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Factors influencing the genetic structure of *Phacelia dubia*, a species with a seed bank and large fluctuations in population size

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The potential factors that may influence the genetic structure of the gynodioecious annual *Phacelia dubia* were assessed using electrophoretic and ecological information at a locality in which the population size changed by about three orders of magnitude. Fluctuations in population size appeared to have little influence on the allelic composition, heterozygosity and mixed mating system of the population. Despite wide fluctuations in total population size, the estimated effective population size during ($N_e = 20$) and after the bottleneck ($N_e = 28$) was little changed. Also, a significant spatial substructuring, evidenced by a cline in MDH and by the *F*-statistics, was observed before and after the bottleneck. The recovery of the population and the preservation of genetic diversity was attributable in part to the seed bank in the soil. Spatial gene flow via seeds was small compared with pollen flow and both were restricted. Nevertheless, substructuring contributes to a small portion of the total inbreeding. Also, the rate of apparent selfing of male steriles did not provide evidence of biparental inbreeding. Most of the inbreeding, however, was within subpopulations and autogamy appears to be the major contributor. It was concluded that the mating system is the leading factor determining the genetic structure and that the seed bank ensures genetic constancy in time in the face of large fluctuations in population size.

Keywords: bottlenecks, breeding systems, effective population size, gene flow, gynodioecy, population structure.

Introduction

Population genetic structure has been considered a major determinant of the rate of evolution (Wright, 1932, 1977, 1988). Indeed, structured populations can evolve faster than panmictic populations as the former have gene flow as an additional source of favourable combinations of genes which might have originated in other local populations (see also Slatkin, 1987). Among the multiple factors that modify population genetic structure, the mating system, by being a genetic link between generations, can be the most important (Allard, 1975; Loveless & Hamrick, 1984), but other factors may also be influential. Genetic drift is enhanced in small-sized populations or when dispersal is limited resulting in an overall increase in homozygosity (e.g. Turner *et al.*, 1982; Lacy, 1987). If population size fluctuates widely, the influence of drift can be intense as the generations with the smallest numbers have the most effect (Wright, 1969). On the other hand, factors such as the seed bank in the soil (Templeton & Levin, 1979) and gene flow among populations (Slatkin, 1987) are expected to offset the effects of drift. Also, in partially inbred populations, mutational load and heterozygote advantage can increase the levels of heterozygosity of the populations even at neutral loci unlinked to those directly affecting fitness (Haldane, 1949; Ohta & Cockerham, 1974; Hedrick, 1980; Charlesworth, 1991). Hence, understanding population genetic structure can be a useful tool to assess the action and interaction of various evolutionary forces in natural populations (Selander & Whittman, 1983).

There is a considerable amount of information on the genetic structure of plant populations (e.g. Clegg & Allard, 1972; Schaal & Smith, 1980; Schwaegerle *et al.*, 1986; Dewey & Heywood, 1988; Soltis & Soltis, 1988) and assessing the factors determining such structure is an active area of research. More studies are

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needed that integrate ecological and genetic information to provide a better picture of how genetic structure is shaped. For instance, fluctuations in population size are expected to be common in annual plants and in other species (Mac Arthur & Wilson, 1967) but only a few studies have documented how the population structure changes during such fluctuations (Epling *et al.*, 1960; Gottlieb, 1974). As many factors appear to act simultaneously in determining population genetic structure, it is necessary to consider as many potential factors as possible before we can draw conclusions about their relative importance.

This paper documents the spatial and temporal variation in population genetic structure in a gynodioecious annual plant over a 4-year period using electrophoretic markers. I also combine ecological and genetic information to explore the role of the different factors that might affect such structure. Specifically, I examine how the breeding system, population subdivision, gene flow and fluctuations in population size contribute to the levels of inbreeding and genetic variability of the population. The existence of male sterile individuals allowed estimations of biparental inbreeding and pollen flow. Of particular interest is the fact that during the study time the total population size fluctuated over several orders of magnitude. I also provide evidence of the importance of the seed bank in the soil in maintaining population size and genetic structure.

Materials and methods

Study system

Phacelia dubia (L.) Trel. (Hydrophyllaceae) is a winter annual distributed in the southeastern U.S.A. It grows in diverse habitats ranging from granite outcrops to forests (Putman, 1964; Murdy, 1966). Flowering at the study site takes place from mid-April to the first week in May. Most of the plants are hermaphroditic and self-compatible (Levy, 1988; del Castillo, 1992, 1993) but male sterile plants, characterized by their lack of pollen production, have been observed in disturbed places near railroad embankments in south western Virginia (Levy, 1991; del Castillo, 1993). Male sterility appears to be controlled by nucleocytoplasmic factors (F. Levy, L. E. Broaddus, and R. F. del Castillo, unpublished data). The flowers are visited by syrphid flies and a variety of polylectic bees, including halictid bees which appear to be the major pollinators. Neither the seeds nor the fruits have any obvious adaptations for either wind or animal dispersal. The fruit is a capsule which discharges the seeds during and after the plants have senesced. Apparently, the desiccation of the fruit creates tension inside the fruit wall that forces out the seeds. Most of the seeds fall in the immediate neighbourhood of the parent, as indicated by observations of the trajectory of the seeds when discharged from the fruits and the distribution of seedlings in the field (R. F. del Castillo, personal observation). Seeds usually germinate in September and October; however, Spring germination has been observed occasionally (Murdy, 1966; R. F. del Castillo, personal observation). The seeds can remain dormant in the soil for several years (Baskin & Baskin, 1973, 1978). In an early study of isozyme variation in this species carried out on 12 localities, Levy (1989) detected 38–60 per cent of polymorphic loci based on 14 enzymes assayed.

Study site

The study site is located in Montgomery County, approximately 1.5 km west of Radford Arsenal, VA. Here the individuals of *P. dubia* are distributed in patches (clusters of plants) along a strip of gravel and soil, approximately 860 m long and 0.5–4 m wide, situated between a railroad track and hardwoods. This set of patches was considered as the population for the purposes of this study. Other plants of the same species have been found along this railroad and in nearby woods but they are separated by at least 1.5 km from the study site. *P. dubia* occurs in association with *Acer negundo, Actinomeris alternifolia, Cardus nutans, Cardamine hirsuta, Festuca obtusa, Specularia perfoliata* and *Veronica peregrina*. This disturbed habitat, unusual for *P. dubia* (Levy, 1991), and the existence of male sterility without any detectable fitness advantage (del Castillo, 1993) suggest that the occurrence of this species is relatively recent at the study site.

Population size, density and frequency of male sterility

Figure 1 shows the positions of the patches for which distribution and number of individuals were monitored, during the flowering season, over 5 years. Each cohort established in the Fall, together with the occasional individuals that germinated in the Spring, is identified by the year of flowering. The population size was estimated from measurements of the length and width of each patch and from estimates of plant density within each patch based on 20–100 quadrats of 0.25 or 0.70 m^2. At each quadrat, the numbers of male steriles and hermaphrodites were tallied. In 1989, all members of the population were mapped. The estimates of population size were based on flowering individuals, which essentially corresponded to the entire population, at the time of the tallies.

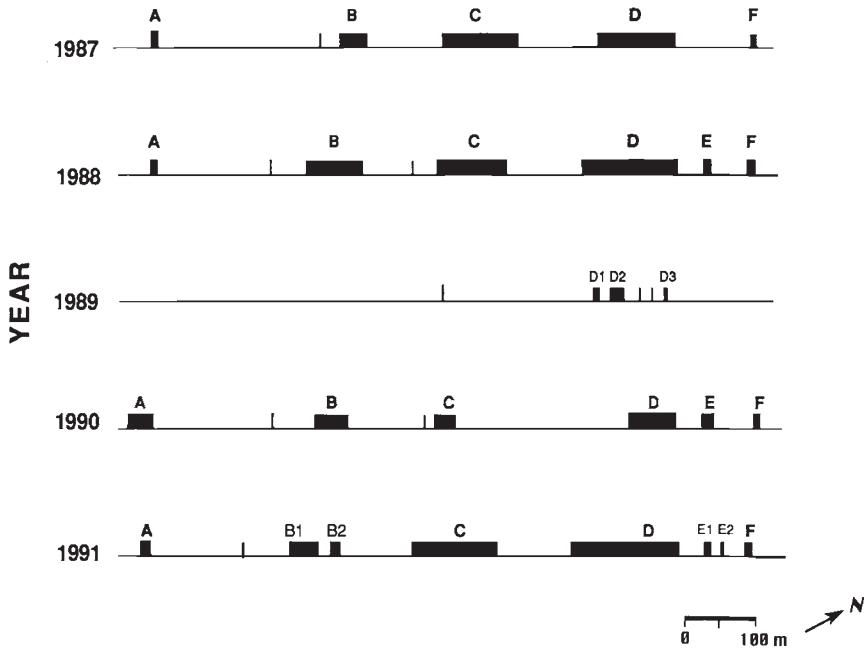

Fig. 1 Dynamics of patch distribution of *Phacelia dubia* near Radford, VA from 1987 to 1991. The relative position of the patches at flowering time is indicated. The rectangles indicate presence of patches and vertical lines indicate the position of isolated individuals. Letters above the patches indicate the subdivision used in the *F*-statistics analysis. I did not sample plant material for electrophoretic analyses in 1987.

Seed and pollen dispersal

In 1988, I estimated pollen dispersal in the field by constructing an experimental population approximately 500 m from the study site but along the same railroad track. Ten hermaphrodite individuals, to be used as a pollen source, were collected at different positions from the study site and transplanted at the beginning of the flowering season into one quadrat (50 cm × 50 cm) at the experimental site. On one side of the hermaphroditic plants, pairs of male sterile individuals were planted at intervals of 0.4 m in a 12-m linear array. As some male steriles did not survive the planting, one plant from each pair was removed, leaving only one plant per interval. All the flowers and fruits of the male sterile plants were detached leaving only flowering buds at the time of planting. The plants were left until the end of their life cycle and then collected. Effective pollen dispersal was estimated by the percentage of fruit set (fruit to flower ratio) determined on the male sterile plants as a function of the distance from the pollen source.

To estimate the seed dispersal curve, five individuals were collected from the field at the end of the fruiting season. These plants with mature fruits on them were positioned individually on an unobstructed surface so that their heights were the same as those in the field and the capsules were allowed to dehisce and discharge the seeds. I measured the dispersal distances of 240 seeds from the stem of the maternal plant to construct a frequency distribution of dispersal distances.

Collection of material for electrophoresis

In 1988, 1989 and 1990, seeds were obtained from 5 to 10 capsules per individual from 90, 27 and 38 naturally occurring plants. After germination in a cold chamber, the seedlings were transplanted to plastic pots and placed in the greenhouse or growth chamber, as described in detail elsewhere (del Castillo, 1992). From each maternal plant, the leaves of randomly selected offspring in early rosette stage were used for electrophoresis. To compare the genetic structure at two different stages of the life cycle, I also analysed electrophoretically the 38 maternal plants of the 1990 seeds and a sample of 100 plants at flowering stage collected in the field in 1991.

Electrophoresis

Horizontal starch gel electrophoresis was performed on enzymes extracted from young leaf tissue. The enzymes were extracted as described in del Castillo (1992). The electrophoretic procedures, buffer recipes and staining protocols follow Soltis *et al.* (1983) and Levy (1989). The following four polymorphic enzymes were scored and interpreted: phosphoglucoisomerase-2 (PGI, E.C. 5.3.1.9), phosphoglucomutase (PGM, E.C. 2.7.5.1), isocitrate dehydrogenase-2 (IDH, E.C. 1.1.1.42) and malate dehydrogenase (MDH, E.C. 1.1.1.37). PGI and PGM were assayed in a LiOH-borate pH 8.3 gel buffer system at 40 mA and 80–100 V. IDH and MDH were assayed in a histidine-

citrate pH 5.7 buffer system at 30 mA and 150 V (maximum). Gels ran for 6–7 h at 4°C. Genetic interpretation was based on segregation of progenies of naturally and artificially pollinated plants which suggested Mendelian inheritance (R. F. del Castillo, unpublished data; Levy, 1989 and personal communication). The loci were diallelic, except for PGI which was triallelic, with two common and one rare allele (slow, frequency ≤ 0.027). No evidence of linkage disequilibrium was detected in any of the pair-wise combinations of allozyme loci utilized (del Castillo, 1992).

Analysis

Population genetic structure was examined with log-linear models for categorical data analysis (Freeman, 1987) and Wright's *F*-statistics (see Wright, 1969). The former analysis was performed using genotypic frequencies as data entries and the procedure CATMOD of SAS (option ML, SAS Institute, 1987). The frequencies were pooled across years, excluding the juveniles of 1990 as they were derived from the sampled adults of that year and, therefore, did not provide independent information.

Spatial genetic structure was also analysed with Wright's *F*-statistics using the analysis of variance approach of Weir & Cockerham (1984). In this analysis, F is the overall inbreeding coefficient, i.e. the correlation of alleles within individuals in relationship to all populations (patches in this study), Θ measures the degree of differentiation between populations and f measures the correlation of alleles within populations. These three parameters are related to Wright's *F*-statistics as $F = F_{IT}$, $\Theta = F_{ST}$ and $f = F_{IS}$ (Cockerham, 1969). Means of the estimates of these parameters were calculated by jackknifing over loci and 95 per cent confidence intervals were constructed from 1000 ordered bootstrap estimates, using loci as a resampling unit (Weir, 1990). This allowed a test of the null hypothesis that the value of the parameters is zero. The subdivision units were the patches observed in the population but in 1989, when the population numbers were small, the analysis was carried out at a level of subdivision (subpatches) that was at a smaller scale than that in the other seasons (Fig. 1). Patches labelled with the same letters corresponded to the same location across years and were pooled as a single patch for the pooled analyses. *F*-statistics analyses, where appropriate, were performed both for each season for juveniles (young rosette stage) and adults and by pooling the data available across years for each of the two stages of the life cycle studied. To avoid bias due to differences in offspring number sampled per plant, for the estimates of allelic frequency and for the *F*-statistics analyses one plant from each progeny was randomly chosen.

Single locus and multilocus estimates of outcrossing and their variances were obtained using the mixed mating model of Ritland & Jain (1981). Outcrossing estimates during 1989 and 1990 were based on the progenies of 27 and 38 hermaphroditic plants, respectively, with an average of five and 10 offspring per family, respectively. In 1990, the genotypes of the parental plants were determined. Homogeneity of the single locus estimates of outcrossing for the 2 years studied was tested with a chi-square test developed for maximum likelihood estimates (Bailey, 1961).

Genotypic correlations in outcrossing matings can be an additional source of inbreeding and a factor that biases downwards outcrossing estimations based on the mixed mating model (Ennos & Clegg, 1982; Ritland, 1984). One way to detect such genotypic correlations is to estimate the rate of apparent selfing in male sterile plants (Sun & Ganders, 1988). An additional outcrossing estimation was made, therefore, in 1990 using all 11 male sterile plants that produced viable seeds in the study site in that season. An average of 12 individuals per maternal plant was used in this study. The genotypes of the male sterile maternal plants were also determined.

Results

Fluctuations in population size and frequencies of male sterility

During the study period, the total population size fluctuated over three orders of magnitude. Flowering plants were abundant in 1987 and 1988 but this was followed by a population crash in 1989. A partial recovery in population size was observed in 1990 and a full recovery in 1991, with an estimated population size of the same order of magnitude as in 1987 and 1988 (Fig. 2). The plants were distributed in a series of patches of relatively constant position throughout all the studied seasons (Fig. 1). The exception was the plants of 1989 which were distributed in small patches in what corresponded to patch D in the other years. The low population size in 1989 was probably due to a severe drought in 1988 (Trenberth *et al.*, 1988). The proportion of male sterile plants also changed considerably, being close to 10 per cent in 1988 and in 1989, less than 1 per cent in 1990 and close to 5 per cent in 1991 (Fig. 3).

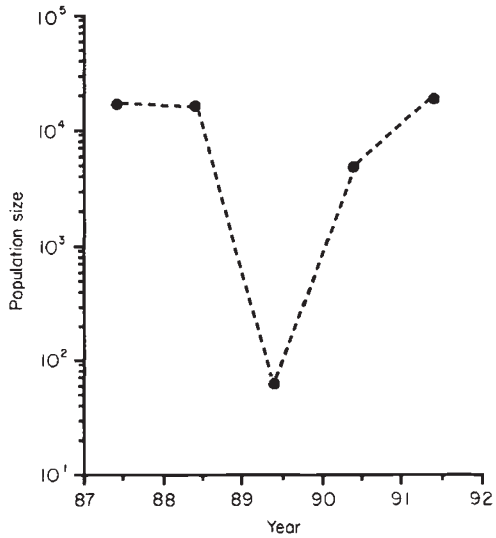

Fig. 2 Changes in population size from 1987 to 1991 (estimated number of flowering plants, see text).

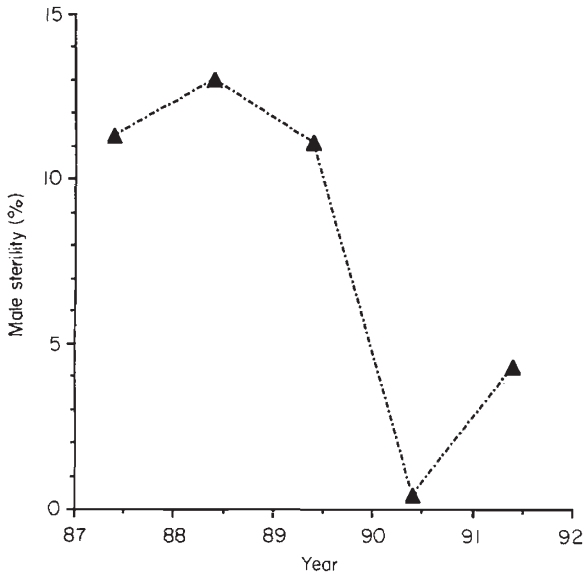

Fig. 3 Frequency of male sterile plants from 1987 to 1991.

Pollen and seed dispersal

The shapes of the curves of pollen and seed dispersal did not differ significantly from that of a normal distribution ($P > 0.4$, Shapiro–Wilk Statistics, Conover, 1980). Fruit set declined as the number of individuals between the pollen source and the recipient plant increased and plants separated by more than eight individuals from the pollen source did not set fruit (Fig. 4). The standard deviation of pollen dispersal in the experimental population was 1.3 m or 3.4 individuals from the source plant in a given direction. The latter quantity is more informative as pollinators usually tend

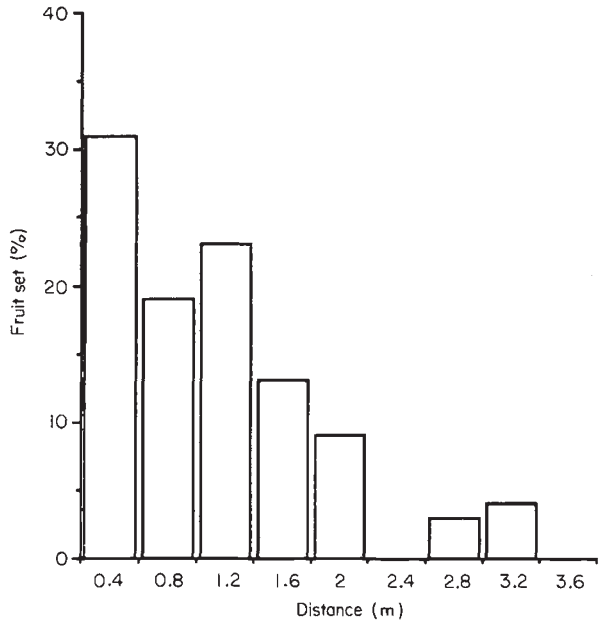

Fig. 4 Relationship between pollen dispersal, expressed as fruit set on male sterile plants, and distance from the pollen source.

to forage shorter distances when plants are dense (Levin & Kerster, 1974; Handel, 1983a; Fenster, 1991). This appears to be the case for *P. dubia* at the study site as no other major alternative floral resources were available for pollinators during its flowering period. Seed dispersal was even more restricted: 94 per cent of the seeds were dispersed within the first 0.4 m from the maternal plant and no seeds were detected beyond 0.8 m (Fig. 5). The standard deviation of seed dispersal was 0.2 m.

Allelic frequencies

Despite the changes in population size, polymorphism at all isozyme loci monitored was maintained during all years in the juveniles and adults analysed. Indeed, for *PGI*, *MDH* and *IDH* the allelic frequencies were remarkably constant over time (Figs. 6 and 7). *PGM-f* showed a trend towards reduction in frequency with time (Fig. 6) but the population bottleneck of 1989 had no visible effect.

Spatial subdivision and F*-statistics*

The frequency of the fast allele of *MDH* increased from south to north (Fig. 8). This trend was consistent across seasons and was also evident in the log-linear analysis after the Bonferroni correction for experiment-wise error considering that four enzymes were analysed (Weir, 1990; $\chi^2_{[2]} = 15.35$, adjusted $P < 0.003$).

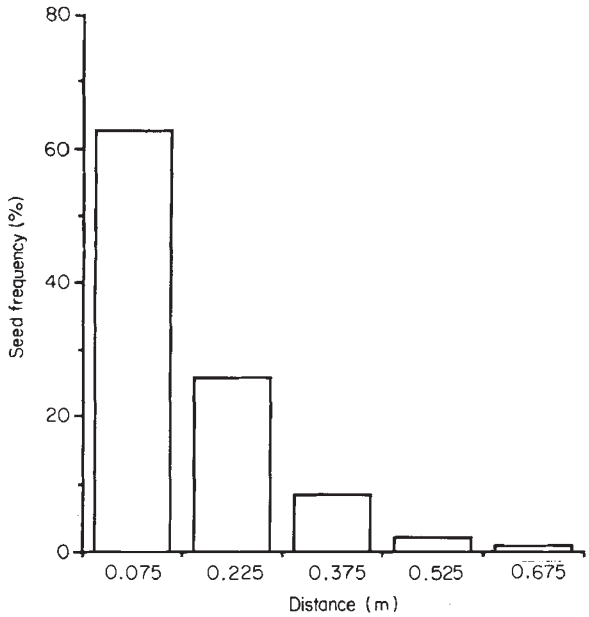

Fig. 5 Distribution of seed dispersal distances from the maternal plant.

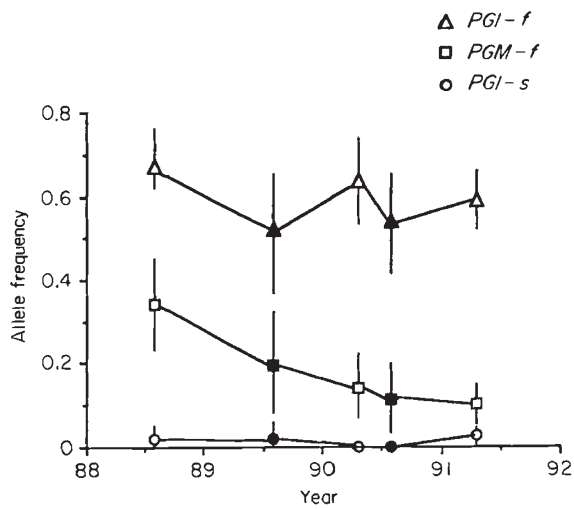

Fig. 6 Allelic frequencies and 95 per cent confidence intervals from 1988 to 1991 of *PGI* fast and slow and *PGM* fast. The confidence intervals were constructed by bootstrapping over individuals. Open figures represent seed samples taken from plants collected in the field. Solid figures represent samples of field flowering individuals.

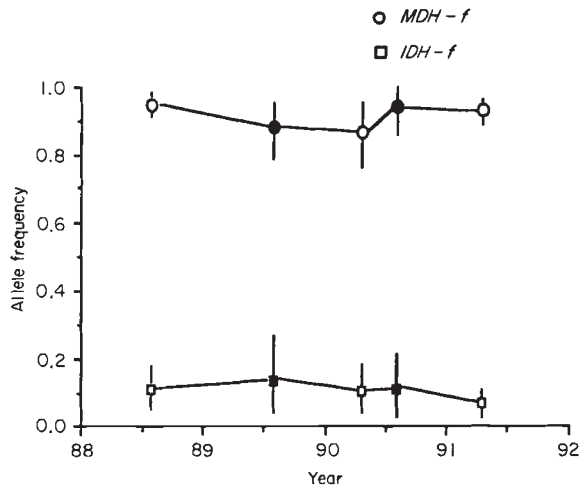

Fig. 7 Allelic frequencies of *IDH* fast and *MDH* fast and 95 per cent confidence intervals from 1988 to 1991. Symbols and confidence intervals as in Fig. 6.

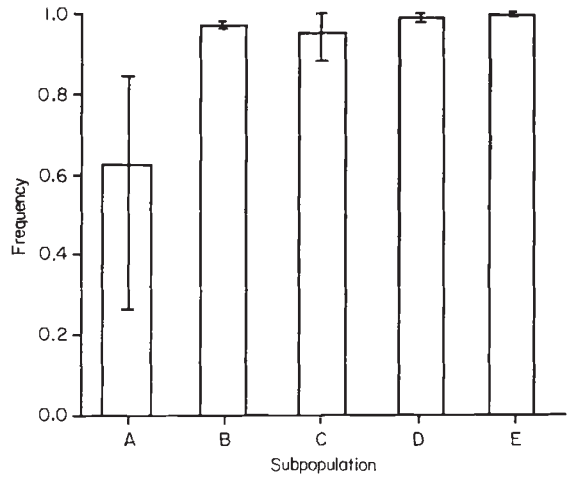

Fig. 8 Means of frequencies of the fast allele of *MDH* in each of the subpopulations (patches). Letters correspond to the subpopulations as shown in Fig. 1. Subpopulations with common letters were pooled. Vertical bars are bootstrapped 95 per cent intervals of sampling periods (= ranges).

No relationship between the spatial arrangement of the patches and genotypic frequencies was detected for any of the other loci studied (analyses not shown).

The combined estimates over all the alleles sampled of F, the total inbreeding coefficient, revealed heterozygote deficiency in all seasons and ontogenetic states (Table 1). This deficiency tended on average to be higher in juveniles than in adult plants. The primary component of F was at the level of individuals within patches and was significantly different from zero in all the cases studied. Also, there was a smaller component of F due to population subdivision at the level of patches as seen in the pooled analysis on the juveniles and in the analyses on the adults.

The relative strengths of drift in population substructure can be assessed by the estimation of $N_e m$, where N_e is the effective size of the population and m is the migration rate (Wright, 1932). In an n-island model, for neutral alleles, and provided that m is small, Θ approaches the formula:

$$\Theta = (4N_e m\alpha + 1)^{-1}, \quad (1)$$

where $\alpha = [n/(n+1)]^2$ (Crow & Aoki, 1984). Values of $N_e m > 1$ indicate that gene flow is enough to prevent substantial differentiation among subpopulations caused by drift. At the level of patches and using juvenile plants, the F-statistics analysis for 1990 gives an estimate of $N_e m$ of 1.2. Given the short dispersal distances and the linear arrangement of the patches, it is likely that migration occurs mostly between adjacent subdivisions. The studied populations are more similar to a stepping stone structure. In this case, eqn 1 underestimates the actual value of $N_e m$.

Table 1 F statistics of *Phacelia dubia* near Radford, VA. Means were jackknifed over loci and significance tests were based on 95 per cent confidence intervals estimated by bootstrapping over loci. The juveniles were derived from seeds collected in the year indicated

Year	F	Θ	f
Juveniles			
1988	0.270*	0.023	0.244*
1989	0.172*	0.008†	0.162*
1990	0.220*	0.127	0.111*
Pooled	0.207*	0.049*	0.167*
Flowering adults			
1990	0.223*	0.127*	0.103*
1991	0.053*	0.026*	0.026*
Pooled	0.110*	0.023*	0.087*

*Estimates significantly different from zero at 5 per cent.
†Population subdivision in this season was at a lower spatial scale (subpatches) than in the other seasons (see Fig. 1).

Table 2 Outcrossing rate estimates and standard errors of *Phacelia dubia* hermaphrodites near Radford, VA, for 2 years based on the Ritland & Jain (1981) mixed mating model

	Year			
	1989		1990	
Locus	T	(S.E.)	T	(S.E.)
PGI-2	0.654	(0.129)*	0.867	(0.071)
PGM-1	0.644	(0.194)	0.629	(0.102)*
IDH-2	0.554	(0.175)*	0.669	(0.112)*
MDH-1	0.631	(0.188)*	0.501	(0.127)*
Mean†	0.626	(0.082)*	0.726	(0.048)*
Multilocus	0.672	(0.081)*	0.683	(0.043)*

†Minimum variance mean.
*Rejection of the null hypothesis that $T = 1$ at 5 per cent.

Breeding systems

During the two seasons where this was possible, the multilocus estimations of outcrossing indicated that *P. dubia* hermaphrodites have a mixed-mating system; the estimates differed significantly from random mating and from complete selfing (Table 2). The multilocus estimate of 1989, when the population size was small, was lower than that of 1990, both for the cohort as a whole as well as for patch D ($T = 0.824 \pm 0.069$ mean and S.E.) but in neither case was the difference significant ($\chi^2_{[1]} = 0.01$ and $\chi^2_{[1]} = 2.01$). Violation of the mixed-mating model assumptions can generate significant differences between the mean outcrossing rates of single locus estimates and the multilocus estimates (Ritland & Jain, 1981; Shaw *et al.*, 1981). As the single locus estimates are estimates of a single parameter, these estimates were combined by weighting their values by their variances (Mather, 1946). These minimum variance means were not significantly different from the multilocus estimates (Table 2). Also, heterogeneities among single locus estimates indicate departures from the mixed-mating model due to effects such as selection or mating among relatives (Clegg, 1980; Ritland & Jain, 1981; Shaw *et al.*, 1981). No heterogeneity among single locus estimates was detected in 1989 ($\chi^2_{[3]} = 0.15$, n.s.) but in 1990, the estimates were significantly heterogeneous ($\chi^2_{[3]} = 9.17$, $P = 0.028$).

The fitness differential between self and outcross progenies biases the outcrossing estimates (see for example Snyder *et al.*, 1985). In *P. dubia*, the fitness of the self progeny relative to that of the outcross progeny, from seed to juvenile stages (ws), is 0.86 under the same conditions as those in which the progenies for outcrossing estimates were grown (36 families analysed, R. F. del Castillo, unpublished data). The multilocus estimates weighted by this value were 0.578 in 1989 and 0.587 in 1990.

For the multilocus estimate of the outcrossing rate of male steriles, *MDH* was excluded from the analysis owing to the low frequency of the slow allele in the progenies of these plants (0.04). The multilocus estimate was 1.278 ± 0.070 (mean and S.E.), confirming that male sterile plants are outbred. This analysis, therefore, does not provide evidence of biparental inbreeding in *P. dubia*.

A population estimate of the outcrossing rate can be obtained by weighting the outcrossing estimates of hermaphrodites (multilocus estimate weighted by ws) and male steriles ($T = 1$) by the frequency of each sex morph. Thus, the population estimates of outcrossing were 0.625 and 0.589 in 1989 and 1990, respectively.

Effective population size

The degree to which genetic drift can change the rates of allelic fixation depends on the effective size of the population N_e. This value depends on two quantities: (i) the number of breeding individuals per unit area (δ) and (ii) the dispersal rate (σ). Assuming a random mating population with stationary gene flow, i.e. no net movement of gametes (Levin & Kerster, 1974), Wright (1946) found a method of relating N_e to δ and σ. For gynodioecious populations, like those of *P. dubia*, in which (i) the dispersal rates of seeds (diploid, σ_o) and pollen (haploid $\sigma_p/2$, Levin & Kerster, 1974) are different and normally distributed, (ii) a fraction $(1-ms)$ of the breeding plants produces pollen and (iii) part of the progeny is the product of selfing, N_e can be estimated as:

$$2\pi(1-ms)\delta r(\sigma_o^2+\sigma_p^2/2),$$

where r is the proportion of random mating. The estimated density in 1989 was 0.523 plants/m^2 whereas that in 1990 was 48.3 plants/m^2. If N_e is relatively large, r can be equated to the total outcrossing rate of the population. If pollinators adjust their flight distances as a function of plant density (Levin & Kerster, 1974; Handel, 1983a; Fenster, 1991), the standard deviation of pollen dispersal in metres (σ_p) can be approximated as: $\sigma_i\delta^{-1/2}$, where σ_i is the standard deviation of pollen dispersal of the experimental population expressed as the number of individuals from the source plant in a given direction. The estimates of N_e were $20(\sigma_p=4.6$ m) in 1989, and $28(\sigma_p=0.48$ m) in 1990. Using the latter estimate and eqn 1, migration rate among patches (m) was estimated as 0.04.

Discussion

Fluctuations in population size

The decrease in total population size to less than 100 plants in 1989 suggests a potential opportunity for genetic drift; therefore, a loss of genic variation, including a reduction in the number of alleles per locus, is expected (Nei *et al.*, 1975). The individuals present during the bottleneck were distributed in a single patch; consequently, given the low seed dispersal rates, they all were likely to be derived from plants of the same patch. Thus, they are expected to be more related among themselves than a random sample of individuals derived from all the patches of the previous year. This creates an additional component of drift (Slatkin, 1977) which suggests even greater chances of genetic loss. There was, however, no loss of alleles after the bottleneck for all the enzymes assayed. *PGI-s*, a rare allele, could not be detected in the sampled adults of 1990 but was detected in their seeds and in the adult samples of the following year.

The decrease in frequency of male steriles in 1990 could have been attributed to the bottleneck effect. In *P. dubia*, the genetic determination of sex appears to be controlled by cytoplasmic factors causing male sterility and by nuclear genes (restorers) that re-establish male fertility on a particular cytotype (F. Levy, L. E. Broaddus and R. F. del Castillo, unpublished data). Therefore, chance over-representation of particular cytotypes and particular nuclear restorers provides an explanation for the observed shift in the frequency of male sterile plants from 1989 to 1990 (Fig. 3). Nevertheless, oscillations in the dynamics of male sterility in gynodioecious species can be reproduced in numerical models in systems controlled by nucleocytoplasmic factors, as is the case of *P. dubia*, without including bottleneck effects (Gouyon *et al.*, 1991; del Castillo, unpublished data).

In the absence of other forces, the bottleneck effect at generation t in a species with a mixed mating system is expected to increase the F value at generation $t+1$ to:

$$F_{t+1}=\tfrac{1}{2}(1+F_t)S+(1-S)G_t,$$

where $S=1-T$ is the selfing rate of the population and G_t is the coancestry coefficient at time t (after Crow & Kimura, 1970). Selfing rates include a component of selfing due to random mating in a finite population. This component is inversely proportional to $2N_e$, where N_e is the effective population size (see Crow & Kimura, 1970). As N_e approaches zero, selfing rates are expected to change widely with small changes in N_e but the opposite is true as N_e increases above 15–20. Thus, the slight and non-significant differences observed in the estimates of S and F in 1989 and 1990 and the estimates of N_e suggest that the decrease in N_e was not as dramatic as the decrease in total population size in 1989. Moreover, the low and non-significant value of Θ suggests that the population was panmictic or nearly so in 1989.

Past bottlenecks appear to reduce electrophoretic variation in natural populations of some species of plants (Fowler & Morris, 1977; Lesica *et al.*, 1988). In other species, however, founder events decrease only slightly allelic polymorphism and have a minimal effect on heterozygosity (Taggart *et al.*, 1990; Betancourt *et al.*, 1991). Also, previous studies on bottlenecks have failed to detect any effect in populations of *P. dubia* occurring along railroad tracks in Virginia (Levy, 1989). Thus, reductions in population size do not necessarily lead to a genetic depauperation pre-

sumably because other factors in the biology of the species act to preserve genetic variation.

Seed bank in the soil

In *P. dubia*, the seed bank may play a major role in ameliorating the genetic consequences of the bottleneck and in recovering the population following the bottleneck. In the field, seeds stored in the soil for 1 year showed an average of 10 per cent of germination when the soil was watered (5.0 per cent, selfed seeds; 12.6 per cent, outcrossed seeds, R. F. del Castillo, unpublished data). Considering the average seed production per plant in the field (250 seeds, del Castillo, 1992, 1993) and the population size the year before the bottleneck (1988, Fig. 2), at least 400,000 viable seeds from the 1988 cohort were present in the soil in the fall of 1989. Therefore, an insufficient number of safe sites rather than an insufficient number of viable seeds may explain the low rates of establishment even in good years. In the study site, the seed pool is almost certainly responsible for the consistency of patch distribution through seasons. It is unlikely that the recovery of patches A, B, C, E and F following their disappearance in 1989 can be explained by colonization from seeds derived from patch D, given the limited seed dispersal distances observed in this species (Figs 1 and 5).

The seed bank can function as an 'evolutionary memory' which stores genotypes for a variable number of years; this memory is selective because it favours those years with large seed crops (Templeton & Levin, 1979). The seed bank, therefore, can help explain not only the preservation of population size after the bottleneck but also the persistence of the genetic diversity and the MDH cline in *P. dubia*. The seed bank has seldom been considered in studies of population structure; however, the constancy in gene frequency detected in other annuals with large fluctuations of population size has been attributed to the seed bank (Epling *et al.*, 1960; Gottlieb, 1974).

The seed population in the soil has a similar effect in time as dispersal does in space. With seed banks, each generation can be considered to result, in part at least, from the transfer of genes from several populations in the past and there is, therefore, an additional source of gene flow. Thus, the seed pool in the soil from favourable years is the temporal equivalent of Pulliam's (1988) source habitats. Although gene flow has been considered a homogenizing agent reducing the genetic variation among populations (Wright, 1969; Slatkin, 1987), in this case gene flow due to the seed bank preserves the spatial genetic structure. Thus, temporal and spatial sources of gene flow can have quite opposite effects in populations.

Pollen and seed dispersal

The dispersal results show that spatial gene flow is rather restricted. This is consistent with the patterns of seed dispersal of species with capsules (Levin & Kerster, 1968; Augspurger, 1980; Cook, 1980; Schaal, 1980; Lee, 1984; Fenster, 1991) and with the dispersal rates of pollen of entomophilous plants (Levin & Kerster, 1968; Augspurger, 1980; Schaal, 1980; Handel, 1983b). Nevertheless, in *P. dubia*, pollen appears to be dispersed at much longer distances than seeds, particularly at low densities as those of 1989. This difference in dispersal is expected to generate a slight excess of heterozygotes (Prout, 1981) and may, therefore, help to prevent a great loss in heterozygosity during bottleneck events.

The rates of seed and pollen dispersal are crude estimates of the actual gene flow. If outcrossing distances affect fitness, then the estimates of gene flow should weight such an effect (Levin, 1981). Outcrossing distances, however, did not have a significant effect on seed viability and seed production in *P. dubia* ($n = 85$, $r^2 \leq 0.11$, n.s., linear and quadratic effects tested; R. F. del Castillo, unpublished data).

The method used here for the estimation of pollen flow probably avoids some of the problems of other methods involving following pollinators or assessing deposition of pollen or pollen analogues on stigmas, as it is based on fruit set which includes not only dispersal but also successful mating. Nevertheless, male sterility may itself affect pollination because the flowers of male sterile plants are smaller in diameter than those of hermaphrodites (0.61 ± 0.02 vs. 0.95 ± 0.02 mm, mean and S.E.) and do not produce pollen. Differences in floral characters determining advertisement and rewards to pollinators affect pollen flow in other species (Waddington, 1983; Waser, 1983; Murcia, 1990; Young & Stanton, 1990). In particular, the average number of flowers reached by donor pollen may be higher in emasculated flowers than in pollen-carrying flowers (Price & Waser, 1982). Thus, male steriles may favour longer pollination flights and, therefore, actual pollen flow might be overestimated with these plants.

Secondary seed dispersal (Phase II of dispersal, Watkinson, 1978) is unlikely to be important in *P. dubia* because: (i) the seeds lack aerodynamic features that favour wind dispersal (see Burrows, 1986); (ii) even under high winds no seed movement was detected in the soil (R. F. del Castillo, personal observation) and (iii) small seed size, as that of *P. dubia* (0.8 mm length), increases the probability of the seed being trapped in soil (Chambers *et al.*, 1991). Nevertheless, seed dispersal is not only affected by the physical properties of the plants and seeds but also by the habitat. Compared

with dense areas, open habitats tend to favour higher dispersal rates (Watkinson, 1978; Lee, 1984; Fenster, 1991). Thus, seeds from individuals in low density stands, as in 1989, are likely to be dispersed further than those in dense stands, as in 1990. This could be an additional factor that minimizes the differences in N_e between the 2 years.

Spatial structure

The coefficient Θ and the MDH cline indicate spatial structuring of genetic variation. Additional evidence of substructuring is provided by the existence of heterogeneity in the germination rates among patches (del Castillo, 1992). Moreover, the heterogeneity detected among the single locus estimates of outcrossing in 1990 may also indicate some assortative mating due to population subdivision (see Clegg, 1980; Shaw *et al.*, 1981). In contrast, the homogeneity of the single locus estimates in 1989 may reflect the lack of structuring in that year when the population was restricted to one patch. Thus, the low *MDH* outcrossing estimate in 1990 is probably due to the spatial cline in this locus, as spatial structure biases outcrossing estimates downwards (Ennos & Clegg, 1982; Ellstrand & Foster, 1983; Fu *et al.*, 1992). Indeed, when *MDH* was removed, no heterogeneity among single locus estimates was detected in 1990 ($\chi^2_{[2]}=4.57$, $P>0.1$); however, the increase in value of the multilocus estimate was not significant ($T=0.771\pm0.047$, $\chi^2_{[1]}=1.91$, $P>0.1$). These results, therefore, provide support for the common finding that natural populations are genetically spatially structured (Wright, 1978; Brown, 1979; Loveless & Hamrick, 1987; Cugen *et al.*, 1988, etc.). In *P. dubia*, the genetic substructuring can be explained in part by very localized seed and pollen dispersal and in part by the large gaps between patches which may serve as barriers to gene flow. Simulation studies have shown that limited gene flow results in high levels of inbreeding, population subdivision and increased homozygosity (Turner *et al.*, 1982). Also, the distribution of the population in a linear arrangement is expected to amplify the effects of population subdivision (Kimura & Weiss, 1964; Wright, 1969; but see Slatkin, 1985).

Despite the limited rates of pollen and seed dispersal, genetic subdivision accounted for only a small portion of the total inbreeding detected. This appears to be common in other plant species (Heywood, 1991) including plants with limited gene flow (e.g. Schaal, 1975). In *P. dubia*, the lack of genetic equilibrium due to the frequent disturbances to which the study site is likely to be exposed may explain this result. Alternatively, the small migration detected between patches may play an important role in overcoming the effects of drift due to population subdivisions, as has been shown theoretically (see Lacy, 1987).

Breeding systems

Most of the inbreeding detected here was at the level of individuals within subpopulations, probably as a result of autogamy. Indeed, the outcrossing estimate in male sterile plants did not provide evidence of biparental inbreeding or other types of assortative mating. Selfing can result from direct action of pollinators as protandry is not absolute and seed production can result from flowers self-pollinated on the first day of opening. Alternatively, 4–6 days after the first opening of each flower, the corolla collapses to push the anthers into contact with the stigma (Levy, 1988). This mechanism, therefore, can promote selfing, particularly without previous pollination. Also, geitonogamy is possible as small pollinators can fly from one flower to another of the same plant (R. F. del Castillo, personal observation).

The use of male sterile plants may underestimate the rates of biparental inbreeding because, as was discussed above, floral morphology may enhance pollination distances. Also, these plants have a higher genetic load than hermaphrodites (del Castillo, 1992, 1993) favouring, therefore, a higher bias towards outcrossing (see Moran & Brown, 1980; Cheliak *et al.*, 1985; Snyder *et al.*, 1985). Thus, genetical and morphological factors in male steriles might not only account for the lack of apparent inbreeding but also explain why the estimates of consanguineous matings using male sterile individuals were actually biased in favour of outcrossing. In any case, assortative mating appears not to be the main component of inbreeding in *P. dubia*.

Inbreeding depression has been detected experimentally in *P. dubia*, particularly at early stages (del Castillo, 1992). This factor, therefore, may contribute to curtail the loss of genetic variation due to the bottleneck effect (see Lesica & Allendorf, 1992).

In conclusion, *P. dubia* has a relatively constant mixed mating system and population genetic structure. Because of the reduction in population size and the distribution of all the individuals in a single patch of the original population, bottlenecks, as those of 1989, can potentially promote loss of genetic diversity. Nevertheless, the effective population size during the bottleneck persisted at a relatively high level presumably because of a density-dependent increase in gene flow. In addition, the bottleneck effect seems to be minimized by the seed bank in the soil. The seed bank buffers changes in population size, helps preserve genetic diversity, and maintains genetic spatial structure.

Moreover, inbreeding depression and gene flow via pollen being much longer than that via seeds may help prevent the decrease of heterozygosity levels during the bottleneck. Restricted pollen and seed dispersal may explain the genetic structuring detected; however, population subdivision accounts for only a minor part of the total inbreeding of the population. In contrast, autogamy appears to be the main contributor to population level inbreeding. The view of Loveless & Hamrick (1984) that the mating system is the main factor in organizing the genetic structure in populations seems to be valid in *P. dubia*.

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