

High Levels of Gene Flow in Bur Oak Revealed by Paternity Analysis Using Microsatellites

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Microsatellite analysis was used to characterize pollen dispersal in a stand of 62 adult bur oaks (*Quercus macrocarpa*) in northern Illinois. Using PCR amplification of four dinucleotide microsatellite loci, pollen donors of 282 acorns collected from three adult trees located in different parts of the stand were determined by exclusion. Overall at least 57% of the acorns were pollinated by trees outside of the stand, because all adult trees in the stand were excluded from paternity. Average pollination distance for within-stand pollinations was 75 m, and pollen donors for two of the three maternal trees were randomly dispersed throughout the stand. These two trees also received more pollen from 50 m away than from near neighbors. Self-pollination was rare or nonexistent. This study provides direct evidence for high levels of long-distance pollination in a wind-pollinated species, and contradicts traditional models of wind pollination which suggest that clouds of pollen dissipate from the source to ineffectively low densities over short distances. Instead, the mating system of bur oak seems to be extraordinarily efficient at producing highly outbred individuals and ensuring long-range pollen flow, perhaps through pollen competition or mate choice favoring distant pollen sources.

The genetic structure of plant populations is determined in large part by the movement of genes via pollen and seed dispersal. Because plants are stationary as adults, the location of an individual plant relative to conspecifics may influence the number of mates a plant has, the relatedness of mates, and relative fitness through male and female function. Pollen dispersal is probably the most important component of gene flow in temperate deciduous trees with large, relatively immobile seeds. Most of these trees (e.g., oak, hickory, and walnut) have wind-dispersed pollen.

Pollination can be studied by following the physical movement of pollen using traps (Greenwood 1986), dyes (Linhart et al. 1987), or the movement of pollinators (Levin and Kerster 1969; Mosquin 1971). Models of pollen movement generally predict that pollen densities will decline rapidly from the source, and most fertilizations will be effected from nearby trees (Faegri and van der Pijl 1979; Levin and Kerster 1974; Whitehead 1983). More recently, pollen traps used to measure pollen densities from many trees have revealed much higher pollen densities than would be predicted based upon dispersal from a point source (Caron and Leblanc 1992) and higher levels of gene flow than

earlier, single-source models (Adams 1992).

Such studies of pollen movement may not, however, accurately reflect actual fertilization and gene flow among local populations. Allozyme studies of insect-pollinated plants have shown that gene flow is usually more extensive than observed pollinator movement (Campbell 1991; Fenster 1991; Schaal 1980). Among wind-pollinated conifers, gene flow appears to be sufficient to prevent differentiation (Epperson and Allard 1989; Govindaraju 1989; Hamrick et al. 1979, 1995; Loveless and Hamrick 1984). Fewer allozyme studies have been conducted on deciduous wind-pollinated trees, but population differentiation is usually absent in these species as well (Berg and Hamrick 1995; Geburek and Tripp-Knowles 1994; Sherman-Broyles et al. 1992). However, allozyme studies generally lack the resolution necessary to accurately estimate levels of gene flow exceeding that needed to simply prevent population differentiation, which may be accomplished by only one or two migrants per generation (Wright 1931). Furthermore, it is difficult to use allozyme patterns to partition gene flow into that effected through pollen movement versus that effected by seed dispersal.

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Here we report the application of a new type of genetic marker, polymorphic microsatellite DNA, for paternity analysis and pollination studies. Microsatellites are short, tandemly repeated simple sequences, 1–6 bp in length, which are highly polymorphic for repeat number (Ashley and Dow 1994). Amplification of the microsatellite region by polymerase chain reaction (PCR) results in fully penetrant, Mendelian inherited, codominant markers than can be precisely identified by length. Unlike allozyme loci, which do not have sufficient variability to determine parentage by exclusion (Chakraborty et al. 1988), each microsatellite locus has many relatively rare alleles, and in most cases all but one adult or all adults in the local population can be excluded from paternity using a few loci (Dow and Ashley 1996; Dow et al. 1995). Movement of successful pollen can then be traced by examining the relative locations of identified maternal and paternal trees, and gene flow from pollen can be estimated from the proportion of pollinations in which all trees in a stand are excluded from paternity.

The only assumptions required for microsatellite paternity analysis are that there are no mutations between parents and offspring and that adults and offspring with matching genotypes are related. The mutation rate of microsatellite sequences has been estimated at 10^{-4} to 10^{-5} mutations per locus per generation (Edwards et al. 1992; Ellegren 1992; Schlötterer and Tautz 1992), which is low enough that the probability of a mutation between parents and offspring is negligible. The probability of cryptic gene flow (an adult within the stand matching an acorn by chance when the acorn was actually fertilized by pollen from outside the stand) can be easily calculated based on allele frequencies (Westneat and Webster 1994).

We apply microsatellite analysis to characterize successful pollen donors of individual seed parents. All adults in a stand of bur oaks (*Quercus macrocarpa*) in northern Illinois were genotyped at four variable microsatellite loci, and offspring (acorns) of trees located in different parts of the stand were scored at these loci as well. The objectives of this study were to (1) evaluate the utility of microsatellite analysis for studying paternity and successful pollination; (2) assess the amount of long-distance gene flow by determining the proportion of fertilizations effected by trees not in the stand; (3) characterize the spatial patterns of pollen movement for fertilization occurring between paternal

and maternal trees within the stand; and (4) determine the rate of self-pollination in bur oak.

Materials and Methods

Study Site and Species

The study site is part of an abandoned farm located in McHenry County near Harvard, Illinois, which was released from agriculture approximately 20 years ago. The stand occupies an area approximately 200 m × 250 m and consists of 62 mature bur oaks (*Q. macrocarpa* Michx.) and 16 red oaks (*Q. rubra* L.), and likely represents a fragment of presettlement oak savanna. All adult trees were mapped using angle and distance.

Q. macrocarpa is a monoecious, dicogamous, deciduous tree. On a single tree, male flowers mature and release pollen before female flowers become receptive to pollen. *Q. macrocarpa* is known to hybridize with *Q. alba* and *Q. muhlenbergii*, but is not known to hybridize with *Q. rubra* (Jones 1963). Oaks are masting species, producing variable crops of acorns in different years. Every tree produced pollen every year in the 3 years that observations were made. Acorn production varied among individual trees. A few trees produced large crops of acorns every year, but most trees had no observable acorn production in at least 1 year. A coordinated reproductive effort among all trees was not observed.

Sample Collection and Microsatellite Amplification

In early May 1992 and 1994, young leaves (0.5–2.0 cm long) from each of 62 adult bur oaks at the study site were collected just after bud break, when tannin content is lowest (Feeny 1970), quick frozen and stored at -70°C . Because our purpose was to develop a detailed understanding of pollen sources used by individual trees, our sampling strategy was to examine as many acorns as possible from each of a few trees located in different parts of the stand. This strategy was expected to reveal any directional bias in pollination (e.g., fewer within-stand pollen donors on the upwind side of the stand), distribution of pollen donors around the seed parents and fertilizations from outside of the stand, which we assumed would be rare. Acorns were collected from 11 trees, with the largest crops in late August and early September 1992. Equal numbers of acorns were collected from all sides of the tree.

Sound acorns were separated from in-

sect-infested acorns by flotation, stratified in damp sand at 4°C for 2–3 months (USDA 1974), and grown in a greenhouse. Three groups of half-sibs were found suitable for this study based on a sample size of 96 surviving seedlings and location of maternal tree. One maternal tree was located on the east side (tree 3E), one was in the middle (tree 17M), and one was on the west side (tree 33W) of the stand. Germination rates of acorns from these trees were 92%, 98%, and 95%, respectively. All of these trees were at least 100 m from any tree outside of the stand to the north and southeast, and greater than 300 m from conspecifics in any other direction (Figure 1). Fully expanded leaves of seedlings were quick frozen and stored as above.

DNA was extracted from frozen leaves of adults and seedlings using previously published protocols (Dow et al. 1995). Amplification of microsatellite loci was performed using four primer pairs (MSQ3, MSQ4, MSQ13, and MSQ16), and genotypes were scored by PCR product length at each locus as described previously (Dow and Ashley 1996; Dow et al. 1995).

Differences in allele frequencies between adults and acorns was tested by using the Kolmogorov–Smirnov test as in Morin et al. (1994). The Kolmogorov–Smirnov test is a nonparametric test in which frequency distributions are arranged cumulatively, then compared between two groups. The largest difference in cumulative frequency distribution is the test statistic D , which has associated critical values (Ebdon 1985).

Assignment of Parentage

Because Mendelian inheritance had been previously established for the microsatellite loci (Dow et al. 1995), each acorn was expected to have two alleles per locus for four loci, one allele derived from each of that individual's parents. The four loci used as markers were not linked. To match the acorn paternal alleles with the putative pollen donor, all possible gametes for each adult (maximum for complete heterozygosity = $2^4 = 16$) and the paternal alleles of each acorn were entered into a database. If an offspring matched both maternal alleles at a locus, making it impossible to distinguish which allele had come from the pollen donor, both alleles were entered into the database with all combinations of its other alleles. Alleles were grouped at each locus in turn using the “sort” function of the database, which resulted in the matching of like genotypes over all four loci. If the pa-

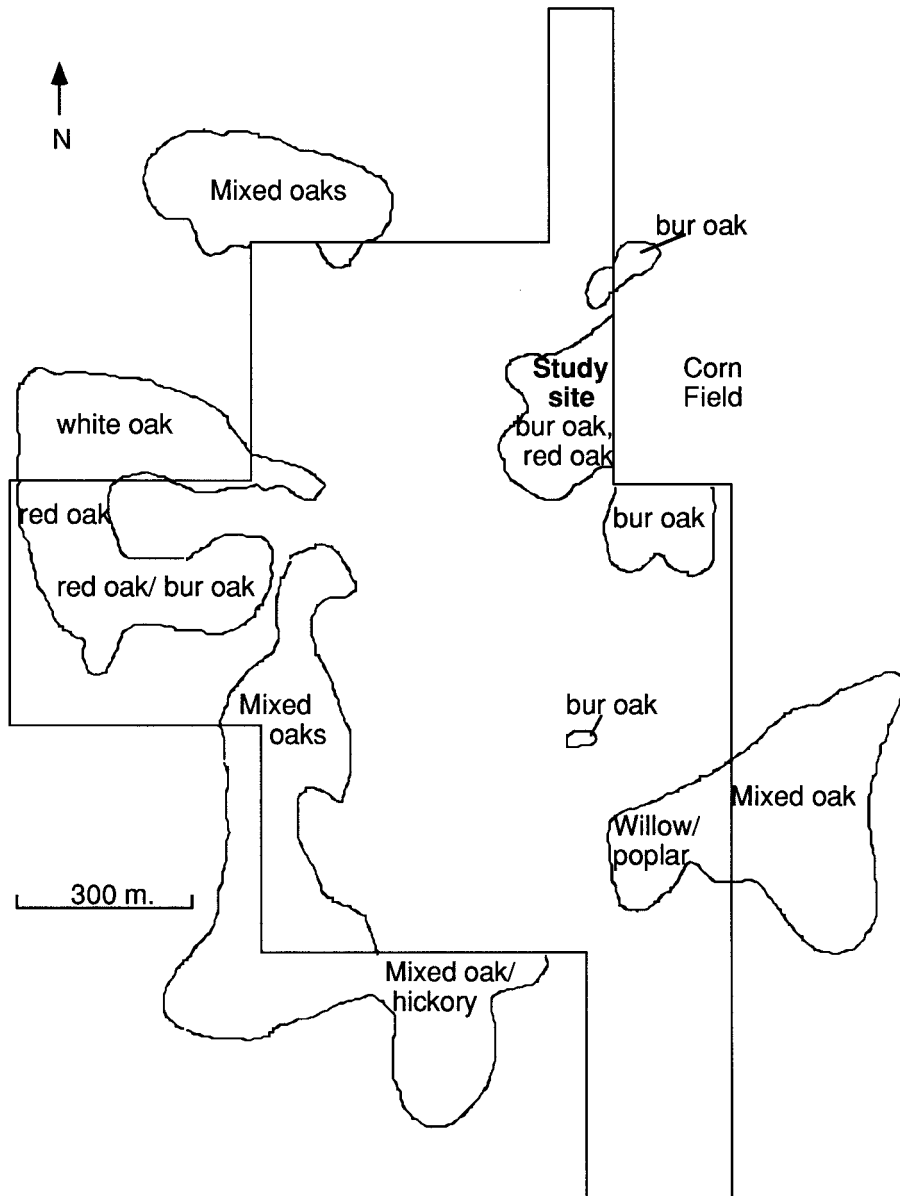


Figure 1. Map of study site and surrounding stands of trees. Straight lines indicate property boundaries. Bur oak = *Q. macrocarpa*; red oak = *Q. rubra*; white oak = *Q. alba*.

ternal parent of an offspring was in the stand, it could be identified. In paternity analysis, there is a chance that two individuals share the same genotype by chance rather than because they are related. If a tree within the stand matches an acorn by chance when in fact that acorn was fertilized by pollen from outside the stand, this gene flow event will be undetected, or "cryptic" (Broyles et al. 1994). The probability that an adult matched an unrelated offspring was calculated following Westneat and Webster (1994). This calculation has been completely described elsewhere (Dow and Ashley 1996). The probability of cryptic gene flow was calculated for each parent-offspring match.

The sum of these probabilities represents the probable number of cryptic gene flow events among all the acorns of a particular tree. Six offspring (four from tree 3E and two from tree 17M) were excluded from all analyses because PCR amplification was unsuccessful at one locus.

Spatial Analysis

In order to test whether pollen donors were clustered around the seed parent, we tested the relationship between distance and pollination success in four ways. First, to test the relative pollination success of neighbors, a chi-square test for association was used to test if numbers of near (<50 m) and far (≥ 50 m) pollinations var-

ied among trees. Second, Kruskal-Wallis *H* tests were used to compare the pollination distances of each tree to its pollen donors. This test is a nonparametric method of determining if the three trees had different overall distances to pollen donors. Third, to examine differential pollination success at all distances for all pollen donors, Spearman rank correlation coefficients were determined for the number of acorns fertilized by each pollen donor (0–9) and the distance of each pollen donor from the maternal tree. Fourth, join-count spatial autocorrelation (sampling without replacement) was used to determine if pollen donors were clustered around the maternal tree. Join-count spatial autocorrelation does not rely on absolute location of individuals but relative location of neighbors, and is therefore an appropriate test of random dispersion of values over an existing set of points, such as location of a tree fixed in a stand (Sakai and Oden 1983; Sokal and Oden 1978). Using a map of the trees in the stand, a join-count network was made by joining each tree to adjacent trees up to 50 m away, and the analysis performed as described by Ebdon (1985).

Results

Null Alleles

Anomalies appeared in the offspring of tree 3E at the MSQ3 locus which suggested the presence of a null allele. The existence of null alleles at low frequency is not unexpected because substitutions or deletions in the flanking region may prevent binding of PCR primers or interfere with primer extension (Callen et al. 1993; Paetkau and Strobeck 1995; Pemberton et al. 1995). Although the offspring of tree 3E all shared at least one band with the maternal tree at the other three loci, 49 of 93 offspring did not share a maternal band at MSQ3. Tree 3E, being heterozygous for an amplified allele and a null allele, would transmit the null allele to about half its offspring, and a small proportion of these, receiving a second null allele from a pollen donor, would appear to have no microsatellite bands. All amplifications produced artifact bands that were longer than the target microsatellite region, and the presence of these artifact bands in individuals that were homozygous for the null allele ruled out the possibility that the PCR had failed.

Another null allele was suspected at the MSQ4 locus when a statistically significant excess of homozygotes was found among

the adults and in all groups of offspring. The presence of this allele was confirmed by extracting and amplifying DNA from 16 offspring of tree 14, an apparent homozygote. Eight of the offspring shared a band with the maternal tree; eight did not. Among the adults, there were 17 trees that were either homozygous for an amplified allele or heterozygous for the null allele at locus MSQ3; there were 14 such trees at locus MSQ4. For the purposes of matching parents to offspring, all apparently homozygous individuals were considered heterozygous for the null allele. This method will overestimate the number of matches within the stand but will not exclude any true parents. Because there was allele information at the other loci, the number of offspring matches that included inferred null alleles was low. To estimate allele frequencies, parents that matched any offspring for a null allele were considered heterozygous and those that did not match any offspring for a null allele were assumed to be true homozygotes.

Allele Frequencies

The total number of alleles at each locus ranged from 14 to 24 (Table 1). At all loci, a few alleles were found among offspring genotypes that were not present in the adults. Heterozygosities ranged from 0.70 to 0.95. The lowest heterozygosities were generally found among the acorns of 33W. A large majority of alleles occurred at frequencies of less than 0.10, and there were only 6 alleles (of 83 total) that had frequencies higher than 0.20.

Kolmogorov-Smirnov tests were used to compare the frequencies of paternal alleles of each group of offspring to that of the parent population. These comparisons showed no significant differences for either MSQ3 or MSQ13. These results indicate that the paternal contribution to the offspring at these loci is indistinguishable from a random sample of all the variation present in the local adult population. There were no significant differences for any locus in the offspring of tree 33W. The offspring of tree 17M had allele frequencies that differed significantly from the parent population at loci MSQ4 and MSQ16, and the offspring of tree 3E differed significantly at locus MSQ4. Differences in allele frequencies indicate that a disproportionate number of fertilizations are coming from either a few trees within or from outside of the stand. These two alternatives will be considered in more detail following presentation of the paternity analysis.

Table 1. Frequency of alleles and heterozygosity at four microsatellite loci of 62 adults and offspring from three trees

Locus/allele	Adults	3E acorns	17M acorns	33W acorns
MSQ3				
191	0.016	0	0	0
193	0	0	0.011	0
195	0.008	0.011	0.011	0.021
196	0.008	0	0	0
197	0	0	0	0.010
199	0.008	0	0	0
201	0.016	0.011	0.011	0.010
203	0.065	0.043	0.064	0.021
205	0	0	0	0.010
207	0.081	0.161	0.064	0.062
208	0	0	0	0.010
209	0.056	0.086	0.085	0.042
211	0.056	0.075	0.160	0.083
212	0	0	0	0.010
213	0.056	0.011	0.021	0.177
215	0.137	0.086	0.170	0.094
217	0.032	0.054	0.021	0.042
219	0.113	0.108	0.128	0.104
221	0.081	0.043	0.043	0.146
223	0.032	0.075	0.053	0.010
225	0.040	0.043	0.011	0.052
227	0.016	0.032	0.085	0.031
229	0.056	0.032	0.021	0
231	0.024	0.065	0.032	0.010
Null	0.097	0.065	0.011	0.052
Heterozygosity	0.90	0.95	0.86	0.81
MSQ4				
201	0	0.010	0	0
202	0.008	0.010	0.073	0.010
203	0.121	0.156	0.135	0.125
204	0.161	0.177	0.083	0.260
205	0.121	0.062	0.031	0.021
206	0.032	0.042	0.010	0.062
207	0.073	0.115	0.083	0.052
208	0	0	0.021	0.052
209	0.056	0.104	0.021	0.010
211	0.024	0.073	0.042	0.042
212	0.008	0	0	0
213	0.048	0.042	0.031	0.042
215	0.065	0.073	0.062	0.042
216	0.008	0	0	0
217	0.065	0.062	0.042	0.010
219	0.048	0.042	0.021	0.010
221	0.065	0.010	0.052	0.208
223	0.008	0	0.021	0.010
225	0.008	0.010	0.146	0
227	0.008	0	0	0
229	0	0	0.010	0
Null	0.073	0.010	0.115	0.042
Heterozygosity	0.90	0.93	0.81	0.78
MSQ13				
202	0	0	0.010	0
222	0.008	0	0.010	0
224	0.008	0	0	0
226	0.016	0.042	0	0.052
228	0.065	0.052	0.052	0
230	0	0	0.021	0.042
232	0.234	0.219	0.250	0.167
234	0.242	0.229	0.125	0.302
236	0.129	0.167	0.219	0.208
238	0.089	0.031	0.125	0.083
240	0.056	0.115	0.042	0.073
242	0.097	0.042	0.104	0.052
244	0.032	0.073	0.010	0.010
246	0.024	0.031	0.031	0.010
Heterozygosity	0.74	0.95	0.92	0.70
MSQ16				
178	0	0.011	0	0.010
180	0.008	0	0	0.010
181	0	0	0.010	0
182	0.016	0.032	0.010	0.010
183	0.024	0	0.052	0.010
185	0.242	0.221	0.188	0.229
186	0	0	0.031	0.021
187	0.081	0.074	0.094	0.115
188	0	0	0.010	0

Table 1. Continued

Locus/allele	Adults	3E acorns	17M acorns	33W acorns
189	0.145	0.211	0.260	0.135
191	0.145	0.084	0.073	0.167
193	0.065	0.074	0.042	0.104
195	0.073	0.053	0.052	0.031
197	0.048	0.011	0.073	0.031
199	0.056	0.032	0.042	0
201	0.032	0.032	0	0.021
203	0.024	0.084	0	0.031
205	0.008	0.053	0.031	0.021
207	0.016	0	0	0.031
209	0.016	0.021	0.021	0.021
219	0	0.011	0	0
221	0	0	0.010	0
Heterozygosity	0.82	0.81	0.92	0.89

Alleles are named by base pair length of PCR product. Frequencies of offspring alleles include only the paternal contribution to the genotype. Frequencies and heterozygosities for loci MSQ3 and MSQ4 could not be precisely determined because of the presence of null alleles (see text for estimation procedure).

Paternity Analysis

All three trees showed remarkably high numbers of pollinations from outside the stand. Tree 3E, at the eastern edge of the stand, had the lowest numbers of outside pollinations. The genotypes of 47 of 92 acorns (51%) of tree 3E did not match any adult in the stand. The probable number of cryptic gene flow events in the remaining 45 acorns was 3.5. In other words, 3–4 of these 45 acorns may not have been pollinated by an individual of a matching genotype, and therefore must have been pollinated from outside the stand. Correcting the previous figures for cryptic gene flow raises the number of outside pollinations to approximately 51 (55%) and lowers the number of within-stand pollinations to 41 (45%). Tree 17M, in the middle of the stand, had the highest number of outside pollinations at 59 of 94 (63%). Estimated cryptic gene flow was 3.7, bringing the probable number of outside pollinations to 63 (67%). Tree 33W, at the western edge, was intermediate at 56 of 96 acorns (58%) pollinated from outside the stand, or 60 (62%) after correction for cryptic gene flow. Over all samples, corrected for cryptic gene flow, 174 of 282 acorns, approximately 62%, were pollinated from outside the stand. Using the corrected numbers of acorns, a chi-square test of association was done comparing the offspring of the three trees. No significant difference in the proportion of acorns pollinated from within the stand and outside the stand was found among the three trees ($\chi^2 = 2.68$, $df = 2$, $P > .25$).

The likelihood of a cryptic gene flow event varied from 0.0008 to 0.42 for individual acorns. The frequency distribution

Table 2. Frequency distribution of the probabilities that a matching haplotype is an unrelated individual for all matches between putative pollen donors and offspring cohorts from three trees

Probability	Count	Percent
$P < .05$	49	40.8
$.05 \leq P < .10$	28	23.3
$.10 \leq P < .15$	19	15.8
$.15 \leq P < .20$	11	9.2
$.20 \leq P < .25$	4	3.3
$.25 \leq P < .30$	6	5.0
$.30 \leq P < .35$	2	1.7
$.35 \leq P < .40$	0	—
$.40 \leq P < .45$	1	0.8

of these probabilities over all matching acorns is shown in Table 2. Although a few matches had uncertainties greater than 0.20, about 80% of the matches had uncertainties of less than 0.15. These probabilities were used to generate a range of values representing the most conservative (“unambiguous”) estimates at the conventionally accepted 95% significance level to the most inclusive estimate, using all potential matches. Additionally the presence of a few common alleles resulted in 13 offspring (of 120 matches or 282 total) with 2 potential pollen donors. These individuals were excluded from the unambiguous analyses and the matching tree closest to the seed parent was considered the pollen donor for the inclusive analyses. In most of the analyses, there was no difference in the results between the unambiguous and the inclusive groups. We will only present the inclusive results except in the case where the results of the two analyses differed.

Within the stand, tree 3E had 20 possible mates, tree 17M had 17 possible mates, and tree 33W had 18 possible mates. These numbers were not significantly different in a chi-square test of association ($\chi^2 = 0.36$, $df = 2$, $P > .75$). Overall, 38 of the 62 trees in the stand may have contributed pollen to these three trees. Relative numbers of fertilizations from each pollen donor are shown in Figure 2. The highest number of fertilizations from a single donor for tree 3E was 7, for tree 17M was 5, and for tree 33W was 9. Self-pollination was possible for one acorn of tree 33W, but this individual also matched a possible gamete for a tree to the southwest, so selfing cannot be confirmed. It is interesting to note that pollen flow from two trees to the northwest of tree 3E was not apparently impeded by the presence of a red oak between them and tree 3E (Figure 2A). Because no pollen donor had a disproportionately high num-

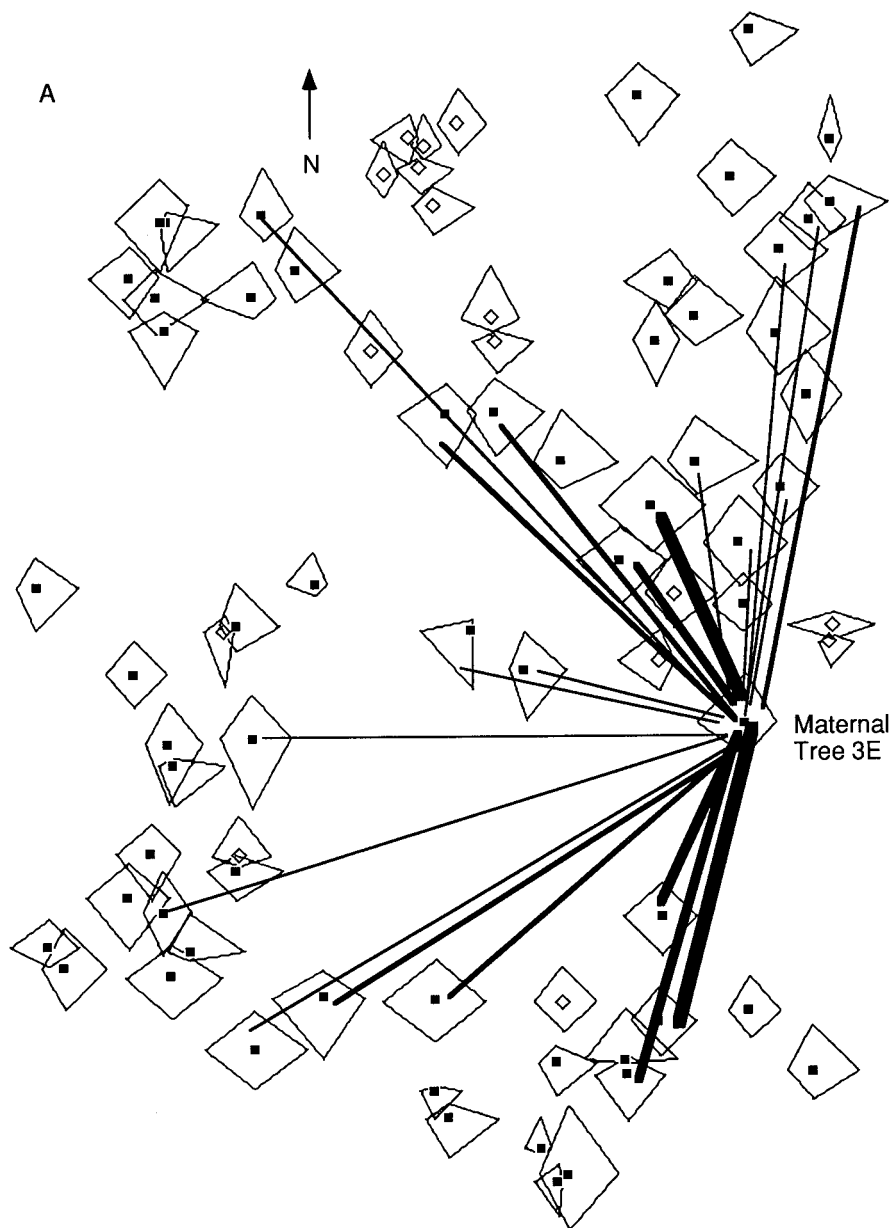


Figure 2. Locations of pollen donors for three seed parents: (A) tree 3E, (B) tree 17M, and (C) tree 33W. Filled squares represent bur oak trunks, open diamonds represent red oak trunks, and polygons represent approximate crown size. Width of line indicates number of pollinations from 1 (thinnest line) to 9 (thickest line).

ber of fertilizations, differential pollination success of nearby trees was not sufficient to explain observed differences in allele frequencies between maternal tree and acorns, discussed previously for trees 17M and 3E. It is more likely that these differences arose from the large influx of pollen from other stands.

Spatial Analysis

The pattern of fertilization of the three trees is shown in Figure 2. A join-count spatial autocorrelation of each tree with its respective pollen donors indicates that pollen donors for trees 3E and 17M are randomly dispersed within the stand. Pol-

len donors for tree 33W are significantly clustered ($P < .01$). The clustering of pollen donors around tree 33W may account for the low heterozygosity found at three of the loci in the offspring cohort (Table 1) because many of the trees nearby shared at least one allele with tree 33W.

To further test differences among the trees, a chi-square test of association was done to determine if the number of acorns pollinated by near neighbors varied among the trees. “Near” was defined as 0–50 m because this range gave adequate expected values, and a chi-square test showed no significant differences in number of trees within 50 m of the three ma-

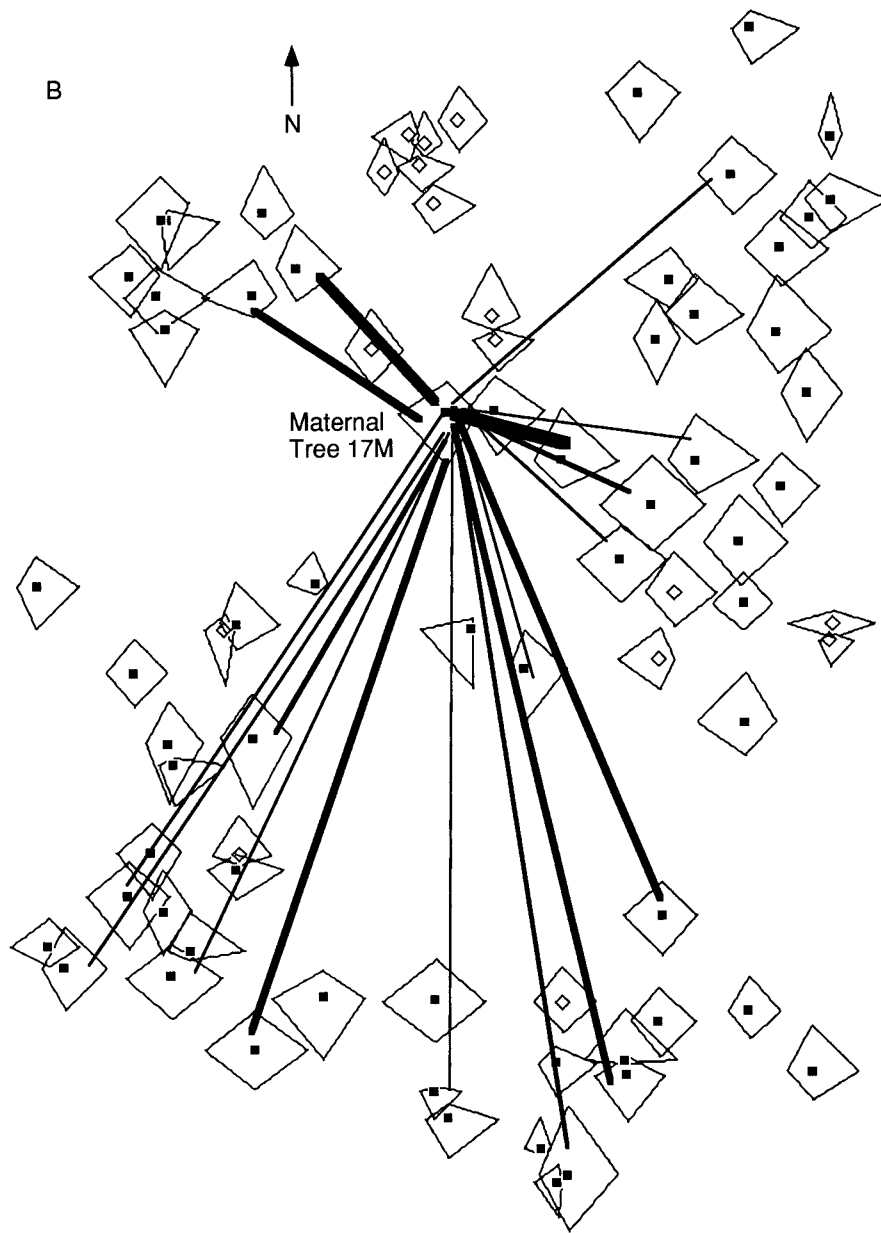


Figure 2. Continued.

ternal trees ($\chi^2 = 1.3$, $df = 2$, $P > .4$). Therefore differences in pollinations would not be a direct result of more or fewer neighboring trees. Tree 3E had 11 pollinations from near trees and 36 pollinations from within-stand trees more than 50 m away ("distant"). Tree 17M had 8 near and 15 distant pollinations, and tree 33W had 23 near and 12 distant pollinations. A chi-square test of association for the number of pollinations in the two distance classes revealed significant differences in the number of apparent pollinations from trees 50 m or closer and those within the stand but more than 50 m away ($\chi^2 = 15.3$, $df = 2$, $P < .001$). Tree 3E had fewer near pollinations than expected by

chance, tree 17M was close to expected, and tree 33W had more than expected near pollinations. Although the number of trees within 50 m was not significantly different, the spatial pattern of these trees was not the same. Trees around tree 33W were closer overall than neighbors of the other trees. The mean distance between tree 33W and trees within 50 m is 18.4 ± 10.1 m. (SD) while mean distances to neighbors within 50 m is 44.3 ± 9.5 m for tree 17M and 44.7 ± 4.3 m for tree 3E. These distances are significantly different (Kruskal-Wallis H test, $P = .0089$). Tree 33W, then, is the only tree to receive pollen frequently from its neighbors, and its neighbors are closer than neighbors of the

other two trees. These results suggest that a near-neighbor advantage may exist over very short distances, but disappears within about 45 m.

The mean distances of pollen donors from trees 3E and 17M were longer than for tree 33W. The mean distance between tree 3E and its pollen donors was 68.6 ± 22.9 m (SD) for unambiguous matches and 75.3 ± 28.7 m for all matches. For tree 17M, mean distance was 79.6 ± 44.7 m (unambiguous) or 88.8 ± 49.6 m (all). Tree 33W had the shortest mean distances and the highest variability at 64.3 ± 82.4 m for unambiguous matches and 63.0 ± 70.8 m for all matches. These differences are not significant for the unambiguous matches, but are highly significant for all matches (Kruskal-Wallis H test, $P = .0044$).

The Spearman rank correlation coefficient was used to examine the relationship between individual pollination success and distance from the maternal tree. The number of acorns pollinated by individual trees in the stand ranged from 0 to 9, with a mode of 0, so there were many tied ranks. It was therefore appropriate to correct the correlation coefficient for ties (Ebdon 1985). Given the previously discussed clustering of pollen donors around tree 33W, it was not surprising that the Spearman coefficient was significant for the association of fertilization and distance from the maternal tree ($r_s = -0.35$, $P = .008$). The negative value of the correlation indicates an inverse relationship, that is, closer trees fertilize more seeds. Also as might be expected, pollen donors of tree 17M showed no significant correlation between distance and the number of fertilizations ($r_s = -0.05$, $P = .72$). Although tree 3E seemed similar to tree 17M in tests of clustering and neighbor fertilizations, trees contributing pollen to this tree had the strongest association of distance and number of fertilizations of the three trees studied, although the relationship was still fairly weak ($r_s = -0.45$, $P = .0005$). Thus, while a large proportion of effective pollen did not come from trees within 50 m of the tree, the overall trend for the entire stand indicates that near trees had a fertilization advantage over distant trees in mating with tree 3E. In this analysis of fertilization from within the stand, it is important to bear in mind that more than half of the acorns from any tree were fertilized by pollen from outside the stand.

Discussion

Each microsatellite locus in this study was highly variable. The number of alleles per

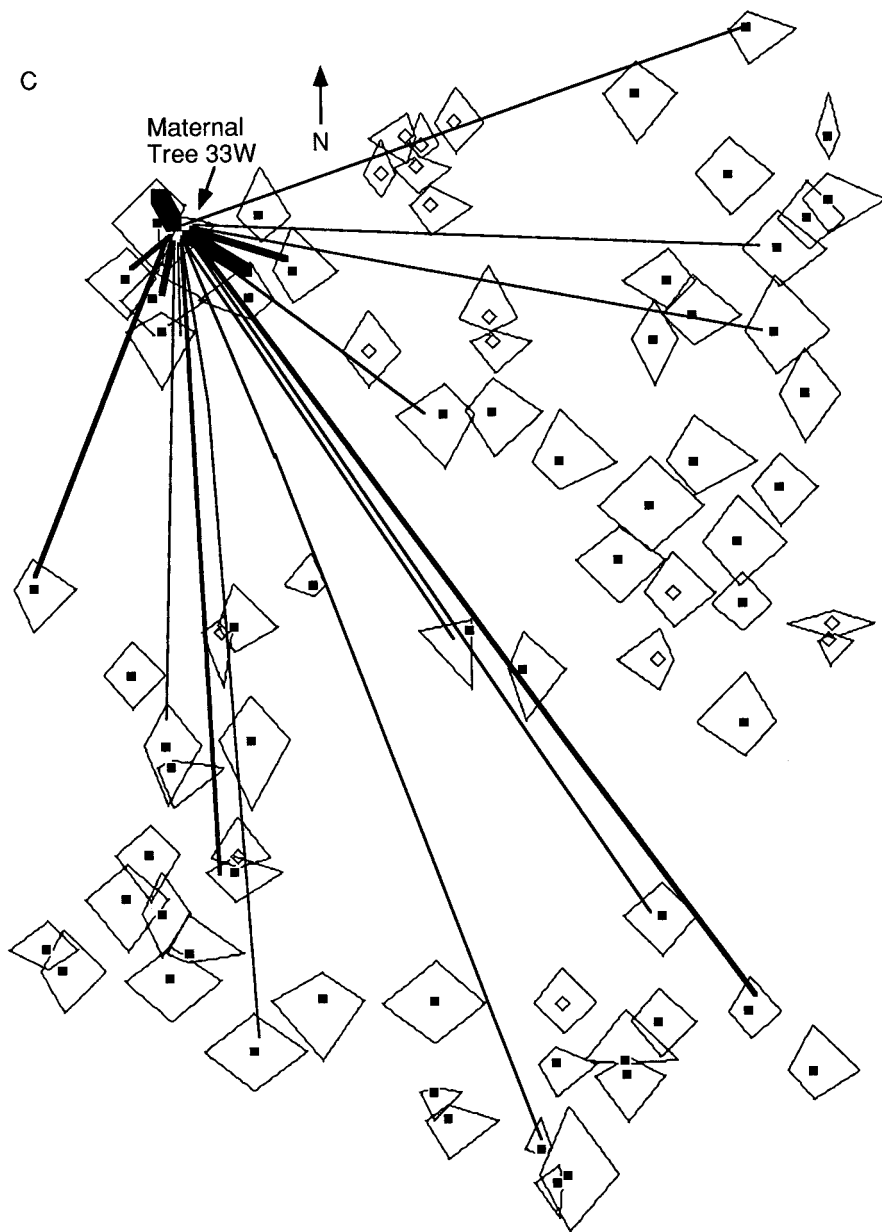


Figure 2. Continued.

locus ranged from 14 to 24, with a total of 83 alleles over four loci. The most common allele had a frequency of 0.24. More than half (55.6%) of these alleles were present at frequencies of less than 0.05, meeting the general criteria for rarity. Microsatellites therefore have the large numbers of rare alleles that are required to perform paternity analyses by exclusion. Of 282 offspring studied 269 (95%) matched either one or no paternal parent in the stand. In the remaining 13 cases (5%), assignment of paternity could be narrowed down to two adults.

The presence of a few relatively common alleles resulted in some of the matches between parent and offspring being

somewhat uncertain (probability of matching by chance was greater than 0.05). Even with this uncertainty, however, microsatellite paternity analysis has several advantages over allozyme methods. In most-likely paternity analysis using allozymes, assignment of paternity is biased toward homozygous individuals so that a heterozygous individual may not be matched to its true offspring (Adams et al. 1992). Furthermore, a high proportion of offspring often must be discarded from allozyme analysis of parentage because alleles are too common to be informative (Godt and Hamrick 1993; Meagher 1986). Paternity exclusion using microsatellites will not exclude a true parent, even if cer-

tainty is sometimes relatively low, and all offspring can be included in the analysis. Using four microsatellite loci, 80% of the parent-offspring matches had certainties of 85% or better, which reflects the correct match most of the time. An additional locus of similar variability would probably lower the likelihood of an unrelated match to 0.05 or less for all offspring.

Our analysis of paternity in bur oaks has revealed several remarkable aspects of wind pollination. Primary among these is that at least 57% of all the acorns included in the study were fertilized by trees outside the stand, the pollen traveling at least 100 m from the pollen donors. This value increases to 62% when corrected for the probability of cryptic gene flow. This proportion of alien pollinations was within the range of pollen contamination (32–83%) measured by pollen traps in a wind-dispersed conifer orchard (Caron and Leblanc 1992). As with all pollen trap studies, no information was gained on what pollen does within the stand. Microsatellite analysis combined with pollen trap studies could better establish the relationship between pollen movement and effective pollination, which will lead to better predictions of genetic structure based on pollination mechanism (Loveless and Hamrick 1984).

Our estimate of gene flow is substantially higher than that reported in allozyme studies of conifers (16 migrants per generation in ponderosa pine, Hamrick et al. 1989; 11 migrants per generation in limber pine, Schuster et al. 1989) and elm (2–3 migrants per generation, Sherman-Broyles et al. 1992). Even though the number of migrants refers to individuals that survive to reproductive maturity, our results suggest that, in the absence of selection for local genotypes, over half of the members of each generation will have paternal alleles from outside the stand.

Studies of crops have shown that pollen flow from outside the field was limited to the windward side. Because a pollen trap study suggested a southeast bias in pollen deposition when averaged over several days (Dow 1995), we expected that tree 33W, on the northwest side, would have the highest proportion of seeds pollinated from outside the stand, and tree 3E, on the east side, would be pollinated primarily by trees from inside the stand. Pollen deposition data from individual days indicated variable directions in peak deposition (Dow 1995), therefore an alternative hypothesis was that the number of pollinations from outside the stand may be strict-

ly related to the distance from the nearest stand. In this case, tree 3E would be expected to have more outside pollinations than tree 33W, because the distance between tree 3E and the nearest tree outside the stand was approximately 100 m (southeast), while this distance for tree 33W was about 200 m (northeast). However, the number of alien pollinations was not significantly different for trees in different parts of the stand, suggesting that neither direction nor distance from neighboring stands were major determinants in pollination success.

Microsatellite analysis can be used not only to estimate gene flow but to determine the pattern of pollen dispersal within the stand. Because individual pollen donors are identified, actual distance of pollen movement within the stand can be measured. The mean pollen dispersal distance exceeded 60 m for all maternal trees. The distance between potential pollen donors and tree 17M was not correlated with the number of acorns fertilized. The distance to pollen donors and the number of acorns fertilized was significantly correlated for trees 3E and 33W, but the relationship was not strong. Distance between trees may influence pollination, but it is probably not a major determinant of pollination success. Successful self-pollination in bur oaks, if it occurs at all, is exceedingly rare.

The spatial relationship among maternal trees and their pollen donors varied. The pollen donors of two trees, tree 3E and tree 17M, appeared to be dispersed at random throughout the stand, and neighboring trees had no apparent fertilization advantage. Tree 33W, which had closer neighbors than tree 3E or 17M, had a higher proportion of neighbor matings than the other two trees, and also showed significant clustering of pollen donors. This was the only evidence of nonrandom mating in the population, and the acorns fertilized by neighbors comprised less than 24% of the total sample for this tree. Neighboring trees may have an advantage at very short distances, but this effect apparently diminishes by about 45 m from the maternal tree. Another factor which may be important in nonrandom mating is that this tree was farther from the nearest neighboring stands than either tree 17M or 3E. However, this greater distance would lead to an expectation that there would be fewer pollinations from outside the stand, and there was no difference among the three trees for this variable. This result also suggests that the presence

of near neighbors does not inhibit pollen flow from distant sources. It would be interesting to determine if gene flow is inhibited in large, dense stands of trees.

The generally low incidence of neighbor pollinations and high incidence of gene flow suggests that pollination success of wind-dispersed pollen does not follow standard models based on air flow dynamics of dispersal from a single source. Over evolutionary time, pollen grains have been selected for size, mass, buoyancy, and other characteristics, but the fact remains that pollen will become diluted in air as it travels from its source (Faegri and van der Pijl 1979; Levin and Kerster 1974). A limited pollen trap study at this site suggested that pollen densities did fall off rapidly with distance from the source tree (Dow 1995). We have shown that much of the pollen that effectively fertilizes seeds comes from a source some distance from the maternal tree. One possible explanation is that theoretical models of pollen dispersal from a single source do not represent actual pollen movement from many individuals, and that pollen densities do not drop as sharply as these models predict. The leptokurtic tails of the distributions of single trees may overlap to form a pollen cloud around a maternal tree (Adams 1992) that would provide that tree with access to many pollen donors. It is also possible that clustering of pollen grains may cause the distribution of fertilizations to be flatter and broader than the distribution of pollen (Tonsor 1985).

Another explanation for the high number of fertilizations from outside the stand is that there may be some mechanism by which a tree can "choose" pollen from distant sources over the more dense pollen from nearby. If trees within the stand shared more alleles with each other than with trees outside the stand, pollen from outside the stand could then be favored over within-stand pollen based on genetic dissimilarity. Selection in oaks may occur by delayed fertilization, selective abortion, or embryo competition (Willson and Burley 1983). Germination rates of developed acorns were greater than 90% for all samples, suggesting that the selective process occurs prior to acorn maturation.

The timing of pollen release is a third possible explanation for the observed fertilization pattern. Pollen release is generally believed to be regulated by environmental cues (Whitehead 1983), yet the principle of dichogamy is that some trees flower slightly ahead of others so that the pollen of a tree is released before its stig-

mas are receptive, but while another tree is receptive. Thus observed pollen donors may have been the only trees releasing pollen when the maternal trees had receptive stigmas. We do not have accurate field observations of the flowering release times of all the trees in the stand, so we cannot determine if this was a factor in the observed within-stand pollinations. However, as Willson and Burley (1983) suggest, the stigma may be able to sample pollen for a month, which is as long or longer than the period in which all pollen release occurs. Thus staggered times of pollen release, while effective in preventing self-fertilizations, may not be sufficient to explain the low incidence of within-stand fertilizations.

Conclusions

Wind pollination in bur oaks seems to be extraordinarily efficient at producing highly outbred individuals and ensuring long-range pollen flow. The high numbers of fertilizations from outside the stand contradict traditional models of pollen movement, but can explain the results of allozyme studies showing little population differentiation in wind-pollinated plants. Because paternity could be determined by exclusion, a very detailed picture of fertilization can be obtained through the use of microsatellite analysis.

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