It is unclear how much of the inbred seed would survive the rigors of the early years after outplanting. If a substantial number of selfs survived early mortality, growth and yield of the resulting plantation could be affected. However, this seed from selected mothers and a high proportion of selected fathers still may be better than current operational seed.

Detailed studies comparing phenology of clone bank clones with surrounding stands would be very helpful in understanding pollen dynamics in these clone banks. Further sampling and electrophoretic analysis of clone bank seed could elucidate possible heterogeneity in the effective pollen cloud at Harmac. However, every effort must be made to ensure that operational seed orchards come as close as possible to the ideal situation. Knowing that some assumptions may not be met, it is important to evaluate parameters, such as pollen contamination and outcrossing rate, before calculating genetic gains and subsequently expecting those gains to be realized operationally.

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