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Variation in allele frequencies in the outcross pollen pool of *Eucalyptus regnans* F. Muell. throughout a flowering season

Y. J. Fripp,*
A. R. Griffin† and
G. F. Moran†

* Department of Genetics and Human Variation, La Trobe University, Bundoora 3083, Australia and † CSIRO Division of Forest Research, PO Box 4008, Canberra 2600 Australia.

Individual trees within a stand of *Eucalyptus regnans* were found to vary in fecundity and time of flowering. Allele frequencies at three enzyme loci were estimated for the outcross pollen pool taking these differences into account, and were found to vary with time. These frequencies were compared to those estimated using methods that assume allele frequencies in outcross pollen pools are constant throughout a flowering season. For times when the bulk of trees were in flower the allele frequencies estimated by all methods were similar, but early and late in the season they differed dependent on the genotypes of the trees in flower. The demonstrated temporal heterogeneity of allele frequencies did not have a major effect on single locus, maximum likelihood estimates of the level of outcrossing in this population.

INTRODUCTION

Flowering time differences between plants have been found in populations or many species (*e.g.*, Ashton, 1975; Bawa, 1983; Cooper, 1959; Florence, 1964; Frankie *et al.*, 1974; McNeilly and Antonovics, 1968; Medway, 1972; Stern and Roche, 1974; Westerman, 1971). In such populations the potential exists for allele frequencies in the outcross pollen pool to change during the flowering season as the flowers open at different times may show different genotype frequencies.

Previous authors have discussed this issue (Bijlsma *et al.*, 1986; Friedman and Adams, 1984; Schoen and Clegg, 1984; Stam, 1983; Stern and Roche, 1974), but have not attempted to measure the degree of such temporal heterogeneity in specific populations. In this paper a method for taking inter-plant variation in flowering phenology and fecundity into account when estimating allele frequencies in outcross pollen pools is described.

This method is applied to data from a stand of the mass-flowering, preferentially outcrossing and predominantly insect-pollinated forest angiosperm, *Eucalyptus regnans* F. Muell. Pollen allele frequencies at three enzyme loci are first estimated as a function of time taking into account the differences in flowering phenology and ignoring the differences in fecundity. Frequencies are then estimated taking account of the differences in both phenology and fecundity. These allele frequencies, and associated estimates of the average level of outcrossing for the stand (t), are then compared to those derived by three alternative procedures which assume that allele frequencies are constant throughout a flowering season.

MATERIALS AND METHODS

1. Collection of data

The 30 plants studied form a discrete sub-unit of a remnant natural stand at Narracan, Victoria, Australia. The flowering phenology of this stand had been monitored over a number of years and these trees were known to differ in their dates of first, maximum and last flower production (Griffin, 1980). The flowering time differences between specific trees are consistent over years (Griffin, 1980; Griffin and Fripp, unpublished). The trees also differed in crown size (fig. 1). The genotype of each tree had been determined previously for a number of loci polymorphic for enzymes present in embryos or newly germinated seedlings (Moran and Bell, unpublished).

The nearest *E. regnans* trees to this study group were separated by 85 m at the closest point (see fig. 1 in Griffin, 1980) and have been shown to

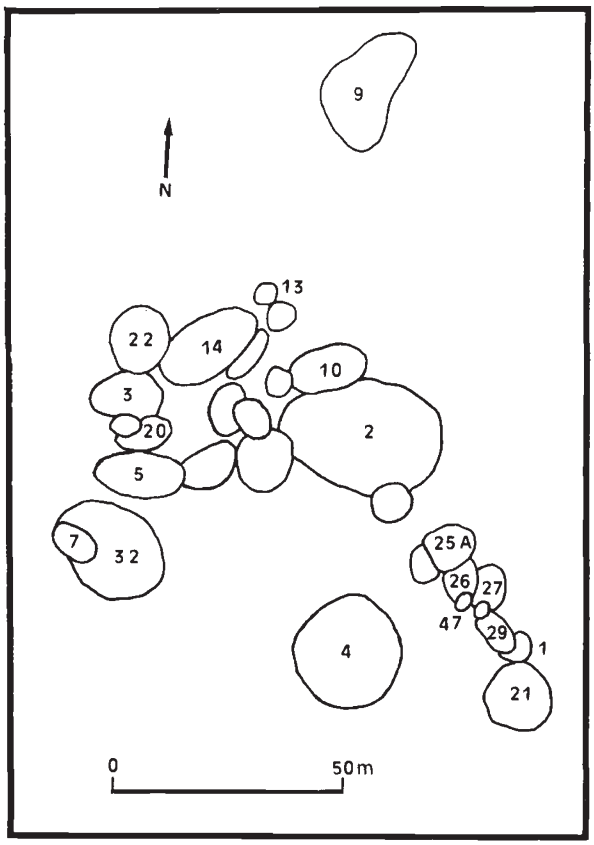

Figure 1 Map of the study area indicating the spatial relationships and relative crown sizes of the 30 trees studied. The 19 trees from which progeny samples were taken are numbered.

flower later than the study trees closest to them (Griffin, 1980). These trees were assumed to make a negligible contribution to the outcross pollen pool in the study area.

The dates on which open flowers were first and last present on each tree in the 1982 flowering season were estimated by scanning the crowns of all 30 trees using binoculars at approximately 3 day intervals and recording the presence of open flowers. The date of maximum flowering (greatest number of flowers open at one time) was also recorded for each tree. As pointed out by Griffin (1980) this date is subjective as it depends on the observer's perception of the change in flower density from one date to the next. The total number of flowers produced by each tree was estimated by counting the number of buds, open flowers and developing capsules on one large and typical branch (flowering unit) at maximum flowering and then multiplying this figure by an estimate of the number of such flowering units on the tree.

In January 1983 samples of capsules from the 1982 flower crop were collected from the 19 trees with good capsule crops and readily accessible crowns. For 15 of these trees samples were taken from two or more positions. Random sampling over the entire crown was not possible in this tall forest species. Embryos or cotyledons from ripe seeds of each of these 19 trees were then assayed for an alcohol dehydrogenase locus, *Adh*-1, a malate dehydrogenase locus, *Mdh*-2, and an aspartate aminotransferase (glutamic-oxaloacetic transaminase) locus, *Aat*-2 (Moran and Bell, 1983) following standard starch gel electrophoretic procedures (Shields *et al.*, 1983). For the *Adh* and *Mdh* enzymes, embryos from seeds soaked overnight were ground up in buffer and the extracts run on histidine gels (Fripp, 1982; Moran and Bell, 1983). For *Aat*, seeds were germinated and extracts from young cotyledons run on lithium borate gels (Moran and Bell, 1983). The extraction buffers and staining solutions were as described by Fripp (1982), except that the cotyledons were extracted in 0·5 M borate buffer *p*H 9·0 containing dithiothreitol (1 mg/ml) and polyvinylpyrrolidone (Sigma PVP 40T, 10 mg/ml).

2. Estimation of allele frequencies in the pollen pool as a function of time

Flowering of individual plants

The present field observations and previous data on the percentage of flowers open at successive time intervals on individual trees (Griffin, 1980) indicated that the opening of flowers on a *Eucalyptus regnans* tree follows a unimodal pattern of few open flowers initially, then a steady increase until the maximum number of open flowers is reached, followed by a decline to few and then zero open flowers. Both the normal and beta frequency distributions were considered as approximations of this process, but the beta was selected for two reasons. Firstly, a beta distribution need not by symmetrical about its mode and for some of the trees studied the number of days from first flower to maximum flowering was markedly different from that for maximum to last flower. Secondly, a beta distribution is defined over a finite range and the parameter values at which the density is to become zero are readily specified.

Thus, $f_i(x)$, the density of flowers opening at time x for tree i was estimated as:

$$f_i(x) = k_i(x - a_i)^{\alpha_i - 1}(b_i - x)^{\beta_i - 1} \qquad (1)$$

where k_i is the constant

$$\frac{\Gamma(\alpha_i+\beta_i)}{\Gamma(\alpha_i)\Gamma(\beta_i)(b_i-a_i)^{(\alpha_i+\beta_i-1)}}$$

and $\Gamma(\cdot)$ is the gamma function. $f_i(x)$ is defined to be zero for all x values $\leqq a_i$ or $\geqq b_i$. a_i and b_i were estimated as three days before (a_i) and after (b_i) open flowers were first and last seen on tree i. α_i and β_i are positive shape parameters. A beta distribution is symmetrical about its mode when $\alpha_i = \beta_i$, skewed to the right for $\alpha_i > \beta_i$, and skewed to the left for $\alpha_i < \beta_i$ (Johnson and Kotz, 1970).

The mode (y_i) of $f_i(x)$ is:

$$y_i = \frac{(\alpha_i-1)b_i+(\beta_i-1)a_i}{(\alpha_i+\beta_i-2)}. \qquad (2)$$

Estimation of α_i and β_i: The observed date of maximum flowering for tree i was the mode (y_i) of the beta distribution fitted to tree i. This value and a_i and b_i were substituted into equation 2, and the β_i values corresponding to α_i values ranging from 1 to 4 calculated. (A value around 2 for α_i was suggested by graphs in Johnson and Kotz, 1970). The resulting beta distributions were plotted and those based on $\alpha_i = 1{\cdot}5$ selected for use in the following equations. (A subjective decision on the α_i value had to be made here as observations on the proportion of flowers open on individual trees at successive time intervals were not available. The α_i values does not appear to be critical, however, as the allele frequencies estimated for the pollen pool of this stand did not differ substantially between α_i values of 1, 1·5 and 2.)

Pollen allele frequencies at time (x) for the entire population

$P_l(x)$, the frequency of allele l at time x in the total pollen pool of the stand was estimated as:

$$P_l(x) = \frac{G_l(x)}{\sum_{l=1}^{m} G_l(x)} \qquad (3)$$

where

$$G_l(x) = \sum_{i=1}^{n} f_i(x) N_i A_{il}$$

$f_i(x)$ = flower density at time x for tree i.

n = number of trees in the population.

m = number of alleles.

N_i = total flowers on tree i.

A_{il} = allele weighting of tree i for allele l.

The allele weighting, A_{il}, is 2 if tree i is homozygous for allele l, 1 if it is heterozygous for allele l, and 0 if it is homozygous or heterozygous for alleles other than l.

Allele frequencies in outcross pollen pool of tree i

$p_{il}(x)$, the frequency of allele l at time x in the outcross pollen pool of tree i was estimated by:

$$p_{il}(x) = \frac{g_{il}(x)}{\sum_{l=1}^{m} g_{il}(x)} \qquad (4)$$

where

$$g_{il}(x) = \sum_{j=1}^{n} f_j(x) N_j A_{jl} W_{ij}.$$

j refers to the tree whose contribution to the outcross pollen pool of tree i is being assessed.

W_{ij} specifies the relative contribution of the jth plant to the outcross pollen pool of tree i. $W_{ij} = 0$ for $j = i$. For the present set of data, the other $(n-1)\,W_{ij}$ values for each tree (i.e., $j \neq i$) were set equal to 1 and the assumption made that each tree contributed equally to the outcross pollen of every other tree in the stand. This was necessary as the relationship between pollen dispersion and factors such as inter-tree distance and canopy structure was unknown for this or similar species.

'Mean' allele frequencies in the outcross pollen pool of tree i over an entire season

ρ_{il}, a single allele frequency summarising the occurrence of allele l in the outcross pollen pool of tree i over its entire fertilisation period was estimated by:

$$\rho_{il} = \frac{z_{il}}{\sum_{l=1}^{m} z_{il}} \qquad (5)$$

where;

$$z_{il} = \int_{a_i}^{b_i} f_i(x) p_{il}(x+\theta)\, dx.$$

$f_i(x)$ was the density of flowers opening on tree i at time x (estimated using equation (1)).

$p_{il}(x+\theta)$, estimated using equation 4, was the frequency of allele l in the outcrossing pollen pool of tree i at the time $(x+\theta)$ when the stigmas in the flowers opening at time x were receptive.

Eucalyptus regnans is protandrous with stigmas receptive approximately 10 days after anthesis (Griffin and Hand, 1979). Flowers opening at time x would have been pollinated by the pollen pool present at day $(x+10)$. Thus, a θ value of +10 was used. (θ would be zero for species where stigma receptivity and pollen release occur simultaneously, and negative for protogynous species.)

Numerical integration was used to calculate the above integral.

3. Comparison with other methods of estimating allele frequencies in outcross pollen pools

The pollen allelel frequencies estimated for the stand as a whole using the above formulae (methods 4 and 5 below), were compared to those from three other procedures.

Summary of the methods

Allele frequencies in the outcross pollen pool were estimated:

(1) From a maximum likelihood analysis for jointly estimating pollen allele frequencies (p_l) and level of outcrossing (t), assuming a mixed mating model (Brown *et al.*, 1975; Clegg *et al.*, 1978). The data used were the observed genotype frequencies in progeny arrays from 19 of the 30 trees. (*Progeny Genotypes, Ml Estimation*).

(2) From the genotypes of the 30 parent trees in the stand. (*Parent Genotypes*).

(3) From the genotypes of the 30 parent trees, after weighting each tree's genotype by the tree's relative fecundity. (*Parent Genotypes + Fecundity*).

(4) From the genotypes of the 30 parent trees and accounting for phenology using equations 1 and 3 (entire stand), or 1 and 4 (tree i). All N_i were set to 1 in equations 3 or 4. (*Parent Genotypes + Phenology*).

(5) As for method 4 but taking the estimated fecundities of the trees (N_i) into account. (*Parent Genotypes + Phenology + Fecundity*).

Methods 1, 2 and 3 assume that the allele frequencies are the same throughout a flowering season and thus make the implicit assumption that there are no flowering time differences between genotypes. Method 3 also assumes that the proportional contribution of each tree depends directly on its relative fecundity. Methods 2 and 4 both assume that each tree contributes the same number of pollen grains to the pollen pool.

Fit of observed and predicted progeny arrays

The allele frequencies estimated by the above five methods were further compared by examining their ability to predict the genotypic frequencies observed in progeny arrays from the 19 trees sampled. *Eucalyptus reganans* has a mixed mating system (Moran and Bell, 1983; Griffin *et al.*, in press), and the expected genotype frequencies for this comparison were obtained using the conditional probabilities in Clegg *et al.*, 1978). These formulae require an estimate of the level of outcrossing (t).

A population $\hat{t}$ was estimated by maximum likelihood for each of the five methods of estimating pollen allele frequencies. The first method (Progeny Genotypes, ML Estimation) estimates t jointly with the pollen allele frequencies. For the other four methods the pollen allele frequencies estimated for each tree were substituted into the formulae for the conditional probabilties of possible progeny genotypes, giving 19 sets of equations. The $\hat{t}$ giving the maximum likelihood of the observed 19 arrays of progeny genotype frequencies was then found.

For methods 4 and 5, the "mean" frequency (ρ_{ij}) estimated from equations 1, 4 and 5 was used in estimating the population t and in predicting genotypic frequencies in the 19 progeny arrays.

For the *Mdh*-2 and *Aat*-2 loci χ^2 goodness-of-fit tests were then carried out on the observed and predicted (expected) progeny data. The data for the *Adh*-1 locus contained numerous very low expected numbers of progeny (<1) and were thus not suitable for χ^2 analysis. For this locus, the five methods were compared using the total log likelihoods over all 19 trees (Kendall and Stuart, 1961).

The *Mdh*-2 locus has only two alleles and the frequency of heterozygotes in the progeny of heterozygous maternal trees is thus expected to be 0·5 (see Brown *et al.*, 1975). Hence, for the heterozygous trees, the χ^2_2 was partitioned into two χ^2_1, one χ^2_1 testing the null hypothesis that heterozygotes : homozygotes = 1 : 1, and the other testing that the ratio of the two homozygous genotypes was as expected from the allele frequencies. Only the latter χ^2_1 was included in the final pooled χ^2.

Allele frequencies in the outcross pollen pool of plants whose flowers open and are fertilised outside the main flowering period of a population are likely to differ from those for the rest of the population (Stern and Roche, 1974). In this protandrous species this prediction applies to the late flowering trees. Thus each total χ^2 or log likelihood was partitioned into two subtotals, one for the five

"late" trees (3, 14, 20, 22, 26) and one for the "bulk" of the trees sampled.

In all analyses there were equal numbers of progeny from each tree. The numbers per tree were 66, 65 and 60 for *Mdh*-2, *Adh*-1 and *Aat*-2, respectively.

RESULTS

Fecundity and flowering phenology

The 30 trees differed markedly in fecundity, the estimated number of flowers per tree varying from 500 to $1{\cdot}4 \times 10^6$ (table 1). The three largest trees each contributed more than 10 per cent of the total flowers compared with 0·005 per cent for the smallest. The cumulative percentages indicated that the six largest trees together contributed 62 per cent of the total flowers, and the six smallest, only 0·66 per cent.

The trees also differed in their dates of first, maximum and last flower production in 1982 (table 1), with no overlap at all between the earliest (tree 29) and latest (tree 14). The number of days over which individual trees flowered differed markedly, varying from 9 days (tree 30) to 78 (tree 22).

The beta distributions fitted to individual trees as approximations of their frequency distributions of flowering over time are illustrated for five trees in fig. 2.

The frequency distribution of open flowers for the stand as a whole, estimated from the beta distributions and estimated fecundities of the 30 trees, was unimodal with the maximum density at day 38 (fig. 3). This was very similar to the pattern observed in 1977/79 by Griffin (1980). Estimation of the relative contributions of individual trees to this estimated flower pool (fig. 3) as a function of time indicated a pattern of flower production likely to lead to temporal heterogeneity of pollen allele

Table 1 Observed number of flowers and floral phenology data for the 30 trees

Tree	Flowers			Flowering Times*			
		Percentage					
No.	Number	†	‡	First	Peak	Last	Period
2	1,470,000	14·86	14·86	17	48	74	57
4	1,433,500	14·49	29·35	20	34	55	35
9	1,032,000	10·44	39·79	20	41	61	41
32	881,500	8·91	48·70	17	34	49	32
14	745,500	7·54	56·24	49	72	100	51
10	606,000	6·13	62·37	23	41	63	40
5	519,500	5·25	67·62	27	48	70	43
22	465,500	4·71	72·33	32	62	110	78
19	310,500	3·14	75·47	20	38	65	45
21	273,500	2·77	78·24	17	37	54	37
3	270,500	2·73	80·97	33	55	70	37
16	253,000	2·56	83·53	17	38	57	40
7	236,500	2·39	85·92	27	36	62	35
1	198,000	2·00	87·92	30	48	64	34
11	163,500	1·65	89·57	17	31	62	45
27	142,000	1·44	91·01	12	41	66	54
29	138,500	1·40	92·41	11	21	41	30
25A	129,100	1·30	93·71	20	27	50	30
20	115,500	1·17	94·88	20	51	85	65
26	91,500	0·93	95·81	32	48	80	48
18	91,000	0·92	96·73	27	41	55	28
13	78,400	0·79	97·52	17	27	47	30
24	57,500	0·58	98·10	27	46	64	37
15	57,200	0·58	98·68	30	48	65	35
17	50,500	0·51	99·19	17	34	53	36
12	47,500	0·48	99·67	46	64	83	37
47	14,000	0·14	99·81	18	34	61	43
25B	12,900	0·13	99·94	20	27	50	30
30	5,000	0·05	99·99	27	32	36	9
28	500	0·005	100·00	17	26	41	24

* Number of days after February 1, 1982.
† Percentage of total flowers.
‡ Cumulative percentage.

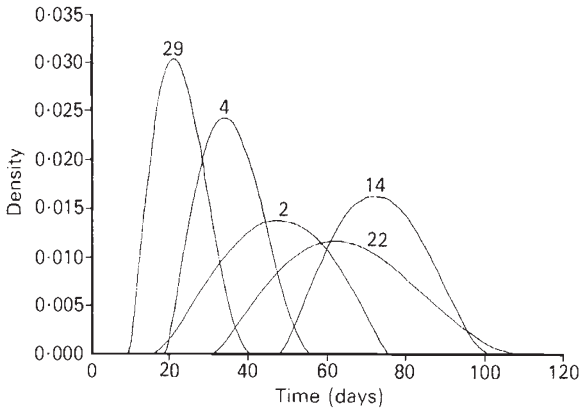

Figure 2 The beta distributions fitted to trees 2, 4, 14, 22 and 29 as approximations of the frequency distribution of their flowering over time.

frequencies. A high proportion of the flowers estimated to be open at any time during the 1982 flowering season were produced by one to few trees, and different trees were the dominant flower producers at different times. For example, trees 4 and 32 together were estimated to produce 54 per cent of the flowers at day 30, but only 0·04 per cent at day 55. In contrast, tree 2 was estimated to produce only 5 per cent of the total flowers at day 30 but 35 per cent at day 55. From day 86 onwards, trees 14 and 22 were the only ones flowering.

Population allele frequencies

The allele frequencies estimated for the pollen pool of the entire stand taking into account differences

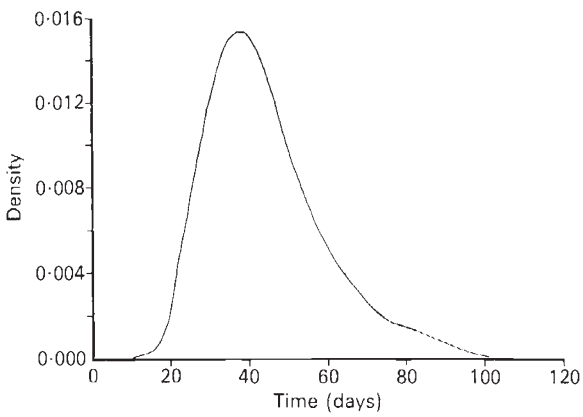

Figure 3 Estimated frequency distribution of flowering over all 30 trees.

in flowering phenology (method 4, dotted curve) and flowering phenology plus fecundity (method 5, solid curve) are plotted as a function of time in fig. 4 (*Mdh*-2), fig. 5 (*Aat*-2) and fig. 6 (*Adh*-1). The frequencies estimated using methods 1 to 3 are also shown.

For the *Mdh*-2 locus, the estimated frequencies taking phenology into account (methods 4 and 5) varied during the flowering season (fig. 4). For example, with method 5 the estimated frequency for the common allele (*Mdh*-2-1) commenced at 0·5 around day 12, increased up to 0·91 at day 30, then decreased back to 0·5 and remained there from about day 75 until flowering ceased. From day 25 to day 60 approximately, however, the frequencies did not differ substantially from the estimates of 0·84, 0·80 and 0·85 for methods 1, 2 and 3 respectively. From fig. 3 it was estimated that 80 per cent of all flowers opened and that 71 per cent of all stigmas were receptive in this 35 day period. Thus for times when the bulk of pollinations would have occurred, all five methods gave similar estimates of the allele frequencies in the total outcross pollen pool of the stand.

For the *Aat*-2 locus, the frequencies estimated using methods 4 and 5 changed with time but the changes were minor from day 20 to day 70 approximately when the bulk of pollinations would have occurred (fig. 5). All methods led to similar allele frequencies for ths main flowering period.

For the *Adh*-1 locus, the estimated allele frequencies in the outcross pollen pool from methods 4 and 5 (fig. 6) also changed during the season, with only the common allele present in the pollen after day 65. However, the major differences found at this locus were associated with taking into account the estimated fecundities of the trees. Allowing for fecundity gave very high frequencies for allele 1 (0·97–1·0) throughout the season; ignoring fecundity gave frequencies of 0·9 or lower for the main flowering period.

Estimates of t

For the *Mdh*-2 and *Aat*-2 loci and all methods of estimating frequencies, the maximum likelihood estimates of t were reasonably consistent with a median value of 0·55 (tables 2 and 3). For *Adh*-1, the estimates differed markedly between methods (table 4). The $\hat{t}$ from methods 3 and 5 were comparable to those from the *Aat*-2 and *Mdh*-2 data. Those from methods 1, 2 and 4, which gave higher frequencies for the rare allele *Adh*-1-2, were considerably lower.

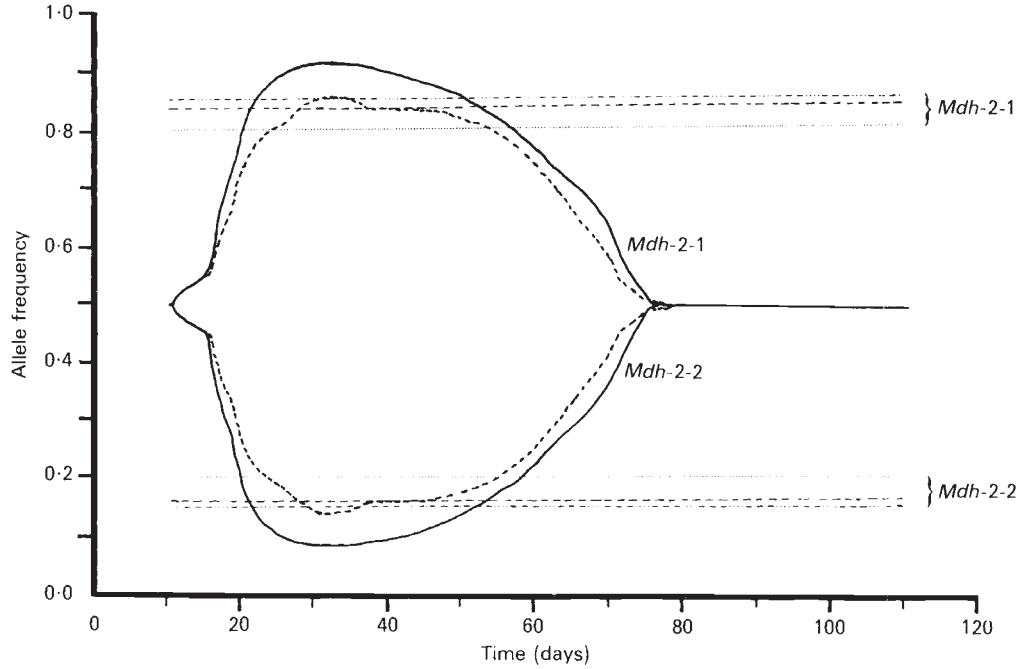

Figure 4 Estimated allele frequencies at the *Mdh*-2 locus for the outcross pollen pool of the entire group of 30 trees. The lines parallel to the *x* axis indicate the frequencies estimated using methods 1(– – –), 2 (· · · ·) and 3(– · · –). The curves show the estimated frequencies when account was taken of differences between trees in phenology (method 4, dashed curve) or both phenology and fecundity (method 5, solid curve).

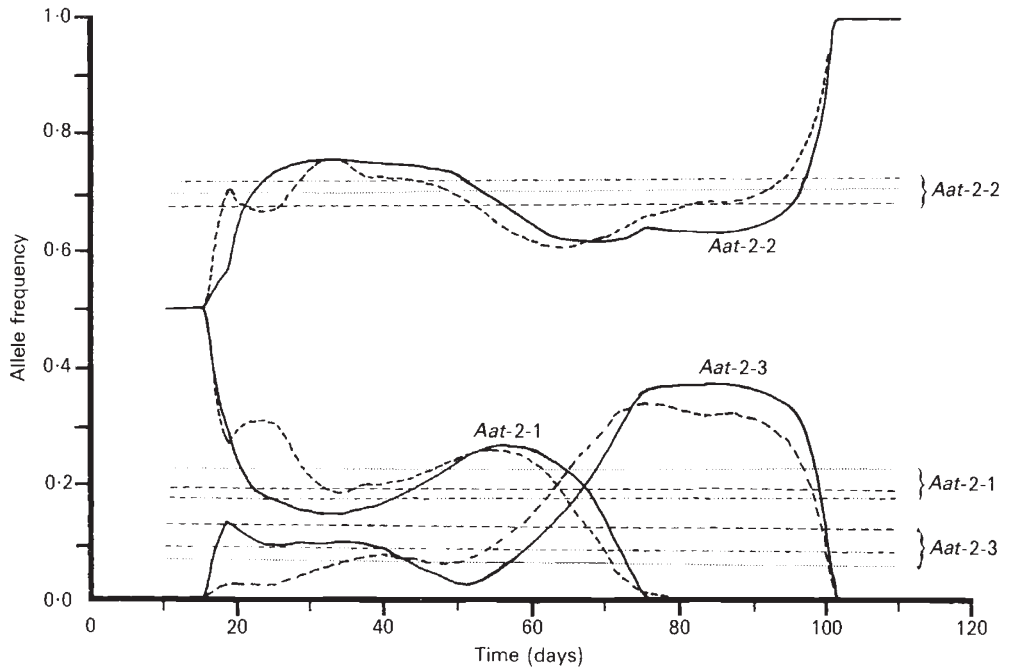

Figure 5 Estimated allele frequencies at the *Aat*-2 locus for the outcross pollen pool of the entire group of 30 trees. The lines parallel to the *x* axis indicate the frequencies estimated using methods 1 (– – –), 2 (· · · · ·) and 3 (· · – · · ·). The curves show the estimated frequencies when account was taken of differences between trees in phenology (method 4, dashed curve) or both phenology and fecundity (method 5, solid curve).

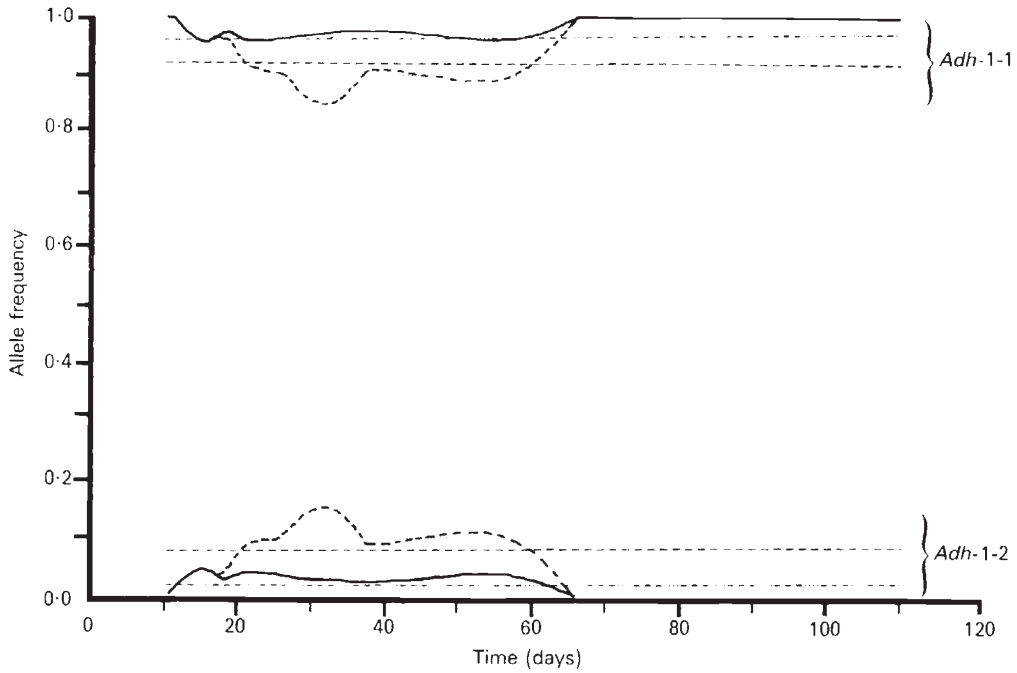

Figure 6 Estimated allele frequencies at the *Adh*-1 locus for the outcross pollen pool of the entire group of 30 trees. The lines parallel to the *x* axis indicate the frequencies estimated using methods 1 (– – –), 2 (· · · · ·) and 3 (· · – · ·). The curves show the estimated frequencies when account was taken of differences between trees in phenology (method 4, dashed curve) or both phenology and fecundity (method 5, solid curve).

Fit of observed and predicted progeny arrays

For the *Mdh*-2 data, taking the flowering time differences among trees into account led to significantly smaller total χ^2 with significance probabilities of 0·05 (method 4) and 0·14 (method 5) compared to values $\leqq$0·004 for methods 1 to 3 (table 2). The improvement was due to better predictions for the progeny of the five "late" trees. For this locus taking estimated fecundities of the trees into account together with their differences in flowering phenology further improved the predictions. Allowing for fecundity differences alone as in method 3 led to the best predictions for the "bulk" subgroup of trees, but the total χ^2 for this method was the largest due to very poor predictions for the late trees.

For the *Aat*-2 data, methods 1, 3, and 4 gave similar total χ^2 which were slightly lower than those for methods 2 and 5 (table 3). The significance probabilities for all five methods were extremely small ($P < 0{\cdot}00001$).

For the Adh-1 locus, the best total log likelihood was obtained using method 4 (table 4). Methods taking into account the estimated fecundities of the trees led to poorer predictions.

DISCUSSION

The present results, in particular those for the *Mdh*-2 locus, demonstrate that flowering time differences between plants may lead to temporal heterogeneity of pollen allele frequencies during a flowering season. In this *Eucalyptus regnans* stand the allele frequencies in the outcross pollen pool at times when the bulk of trees were in flower were different from those early and late in the season.

For *Mdh*-2 and *Adh*-1, the allele frequencies estimated taking flowering phenology into account gave predictions of genotype frequencies in progeny arrays that were better than those estimated from alternative methods which assume that allele frequencies in outcross pollen pools remain constant throughout a flowering season (tables 2 and 4). For the *Aat*-2 locus no predictions were good, but those when flowering phenology was taken into account (method 4) were best together with those from methods 1 and 3 (table 3).

For the *Mdh*-2 locus, taking the fecundity differences between the trees into account as well as their flowering time differences further improved these predictions (table 2). For *Aat*-2

Table 2 Goodness of fit χ^2 for the *Mdh*-2 locus, comparing observed progeny arrays with those expected from 5 alternative methods of estimating allele frequencies in outcross pollen pools (see text)

Estimation Method	$\hat{t}$*	χ^2 Total 19d*f*	Bulk 14df	Late 5df
1. Progeny Genotypes, ML Estimate	0·61	46·91 0·0004†	21·17 0·0974	25·74 0·0001
2. Parent Genotypes	0·54	46·34 0·004	28·58 0·0119	17·76 0·0033
3. Parent Genotypes + Fecundity	0·63	48·54 0·0002	19·76 0·1379	28·78 <0·0001
4. Parent Genotypes + Phenology	0·52	29·91 0·0530	26·49 0·0224	3·42 0·6355
5. Parent Genotypes + Phenology + Fecundity	0·60	25·50 0·1447	22·69 0·0655	2·81 0·7292

* Maximum likelihood t for the particular set of allele frequencies.
† Significance probability for the above χ^2.

Table 3 Goodness of fit χ^2 for the *Aat*-2 locus, comparing observed progeny arrays with those expected from 5 alternative methods of estimating allele frequencies in outcross pollen pools (see text)

Estimation Method	$\hat{t}$*	χ^2 Total 49d*f*	Bulk 36df	Late 13df
1. Progeny Genotypes, ML Estimate	0·53	112·77 <0·00001†	85·03 <0·00001	27·74 0·0098
2. Parent Genotypes	0·56	125·27 <0·00001	83·50 <0·00001	41·77 0·00007
3. Parent Genotypes + Fecundity	0·57	110·96 <0·00001	83·15 0·00004	31·76 0·0026
4. Parent Genotypes + Phenology	0·52	111·31 <0·00001	89·57 <0·00001	21·74 0·0595
5. Parent Genotypes + Phenology + Fecundity	0·52	128·04 <0·00001	100·80 <0·00001	25·74 0·0184

* Maximum likelihood t for the particular set of allele frequencies.
† Significance probability for the above χ^2.

Table 4 Total log likelihoods for the Adh-1 locus, comparing observed progeny arrays with those expected from 5 alternative methods of estimating allele frequencies in outcross pollen pools (see text)

Estimation Method	t*	—Log Likelihood Total	Bulk	Late
1. Progeny Genotypes, ML Estimate	0·41	392·41	370·60	21·81
2. Parent Genotypes	0·33	392·94	371·08	21·86
3. Parent Genotypes + Fecundity	0·53	398·69	380·10	18·58
4. Parent Genotypes + Phenology	0·38	386·68	369·12	17·56
5. Parent Genotypes + Phenology + Fecundity	0·54	396·55	379·29	17·26

* Maximum likelihood t for the particular set of allele frequencies

and *Adh*-1, however, taking account of fecundity as well as phenology led to larger χ^2 or log likelihood totals (tables 3 and 4). Examination of the χ^2 or log likelihood values for individual trees indicated that these increases were due to poorer predictions for a group of small trees with much higher than average frequencies of the less common *Aat*-2-1 and *Adh*-1-2 alleles. Because the relationship between level of gene dispersion and actors such as inter-tree distance is not known for forest species such as *Eucalyptus regnans*, it was assumed in the present analyses that the contribution that a tree made to the outcross pollen pool of any other tree was independent of the distance between the trees (i.e., $W_{ij} = 1$ in equation (4)). When differences in fecundity between trees were taken into account under this assumption, the genes these small trees contributed to each other's outcross pollen pools were swamped by those from the highly fecund trees some distance away which were homozygous for the common alleles *Aat*-2-2 and *Adh*-2-1. W_{ij} values that take into account spatial relationships of the trees would allow more realistic treatment of fecundity differences than was possible here.

Spatial heterogeneity of allele frequencies in the outcross pollen pool of a population has been shown to lead to underestimates of t using single locus estimation procedures (see Brown *et al.*, 1985; Ennos and Clegg, 1982). Temporal heterogeneity of pollen allele frequencies might also lead to biased estimates of t, and Bijlsma *et al.*, (1986) have recently suggested temporal heterogeneity as one possible reason for the lower $\hat{t}$ observed for two enzyme loci in their study of an open-pollinated population of maize. The effect of the present temporal heterogeneity on the estimation of t was examined for the *Mdh*-2 and *Aat*-2 loci by making single locus maximum likelihood estimates of t separately for the five "late" trees and the other 14 trees sampled. The Adh-1 data were excluded because all the late trees were the same genotype (*Adh*-1-1 *Adh*-1-1) and because of the extreme spatial heterogeneity of the distribution of *Adh*-1 genotypes in this stand. For the *Mdh*-2 locus, t estimates of $0{\cdot}61 \pm 0{\cdot}05$, $0{\cdot}69 \pm 0{\cdot}06$ and $0{\cdot}56 \pm 0{\cdot}10$ were obtained from all 19 trees sampled, the main group of 14 trees, and the five "late" trees, respectively. For the *Aat*-2 locus, the corresponding estimates were $0{\cdot}53 \pm 0{\cdot}05$, $0{\cdot}54 \pm 0{\cdot}06$ and $0{\cdot}57 \pm 0{\cdot}11$. This separation of the trees into two subgroups based on their flowering times did not lead to consistent nor significant changes in t. Thus there is no evidence in these data of biased single-locus t estimates due to the demonstrated temporal heterogeneity of allele frequencies.

The trees studied here, and stands of *Eucalyptus regnans* in general, have a single flowering period each year (Ashton 1975; Griffin, 1980), with the bulk of trees in any stand in flower together in the main flowering period, but with periods at the beginning and end of the season when there are only a few trees with open flowers. The present data indicate that for populations with such a pattern of flowering, estimates of allele frequencies based on the assumption that the outcross pollen pool is constant throughout a flowering season should be reliable for the majority of parent plants but are likely to be unreliable for any individuals that set a majority of seeds early or late in the season.

Without further studies the present conclusions are clearly only applicable to the particular distribution of flowering characteristics found in this stand of *Eucalyptus regnans*. Many plants have more irregular or protracted flowering periods than *E. regnans*. For populations of these species the biasses introduced by the assumption that the outcross pollen pool is constant throughout the flowering season might be more severe than indicated here.

Making observations on the relative flowering times of individual plants when carrying out studies of mating systems or gene flow in populations is recommended. This would enable plants that flower at different times to be recognised, and the differences in flowering times taken into account when sampling the population and analysing the data.

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