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Pollen pool heterogeneity in shortleaf pine, *Pinus echinata* Mill.

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Abstract

Pollen is the dominant vector of gamete exchange for most temperate tree species. Because pollen movement influences the creation, maintenance and erosion of genetic structure in adult populations, it is important to understand what factors influence the process of pollen movement. Isolation by distance in pollen donor populations can create highly structured pollen polls by increased sampling of local fathers. Extrinsic factors, such as the intervening vegetative structure and local pollen donor densities, can also influence the genetic composition of local pollen pools. Using paternally inherited chloroplast microsatellite markers, we examined the structure and diversity of pollen pools in *Pinus echinata* Mill. in southern Missouri, USA. Our analysis is based on a multivariate AMOVA analysis of stands (≈ 1 ha; six per region) nested within regions (≈ 800 ha; four each). Significant multilocus structure of the pollen pool within regions (ϕ_{SR} = 0.095), but not among regions (ϕ_{RT} = 0.010), indicates that pollen movement is relatively restricted. Furthermore, the significant correlation between pairwise genetic and physical distances (Mantel correlation; ρ = 0.32) provided support for the isolation by distance hypothesis. Our results indicated that availability of pollen donors did not affect diversity of the pollen pool, measured by the number of unique multilocus genotypes at each stand. However, pollen pool diversity was negatively associated with vegetative structure, measured as total forest tree density. Our findings indicated that on-going pollen movement within continuous forest is relatively restricted as a result of both isolation by distance and vegetative structure.

Keywords: cpSSR, density, genetic, *Pinus*, pollen, structure

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Introduction

Pollen, the dominant vector of gamete exchange for most temperate tree species (Ennos 1994), determines the distribution of genotypes within and between populations. Restrictions in pollen movement can result in increasing isolation by distance (IBD) among populations (e.g. Wright 1943; Malécot 1973; Turner *et al*. 1982) with pollen pools having increased proportions of gametes from local pollen donors. Furthermore, extinction/recolonization events may further promote the creation of genetic structure in a pattern conforming to IBD (e.g. Slatkin 1977; Whitlock & McCauley 1990). Conversely, extensive pollen movement overcomes local genetic differentiation resulting in populations that are in genetic equilibrium. Because pollen movement influences the creation, maintenance and erosion of genetic structure in adult populations, it is important to understand what factors influence the process of pollen movement (Gregorius 1987). Only through direct examination of the genetic structure of the pollen pool, can we gain insight into the scale of and the factors that influence pollen.

One factor that can modify the genetic structure of local pollen pools is the physical structure of the surrounding stand. Site-specific conditions such as total stand density and/or basal area influence the movement of wind-dispersed pollen by modifying the turbulence of the surrounding air. Okubo & Levin (1989) outlined a theoretical framework for pollen dispersal distances based upon intervening turbulence with the prediction that increased turbulence will significantly reduce the distance that pollen is dispersed. While the influence of air turbulence on the genetic composition of the pollen pool has not been

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examined directly, it has been shown in *Pinus pungens* and *Picea sitchensis* that the position of the cone along the vertical axis of the tree influences the pollen allele frequencies and outcrossing rates (Gibson & Hamrick 1991; Chaisurisri *et al*. 1994). We interpret these findings as being consistent with the air turbulence framework provided by Okubo & Levin (1989), wherein the physical structure of the canopy increased air turbulence resulting in structured pollen pools at the scale of a single tree. Furthermore, the removal of intervening sources of turbulence may result in increased pollen movement. For example, Young *et al*. (1993) found that seeds collected from fragmented patches of *Acer saccharum* had higher genetic variation than did those from sites within a continuous stand. Young *et al*. suggested that pollen movement is greater between fragments because of the lack of interference caused by intervening forest canopy. At this time, we lack empirical evidence for the association between vegetation structure and pollen movement in wind-pollinated trees.

A second factor that influences the genetic structure of local pollen pools is the density of flowering conspecifics. Higher densities of pollen donors create a more diverse pollen pool. The influence of pollen donor densities has repeatedly been shown to influence the outcrossing rates in conifer species (e.g. Shea 1987; Murawski & Hamrick 1991; Murawski *et al*. 1994), in which low-density stands have reduced outcrossing rates. It is suggested that in low-density stands, each individual has a larger proportion of their pollen in the local pollen pool, thereby increasing the opportunity for selfing events. Conversely, in high-density stands each individual has a smaller proportion of their pollen in the local pollen pool resulting in increased outcrossing.

Our central question regards the extent to which spatial separation and local stand characteristics influence the genetic structure and diversity of pollen pools. We investigate the structure and diversity of the pollen pool by sampling first-year seedlings in natural stands of shortleaf pine, *Pinus echinata* Mill. using a multivariate analysis of chloroplast microsatellite markers. *P. echinata* is an ideal species to address our question because its pollen dispersal mode is typical for many temperate tree species. Furthermore, the strictly paternal inheritance of the chloroplast genome (e.g. Wagner 1992) provides an unambiguous representation of the pollen donor haplotype in each seedling assayed. Thus, the collections of seedlings provide estimates of the genetic structure of the local pollen pool. In this study, we assayed the pollen donor haplotypes to ask two specific questions. First, are pollen pools genetically differentiated and if so, does the pattern of differentiation conform to the model of isolation by distance? Second, assuming that pollen pool diversity reflects pollen movement, is the diversity of sampled pollen pools influenced by vegetative structure or pollen donor densities?

Materials and methods

Study organism and sampling

Shortleaf pine, *Pinus echinata* is widely dispersed throughout the eastern US (Little & Critchfield 1969). Shortleaf pine can grow in a variety of elevation and soil types that range from dry, nutrient poor or eroded soils to moist, rich and wet soils. The latitude in growing conditions results in a much larger geographical distribution than other southern pines (Wakeley 1954).

The genetic structure of *P. echinata* was recently surveyed in 15 populations across much of the species' natural range by Raja *et al*. (1997). Moderate levels of among population genetic variation ($F_{ST} = 0.089$) measured using allozymes suggests that gene flow is extensive throughout much of the species range ($Nm = 2.56$). Furthermore, populations along the western-most portion of the species' range (west of the Mississippi River) contained more genetic variation than eastern populations.

Our study site is part of a larger, multidisciplinary ecosystem-level study, the Missouri Ozark Forest Ecosystem Project (MOFEP), being conducted by the Missouri Department of Conservation (MDC; Brookshire & Shifley 1997). Shortleaf pine grows throughout the Missouri Ozarks where it was once the dominant canopy tree species (Guyette & Dey 1997). Within this landscape, shortleaf pine currently ranks fourth in relative importance behind *Quercus velutina*, *Q. coccinea* and *Q. alba* (Brookshire *et al*. 1997). The forest structure is predominately upland oak/hickory/pine forests (> 90%), with small portions of lowland and wet lowland forest (Brookshire *et al*. 1997).

We sampled four regions, ranging in size from 750 ha (region 3; Fig. 1) to 900 ha (region 1; Fig. 1). Within each region, we randomly selected six stands (≈ 1 ha each) from all the sites that have shortleaf pine. At each stand, we sampled needle tissue from 10–12 first-year seedlings. All tissue was placed in drying silica gel and transported back to the University of Missouri, St. Louis.

Laboratory methods

Total DNA was extracted from needle tissue from each individual following the microprep variation of Doyle & Doyle (1989) presented by Dellaporta *et al*. (1983). Following extraction, chloroplast microsatellite (cpSSR) loci were surveyed following Vendramin *et al*. (1996) and Powell *et al*. (1995) and all assayable loci were used for the analysis. cpSSR primers were obtained from Research Genetics (Huntsville, AL, USA). Loci used in this analysis were multiplexed on a Perkin–Elmer thermocycler (Sigma Products, St. Louis MO, USA) following the thermal regime of Powell *et al*. (1995). Haplotypes were assayed on

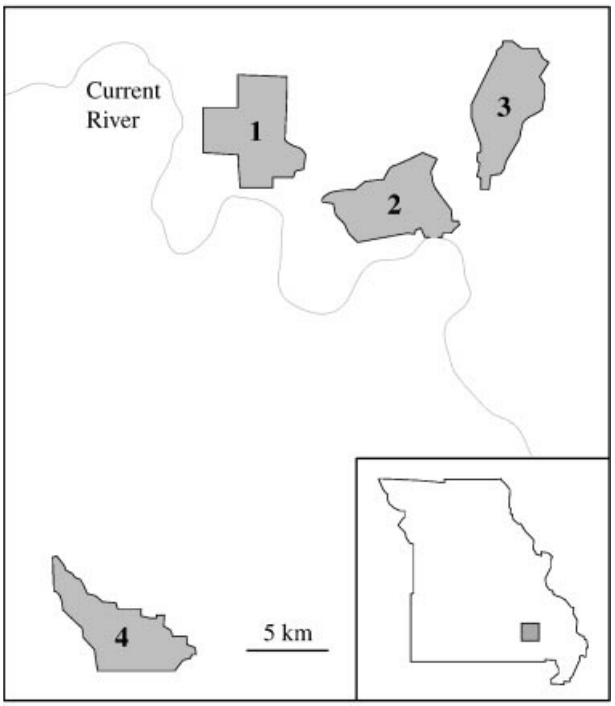

Fig. 1 Map of study area indicating the spatial arrangement of regions 1–4. Within each region, six populations were sampled (not shown).

a 4% MetaPhor high-density agarose (FMC Bioproducts, Kaysville UT, USA) on a vertical gel rig (Sigma).

Data analysis

To evaluate the overall heterogeneity of the cpSSR loci across the sampling landscape, we used a nested AMOVA with sampling sites nested within regions. The AMOVA analysis is similar in structure to the variance decomposition proposed by Weir & Cockerham (1984). The AMOVA analysis does not rely upon allele frequencies for the decomposition of genetic variance rather it uses a multilocus, pairwise genetic distance matrix approach. Analysing all loci simultaneously instead of averaging estimates across loci accumulates small differences in allele state across the loci providing a more powerful genetic discrimination between populations (e.g. Smouse *et al*. 1982).

The AMOVA analysis requires multilocus genotypes to be coded as a pairwise squared multivariate distance matrix prior to analysis. The coding convention used follows Smouse & Peakall (1999) in which the number of nonsimilar alleles is the distance metric for each pair of individuals as:

$$d_{ij}^2 = \sum_{l=1}^{n} k_l^2 \left\{ \frac{k = 1 \text{ if alleles are not the same}}{k = 0 \text{ otherwise}} \right\} \quad (1)$$

where d_{ij}^2 is the squared genetic distance between individuals i and j. We used the multilocus AMOVA model because analysing loci jointly provides a more precise estimate of population differentiation than averaging differences across loci (Smouse *et al*. 1982).

The overall model is a nested, random effect AMOVA (Excoffier *et al*. 1992) in which we tested for differences between sampling sites (nested within regions) and differences between regions. The statistic of differentiation, ϕ, is based upon the ratio of the appropriate variance components for the strata being evaluated, similar to those described in Cockerham (1969, 1973). While the ϕ statistic is analogous to a multilocus, hierarchical *F*-statistic, it is free of the assumptions embedded in *F*-statistic models (see Neigel 1997 and Bossart & Prowell 1998 for review). The strengths of this technique are that it is based upon a multivariate distance metric and is constructed upon a hierarchical partitioning of within vs. among variance estimates. Significance testing is conducted by means of permuting the individuals among the various hierarchical levels and recalculating the null distribution of the test statistic (Excoffier *et al*. 1992). The observed test statistic is then compared with the null distribution to determine significance. In terms of a linear model, the AMOVA analysis can be denoted as:

$$x_{jig} = \mu + \gamma_g + \tau_{ig} + \varepsilon_{jig} \quad (2)$$

where x_{ijg} indexes the jth individual, μ is the overall mean, γ represents the group effect (regions in our model), τ is the treatment effect (stands nested within regions) and ε is the error term derived from the individuals within populations. The genetic analyses were conducted in GeneticStudio written by RJD (available at http://www.GeneticStudio.com).

To evaluate the sample sizes needed to test the treatment effect (τ), we conducted a permutation analysis following Chernick (1999). Multilocus genotypes were sampled 1000 times (with replacement) from the empirical data set with increasing numbers of individuals allocated to each sampling site. We started with two randomly selected individuals per stand and increased the sample size following each set of permutations. The null distribution of the bootstrap statistic $\hat{\phi}_{SR}$ was estimated from the 1000 permutations. The mean and 95% confidence interval of the test statistic $\hat{\phi}_{SR}$ was determined for each sample size using the percentile method after verifying that the permuted estimates of the test statistic were $\approx N(\bar{\phi}_{SR}, \sigma^2)$ as defined in Efron & Tibshirani (1986). The bias of the bootstrap estimates (Chernick 1999) was evaluated to determine the adequacy of the estimated null distribution, $\hat{\phi}_{SR}$. The sample size beyond which the mean and variance stabilize indicates the sample sizes for each sampling site that are sufficient to adequately describe the differentiation between pollen pools and show the magnitude of the treatment effect.

We evaluated the power of the analysis as we genotyped individuals. We randomly selected two individuals per

sampling site, assayed the multilocus genotypes and analysed the power of the analysis. We then randomly selected another individual from each sampling site, assayed their multilocus genotype and re-evaluated the power of our analysis. This process was repeated until both the mean and variance of the permuted null distribution were sufficiently stabilized, at which time we stopped adding individuals. If the mean and variance of the null distribution did not stabilize once we reached all the individuals sampled, we concluded that we could not adequately describe the among-population variation in pollen poll diversity. However, if the both the mean and variance did stabilize, we concluded that we had sufficient power to describe the among-population component of genetic variation in pollen pools (the τ term in eqn. 2).

We tested the hypothesis of isolation by distance using the genetic distance matrix and the AMOVA analysis. Under the AMOVA model, the genetic distance between two sampling sites is half the among population component of variation (σ_A^2). The association between the two squared distance matrices (pairwise genetic distances between stands and pairwise physical distances separating stands) were then analysed via a Mantel test (Mantel 1967). These pairwise genetic distances are presented as a function of the $\log_5$ of the physical separation (in km) to visually partition the data into spatial scales approximating the within region ($\log_5 \leq 1.0$) and between regions ($\log_5 > 1.0$) components.

To assess the interaction of vegetative structure and pollen donor density on haplotypic diversity, we used stepwise regression (Draper & Smith 1981). The surrogate measure of vegetative structure was estimated as total stand density (≤ 10 cm dbh and > 10 cm dbh) as well as total basal area (m / ha). For each stand, we estimated density of pollen donors as number of conspecifics ≥ 10 cm dbh / ha. These data were collected and provided by MDC (Brookshire *et al*. 1997). The haplotypic diversity at each sampling site was determined by tallying the number of unique multilocus haplotypes. Significant regressions on any of the local stand variables would suggest that pollen pool diversity is influenced by local site-specific factors.

Results

Spatial heterogeneity

Ten cpSSR primer pairs were assayed, nine of which produced product. However, reliable scoring of dinucleotide repeat differences was difficult when the total fragment size was below ≈ 100 bp on MetaPhor agarose. Consequently, for this analysis three cpSSR loci (with three to four alleles) were used (Table 1): *pt100783*, *pt30204* and *pt100517*.

Permutations of the empirical data revealed that the sample sizes required to describe the degree of differentiation between stands were surprisingly small. The mean $\hat{\phi}_{SR}$ is relatively flat throughout the entire range of sample sizes (Fig. 2). More importantly, the 95% confidence interval on the mean were asymptotic beyond approximately five individuals per sampling site. We therefore concluded that seven individuals per sampling site would be adequate in describing the among-population variation in pollen pools and did not assay additional individuals. The bias of the bootstrap estimates (Chernick 1999) were sufficient small (range: 0.0006–0.0018) to suggest adequate estimates of the null distribution, $\hat{\phi}_{SR}$.

Analysis of molecular variance (AMOVA) revealed significant structure within the sampled pollen pools across

Table 1 Allele frequencies, by region, for three chloroplast microsatellite loci in *Pinus echinata*

		Allele			
Locus	Region	1	2	3	4
Pt 100783	1	0.31	0.30	0.39	
	2	0.06	0.53	0.41	
	3	0.58	0.29	0.13	
	4	0.37	0.26	0.37	
Pt 30204	1	0.91	0.09		
	2	0.72	0.28		
	3	0.59	0.26	0.15	
	4	0.71	0.29		
Pt 100517	1	0.54	0.46		
	2	0.62	0.38		
	3	0.51	0.43	0.06	
	4	0.35	0.29	0.27	0.09

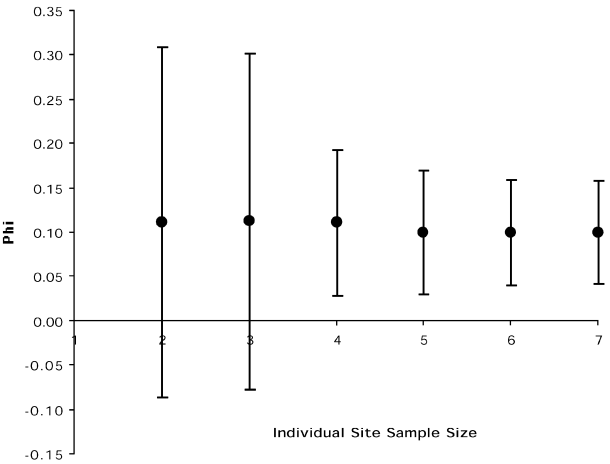

Fig. 2 Permutation analysis of the degree of differentiation $\hat{\phi}_{SR}$ as a function of individual site sample size. The mean and 95% confidence intervals are based upon 1000 permutations of the original data.

Table 2 AMOVA table for the evaluation of genetic differences between sampling sites and regions. Significance was tested by permuting the appropriate hierarchical level and recomputing the test statistic 1000 times

Source	df	ϕ	*P*
Regions	3	0.010	NS
Sites	20	0.095	< 0.01
Error	145		

the range of sampling sites (Table 2). Nested within a region, sampling sites were significantly differentiated ($\phi_{SR} = 0.095$; $P < 0.01$). Regions were not significantly differentiated ($\phi_{RT} = 0.010$; $P > 0.05$). Variance decomposition showed that the majority of the variation was contained among sites nested within regions (10%) and within sampling sites (89%) with little attributable to among regions.

Isolation by distance

The overall correlation between the physical and genetic distance matrices was $\rho = 0.32$ and significant at $P < 0.001$ (Mantel; 1000 permutations). A scatter plot of pairwise genetic and physical distance illustrates the increase in genetic distance between populations at increasing physical distances (Fig. 3). Isolation by distance within sampling regions, as indicated by pairwise contrasts $< \log_5 = 1$ (Fig. 3), was also significant ($\rho = 0.22$; Mantel; $P < 0.001$; 1000 permutations).

Pollen pool diversity

The number of unique multilocus haplotypes in each stand ranged from three to seven (mean five; mode four). We found evidence that vegetative structure influences pollen pool diversity. Total density of all individuals (> 10 cm dbh; range 163–547 trees per ha) was significant when regressed upon the number of unique multilocus haplotypes (df = 1,24; $F = 7.001$; $P = 0.014$; $R^2 = 0.241$). The negative slope showed increases in total stand density associated with a concomitant reduction in the number of unique multilocus genotypes (Fig. 4). In contrast, total stand density (< 10 cm dbh) was not significant when regressed on haplotypic diversity. Both total basal area and pollen donor densities were not significant when regressed on the number of unique multilocus genotypes.

Discussion

Hierarchical analysis of pollen pool variability in *Pinus echinata* shows substantial variation among sampling sites on a relatively small spatial scale. The magnitude of this differentiation, $\phi_{SR} = 0.095$, indicates that pollen dispersal within a single reproductive bout is not homogenizing spatially separated pollen pools. If pollen dispersal were as extensive as suggested Raja *et al*. (1997; $Nm = 2.56$) for *P. echinata* sampled from a much larger spatial scales, we would not have found such high local differentiation. Furthermore, because our markers are inherited exclusively through the pollen, factors such as maternal effects or selection on nuclear loci do not influence our estimates of pollen pool structure.

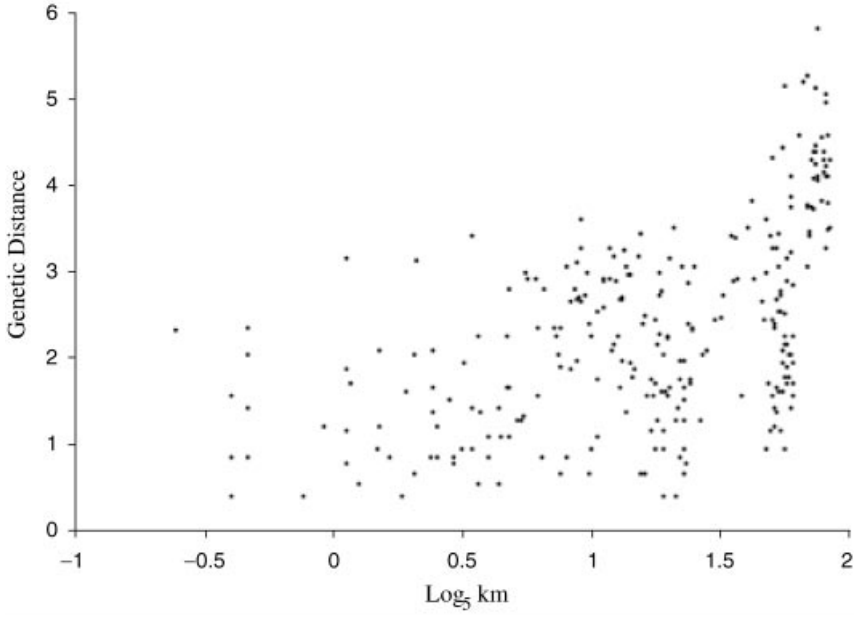

Fig. 3 Scatter plots of genetic distance as a function of the log (base 5) of physical separation (km) between sampling sites. Overall correlation for all pairwise contrasts is significant (Mantel test; $\rho = 0.32$; $P < 0.001$; 1000 permutations).

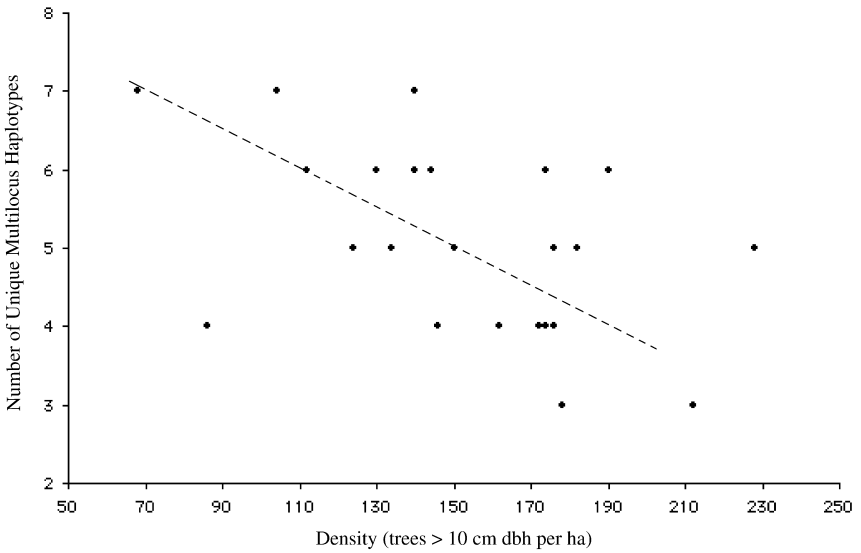

Fig. 4 Number of multilocus haplotypes as a function of total stand density (trees > 11 cm dbh/ha). The dashed line is the predicted value from the regression equation ($F = 7.001$; $P = 0.015$; $R^2 = 0.241$).

The amount of pollen pool structure observed is similar to that reported for *Pinus resinosa* ($G_{ST} = 0.121$) by Echt *et al*. (1998), who also used cpSSR markers. They reported a higher level of differentiation sampled from a much larger spatial scale. However, because Echt *et al*. (1998) did not sample at local spatial scales comparable with ours, it is impossible to determine whether the genetic structure they observed was due to pollen movement on a local scale such as ours or on a larger scale. Results of a previous analysis of population structure in *P. echinata* by Raja *et al*. (1997) suggested that gene flow should be relatively extensive throughout much of this species range. Our findings do not support this interpretation, and instead suggest that contemporary pollen movement is relatively restricted.

Genetic differentiation is proportional to physical distance supporting the hypothesis of isolation by distance in pollen pools ($\rho = 0.32$; $P < 0.001$; Fig. 3). The pattern of isolation by distance (IBD; Fig. 3) can be partitioned into within region and among region components, both of which are significant (see Fig. 3 and above). The within-region IBD most likely reflect the patterns of current pollen movement during a single reproductive event. However, the between-region components are most likely a mixture of historical genetic differentiation in the adult pollen donor populations as well as current pollen movement. We do not suggest that the lack of regional differences in pollen pool composition preclude occasional long-distance pollen dispersal events, rather the impact of long-distance dispersal on pollen pool structure is relatively insignificant when compared with that of localized (within region) pollen movement.

Genetic dissimilarity, under the model of isolation by distance, is asymptotic (e.g. Epperson 1995a,b; Epperson & Li 1996) because there is a maximum degree of differentiation possible given any set of populations. Therefore, if sampling is conducted at distances beyond the asymptote, as may have been the case when Raja *et al*. (1997) previously studied the genetic structure of *P. echinata*, no isolation by distance will be detected. We conclude that isolation by distance is prevalent within our sampling scale and contributes to our highly structure pollen pools.

Local vegetative structure influences the genetic diversity of sampled pollen pools. Total stand density may have affected dispersing pollen by creating a more turbulent dispersal environment. Under the assumption that local stand density is an adequate surrogate for air turbulence, our results support the theoretical framework provided by Okubo & Levin (1989) describing the inverse relationship between turbulence and pollen dispersal distances. While the effects of vegetative structure on pollen pool diversity have been examined at the individual tree level (e.g. Gibson & Hamrick 1991; Chaisurisri *et al*. 1994), our study is unique in demonstrating that vegetative structure, including nonspecific species, may influence the lateral movement of pollen in closed canopy forests. Our data imply that dense vegetative structure impedes the movement of pollen into the stand. Therefore, trees within continuous forests may have alternate pollen dispersal distances depending upon differences in intervening vegetative structure. Inclusion of ecological variables, such as vegetative structure, may provide additional insights into pollen movement dynamics.

In this study, we did not find that the density of local pollen donors has an impact on pollen pool structure in this system. An increase in the number of local pollen donors is predicted to increase both pollen pool structure and diversity because high-density stands contain gametes from more potential donors (Mitton 1992). While density effects on pollen pool structure have often been shown (e.g. Farris

& Mitton 1984; Knowles *et al*. 1987; Shea 1987), significant relationships are not found consistently (e.g. El-Kassaby & Jaquish 1996). In our analysis, there was a positive, albeit nonsignificant, relationship between pollen donor density and pollen pool diversity, which indicates that the effect was weak if it was present at all.

Finally, we would like to comment on the benefits of evaluating the sample sizes required to describe genetic structure. Most population genetic statistics are tested by means of permutation of the empirical data set (e.g. Weir & Cockerham 1984; Excoffier *et al*. 1992) thereby preventing the use of typical parametric power tests. By evaluating the sample sizes using methods common to quantitative genetics, we were able to determine the sample sizes sufficient to describe the genetic structure of spatially separated pollen pools. In our results, the significance test conducted in the AMOVA analysis showed that test statistic, ϕ_{SR} describing the distribution of multilocus genotypes nested within regions, is significantly large given the permuted null distribution provided by the AMOVA analysis (Excoffier *et al*. 1992). The addition of the permutation test on increasing sample sizes clearly shows the asymptotic behaviour of both the mean and 95% confidence interval of the permuted test statistic, $\hat{\phi}_{SR}$. We conclude that the treatment effect (i.e. the spatial partitioning of multilocus genotypes) is sufficiently large that a sample size of seven individuals per site adequately describes the genetic differentiation.

We recommend that sample sizes should be evaluated in every study for two reasons. First, if the sample sizes collected do not stabilize the mean and variance of the test statistic, then the results must be interpreted with caution. However, if a subset of individuals sufficiently describe the genetic structure (Fig. 2) then the addition of individuals provides little additional information on the degree of genetic differentiation. In this latter case, the resources may better be served by increasing the replication across populations rather than increasing the point estimates within populations. Clearly, the sample sizes for any study depend upon the degree of differentiation among populations, the genetic variation present in the study organism, and the test statistic being used.

In conclusion, both IBD and vegetative structure within stands contributed to highly structured pollen pools in *P. echinata* in Missouri Ozark forests. Across generations, these factors may be responsible for genetic structuring of the adult populations. To effectively use genetic structure information for studies of pollen movement, one must sample on spatial scales that are sufficiently small to detect current patterns of dispersal. Moreover, to understand external factors that influence pollen movement, studies should incorporate ecological variables, such as vegetative structure and pollen donor availability, that are appropriate to the dispersal vector.

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