Theor. Appl. Genet. 57, 101-105 (1980)

Temporal Heterogeneity of Outcrossing Rates in Alpine Ash (*Eucalyptus delegatensis* R.T. Bak.)

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Summary. Three seed crops of a *Eucalyptus delegatensis* population were assayed for their allozyme genotype at three loci to determine estimates of mating system parameters. In the pollen the allelic frequencies at each of the three loci were similar to those in the parents and the progeny. Overall there was a significant amount of inbreeding (23%) in the population. The levels of outcrossing in each crop were significantly different from each other indicating apparent temporal variation in outcrossing rates. The outcrossing rate was greatest in the oldest crop (85%) and lowest in the most recent crop (66%). Mean heterozygosity in the progeny of all three crops was less than the heterozygosity in the parents indicating that selection favours heterozygotes during the life cycle. The implications of a 'balanced' mixed mating system for a eucalypt breeding program are discussed.

Key words: *Eucalyptus* – Allozyme variation – Breeding system – Inbreeding – Life cycles

Introduction

Flowering plants exhibit a diversity of genetic population structures and central to this diversity is the evolutionary role of their breeding systems. Recent reviews (Gottlieb 1977; Hamrick et al. 1979; Brown 1979) have emphasized that generally there is considerable genetic variation both within and between populations of plant species. Tree species have so far been found to have as high a level, if not higher, of genetic variation as any other plant group (Hamrick 1978). This is undoubtedly, linked with the fact that most trees are predominantly outcrossing (Fryxell 1957; Stern and Roche 1974; Squillace 1974), but quantitative estimates of outcrossing rates for most species are still rare. However the advent of electrophoretic techniques has allowed the mating system parameters of tree populations to be estimated relatively easily (Brown et al. 1975; Phillips and Brown 1977; Mitton et al. 1977; Moran et al. 1980).

In outcrossing self-compatible species, it is reasonable to assume that the rate of outcrossing is associated with a wide potential range over seasons (temporal) and localities (spatial). Studies of several plant species have shown that populations of a species can differ markedly in their levels of outcrossing (Harding and Barnes 1977; Rick et al. 1977). However the extent to which the mating system varies over different seasons in natural populations is largely unknown. Some possible theoretical consequencies of the temporal fluctuation in outcrossing were raised by Jain and Marshall (1968) and Nei (1975).

Eucalypts have been considered to be predominantly outbreeding (Pryor 1976) and recent estimates using allozyme variants have confirmed this for two species, e.g. *E. obliqua* (Brown et al. 1975) and *E. pauciflora* (Phillips and Brown 1977). In *E. obliqua* it was estimated that on the average 24% of the viable seed was derived from self-fertilisation while in *E. pauciflora* the selfed seed fraction was sometimes as high as 37%. In this paper we report quantitative estimates of the mating system of alpine ash, *Eucalyptus delegatensis*. This species was studied for two reasons. Firstly, it is one of Australia's economically important hardwoods. Second, *E. delegatensis* tends to retain its fruit crops on the tree, sequentially in distinct clusters, for more than one season. This is a common feature of many eucalypts from southeastern Australia, especially in members of the subgenus *Monocalyptus* (Pryor and Johnston 1971). A random collection of capsules at any time is therefore likely to be heterogenous with respect to the season in which fertilisation occurred. The question arises as to whether there would be any heterogeneity of outcrossing rates measurable between crops in such a harvest, and hence any important differences in the genetic composition of the crops. Differences in outcrossing rates between crops are evidence of tem-

0040-5752/80/0057/0101/$ 1.00

Table 1. Frequencies of alleles at three seed loci in three crops of the Pilot Hill population

		Progeny ($\hat{P}$)					Pollen ($\hat{P}'$)		
Locus	Allele	A	B	C	Total	Parent ($\hat{P}_m$)	A	B	C
Mdh-1	F	0.40	0.46	0.43	0.43	0.42	0.36 ± 0.04	0.65 ± 0.08	0.48 ± 0.04
	S	0.60	0.54	0.57	0.57	0.58			
Mdh-2	B		0.01	0.01	0.01				
	F	0.77	0.79	0.72	0.75	0.72	0.87 ± 0.03	0.85 ± 0.02	0.78 ± 0.04
	S	0.23	0.20	0.27	0.23	0.28			
Aph-3	F	0.66	0.67	0.68	0.67	0.65	0.64 ± 0.05	0.66 ± 0.04	0.65 ± 0.04
	S	0.34	0.33	0.32	0.33	0.35			

A = most recent crop; C = oldest seed crop

poral fluctuations in outcrossing and would affect the population genetic structure of such species, as well as the collection of seed for forest plantations.

Materials and Methods

E. delegatensis has a wide geographic range over the alpine country of S.E. Australia. Field observations had indicated that the population of *E. delegatensis* at Pilot Hill on Bago State Forest near Batlow in N.S.W. was a consistently good seed production area. Therefore at this site 30 randomly chosen trees were sampled simultaneously for separate seed collections from at least three consecutive crops. The capsules of each crop are clustered on a branch. Vegetative growth between flowering seasons separates the different crops from one another. The species normally flowers once a year about February. It takes two years from bud initiation to seed maturation.

Single seeds, after moist stratification for 5 weeks at 4° C, were crushed in one drop of 0.1M phosphate buffer (pH7.0) containing 1 mg dithiothreitol per ml and the extracts absorbed onto paper chromatography wicks (6 mm X 5 mm). The wicks were inserted in a 12.5% starch gel buffered with 5 m M histidine titrated to pH 8.0. Electrophoresis was conducted for $4\frac{1}{2}$ hours at 7 v cm^{-1} and the connecting buffer was 0.41M sodium citrate titrated to pH 8.0 with 0.41M citric acid. The horizontal slices were assayed for malate dehydrogenase (EC 1.1.1.37) and acid phosphatase (EC 3.1.3.2) by standard procedures (Brown et al. 1974). At least 10 seeds from each of the three crops from each of the thirty trees were scored for electrophoretic variants as revealed by these assays.

As in the closely related species, *E obliqua* and *E. pauciflora* (Brown et al. 1975; Phillips and Brown 1977) there were two alleles segregating at both the loci *Mdh-1* and *Aph-3*, and three alleles at the *Mdh-2* locus. The *Aph-3* locus corresponds with the *Phos* locus in *E. obliqua* and to the *Phos*$_2$ locus in *E. pauciflora*. This locus was designated *Aph-3* in *E. delegatensis* because there are two faster migrating loci in seedlings which are not assayable in seeds and hence were not used in this study. A third polymorphic *Mdh* locus was not used since the genotypes of all progeny could not be determined consistently. Methods for the classification of maternal genotypes and estimation of genetic parameters were those previously published (Brown et al. 1975). In addition, multilocus estimates of the outcrossing rate (t) were obtained by maximum likelihood procedures from gametic frequencies (Green et al. 1979).

Results

Table 1 gives estimates of the frequencies of all alleles in the progeny and the maternal trees for the three loci in the three seed crops. A is the most recent crop and C is the oldest crop. In the pollen pool only the frequency of the *F* allele at each locus has been estimated. The allelic frequencies in the progeny and in the maternal trees are based on at least 300 progeny and 30 maternal genotypes respectively.

At the three loci the allelic frequencies in the progeny of the three crops and in the parents are very similar. Likewise the frequencies of the F alleles in the pollen pool are on the whole similar to the corresponding parental and progeny frequencies with the marked exception of the F allele at *Mdh-1* in the B crop. The data indicate that the commonest allele at *Mdh-2* is at a significantly higher frequency in the pollen pools than in the parents and progeny of the two younger crops.

The heterozygosities observed at the three loci in both the maternal trees (H_m) and the progeny (H) (Table 2) indicate that the mean heterozygosity in the progeny of all three crops was significantly ($P<0.01$) less than that of the parents. The heterozygosity is least in the most recent crop but the differences between crops are not statistically significant.

Table 2. Observed heterozygosity for each locus in each crop

		Observed heterozygosity			
		Progeny (H)			Parent (Hm)
Locus	Crop.	A	B	C	
Mdh-1		0.45	0.48	0.43	0.57
Mdh-2		0.28	0.32	0.40	0.50
Aph-3		0.28	0.31	0.31	0.57
	Mean	0.34	0.37	0.38	0.55

Table 3. Estimates of Wrights Fixation Index ($\hat{F}$) for each locus and each crop and the parents

	Crop				
Locus	A	B	C	Total	Parent
Mdh-1	0.06 ± 0.06	0.02 ± 0.06	0.12 ± 0.06	0.07 ± 0.04	−0.17[a] ± 0.03
Mdh-2	0.21[a] ± 0.11	0.05 ± 0.04	0.02 ± 0.09	0.09 ± 0.09	−0.24[a] ± 0.04
Aph-3	0.38[a] ± 0.08	0.29[a] ± 0.08	0.29[a] ± 0.09	0.29[a] ± 0.07	−0.25[a] ± 0.06
Mean	0.21[a]	0.13[a]	0.14[a]	0.15[a]	−0.22[a]
Fe.	0.21	0.12	0.08	0.13	

[a] Rejection of the null hypothesis that F = 0 at the 5% level

To test whether the observed differences in heterozygosity were due to different inbreeding levels, estimates of the fixation index (F) were made (Table 3). All estimates were positive and all three crops showed statistically significant positive values of $\hat{F}$. There was a deficiency of heterozygotes among the progeny compared to Hardy-Weinberg expectations. Moreover the deficiency was greatest in Crop A suggesting that there was apparently more inbreeding in this crop.

The extent of linkage disequilibrium between loci was determined by calculating the values of disequilibrium ($\hat{D}$) for the three pairwise combinations of loci by the zygotic method (Brown 1975) (Table 4). Although a couple of comparisons for individual crops were statistically significant, overall estimates were essentially zero. This is confirmed by the values of D′, the proportionate disequilibrium, which give the observed disequilibria as a proportion of the maximum possible for the given gene frequencies. Therefore it can be assumed that the three loci segregate independently and that alleles combine at random to make gametic combinations.

Tabel 4. Estimates of linkage disequilibrium ($\hat{D}$), its standard error and the corresponding standardised disequilibrium (D′) for three pairs of loci

		Crop			
		A	B	C	Total
Mdh-1^F/*Mdh-2*^{F}	$\hat{D}$	+0.015	−0.009	−0.005	−0.000
	SE($\hat{D}$)	±0.012	±0.012	±0.012	±0.007
	D′	+0.050	−0.026	−0.017	−0.000
Mdh-1^F/*Aph-3*^{F}	$\hat{D}$	−0.009	+0.021	−0.004	+0.003
	SE($\hat{D}$)	±0.013	±0.013	±0.013	±0.008
	D′	−0.004	+0.070	−0.014	+0.011
Mdh-2^F/*Aph-3*^{F}	$\hat{D}$	−0.027	−0.017	−0.035	−0.000
	SE($\hat{D}$)	±0.01	±0.01	±0.011	±0.007
	D′	−0.051	−0.032	−0.076	−0.000

The expected gametic frequencies could thus be tabulated from the maternal three-locus genotypes and the maximum likelihood estimates of the outcrossing rate (t) could be made by comparison of these expected values with the observed frequencies. These estimates and their standard errors are given in Table 5 for the three crops and the total crop along with the single-locus estimates. The single-locus estimates of outcrossing were heterogeneous among loci within crops. Such heterogeneity is commonly found (Allard et al. 1977), and arises because single-locus estimates are sensitive to other deviations from panmixia besides that due to selfing. Nevertheless the means of the single-locus estimates for the three crops agreed closely with the corresponding multilocus gametic estimates. The overall estimate was of 23% selfing in the population but the estimates for individual crops were

Table 5. Estimates of the outcrossing rate ($\hat{t}$) and their standard errors in the three crops from single-locus (SL) and multi-locus (ML) procedures

		Crop			
	Locus	A	B	C	Total
SL	*Mdh-1*	0.79 ± 0.08	0.60 ± 0.08	0.89 ± 0.08	0.76 ± 0.05
	Mdh-2	0.58 ± 0.08	0.97 ± 0.08	0.80 ± 1.0	0.80 ± 0.05
	Aph-3	0.57 ± 0.09	0.75 ± 0.09	0.78 ± 0.09	0.70 ± 0.05
	Mean	0.65	0.77	0.82	0.75
ML		0.66	0.78	0.85	0.77
	S.E.	± 0.05	± 0.05	± 0.06	± 0.03

significantly different from each other. Thus $\hat{t}$ increases significantly from 0.66 in the youngest crop A to 0.85 in the oldest crop C. These results suggest that there is both a significant level of inbreeding in the population, and considerable variation in the amount of outcrossing between different crops.

Table 3 gives the fixation indices for the maternal trees and highlights marked changes that apparently occur in the heterozygosity levels through the life cycle of this population. The F values based on the heterozygosity levels in the parents were significantly less than zero at all three loci. This indicated that there was a significantly greater proportion of heterozygotes observed in the parents at the three loci than expected on the basis of random mating. These data suggest that there may be heterotic selection associated with the high competition occurring in the seedling and subsequent adult stage in natural eucalypt populations.

Discussion

The overall estimate of outcrossing in the Pilot Hill population of *E. delegatensis* was 77%. As in *E. obliqua* and *E. pauciflora*, the breeding system is apparently one of predominant outbreeding, but with a significant inbreeding component making it a definite mixed mating system. The fate of the zygotes derived from selfing in the subsequent generation may have important implications for the maintenance of this mixed mating system. The maintenance of a mixed mating system would seem to be necessary from theoretical considerations since Fisher (1941) showed that when a gene permitting self-fertility arises it has an immediate evolutionary advantage and that some balancing force is necessary to prevent fixation of this gene.

There was also a considerable difference between the outcrossing rate estimated in the youngest crop (66%) and the oldest (85%). Two factors could account for this difference. The first would be differences in the rate of outcrossing in the different seasons. Under this hypothesis, the fact that the oldest crop had the highest rate would be mere coincidence. The second factor could be differential viability of inbred as opposed to outcrossed seed since the fertilisation events in the oldest crop. The present data do not discriminate between these factors. However the second hypothesis of constant outcrossing, but a seed viability advantage for outcrossed seeds, requires that the viability advantage be quite large. Under this hypothesis, if all the differences among crops were due to differential viability, the probability that a selfed seed (relative to that for an outcrossed seed) which is viable at crop A, would be viable two seasons later is (85-66)/34 = 0.56.

Eucalypts are vector pollinated, in contrast to the majority of Gymnosperms which are wind pollinated. Apparently for most eucalypt species there are numerous non-specific vectors (Pryor 1976; Ashton 1975). In the ash group of eucalypts, most of these vectors are insects, and several different types of species are involved including many very localised non-flying species. With these latter vectors selfing would be enhanced by the large crowns with numerous flowers on mature eucalypt trees. Perhaps the mixed mating system is at least in part a consequence of the generality of the vector system, with the latter maximising a strategy of high fecundity wherever possible. It would be of considerable interest to study eucalypt species which were predominantly pollinated by non-insect vectors such as birds, i.e. some winter flowering species such as *E. caesia* (Hopper, personal communication).

From the data it appears that selection occurs in the seed maturation phase of the life cycle but further work is required to estimate viability and fertility selection components in the various stages of the eucalypt life cycle.

Maintenance in natural populations of a mixed mating system by subsequent selection in the next generation has implications for applied breeding programs with eucalypt species. The present trend is towards the use of seed orchards as the source of improved commercial seed. However this procedure may lead to the perpetuation of some of the inbreeding component, which would perhaps not survive in a natural situation.

If the heterogeneity between seed crops is a widespread phenomenon, then when seed collections are made from natural populations either for the purposes of conservation of genetic resources or as a source for plantations, the optimal strategy would be to collect preferentially from the older crops where possible, especially when the number of trees that can be sampled in a population is limited.

Acknowledgement

The seed collections in the Pilot Hill population were made in collaboration with D.J. Boland. We thank J.C. Bell for excellent assistance with the laboratory work. We are grateful to our colleagues D.J. Boland, P.P. Cotterill, K.G. Eldridge and D.R. Marshall for their critical reading of the manuscript.

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Accepted December 6, 1979
Communicated by R.W. Allard

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