

Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis

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Patterns of pollen dispersal were investigated in a small, isolated, relict population of *Pinus sylvestris* L., consisting of 36 trees. A total-exclusion battery comprising four chloroplast and two nuclear microsatellites (theoretical paternity exclusion probability $EP=0.996$) was used to assign paternity to 813 seeds, collected from 34 trees in the stand. Long-distance pollen immigration accounted for 4.3% of observed matings. Self-fertilization rate was very high (0.25), compared with typical values in more widespread populations of the species. The average effective pollen dispersal distance within the stand was 48 m (or 83 m excluding selfs). Half of effective pollen was dispersed within 11 m, and 7% beyond 200 m. A strong correlation was found between the distance to the closest tree and the mean mating-distance calculated for single-tree progenies. The effective pollen dispersal distribution showed a leptokurtic shape, with a large and

significant departure from that expected under uniform dispersal. A maximum-likelihood procedure was used to fit an individual pollen dispersal distance probability density function (dispersal kernel). The estimated kernel indicated fairly leptokurtic dispersal (shape parameter $b=0.67$), with an average pollen dispersal distance of 135 m, and 50% of pollen dispersed beyond 30 m. A marked directionality pattern of pollen dispersal was found, mainly caused by the uneven distribution of trees, coupled with restricted dispersal and unequal male success. Overall, results show that the number and distribution of potential pollen donors in small populations may strongly influence the patterns of effective pollen dispersal.

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Introduction

Pollen dispersal is a major component of gene flow in plant populations. Although it lacks the colonization function of seeds, the potential for long-distance transport of male gametes greatly influences genetic processes that have central evolutionary implications, such as 'isolation by distance' within continuous populations and gene exchange among spatially isolated populations (Wright, 1946; Crawford, 1984; Ennos, 1994). Since early experimental attempts to estimate contemporary pollen dispersal in tree populations using traps or dyes (Koski, 1970; Levin and Kerster, 1974), a number of analytical procedures have been developed to make best use of effective pollen dispersal information derived from molecular marker assays (for a review, see Smouse and Sork, 2004). Among them, those based on paternity analysis have been widely used to characterize spatial patterns of pollen dispersal in tree species (Adams, 1992; Burczyk *et al.*, 1996; Stacy *et al.*, 1996; Dow and Ashley, 1998; Kaufman *et al.*, 1998; Streiff *et al.*, 1999; Konuma *et al.*, 2000; Schuster and Mitton, 2000; Lian *et al.*, 2001; Burczyk *et al.*, 2002).

Two major limitations are inherent in pollen flow studies based on paternity analysis. First, despite increasingly high-resolution genetic markers, total exclusion of all but one male when assigning paternity to offspring from maternal trees remains beyond most practical situations (Chakraborty *et al.*, 1988). Fractional paternity analysis and likelihood-based procedures have been developed to tackle this problem, providing unbiased estimates of male reproductive success (Meagher and Thompson, 1986; Devlin *et al.*, 1992; Smouse and Meagher, 1994). Second, the exhaustive sampling of males required for paternity assignment often limits the spatial extent of analysis, for practical reasons, to the point that a very substantial number of offspring are found to have been sired by unknown males from outside the study area. Consequently, even if unambiguous assignment has been achieved for a portion of the sample, a large gap will exist between the spatial scale of effective pollen dispersal in the population and the scale over which it is actually measured. Inferences about the pollen dispersal distribution will often be problematic (Smouse and Sork, 2004).

Small, isolated populations provide an interesting opportunity for mating and pollen dispersal studies. A reduced number of individuals will potentially allow total discrimination of pollen contributions, enabling unusually categorical paternity analysis. In addition, it will be possible to collect seeds from virtually all trees,

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extending the experimental scale to the whole population and achieving ample individual replication. Additionally, if spatial isolation results in the absence of substantial pollen immigration, most of the observations can be converted into mating distances, and little information will be lost for the estimation of the effective pollen dispersal distribution or other within-population reproductive parameters. Besides these analytical advantages, small population genetic processes are significant in themselves, from an evolutionary and conservation point of view (Ellstrand and Elam, 1993).

An important question arises when inferring the effective pollen dispersal distribution in small populations, the extent to which the results obtained can be compared among different spatial and/or demographic situations. Under the theoretical framework of 'competing pollen sources' (Levin and Kerster, 1974; Adams, 1992), the mating success of a given individual is proportional to its relative contribution to the pollen cloud around different female trees. Different effective pollen dispersal distributions can thus be expected when population size ranges from a few tens of scattered individuals to several hundreds or thousands of trees, growing in dense woodlands and adding their long-distance pollen contributions. One way to generalize gene flow measurements is to estimate the mean dispersal probability density function for individual plants (ie the dispersal kernel), which has been used to model gene escape from transgenic fields, colonizing processes of plants, or to draw inferences about factors affecting male reproductive success (Kot *et al*, 1996; Lavigne *et al*, 1996; Clark, 1998). The dispersal kernel describes the probability of a pollen grain travelling a given distance from an individual source. It is by definition independent of the experimental design, providing a test of how effective pollen dispersal is affected by differences in number, density, or spatial distribution of conspecific reproductive individuals (Lavigne *et al*, 1996).

In this study, we investigated spatial patterns of pollen dispersal for Scots pine (*Pinus sylvestris* L.). Scots pine has the widest geographical distribution of any species in the genus, and is often viewed as the paradigm for monoecious, wind-pollinated, predominantly outcrossing conifers (Muona and Harju, 1989; Kärkkäinen *et al*, 1996). Previous studies using pollen traps and tracking marker isozyme alleles suggest Scots pine's potential for long-distance pollen dispersal (Koski, 1970; Yazdani *et al*, 1989). In the present study, we used a combined high-resolution battery of chloroplast and nuclear microsatellites to perform a total-exclusion paternity analysis on seeds collected from all trees in a small isolated stand, providing detailed estimates of the effective pollen dispersal distribution and the dispersal kernel for the species.

Material and methods

Study site and sample collection

The study site is an isolated relict stand of Scots pine, located on the plains of the Northern Meseta plateau, 790 m above sea level, in Segovia province, central Spain. It is one of only two Scots pine populations growing on the dry plains of the Iberian Peninsula at present, which survive thanks to a local phreatic supply of water. The nearest *P. sylvestris* populations is an 8000-tree stand, located 30 km east of the study site (Cuéllar), and the next nearest is a widespread, monospecific woodland, growing in the Guadarrama Chain, 60 km to the south. The entire population consists of 36 Scots pine trees, about 100 years old, scattered within a continuous maritime pine (*Pinus pinaster* Ait.) forest, over an area of approximately 20 ha (Figure 1). Exhaustive (measuring every tree), 10-yearly inventories carried out since the beginning of the 20th century by the regional forest administration have failed to find any other Scots pine in this *P. pinaster* forest. Both Scots pine and the surrounding maritime pines are about 10–15 m high. Pollination of

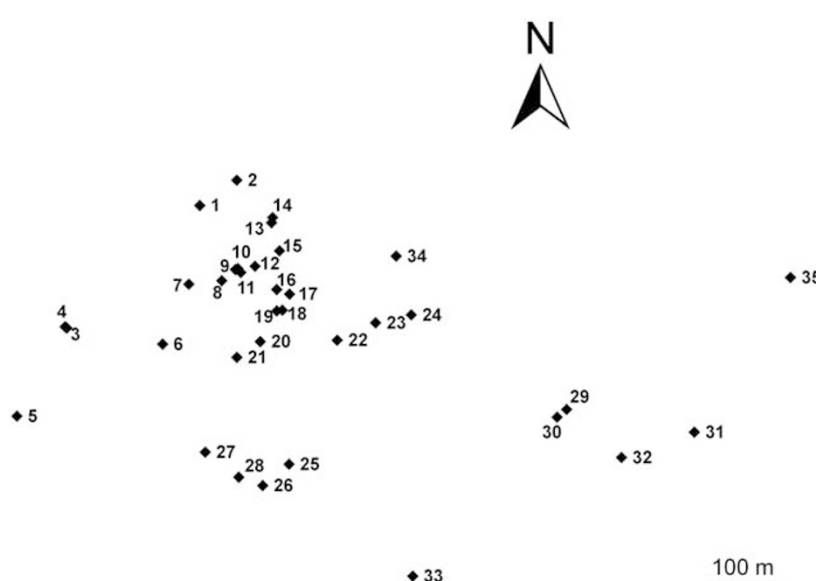


Figure 1 Spatial location of the 36 *P. sylvestris* individuals in the study stand. The area is flat and covered with a continuous *P. pinaster* forest, within which *P. sylvestris* trees are scattered.

P. sylvestris by closely related species (leading to the formation of empty seeds) has been reported (Sarvas, 1962). Flowering of the two pine species in the area does not overlap, however, since maritime pine pollen dispersal (late April–early May) is already finished when Scots pine female strobili become receptive (late May–early June). Significant pollination of the few *P. sylvestris* trees with pollen from the surrounding *P. pinaster* stands can thus be disregarded. Spatial coordinates of all Scots pine individuals in the stand were obtained using Trimble's GeoExplorer® 3 GPS-based mapping system with postprocessing differential correction.

Needles were collected from all 36 trees in the stand to determine the adult genotypes. Climbers collected all accessible mature cones from 35 out of the 36 trees (individual 35 bore no cones). Cones from each tree were open and seeds were extracted, pooled by tree, and stored at 4°C. For the paternity analysis, 22–24 randomly chosen seeds were included from each of the 35 cone-bearing trees. All seeds collected from tree 26 turned out to be empty, and the total sample size for the paternity analysis was finally $n=813$. All the individuals in the population were thus included in the study as potential pollen donors, and all but two as seed donors. The collected mature cones represent a single reproductive season, since *P. sylvestris* is a nonserotinous species. Seeds were soaked in water overnight and then placed slightly buried in moist perlite for a week, prior to DNA extraction.

DNA extraction and microsatellite analysis

Total genomic DNA was extracted from the adult needle tissue and from the germinated embryo tissue of each seed, following the protocol of Dellaporta *et al* (1983). The megagametophyte of each seed was carefully dissected away and preserved separately at –20°C for potential subsequent analysis (see below). For paternity analysis, a combined set of chloroplast and nuclear polymorphic microsatellites was used (cpSSR and nSSR, respectively). Chloroplast microsatellites are haploid and paternally inherited in pines, and their multiple nonrecombinant loci are an effective paternal marker, ideal for pollen flow studies, since they give profiles (haplotypes) that exactly match those of the pollen donors (Plomion *et al*, 2001; Provan *et al*, 2001). The ultimate aim was to employ enough genetic markers to differentiate each of the 36 adult trees in the stand, in order to assign paternity unambiguously to the embryos collected from each individual.

The following seven cpSSR primer pairs were initially tested on the 36 adults: Pt1254, Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, and Pt87268 (Vendramin *et al*, 1996). Only four of them were selected for subsequent analyses (Pt15169, Pt30204, Pt36480 and Pt71936), the other three providing very little extra discrimination. To achieve additional exclusion power, two nSSR (biparentally inherited) markers were also used: SPAC11.4 and SPAC12.5 (Soranzo *et al*, 1998). Polymerase chain reaction (PCR), electrophoresis and PCR fragment scoring were performed according to the protocols described by Gómez *et al* (2003) and Robledo-Arnuncio *et al* (2004) for cpSSR and nSSR, respectively.

Paternity assignment

Paternity assignment was conducted by simple exclusion, based on the cpSSR and nSSR multilocus profiles. First, the 36 adult genetic profiles at both kinds of markers were obtained from the needle tissue collected from each tree in the stand. Second, the male genetic contribution to each of the 813 embryos was inferred. In the case of cpSSRs, the combination of the size variants recovered from the haploid cpSSR sites of the embryo is identical to the corresponding father's profile, and the male genetic contribution is thus observed directly. In the case of the diploid nuclear microsatellites, the male contribution was obtained by subtracting the female contribution from the multilocus genotype of the embryo. In the ambiguous case of both seed parent and offspring being heterozygous and sharing the same alleles at one locus, the maternal contribution was obtained by assessing that locus from the corresponding embryo megagametophyte. This is possible in pine species, in which the megagametophyte tissue of the seed is haploid and genetically identical to the ovule.

The male contribution to each embryo, both at the cpSSRs and nSSRs, was then compared with the genetic profiles of the 36 adults in the stand. Three alternative conclusions are possible after simple exclusion analysis: (i) only one male cannot be excluded, (ii) several males cannot be excluded and (iii) all males are excluded. In those cases where all males were excluded, PCR and electrophoresis were repeated to detect any genotyping errors. When the same result was obtained after this verification, pollen was considered to originate from outside the stand. Among the rest of the embryos, all turned out to be associated with a single male parent within the stand, yielding a total-exclusion paternity analysis. The nonexcluded male was assigned paternity, and the distance of effective dispersal (δ) was recorded as the distance between mates for subsequent analyses. Self-fertilization was considered as zero distance. The axial variance of effective pollen dispersal (σ) was estimated as $\sigma^2 = \sum \delta_i^2 / 2(n-1)$, where n is the number of observations (Crawford, 1984).

The method described by Harju and Nikkanen (1996) was used to estimate the probability of undetected (cryptic) pollen flow from outside the stand, that is, immigrant male gametes from distant populations genetically indistinguishable from those produced by the adults within the stand. The portion of detectable immigrant pollen gametes, f , was estimated from the allelic frequencies determined from a 105-tree sample from the nearest (background) population. The coefficient f was estimated as the sum of the expected frequencies of all possible multilocus gametes in the background populations that are not present among the adult trees of the stand (Harju and Nikkanen, 1996). It was assumed that the frequency of multilocus gametes was the product of their single-locus allele frequencies, considering the nonrecombinant chloroplast genome as an independent single 'locus' and each different chloroplast haplotype as an 'allele'. It is also assumed in this method that immigrant pollen pool frequencies can be estimated from the sampled adult trees of the nearest populations. The actual level of pollen flow from outside the stand was derived by multiplying the detected level by $1/f$.

Pollen dispersal distance analysis

The relationship between effective pollen dispersal and distance was tested in two ways. First, Spearman's rank correlation coefficients were determined for the average effective pollen dispersal distance (excluding selfing), calculated for each seed-tree sibship, and the average pairwise distance from the seed-tree to (i) the closest tree, (ii) the three closest trees, (iii) the 10 closest trees and (iv) all the other trees in the stand. An analogous correlation analysis was also done for the self-fertilization rate within each seed-tree sibship. Spearman's rank correlation coefficients were used, rather than the Pearson product-moment correlation coefficients, because of the non-normal distribution of the variables and the existence of outlying observations (eg tree 36).

Second, data were pooled over all 34 seed-trees, and the frequency distribution of intermate distances was compared with that generated under the null hypothesis of uniform dispersal. To do so, 20 m distance classes were considered (min 0 m, max 760 m), including a separate class for self-fertilization. Owing to the uneven spatial distribution and the small number of trees in the stand, we expect an excess (or shortage) of mating events within particular distance classes containing more (or less) tree-pairs than the expected number under a uniform spatial distribution of individuals. To correct for this effect, the observed number of mating events within a given distance class was divided by the number of tree-pairs existing in that class (yielding the average number of matings per tree-pair within the class) before calculating the relative frequency distribution of mating distances.

Uniform dispersal was simulated by generating a 24-embryo sibship for each seed-tree of the stand, with the male parent of each offspring randomly chosen from among all the 36 potential pollen donors (random mating with self-fertility). In doing so, the probability of effective dispersal is independent of distance, generating a uniform pollen dispersal distribution. The process was repeated 10 000 times to calculate 95% confidence intervals for the number of mating events within each distance class under the null hypothesis.

A more general investigation of the patterns of pollen dispersal was carried out by estimating the individual dispersal kernel, that is, the average pollen dispersal probability density function for individual trees (Wright, 1946). The dispersal kernel describes the probability of a pollen grain being dispersed a given distance from the source individual. A two-parameter bivariate exponential-power function was considered:

$$p(x, y) = \frac{b}{2\pi a^2 \Gamma(2/b)} \exp\left(-\left(\frac{\sqrt{x^2 + y^2}}{a}\right)^b\right) \quad (1)$$

where $p(x, y)$ denotes the probability per unit area of pollen dispersal to a point at a distance $\sqrt{x^2 + y^2}$, Γ is the gamma function, a is the scale parameter for distance and b is the shape parameter (Clark, 1998; Dick *et al.*, 2003). The case of $b=2$ corresponds to a bivariate Gaussian distribution, whereas $b=1$ corresponds to a bivariate exponential distribution. The average dispersal distance is given by $a\Gamma(3/b)/\Gamma(2/b)$. We employed a recently developed maximum-likelihood procedure (S Oddou-Muratorio, EK Klein and F Austerlitz, unpublished) to fit the dispersal kernel on paternity analysis data. Following

their method, the expected relative contribution (π) of pollen donor i to the progeny of mother j can be described in terms of 'competing pollen sources' (see also Devlin *et al.*, 1992) by

$$\pi_{ij} = \frac{p_{ij}}{\sum_{k \in N} p_{kj}} \quad (2)$$

where p_{ij} denotes the probability, given by the dispersal kernel (1), of a pollen grain travelling the distance that separates trees i and j , and N is the total number of individuals in the stand. From equation (2), and based on the total set of S seeds for which paternity had been unambiguously determined, the kernel parameters a and b were estimated by maximizing the log likelihood:

$$L(a, b) = \sum_{s=1}^S \log(\pi_s(a, b)) \quad (3)$$

in which π_s denotes the expected contribution of the male parent of seed s to the seed parent of s , calculated from equation (2) under a dispersal kernel with parameters a and b . Since there was no available information about phenology or pollen fecundity to include in the model, synchronous phenology and equal male fecundity were assumed. The method also assumes that all trees disperse their pollen similarly.

In order to visualize the fit of the model to observed data, effective pollen dispersal was simulated in the stand under the estimated dispersal kernel. To do so, a 24-seed sibship was generated for the j th seed-tree, the paternity of each of her offspring being assigned to the i th pollen donor with probability π_{ij} , given by equation (2), assuming an exponential-power kernel with the estimated parameters a and b . The resulting frequency distribution of mating distances was then calculated, considering 20-m distance intervals, and 95% confidence limits were obtained for each interval frequency by repeating the process 10 000 times. Finally, results were plotted against the observed frequency distribution.

Pollen dispersal directionality

The null hypothesis of isotropic pollen dispersal in the stand was tested. For this purpose, the neighbourhood of each seed-tree was subdivided into eight equal angular sectors. The average number of mating events per tree-pair was then calculated for each sector by dividing the observed number of effective pollinations by the number of potential pollen donors lying in the sector. The resulting frequency distribution of mating directions was averaged over all maternal trees. The distribution under the null hypothesis was obtained by simulating isotropic dispersal in the stand. Three different simulation sets were performed. First, male gametes were considered to be dispersed in an isotropic uniform fashion, simulated as described previously for random mating with self-fertility. Second, dispersal was assumed to be isotropic but spatially restricted, simulated by using the estimated dispersal kernel, as previously described. And third, isotropic restricted dispersal with unequal male success was considered. Relative male success of the i th pollen donor (λ_i) was estimated as the proportion of the total seed sample sired by the i th individual. In this case, paternity of each of 24 offspring of mother j

was assigned to father i with probability given by

$$\pi_{ij} = \frac{\lambda_i p_{ij}}{\sum_{k \in N} \lambda_k p_{kj}} \quad (4)$$

Each simulation set consisted of 10 000 random boots, which were used to construct 95% confidence intervals for the relative frequency of mating events within the eight sectors under each of the three kinds of isotropic dispersal scenarios.

Results

Paternity analysis

The number of size variants at each cpSSR site ranged from 2 to 5 in the 36-trees adult population, constituting 23 different four-marker chloroplast haplotypes; haplotypic diversity was 0.940. The observed number of alleles and genetic diversity of the two nSSRs were, respectively, 13–21 and 0.843–0.927. Considering the chloroplast genome as a single independent ‘locus’ and each of the observed chloroplast haplotypes as an ‘allele’, the theoretical multilocus paternity exclusion probability (Jamieson and Taylor, 1997) among the adults and over the whole genetic battery was 0.996.

For 35 embryos out of the 813 analysed, no possible male parent was found within the 36 potential pollen donors in the stand, yielding a minimum estimate of effective pollen immigration from outside the stand of 4.3%. Interestingly, the 35 immigrant male gametes must have been drawn from at least 30 different trees, since 30 of them showed a unique chloroplast haplotype. Trees receiving immigrant pollen were located both in the centre and periphery of the stand (see the spatial location of individuals in Figure 1, and the number of embryos from each seed-tree considered to originate from outside the stand in Table 1). For each of the remaining 778 offspring, a single compatible male parent was found within the stand, making possible a categorical assignment of paternity. All the 778 matches were included in subsequent within-population pollen dispersal estimates.

The portion of detectable pollen gametes in the background pollen, f , estimated from the allele frequencies of the nearest populations, was 0.97, which yields a very low estimate of cryptic pollen flow: $4.3(1/f - 1) = 0.13\%$, or about one gamete in an 813-size sample. The high value obtained for f is a logical consequence of the limited number of possible pollen genotypes that can be generated in a population with only 36 adults, necessarily small when compared to the expected number in background pollen putatively originated in large populations. This fact, coupled with the very high polymorphism of the markers employed (average allele frequency = 0.02, maximum allele frequency = 0.14), resulted in this negligible estimate of cryptic immigration in the stand pollen cloud.

Pollen dispersal distance analysis

The average effective pollination distance for single mother sibships ranged from 2.1 m ($\sigma = 5.4$ m) to 149.5 m ($\sigma = 125.3$ m), with a mean of 47.6 m ($\sigma = 52.1$ m; Table 1). Selfing accounted for a very substantial portion of mating events, since a total of 205 offspring (25.2%)

could be unambiguously associated with self-fertilization. The rate of self-fertilization was quite variable among individuals, however, ranging from 0 to 92% (Table 1). When self-seeds were excluded from the analysis, the effective pollination distance calculated for single seed-tree sibships increased sharply, ranging from 10.9 m ($\sigma = 16.6$ m) to 541.9 m ($\sigma = 483.2$ m), with an average of 82.7 m ($\sigma = 76.9$ m; Table 1).

The relative spatial location of the trees had a significant effect on both their self-fertilization rate and average outbred pollination distance (Table 2). The Spearman rank correlation coefficients showed a strong positive association ($r_s = 0.719$, $P = 0.000$) between the distance to the closest individual from a seed-tree and the mean outbred pollination distance calculated within her progeny. The correlation between relative spatial location and outbred pollination distance weakened progressively (although it remained significant) when the average distance from the seed-tree to the three closest, the 10 closest and all the other trees in the stand were considered (Table 2). Self-fertilization rate also showed the strongest association with the distance to the closest neighbour, with a moderate positive correlation ($r_s = 0.608$, $P = 0.000$). These results suggest that spatial isolation of individual trees increased fertilization success of self-pollen, and that seed-trees were mainly pollinated by their nearest pollen-donor. A more detailed analysis of factors affecting male reproductive success in the stand is described elsewhere (JJ Robledo-Arnuncio and L Gil, unpublished).

The observed frequency distribution of mating distances, based on the categorical paternity assignment for 778 offspring, pooled over all 34 seed-trees, showed a large departure from that obtained under uniform dispersal (Figure 2). The shape of the observed distribution was fairly leptokurtic, with a rapid decrease of mating frequencies with distance but with low-frequency successful fertilizations still occurring over long distances (200–700 m). Half of the effective pollen (including selfing) was exchanged between trees located within 10 m from each other, and only 7% was exchanged between individuals more than 200 m apart. There was a significant excess of effective pollinations within the smallest distance classes (0–50 m), and a significant shortage in classes beyond 100 m, relative to a uniform pollination distribution (Figure 2). Consequently, the observed mean effective dispersal distance, 47.6 m, was substantially smaller than the average pairwise distance among trees, 180.4 m, which equals the expected mean pollination distance under uniform dispersal. The null hypothesis of uniform dispersal of effective pollen within the stand could be convincingly rejected.

The maximum likelihood fit of the pollen dispersal kernel for individual trees, based on a categorical paternity analysis of 778 offspring from 36 potential pollen donors, yielded a scale parameter $a = 24.08$ and a shape parameter $b = 0.67$ ($L = 1973.92$), for the bivariate exponential-power model in equation 1 (see Materials and methods). The shape parameter, smaller than unity, indicates a distribution that is quite leptokurtic, relative to the exponential ($b = 1$). The average pollen dispersal distance from individual trees corresponding to the estimated function is 135.5 m. In order to assess the model goodness-of-fit to the observed mating-distances distribution, pollen dispersal was simulated in the stand,

Table 1 Results of the categorical paternity analysis performed on the progeny sampled from each single tree in the stand

Tree	n	n_s	n_i	δ	σ	δ_{out}	σ_{out}
1	24	0	1	50.6	44.5	50.6	44.5
2	24	5	1	67.6	70.0	86.4	80.2
3	24	0	2	18.0	35.2	18.0	35.2
4	24	1	1	95.7	86.3	100.1	88.5
5	24	17	0	65.7	104.1	225.3	215.7
6	24	18	1	17.5	29.0	80.5	74.2
7	24	8	0	36.4	35.3	54.6	44.2
8	24	3	0	27.3	25.8	31.2	27.7
9	23	0	0	26.7	23.7	26.7	23.7
10	24	0	2	25.8	23.7	25.8	23.7
11	24	5	0	15.8	24.9	20.0	28.4
12	24	3	0	25.8	23.4	29.5	25.2
13	24	3	0	9.5	15.4	10.9	16.6
14	24	0	1	27.5	35.6	27.5	35.6
15	22	1	2	59.5	69.2	62.6	71.1
16	24	0	0	44.0	39.2	44.0	39.2
17	24	1	0	55.3	47.4	57.7	48.4
18	24	0	0	50.0	55.9	50.0	55.9
19	24	0	1	40.3	33.7	40.3	33.7
20	24	9	3	40.0	41.2	70.0	56.6
21	24	0	1	75.8	61.3	75.8	61.3
22	24	0	1	83.7	67.5	83.7	67.5
23	24	2	0	55.5	52.4	60.4	54.9
24	24	5	3	55.2	54.7	72.5	63.7
25	24	2	4	149.5	125.3	167.1	132.9
26	—	—	—	—	—	—	—
27	24	17	1	28.8	47.9	110.4	107.5
28	24	22	0	2.1	5.4	25.2	36.0
29	24	13	2	42.0	60.2	102.7	101.2
30	24	1	0	10.8	10.6	11.3	10.9
31	24	12	3	48.8	72.5	113.9	118.9
32	24	20	2	5.0	12.2	55.0	77.8
33	24	16	1	52.8	70.9	173.5	143.5
34	24	2	2	97.1	81.4	106.8	85.8
35	—	—	—	—	—	—	—
36	24	19	0	112.9	184.1	541.9	483.2
Mean		6.0	1.0	47.6	52.1	82.7	76.9

n , number of seedlings analysed; n_s , number of self-fertilized seedlings; n_i , number of seedlings not matching any male parent within the stand; δ , σ , average effective pollen dispersal distance, and axial variance of effective pollen dispersal, within the stand for each single-tree progeny; δ_{out} , σ_{out} , average effective pollen dispersal distance, and axial variance of effective pollen dispersal, within the stand for each single-tree outbred progeny.

All seeds from tree 26 were empty, and tree 35 was not bearing any seed-cone.

Table 2 Spearman's rank correlation coefficients (and P -values) between relative spatial position of each tree and effective pollen dispersal parameters calculated among its progeny

	D_{min}	D_3	D_{10}	D_{mean}
Selfing	0.608 (0.000)	0.502 (0.004)	0.554 (0.002)	0.560 (0.001)
δ_{out}	0.719 (0.000)	0.668 (0.000)	0.585 (0.001)	0.472 (0.007)

Selfing, portion of self-fertilized seedlings; δ_{out} , average effective pollen dispersal distance excluding selfing; D_{min} , distance to the closest tree; D_3 , D_{10} , D_{mean} , average distance to the three closest, the 10 closest, and to all the other trees in the stand, respectively.

assuming the estimated dispersal kernel for each individual tree. The resulting value for the average effective pollination distance was 46.0 m (95% confidence interval 42.1–49.9 m), very close to and not significantly different from the observed value of 46.7 m. Selfing, however, was overestimated by the model, while matings over distances of 0–40 m were significantly higher in the observed distribution (Figure 3). The fit was better in a broad range of intermediate distances (40–500 m), with

most of observed frequencies not significantly different from the simulated values. The few effective matings observed beyond 500 m were slightly (but significantly) underestimated by the model.

Direction of pollen dispersal

The observed distribution of the mean number of matings with individuals lying within eight different angular sectors from seed-trees showed a marked departure from the radially symmetric pattern obtained under isotropic uniform dispersal (Figure 4), with a significant excess of effective pollen coming from the N, NW and NE, and a significant shortage from the E, SE, SW and W. However, when restricted dispersal (following the fitted exponential-power law) was included in the isotropic model, the distribution also showed an asymmetric pattern, more closely related to that observed. Indeed, the directional distribution obtained after simulating isotropic restricted dispersal also showed a significant excess of matings in the N and NW, and a shortage in the E sector, relative to the distribution resulting from isotropic uniform dispersal. Interestingly,

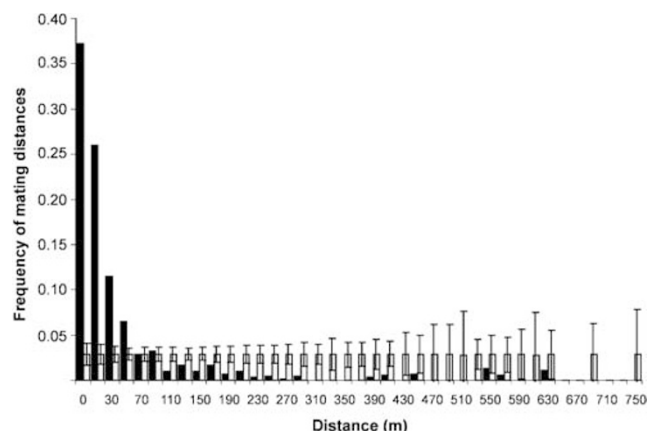


Figure 2 Comparison between the observed (black bars) and the simulated uniform (white bars) mating-distance frequency distributions in the stand. Class 0 corresponds to self-fertilization. The uniform distribution was obtained by simulating random mating with self-fertility within the stand (see Materials and methods). Vertical lines show the 95% confidence intervals for each distance class after 10 000 repetitions of uniform dispersal. Bigger confidence intervals correspond to classes with smaller number of tree-pairs. Four of the distance classes (those without white bars) did not contain any tree-pair.

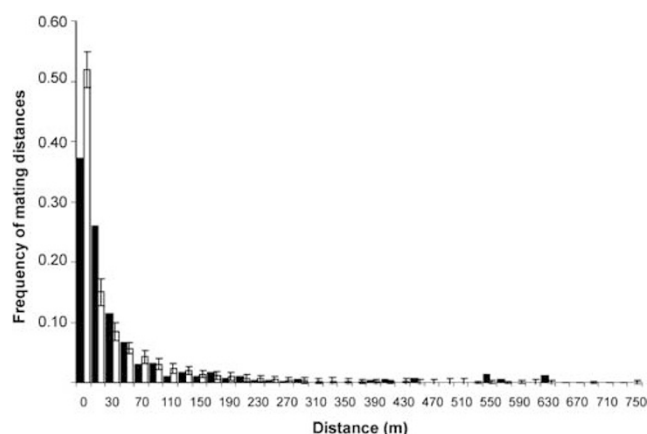


Figure 3 Frequency distribution of mating distances within the stand obtained by simulating isotropic restricted dispersal using the estimated individual dispersal kernel (see Materials and methods). The resulting distribution (white bars), with 95% confidence intervals (vertical lines) obtained after 10 000 repetitions, is plotted against the observed distribution (black bars).

when the estimated values of male success for each potential pollen donor were added to the isotropic restricted model, the resulting distribution approached more closely the shape of that observed (Figure 4), which then showed a smaller (but still significant) shortage of matings from the W and E ($P < 0.01$) and an excess from the NE direction ($P < 0.05$), relative to the isotropic restricted model with unequal male success.

Discussion

Study framework

Two main experimental advantages were derived from choosing a reduced number of isolated and scattered

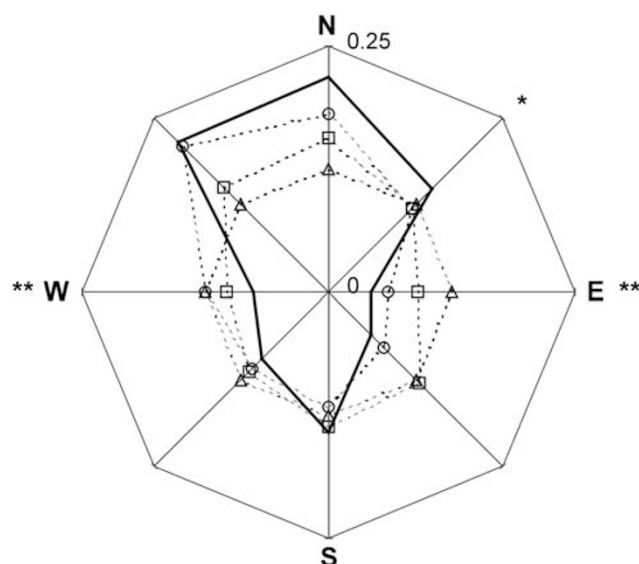


Figure 4 Comparison between the observed (solid line) and isotropic (dotted lines) frequency distributions of mating-directions in the stand. Directions refer to the position of pollinating males relative to the mother tree (origin). Three kinds of isotropic dispersal scenarios were simulated (see Materials and methods): uniform (triangles), restricted (squares), and restricted with unequal male success (circles). Directions in which the observed frequency is significantly ($*P < 0.05$; $**P < 0.01$) different from that obtained under restricted isotropic dispersal with unequal male success are indicated. Distributions are pooled over all seed-trees in the stand.

trees as a model for pollen dispersal study. First, it was possible to eliminate the equivocal paternity determination that is usually associated with pollen flow studies performed within larger and denser tree populations (Smouse and Sork, 2004). This was achieved while maintaining a large scale of analysis, with a maximum within-population potential mating distance of 750 m and a maximum realized dispersal distance of 629 m. Second, 778 out of 813 (95.7%) fertilizations could be assigned to males within the study site, and thus less than 5% of the sampling effort was lost for within-population pollen dispersal analysis. On the other hand, a value of 35 (4.3%) long-distance pollen immigrants in a single year, dispersed from at least 30 different trees, represents an impressive level of long-distance immigration, in evolutionary terms (Slatkin, 1985). A similar pollen immigration rate (6.5%) has been reported for a 450-trees isolated population of *Pinus flexilis* (Schuster and Mitton, 2000), confirming the potential for long-distance pollen flow in pine species.

The same spatial and demographic features peculiar to small isolated stands, which pose evident experimental advantages, must be carefully considered as factors conditioning target reproductive processes. Airborne pollen dynamics in forested areas may be highly affected by wind turbulences associated with horizontal and vertical canopy structure (Di-Giovanni and Kevan, 1991), and it has been suggested that low-density populations may enhance pollen movement (Okubo and Levin, 1989). However, the study site is located within a uniform, flat and continuous forest of several thousand hectares. Even if virtually the whole surface is covered with a different pine species, this is not likely to have a significant effect

on the main underlying fact (from the aerodynamic point of view) that pollen grains are moving over a closed and homogeneous canopy. These singular experimental conditions provided an interesting chance to investigate the isolated effect of the small number and the relative spatial distribution of conspecific individuals on effective pollen dispersal (see below), effect that would have been confounded with that of (low) total vegetation density if the stand was monospecific.

Effective pollen dispersal within a small population

The average self-fertilization rate (0.25) was markedly higher than the reported values for a large population of Scots pine. Outcrossing rates ranging from 0.93 to 0.99 were found in widespread forests of the species, located 60 km south of the present study site (Robledo-Arnuncio *et al.*, 2004), and a similar estimate (0.94) was obtained in natural populations of northern Europe (Muona and Harju, 1989). This contrast suggests a limitation of outbred pollen availability within the stand, likely due to the small number of individuals. Assuming invariant air movement conditions, the relative pollen contribution from a given tree to its own pollen cloud (and subsequent self-fertilization of self-compatible species) can be expected to increase as the number of neighbouring individuals decreases (Loveless and Hamrick, 1984). Indeed, this hypothesis is supported by the moderate positive correlation found between individual self-fertilization rate and distance to the closest tree, which also indicates restricted pollen dispersal. This association is all the more noteworthy when considering that the realized selfing rate at the seed stage is highly determined by early inbreeding depression, itself subject to great individual variation in Scots pine (Kärkkäinen and Savolainen, 1993), and that factors such as among-individual asynchronous phenology or unequal male fecundity can mask the effect of spatial isolation on self-pollination success.

The strong relationship inferred from paternity analysis between outbred mating distance for individual mothers and the distance to the closest tree indicates that most of effective pollen is being contributed by the nearest neighbours and that spatial proximity is an essential factor in determining male reproductive success. Indeed, on average, 25% of outcrossed embryos from each seed-tree were sired by the nearest neighbour, and 42% by the three closest trees (data not shown). This fact was also reflected in the leptokurtic effective pollen dispersal distribution, pooled over all trees in the stand (Figure 2), showing 50% of effective pollen (including selfing) being dispersed less than 10 m away, and a large departure from the expected distribution under uniform dispersal. Although restricted pollen dispersal favouring mating with neighbouring individuals has been reported frequently for wind-pollinated tree species (Burczyk *et al.*, 1996; Dow and Ashley, 1998; Streiff *et al.*, 1999; Lian *et al.*, 2001), the fact that this proximity advantage results in such large individual contributions from near neighbours is unusual. Two studies in large Scots pine populations using the rare marker approach showed that the proportion of offspring sired by individual pollen donors was always low (2–10%), even for the closest seed-trees (cited in Adams, 1992).

In widespread tree populations, the collective pollen contribution from a large number of distant trees may account for an important portion of a seed-tree progeny, especially if dispersal is leptokurtic (has a fat-tailed distribution), and even if the expected contribution of any one of the few near neighbours is proportionately much greater than that of any one of the many distant individuals (Adams, 1992; Ellstrand, 1992). However, this broad spectrum of distant pollen donors does not exist for a small isolated stand, and the collective contribution from a small number of nearby males will represent a greater proportion of the total pollen cloud for any one seed-tree. The high self-fertilization rate and the very restricted effective pollen dispersal pattern found for Scots pine in this study are consistent with this 'competing pollen sources' hypothesis (Levin and Kerster, 1974), and suggest its significant implications for effective pollen movement within small populations.

Individual dispersal kernel

The likelihood procedure used to estimate the individual dispersal kernel incorporates the information relating to number and relative distribution of conspecific individuals under the 'competing pollen sources' framework. It thus takes into account the particular demographic and spatial characteristics of the study site to extract an estimate of the probability density function of pollen dispersal distances from individual trees. It is worth noting that the probability associated with a given distance by the kernel function does not refer to effective (realized) dispersal, but to the movement of pollen from the source tree to the pollen cloud around a target seed-tree, a given distance away (Lavigne *et al.*, 1996). The effective pollen dispersal distribution for the entire population will be determined by both the kernel characteristics and the number and spatial distribution of individual pollen sources (Levin and Kerster, 1974).

A simulation-based study indicates that if trees are Poisson distributed across the landscape, and if trees disperse pollen following a given individual dispersal kernel, assumed to be population-size independent, the effective pollen dispersal distance distribution for the entire population is the same as the individual dispersal kernel beyond a certain population size threshold, which is dependent on the shape of the kernel (Robledo-Arnuncio and Austerlitz, unpublished). Below this threshold, the proximity advantage of near pollen donors increases with decreasing population size, and, as a result, the distribution of mating distances steepens (and the average effective pollen dispersal distance decreases) relative to the potential extent defined by the individual kernel. The marked difference between the estimated kernel and the observed effective pollen dispersal distribution in this study (average dispersal distances of 135 vs 48 m, respectively) is consistent with these theoretical predictions, that is, that small population size, and the consequent relatively small number of distant pollen donors, may increase the probability of mating with near neighbours.

It is thus advisable to use the estimated kernel function, rather than only the observed mating-distance distribution, when comparing the results of this study with those obtained within widespread tree populations. The estimated kernel corresponds to an average

dispersal distance of 135 m, with 50% of the pollen being dispersed more than 30 m from the source, and 5% beyond 180 m. These values are of the same order of magnitude as results obtained from large populations of Scots pine, obtained by tracking labelled pollen or rare isozyme alleles. Koski (1970) calculated that 50% of effective pollen was dispersed within 47 m from source trees, and Yazdani *et al* (1989) reported a value of 54% within 50 m. Using isozyme markers and a fractional paternity approach, Schuster and Mitton (2000) estimated a mean within-population pollen flow distance of 140 m for *P. flexilis*, very close to the 135 m value derived from the individual kernel in this study. In a pollen-flow study involving *P. densiflora* (Lian *et al*, 2001), the average effective pollination distance calculated for an exhaustively sampled single seed-tree sibship was lower, only 68 m, although it is probably an underestimate, since 31% of the offspring were sired by unknown male parents from outside the study site.

The use of the dispersal kernel also proved useful in dissecting factors that determine the apparent directionality of pollen dispersal in the stand. Specifically, the analysis showed that isotropic dispersal from individual trees can result in an asymmetrical distribution of matings in a small population, solely as a consequence of restricted dispersal, coupled with the uneven distribution of individuals and unequal male success. The action of prevailing winds, however, could not be totally discounted. Several studies have reported significant directionality of effective pollen dispersal in wind-pollinated tree species, usually ascribed to wind effects (Burczyk *et al*, 1996; Burczyk and Prat, 1997; Lian *et al*, 2001), while others have found patterns not significantly departing from isotropy or variable among seed-trees (Dow and Ashley, 1998; Streiff *et al*, 1999). These contrasting results seem perfectly consistent with the highly stochastic nature of meteorological phenomena.

Experimental constraints

One major limitation of the present work is that it is based on a single-year observations. Although it may require substantial extra sampling effort, increasing evidence is suggesting the advisability of interannual replication of pollen dispersal experiments in tree populations, since spatial and mating-system parameters show significant temporal variation (Cheliak *et al*, 1985; Schnabel and Hamrick, 1995; Irwin *et al*, 2003), and natural stands regeneration is generally achieved over a long series of years. Results of this study would also have benefited from information on individual flowering characteristics. Although the ample individual replication could compensate to some extent for this lack, it would be worth including phenology and male fecundity factors in future spatial analyses of pollen dispersal in the stand to test if they improve significantly the fit of the dispersal kernel.

Finally, the fit of the dispersal kernel is based on mating events restricted to the experimental spatial scale. Even though this study uses a wider scale than most previous paternity analyses of wind-pollinated trees, the data do not allow reliable estimates of the tail of the distribution, which, as suggested by the observed portion of long-distance immigrants, may extend over tens of kilometres. On the other hand, long-distance

dispersal events are likely to be governed by complex stochastic atmospheric processes that are poorly predicted by standard empirical or mechanistic models (Bullock and Clarke, 2000), and they may require novel experimental and analytical approaches (Nathan *et al*, 2003).

In conclusion, the present paternity analysis confirms that pollen flow operates on a wide scale within and among pine populations. The number of potential pollen donors and the relative distribution of individuals are factors that may strongly determine the extent of effective pollen dispersal and the selfing rate within small stands, deserving careful attention in evolutionary and conservation studies of these species.

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