Non-random contribution of pollen in polycrosses of Pinus radiata D. Don

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Summary

A series of single-pair and polymix crosses were established with one clone as a female and four as male parents to examine the competitive ability of outcrossing male gametes. Three isozyme loci were assayed in viable embryos to test for departures from genotypic expectations in the crosses. In some polymix crosses there were significant departures from expectations which suggested differences in competitive ability of pollen parents. However this competitive ability appeared to depend on the particular clonal combinations of the mixes. Further work is required to determine how common, and important these selective forces are within the reproductive system, and the implications for tree breeding strategies.

Key words: Pinus radiata, polymixes, isozymes, selection.

Zusammenfassung

Bei *Pinus radiata* D. Don wurde eine Serie von Einzelpaar- und Polymix-Kreuzungen mit einem weiblich blühenden Klon und 4 männlich blühenden Klonen durchgeführt, um die Konkurrenzfähigkeit nicht verwandter männlicher Gameten zu untersuchen. Danach wurden die Embryos von den aus Kreuzungen hervorgegangenen Samen an drei Isoenzym-Loci untersucht, um die Kreuzungen auf Abweichungen vom zu erwartenden Genotyp hin zu testen. In einigen Polymix-Kreuzungen gab es solche signifikanten Abweichungen, welche auf Unterschiede bei der Konkurrenzfähigkeit der Pollen-Eltern hindeuten. Es scheint jedoch, daß die Konkurrenzfähigkeit von den einzelnen Klonkombinationen der Gemische abhängt. Um zu bestimmen, wie allgemein und wichtig diese selektiven Kräfte innerhalb eines reproduktiven Systems sind, bzw. die implizierten Folgerungen für die Strategien in der Forstpflanzenzüchtung, sind noch weitere Untersuchungen erforderlich.

Introduction

In populations of higher plants, selection has traditionally been considered of importance in the diploid, sporophytic stage of the life cycle. Recently there has been an upsurge of interest in the extent and evolutionary significance of selection in other parts of the life cycle (Clegg et al. 1978, Mulcachy 1979), especially in various stages of the reproductive cycle (Mulcachy 1975). In the haploid gametophyte stage it is important to determine the size of the selection forces compared to the potentially large random environmental factors. In the gymnosperms these random environmental factors are primarily associated with the direction of pollen dispersal and timing of pollen arrival on the micropyle. Recently it has been postulated that these random effects will be greater in wind-pollinated plants such as gymnosperms compared to insect-pollinated plants (Regal 1977, Mulcachy 1979, Kress 1981).

Clearly, between pollen/ovule formation and viable embryo maturation, selection could occur in both the haploid and diploid stages. In the prezygotic phase gametic selection between clones can occur during pollen germination and pollen tube growth, but non-Mendelian segregation may also be operating. If selection is monitored at the viable embryo stage, postzygotic embryo abortion (random or selective) would also be included. In this stage of the life cycle much of the research has concentrated on selfed versus outcrossed events (Jones 1928, Johnson and Mulcachy 1978), especially in gymnosperms (Squillace and Bingham 1958, Koski 1973, Lndgren 1975). Current evidence suggests that in conifers postzygotic selection within and between ovules against selfs is significant (Sarvas 1962, Sorenson 1982). There is no evidence of strong selection in the haploid phase (Orr-Ewing 1957, Hagman and Mikkola 1963). Polyembryony is common in gymnosperms (Chamberlain 1966, Dogra 1967) and selection within ovules can occur because in general, only one embryo per ovule develops to maturity. This effect is explainable in terms of the increased probability of expression of deleterious alleles in the inbred embryos and is a consequence of the similarity of male and female genotypes rather than selfing *per se*. Similar allelic combinations can also occur as a result of outcrossing. Therefore, it is reasonable to postulate effective competition between a series of outcrossed pollens as assessed at the viable embryo stage. In contrast cytological observations suggest that there is not strong selection against selfs in the haploid phase (Orr-Ewing 1957, Hagman and Mikkola 1963).

From an applied tree breeding viewpoint it is just as important to determine whether selection operates between different outcross pollens in mixtures in either the gametophytic or sporophytic stage to mature seed formation. The polymix mating design has a number of practical advantages in tree breeding but there is an implicit assumption that each male in the cross makes an equal contribution to the progeny. To test this assumption for *Pinus radiata* D. Don a series of polymix crosses were carried out. Isozyme techniques were then used to assess crosses for departures from equal contributions from all male parents.

Materials and Methods

a) Seed production procedures

One clone was used as the female parent and four others as male parents. Pollinations were made with each pollen singly, with the four pollens in a mixture, and with mixtures omitting one pollen at a time. Thus there were four single-pair crosses, four crosses each involving three pollens in mixtures and a fifth (P_5) with all four males in the pollen mix. Crosses were carried out in Saxton's orchard, near Traralgon in Victoria. These clones were used because some were putative heterozygotes or homozygotes for a fused needle trait. Isozyme techniques were developed after the crosses were done, and so the morphological character was not used in the analysis of the crosses.

The importance of careful experimental control in selective fertilisation tests has been emphasised by Squillace and Bingham (1958). Non-genetic, maternal effects (such as ramet vigour) or location of pollinated strobili on the

Table 1. — In vitro viability of test pollens and seed yield from test crosses to two clones.

Trait [b]	Pollen Parent No.[a]			
	50083	80019	12038	30028
Mean germination %	78.7	72.0	77.4	77.7
No. cones from cross to - 12403	8	3	5	2
- 12378	7	5	9	1
No. sound seed/cone - 12403	41	52	35	40
- 12378	78	63	69	92
Weighted Mean	58.3	58.9	56.1	57.3
% sound seed/cone - 12403	67	78	64	74
- 12378	73	76	75	84
Weighted Mean	69.8	76.7	71.1	77.3

a — Ausralian Plus Tree Register No.
b — Pollen parent effects were non-significant for germination %, sound seed/cone and percentage sound seed/cone.

Table 2. — Details of pollen combinations and number of cones and full seeds harvested in crosses with 80055 as the female.

Pollination code	Male parents	Total number cones harvested	Mean no. of full seed/cone
S_1	50083(M_1)	16	89
S_2	80019(M_2)	14	66
S_3	12038(M_3)	7	63
S_4	30028(M_4)	15	68
P_1	$M_2 + M_3 + M_4$	14	51
P_2	$M_1 + M_3 + M_4$	20	73
P_3	$M_1 + M_2 + M_4$	14	49
P_4	$M_1 + M_2 + M_3$	12	49
P_5	$M_1 + M_2 + M_3 + M_4$	18	59

leader or lateral branches may affect cone and seed size. Differential viability of the pollen for non-genetic reasons related to extraction or storage history could also be a major complicating factor. The latter is recognised by some tree breeders who adjust the volume contribution of pollens in test mixes according to their individual performance in *in vitro* germination tests (Kirby and Stanley 1976).

The test pollens, collected the previous flowering season, were germinated in water at 27° C using a modified hanging drop method. Four separate samples of each pollen were set and, after 5 days, two microscope fields containing approximately 100 grains were counted. Mean germination ranged from 72.0 to 78.8% but this differences was not statistically significant *(Table 1).*

An *in vivo* test of pollen viability was carried out by crossing each single pollen parent to two females not directly involved in the experiment, and determining the number and proportion of sound seed per cone which resulted *(Table 1).* The proportion of sound seed is of more direct interest since it compensates for differences in cone size and hence number of ovules per cone. Variation between pollen parents was not significant and it was concluded that each, on its own, should be equally effective. The various experimental pollen mixtures were therefore made up of equal quantities of each constituent pollen by volume.

In order to minimise maternal effects, 5 ramets of the designated female parent, (80055) with at least 9 flowering branches were identified. Nine isolation bags were placed on each ramet and pollen combinations allocated at random to bags. Each bag was pollinated an average of 7 times at 3 day intervals to ensure that all strobili were pollinated when receptive. Pollen combinations, numbers of cones, and full seeds harvested are shown in *Table 2.*

Table 3. — Genotypes at three loci of clones used in pollen mix study.

Locus	Clone				
	80055	50083	80019	12038	30028
ADH-2	MS[a]	FM	FF	SS	MM
PGI-2	MS	MS	MS	MM	MM
AC-1	MM	MM	FF	FM	MM

a — F, M and S refer to the fast, medium and slow alleles at each locus.

b) Isozyme techniques

Seed from the five clones were assayed by starch gel electrophoresis to determine their genotypes at 20 isozyme loci. The genotypes of each clone were deduced from the haploid megagametophytic tissue of open-pollinated seed arrays. Details of electrophoretic techniques, tissue extraction procedures and enzyme assays are given elsewhere (Moran *et al.* 1980, Moran *et al.* 1983). Among the five clones three loci were polymorphic and these produced the enzymes alcohol dehydrogenase (ADH,E.C.1.1.1.1), phosphoglucose-isomerase (PGI, E.C.5.3.1.9) and aconitase (AC,E.C.4.2.1.3). Of the two ADH loci, the second (ADH-2) was polymorphic and could be scored reliably on both the megagametophyte and diploid embryo/seedling material. Similarly, the second of the two PGI loci and the AC locus were assayable and polymorphic *(Table 3).*

From the single-pair crosses (S_1—S_4 in *Table 2),* germinating seedlings and the corresponding megagametophytes were assayed for their genotypes at the three loci. In seedlings from the series of polymix (P_1—P_5) crosses the same loci were assayed to test for equal contributions to the polymix by the pollen of the four clones. The expected

Table 4. — Expected zygotic ratios in polymix crosses.

Cross	Locus	ADH-2					PGI-2			AC-1		
	Genotype	FF	FM	FS	MM	MS	SS	MM	MS	SS	FM	MM
P_1(ex 50083)		-	1	1	1	2	1	5	6	1	1	1
P_2(ex 80019)		-	1	1	3	5	2	5	6	1	1	5
P_3(ex 12038)		-	3	3	3	3	-	2	3	1	1	2
P_4(ex 30028)		-	3	3	1	3	2	2	3	1	1	1
P_5		-	3	3	3	5	2	3	4	1	3	5

Table 5. — Observed genotypic arrays in single crosses.

CROSS	LOCUS	GENOTYPES FM	FS	MM	MS	SS	Total	X^2(df)
S_1	ADH-2 O[a]	88	84	124	104		400	9.92*(3)
	PGI-2 O			101	157	63	321	9.15*(2)
S_2	ADH-2 O	49	53				102	0.16(1)
	PGI-2 O			33	44	27	104	3.16(2)
	AC-1 O	71					71	-
S_3	ADH-2 O				64	77	141	1.21(1)
	PGI-2 O			81	60		141	3.13(1)
	AC-1 O	51		65			116	1.69(1)
S_4	ADH-2 O			42	53		95	1.27(1)
	PGI-2 O			40	50		90	1.11(1)

a O = observed,
* significant at 5% level.

Table 6. — Two-Locus segregation patterns and Chi-square analyses for detection of linkage from open-pollinated seed.

Clone	COMBINATION Locus A	Locus B	No. megagametophytes/class				Total	Segregation at A $\chi^2(1)$	P	Segregation at B $\chi^2(1)$	P	Joint Segregation $\chi^2(1)$	P
			FM	FS	MM	MS							
12236	ADH-2	AC-1	61	49	55	60	225	0.11	0.7-0.8	0.22	0.5-0.7	1.28	0.2-0.3
			MF	MM	SF	SM							
12001	PGI-2	AC-1	53	50	67	67	237	4.05	0.02-0.05	0.38	0.5-0.7	0.04	0.8-0.9

genotypic ratios from the polymix crosses on the basis of equal pollen contribution by the clones are shown in *Table 4.*

Results

The arrays of allozyme genotypes at the three loci from the four single-pair crosses (S_1—S_4) along with Chi-square values, which test for departures from mendelian expectations of equal contributions from all male gamete types, are shown in *Table 5.* In the crosses S_2, S_3 and S_4 the frequencies of the observed offspring agree well with Mendelian expectations based on the parental genotypes for the three loci. However in the S_1 cross, data for both segregating loci show significant departures from expectations. To further examine this cross we first tested whether the three loci segregated independently. Moran *et al.* (1983) from a female megagametophytic analysis of six clones (including 80055) found that ADH-2 and PGI-2 were linked and 26 map units apart. Analysis of two-locus megagametophytic arrays from two other *P. radiata* clones *(Table 6)* indicated that ADH-2 and PGI-2 segregated independently of AC-1. Thus, it is not surprising that both ADH-2 and PGI-2 show aberrant ratios in the S_1 cross, the only double heterozygote. An analysis of the ADH-2 : PGI-2 gametic types in the S_1 seed indicates that in the megagametophytes the segregation at PGI-2 fits a 1:1 ratio, but at ADH-2 is significantly different from a 1:1 ratio at the 5% level *(Table 7).* However, in the other single-pair crosses there was a good fit to 1:1 ratios for ADH-2 and PGI-2 in the megagametophytes. It seems likely, therefore, that there is no segregation distortion in clone 80055 as a female and that results for the S_1 cross merely reflect sampling error.

On the other hand in the pollen from the S_1 cross there was significant preferential recovery of ADH-2 M and PGI-2 M alleles. Distortion at ADH-2 would be expected to be accompanied by a similar phenomenon at PGI-2 since they are linked. However the implication is that in 50083 ADH-2 M is cis with PGI-2 M and ADH-2 F cis with PGI-2 S. But in fact, from genetic analysis of megagametophytes of 50083 it is known that the arrangement is M with S and F with the M allele (Moran *et al.* 1983). Thus there appears to be preferential recovery of MM crossover gametes in the viable zygotes of the S_1 cross, and these derive from the pollen. If the FS gamete is of reduced viability, then the depletion in F-bearing genotypes should be proportional to the frequency of FS crossovers. There

Table 7. — Analysis of ADH-2:PGI-2 gametic types in the S_1 cross.

CLONE	SEX	GAMETIC TYPE ADH-2/PGI-2				SEGREGATION $\chi^2(1)$ ADH-2	PGI-2	JOINT
		MM	MS	SM	SS			
80055	♀	50	123	107	27	4.95*	0.16	76.25***
		MM	MS	FM	FS			
50083	♂	104	79	83	47	8.97**	11.89***	21.25***

*, **, *** significant at the 5%, 1% and .1% levels respectively.

Table 8. — Observed and expected progeny genotypes at three loci in polymix crosses.

CROSS		GENOTYPE					TOTAL	χ^2
		FM	FS	MM	MS	SS		
ADH-2								
P_1	O[a]	52	44	64	101	72	333	9.71
	E	55.5	55.5	55.5	111	55.5		
P_2	O	25	19	42	96	31	213	6.55
	E	17.7	17.7	53.1	89	35.5		
P_3	O	87	77	62	69	-	295	4.70
		73.75	73.75	73.75	73.75	-		
P_4	O	40	53	9	124	119	345	68.70*
	E	86.25	86.25	28.75	86.25	57.50		
P_5	O	68	69	84	147	79	447	15.53*
	E	84	84	84	140	56		
PGI-2								
P_1	O			113	120	22	255	1.01
	E			106	128	21		
P_2	O			77	106	20	203	1.59
	E			85	101	17		
P_3	O			94	139	52	285	0.52
	E			95	142.5	47.5		
P_4	O			149	170	27	346	26.09*
	E			115.4	173	57.6		
P_5	O			84	93	30	207	2.24
	E			78	104	25		
AC-1								
P_1	O	35		78			113	16.36*
	E	56.5		56.5				
P_2	O	19		137			156	2.26
	E	26		130				
P_3	O	101			189		290	0.25
	E	97			193			
P_4	O	154			181		335	2.24
	E	167.5			167.5			
P_5	O	74			115		189	0.20
	E	71			118			

F/102T * significant at the 1% level.
a — O = observed, E = expected.

is an fact a 12% depletion which fits with the frequency of crossovers (26%) since only half of them will be of FS type. Therefore, it is possible that reduced viability of the FS gamete occurs in the haploid pollen phase and is a form of synthetic lethality. Pollen contamination, of course, is another possible explanation of the S_1 data. However a number of S_1 seed were screened for contaminant alleles at 15 loci and none were detected, suggesting that contamination is unlikely. A breakdown of the gametic data by female ramet showed that the excess of MM gametes compared to FS was consistent across ramets. Recent work from other crosses have indicated that in 50083 there is no segregation distortion ovules (Moran *et al.* 1983).

Chi-square tests were used to determine departure of progeny genotypic arrays in the polymix crosses from expected ratios *(Table 8)*. Crosses with significant Chi-squares were examined at the level of individual genotypes. In the P_4 cross, there is an excess of the SS genotype at ADH-2 and the MM genotype at PGI-2, which suggests that M_3 males outcompete both M_1 and M_2. Given the genotypes of the pollen clones at AC-1 it would be difficult to detect differential pollen transmission in P4 using this locus. The apparent competitive superiority of M_3 males is supported by the excess of SS homozygotes at ADH-2 in P_1. In contrast, there is no evidence of differential male transmission in the P_2 cross which also includes M_3. Overall the data indicated a trend in decreasing competitive ability as follows: $M_3 \cong M_4 > M_1 \geq M_2$. Clearly, this sequence does not hold in all crosses and some crosses demonstrate parts of the sequence but not others. Thus P_1 data indicate that M_4 contributes at least as much pollen to viable offspring as M_3 whereas both contribute more than M_1 and M_2. However removal of M_2 (as in P_2) apparently removes any differences between other pollen clones and when M_3 is absent, M_4 males do not appear to have a competitive edge over the other two clones.

No simple model is adequate to explain the data, and an interaction model is more likely. For example, in certain mixtures of clones there is preferential recovery of M_3 gametes in viable embryos (e.g. P_4, P_1, and P_5) but not in other mixtures (P_2) and hence there is positive selection for M_3 males in only some pollen mixes. On the other hand certain clones are clearly competitively inferior in some mixtures (i.e. M_2 in P_1, P_4 und P_5) but not in others (M_2 in P_2 and P_3). Although the three genetic markers are functionally active in pollen, screening at the viable embryo stage does not allow us to discriminate between haploid and diploid selection.

Discussion

In the single-pair crosses one cross exhibited departure from effective random mating. With sampling at the viable embryo stage it was not possible to distinguish at what precise stages of the life cycle non-random departures arose. On the paternal side it could have arisen by non-mendelian segregation or intraclonal gamete competition in pollen tube growth or pollen germination. On the other hand the aberrant ratios could just as likely have resulted from selective embryo abortion subsequent to zygote formation. Non-cleavage polyembryony, which has been shown to occur in *P. radiata* using isozyme markers (Moran unpublished), could be a source of selective competition due to either or both the maternal or paternal components of the embryos. Lill (1974) found more than 91% of the immature seeds of clone 80055 contain more than one embryo, whereas we found for the same clone about 1% at germination. For gymnosperms there is no evidence to suggest that a clone which exhibits mendelian (or non-mendelian) segregation maternally will do the same when acting as a male. Likewise in single-pair crosses one would not necessarily expect any correlation between, for instance, random segregation on the maternal side (as in this study) and the segregation of gametes in the males up to the early prezygotic phase. In fact, both normal segregation and segregation distortion in the pollen gametes of crosses have both been reported with normal segregation in the ovules of the same crosses (Lundkvist 1974, Adams and Joly 1980, Moran *et al.* 1983).

All the above factors could operate in the polymix crosses of this study as well as interclonal competition. The polymix data indicated that certain pollen parents are more successful than other males in contributing to the viable embryo population. However the competitive success of each individual clone depends on the other males present. A paternal interaction model is suggested from these results, yet our study does not discriminate between possible points of interaction such as the haploid pollen phase and/or the diploid zygote-embryo phase. Clearly, more sampling points are required within this stage of the life cycle. In conifers it appears that selection against selfs is commonly associated with polyembryony (Lindgren 1975, Matheson 1980, Sorensen 1982). It will be of interest to determine whether competition between outcros-

sing male gametes also primarily occurs in the polyembryonic stage. There is no good genetic evidence of effective competition in the haploid pollen stage up to fertilisation for conifers, even when selfing is involved.

A question not addressed by this study is whether the genotype of the female parent influences the selection between pollen gametes. In many crosses the male contributing to each embryo could not be determined unequivocally with the isozyme markers available. Studies in progress will hopefully rectify these problems and also allow sampling at a number of points between pollination and the maturation of the embryo.

The polymix mating design for progeny testing select clones is attractive because it costs less in terms of labour and resources than equivalent series of single-pair matings. If pollen parents are not equally represented in the progeny of each female parent then breeding value estimates may be biased. Similarily in a clonal seed orchard situation the selection already proven to exist against selfed embryos may operate against particular outcross combinations. On the positive side there is potential to look for correlations between gametophytic selection and sporophytic performance (Ter-Avanesian 1978, Ottaviano *et al.* 1980): do the best pollen competitors exhibit the best adult growth?

We conclude that our results show sufficient evidence of non-random contribution of pollens to viable seed formation to warrant further study. In particular, results should be obtained for a larger sample of pollen donors and seed parents. Nevertheless, in this study at least, such effects are not so strong as to affect he utility of the polymix mating design in practical tree breeding. One approach to overcome the problem of non-random contribution would be to use a large number of pollens in mixture, thereby reducing the influence of any one male with unusual competitive ability. The disadvantage of such a solution would be the reduced chances of identification of male parents of superior progeny using genetic markers.

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Provenance Productivity in Eucalyptus camaldulensis Dehnh. and its Implications to Genetic Improvement in the Savanna Region of Nigeria

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Abstract

A 13-year provenance trial of *Eucalyptus camaldulensis* Dehnh. at Afaka was analysed for height and girth growth. Highly significant differences were obtained for the two growth characteristics among the sixteen provenances tested. Provenance variation contributed about 28.3 and about 27.5 per cent to the observed variations in height and girth growth respectively. Individual tree differences accounted for about 58.0 and about 65.6 per cent to the variation in height and girth growth respectively.

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