

# Fluorescent dye particles as pollen analogues for measuring pollen dispersal in an insect-pollinated forest herb

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**Abstract** In flowering plants, pollen dispersal is often the major contributing component to gene flow, hence a key parameter in conservation genetics and population biology. A cost-effective method to assess pollen dispersal consists of monitoring the dispersal of fluorescent dyes used as pollen analogues. However, few comparisons between dye dispersal and realized pollen dispersal have been performed to validate the method. We investigated pollen dispersal in two small populations of the insect-pollinated herb *Primula elatior* from urban forest fragments using direct (paternity analyses based on microsatellite DNA markers) and indirect (fluorescent dyes) methods. We compared these methods using two approaches, testing for the difference between the distance distributions of observed dispersal events and estimating parameters of a dispersal model, and related these results to dye dispersal patterns in three large populations. Dye and realized (based on paternity inference) pollen dispersal showed exponential decay distributions, with 74.2–94.8% of the depositions occurring at <50 m and a few longer distance dispersal events (up to 151 m). No significant difference in curve

shape was found between dye and realized pollen dispersal distributions. The best-fitting parameters characterizing the dye dispersal model were consistent with those obtained for realized pollen dispersal. Hence, the fluorescent dye method may be considered as reliable to infer realized pollen dispersal for forest herbs such as *P. elatior*. However, our simulations reveal that large sample sizes are needed to detect moderate differences between dye and realized pollen dispersal patterns because the estimation of dispersal parameters suffers low precision.

**Keywords** Microsatellite DNA · Paternity analysis · Dispersal kernel · *Primula elatior* · Realized pollen dispersal

## Introduction

In flowering plants, gene dispersal occurs through the dispersal of diaspores (seeds, vegetative parts) and pollen. Pollen dispersal is often the major contributing component to gene flow at within-population, local and regional scales (e.g. Levin and Kerster 1974; Schnabel 1998; Petit et al. 2005), because seed dispersal is often spatially localized. Different direct or indirect methods can be used to measure contemporary pollen dispersal. Some of these methods are based on molecular markers (allozymes, DNA), others track the movement of pollen, pollinators or pollen analogues (powdered dyes) (e.g. Handel 1983; Kearns and Inouye 1993; Neigel 1997; Oddou-Muratorio and Klein 2008).

Paternity analysis is a direct molecular approach allowing assessment of realized pollen dispersal (or realized pollen flow), i.e. pollen deposition that has led to successful seed production, which depends on pollen

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movement to stigmas (potential pollen dispersal) and also on post-pollination processes (Levin and Kerster 1974; Campbell 1991; Bernasconi 2003). It involves examining the respective paternal contribution to the progeny array of known maternal plants, and identifying the actual fathers from a number of potential fathers (Smouse et al. 1999; Streiff et al. 1999; Jones and Ardren 2003). Combined with the geographical location of the parents, a paternity analysis allows retracing pollen movements and identifying the direction of pollen flow. Paternity assignment requires sufficiently polymorphic genetic markers, such as microsatellite (simple sequence repeats) DNA (e.g. Queller et al. 1993; Gerber et al. 2000; Hardy et al. 2004). However, paternity analyses are time-consuming and costly, and can be difficult to apply when the number of potential fathers becomes too large because many markers are required to identify the actual pollen donors. Hence, quantifying realized pollen dispersal using paternity analyses might be easier in small populations. In such cases all the potential paternal plants in the population can be screened, and immigrant pollen identified (Marshall et al. 1998; Jones and Ardren 2003).

Using fluorescent dye particles as pollen analogues is an efficient, quick and economic way to explore pollination processes, male reproductive success, pollen dispersal patterns and pollinator movements within and among natural or experimental populations of animal-pollinated plant species (e.g. Price and Waser 1982; Waser and Price 1984; Mitchell 1993; Morris et al. 1994; Townsend and Levey 2005; Van Rossum 2009, 2010). Studies have usually found a good correspondence between the distributions of dye and pollen carryover. For example, dye acted as a pollen mimic when carried by hummingbirds in *Ipomopsis aggregata* (Waser and Price 1982) and *Silene virginica* (Fenster et al. 1996); by bumblebees in *Delphinium nelsonii* (Waser 1988) and *Echium vulgare* (Rademaker et al. 1997); and by bees, wasps and syrphid flies in *Sabatia angularis* (Dudash 1991).

However, there are reasons to question the use of dye to measure pollen dispersal patterns. First, it may be transported differently than pollen: dye was found to move 1.45 times farther than pollen when transported by bumblebees in *Erythronium grandiflorum* (Thomson et al. 1986), whereas dye deposition underestimated pollen dispersal by a factor of 1.8 for *D. nelsonii* when pollinators were hummingbirds (Waser 1988), and for a long sequence of flower visits by bees in *Gelsemium sempervirens* (Adler and Irwin 2006). Second, estimating dye dispersal investigates pollen movement to recipient stigmas (potential pollen dispersal), not what happens after pollination. Post-pollination processes such as pollen competition, female mate choice, and (biparental) inbreeding depression can cause differential pollen success in fertilizing ovules and

differential development of embryos (Ganders 1979; Waser and Price 1993; Streiff et al. 1999; Bernasconi 2003; Sage et al. 2006). As a result, dye dispersal might not accurately reflect realized pollen dispersal. For instance, discrimination against pollen of genetically related neighbours can lead to an increase in the distance of effective pollen flow (Campbell 1991), and discrimination against pollen from genetically dissimilar individuals can lead to a decrease in the distance of effective gene flow (Waser et al. 2000). However, whether dye dispersal patterns can be used to estimate realized pollen dispersal has rarely been examined (Campbell 1991). Besides the potential impact of post-pollination events, estimating and comparing dispersal curves can also be challenging. Many studies have compared the distance distribution of observed dispersal events. However, such distribution not only depends on pollen dispersal ability but also on the experimental setup because the probability of detecting a given dispersal event varies with the distance crossed. To alleviate this issue, data can be fitted to a *forward* (i.e. from pollen donor to arrival points) dispersal model to estimate parameters of a two-dimensional probability distribution function (e.g. Hardy et al. 2004; Oddou-Muratorio et al. 2005).

*Primula elatior* (Primulaceae) is a self-incompatible forest herb that flowers in early spring. It is insect-pollinated, mainly by bees and bumblebees. We investigated pollen dispersal patterns in two small populations of *P. elatior* located in forest fragments of the Brussels urban area (Belgium), using paternity analyses based on microsatellite DNA markers and using fluorescent dyes as a pollen analogue. In this paper we compare the two methods using two different approaches—a test of goodness of fit and a pollen dispersal simulation model—to test whether dye dispersal can be used as an estimator of the spatial patterns of realized pollen dispersal. We also relate these results to dye dispersal patterns investigated in three larger populations, where paternity studies may be hard to perform, to evaluate whether what we find in the small populations might be representative of the pollen dispersal patterns observed in larger populations.

## Materials and methods

### The species

*Primula elatior* is a diploid, long-lived perennial, rosette-forming plant that mainly occurs in moist deciduous forests. It is distributed over western and central Europe, extending northward to Denmark and eastward to central Asia (Valentine 1948). In Belgium, it is a common species (van Rompaey and Delvosalle 1979), but it occurs in ancient deciduous forests, which remain as isolated

fragments. It is however rare and declining in the Brussels Capital Region (Godefroid 2001; Weyembergh et al. 2003). In early spring (April), it produces umbels with five to <20 pale yellow flowers, containing five stamens and an ovary bearing one style overtopped by one stigma. The flowers are insect-pollinated, primarily by Hymenoptera. The most frequent visitors are the solitary bee *Anthophora plumipes* and large bumblebee species, usually queens at the beginning of the flowering period, including *Bombus terrestris* sensu lato, *B. pascuorum*, *B. pratorum* and *B. hortorum*. Other visitors include *Bombus wurflenii mastrucatus*, *Bombus ruderratus*, *Bombus soroeensis autumnalis*, and the solitary bees *Osmia rufa*, *Andrena bicolor*, *Andrena fulva* and *Andrena subopaca*. Diptera, mainly Syrphidae, have also been observed visiting *P. elatior* flowers (Knuth 1909; Schou 1983; Haubruge and Rasmont 2007; Van Rossum, personal observations).

*P. elatior* shows a sexual dimorphism (distyly) characterized by two genetically determined and self-incompatible floral morphs (pin or thrum), with each individual being either pin or thrum (Schou 1983). This promotes disassortative mating (Ganders 1979). Hand-pollination experiments (Wedderburn and Richards 1990) indicated that *P. elatior* could be considered as an obligate outcrosser. Plants mainly reproduce by seeds. Seeds have no particular mechanism for dispersal (Valentine 1948), and therefore their dispersal is restricted to short distances.

#### Studied populations

The five studied populations (small populations, Rivieren and Fonteinen; larger populations, Meerveld A and B and Rood Klooster) were located in the Brussels Capital Region (163 km<sup>2</sup>; 1 million inhabitants), which corresponds to the urban area of Brussels sensu lato (which also encompasses more rural suburbs). Rivieren (4°19'E 50°53'N, north-west Brussels) is a small population ( $N = 33$  flowering individuals, maximum distance between individuals  $d_{\max} = 25.9$  m) located in an isolated urban forest fragment embedded in an urbanized matrix. Fonteinen (4°27'E, 50°48'N;  $N = 58$ ,  $d_{\max} = 148.3$  m) and Rood Klooster (4°27'E, 50°49'N;  $N = 300$ ,  $d_{\max} =$  ca. 200 m) are located in the Sonian forest (south-east Brussels). The two populations are 700 m apart, separated by a road network, and may be considered spatially isolated (Stiers 2005; Van Rossum 2008). Meerveld A and B are located in south-west Brussels (4°15'E, 50°49'N;  $N = 200$ ,  $d_{\max} = 130$  m and  $N = 90$ ,  $d_{\max} = 179$  m, respectively) and are about 200 m apart. All the flowering individuals were mapped in the two small populations (Rivieren and Fonteinen), while only source and sampled recipient individuals used for the dye dispersal experiment (see below) were mapped in the larger populations. The minimal

distance between two individuals was 0.1 m in Rivieren and 0.2 m in Fonteinen. Morph type (pin or thrum) was noted for each flowering individual except for 11 individuals in Fonteinen, whose flowers had been eaten by rabbits.

#### Microsatellite analysis

In the two small populations (Rivieren and Fonteinen), seeds were collected in June 2004 from 20 maternal plants covering the population area and germinated in pots filled with compost and sand. Young leaf material was sampled from all flowering individuals, which included maternal plants and potential pollen donors ( $n = 33$  and 58 in Rivieren and Fonteinen, respectively) and from 50 seedlings (two to three per maternal plant) per population (total: 191 individuals). Leaves were dried at 40°C for 48 h and stored in silica gel until DNA extraction. Genomic DNA extractions were performed on dried leaf material (7 mg dry weight) using the Nucleospin Plant extraction kit (Macherey-Nagel, Düren, Germany). Polymorphism was assayed on each DNA sample at microsatellite loci transferred from *Primula vulgaris* (Van Geert et al. 2006). Two unlinked microsatellite loci (PRIV4, PRIV7) gave interpretable patterns (Van Geert et al. 2006). They were highly polymorphic: (PRIV4, 11 and 13; PRIV7, eight and 11 alleles, for Rivieren and Fonteinen, respectively). No null allele was detected for the two loci (when tested using the software CERVUS; Marshall et al. 1998). Microsatellite loci amplification by polymerase chain reactions and analysis of the amplification products followed Van Geert et al. (2006).

#### Assessing realized pollen dispersal using paternity analysis

Paternity was assigned within each population using the software CERVUS 2.0 (Marshall et al. 1998). For each offspring tested, the paternity likelihood of each candidate pollen donor was calculated using a ratio of probabilities (the LOD score) depending on the genotypes of the offspring, its mother and the candidate father as well as on the population allele frequencies (Meagher 1986). To evaluate the confidence at which the most likely father (i.e. the one with the highest LOD score) is the true father, CERVUS performed 10,000 simulations to estimate the critical  $\Delta$  value—the difference between the highest and second highest LOD scores—at which the most likely father is the true father in 80% (relaxed confidence) or 95% (strict confidence) of cases. As both confidence levels gave similar results, we only considered the relaxed confidence levels for the subsequent analyses. The analyses were performed separately for pin and thrum plants, as only thrum can be pollen donors for pin maternal plants and vice

versa, due to the distylous self-incompatibility system (Rivieren, 20 pins, 13 thrums; Fonteinen, 25 pins, 22 thrums and 11 unknown). In Fonteinen, the 11 individuals with unknown morph type were included as potential fathers in both analyses. As all flowering individuals were sampled in the populations, the proportion of candidate fathers was 1. There were no missing (unsolved) genotypic data, so the proportion of loci typed was set at 1 in the CERVUS analyses. Only one apparent mismatch was observed between the known mother and its progeny for one locus, and so the typing error rate was fixed at 0.005. For each offspring with a father assigned by CERVUS, the distance from the maternal plant to the assigned pollen donor was calculated using map coordinates.

#### Estimating potential pollen dispersal using fluorescent dye

Five colours (orange, yellow, red, UV blue and blue) of fluorescent dye (Radiant Color, Series Radglo R) were used as pollen analogues to estimate potential pollen dispersal. Marking flowers of *P. elatior* with dye did not change the behaviour of visiting insects (Van Rossum, personal observations). A previous study showed no difference in dispersal patterns among the used dye colours (Van Rossum and Triest 2006a) and between morphs when they were used as dye source (Van Rossum, unpublished data). Marking flowers with dye was conducted at the peak of flowering, during 4-day periods of dry sunny weather, in April 2004 (Rivieren, Meerveld A and B) and 2005 (Fonteinen, Rood Klooster). On day 1, dyes were applied once with wooden toothpicks to dehiscing anthers of 30 flowers from one to two groups of plants (=dye source individuals). The dye donor's location was chosen so as to include a wide range of potential distances between the dye donors and recipient individuals, i.e. within the central third of the population, the two dye sources being <40 m apart. For Rivieren, the dye source was located at one side of the population. One group of pin individuals was marked with dye in Rivieren (orange dye), and two groups of plants, one of pin individuals and one of thrum

individuals, were marked with different dye colours in Fonteinen (orange/yellow dye), Meerveld A (orange/yellow dye), Meerveld B (UV blue/blue dye) and Rood Klooster (red/blue dye). On day 4, stigmas receptive during the experimental period were harvested from three to ten flowers on 21–42 recipient individuals per population (Table 1). In Rivieren and Fonteinen, all flowering individuals were sampled, except when flower stalks were eaten by rabbits. In the three larger populations, the sampling of the recipient individuals followed the same scheme, covering the whole population area, including the most extremely located individuals in the population. The stigmas (one per flower) were embedded in a semi-permanent mount of glycerine jelly (50 g gelatine dissolved in a solution of 175 ml distilled water and 150 ml glycerine) on a microscope slide (see Kearns and Inouye 1993 for method). In total, 1,475 stigmas collected from 189 flowering individuals were examined for dye particles at 40× under a fluorescence microscope. Presence or absence of fluorescent dye particles was recorded on each stigma and used to calculate, for each recipient individual and dye colour, the proportion of stigmas showing deposited dye (fraction stigmas with dye = number of stigmas with dye/total number of collected stigmas). The fraction of stigmas with dye has been shown to be highly correlated to the abundance of dye on the stigmas (Pearson's correlation coefficient  $r = 0.860\text{--}0.949$ ,  $P < 0.001$ ) and to show similar dispersal patterns (Stiers 2005). Hence, it can be used as a reliable representation of dye dispersal. For each recipient individual, the distance to dye source was calculated using map coordinates. The range of potential distances from dye source to recipient individuals within each population is given in Table 1.

The pattern of within-population dye dispersal was investigated for each population using a regression analysis, which tested the relationship between the fraction of stigmas showing deposited dye for each recipient individual (dependent variable) and the distance to dye source (predictor variable, log transformed). In the four populations where two dye sources were used, the analyses were performed on the combined data sets, as no significant

**Table 1** Population details and results of the paternity and dye experiment in five populations of *Primula elatior*: mean distance between maternal plants and pollen donors (paternity), mean potential

Population	Distance mother–father (m)	<i>n</i>	Distance to dye source (m)	Distance of dye transfers (m)	Ind. with dye (%)	Fraction stigmas with dye
Rivieren	6.9 (0.2–22.9)	21	14.8 (0.3–25.3)	10.3 (0.3–23.1)	57.1	0.69 (0.25–1.00)
Fonteinen	32.4 (0.2–136.3)	34	38.6 (4.9–124.4)	31.7 (4.9–123.7)	83.8	0.79 (0.10–1.00)
Meerveld A		42–39	26.0 (0.6–100.5)	18.3 (0.6–76.3)	71.6	0.52 (0.11–1.00)
Meerveld B		38–29	32.8 (0.2–144.3)	28.9 (0.2–114.8)	73.1	0.62 (0.12–1.00)
Rood Klooster		35	54.9 (0.2–164.3)	32.2 (0.2–151.4)	44.3	0.46 (0.08–1.00)

*n* Number of sampled recipient individuals per dye source, *Ind. with dye* proportion of recipient individuals showing dye (%)

( $P > 0.05$ ) differences in dye deposition patterns were found between the two dye sources, when tested by tests of homogeneity of slopes (performed on the fraction of stigmas with dye, with dye source as group and the distance to dye source as independent variable).

To investigate whether the observed dye dispersal patterns in small populations can be representative of what happens in larger populations, pairwise tests of homogeneity of slopes were performed to test for differences in within-population dye dispersal patterns (distance to dye source as independent variable, log transformed) between the populations. The analyses were carried out for the same range of distance to dye source, i.e. up to 110 m. Because of within-population distances which were too small, Rivieren was excluded from the analyses. As multiple tests were involved, Bonferroni correction was applied to test for significance (Rice 1989). All analyses were performed using STATISTICA.

To investigate whether dye dispersal might be a good estimator of realized pollen dispersal, the patterns of dye dispersal (distance to dye source) and of realized pollen dispersal (distance paternal–maternal plant) were compared for Rivieren and Fonteinen using two different analyses: a comparison of the distribution of observed dispersal events, and a comparison of best-fitting forward dispersal model parameters.

#### Comparison between observed realized pollen and dye dispersal events

Difference in general shape between cumulative frequency curves of realized pollen dispersal events (paternity analysis) and of relative dye deposition according to dispersal distance were compared using a nonparametric Kolmogorov–Smirnov (K–S) test for goodness of fit (to this end, the distance data were not grouped into classes; Sokal and Rohlf 2000). The observed distributions of pollen or dye dispersal events depend on pollen or dye dispersal capacities, but they also depend on the spatial distributions of sources (potential fathers or dye source individuals) with respect to recipient samples (maternal plants on which seeds were collected or recipient individuals on which stigmas were sampled). Therefore, a significant K–S test would reflect a difference between realized pollen and dye dispersal only if the distance distributions between sources and samples are similar for dye and paternity methods. To check this assumption, the expected cumulative frequencies over a set of distance classes were calculated under random mating, i.e. for random adult-offspring or for random source-recipient pairs of individuals (summation of the observed proportions over all recipient individuals divided by the total number of samples; Hardy et al. 2004). In Fonteinen, the paternity analysis and the dye dispersal experiment were not performed the same

year (2004 and 2005, respectively). Although we assumed that pollen dispersal kernels were constant between years, higher flower herbivory by rabbits in 2005 resulted in longer distances between the remaining flowering individuals, with a distance to dye source  $\geq 4.95$  m. Therefore, the comparison between dye dispersal and pollen flow based on paternity analysis was realized in Fonteinen for distances  $\geq 4.95$  m. The analyses were performed using STATISTICA.

#### Modelling realized pollen and dye dispersal

We inferred the shape of the pollen and dye dispersal distribution around each plant (i.e. dispersal kernels), based on the method described by Hardy et al. (2004). Pollen dispersal events obtained by paternity analysis and dye deposition events estimated by the fraction of stigmas with dye were used to fit a function characterizing pollen and dye dispersal, respectively, in each population. The pollen (or dye) dispersal kernel,  $f(x,y)$ , represents the probability that a pollen (or dye) grain moves by distances  $x$  and  $y$  along two orthogonal axes from the pollen (or dye) donor. Pollen and fluorescent dye were assumed to disperse isotropically following a two-dimensional exponential power function (Tufto et al. 1997):  $f(\alpha, \beta; x, y) = \beta \exp[-(r/\alpha)^\beta] / [2\pi\alpha^2\Gamma(2/\beta)]$ . In this equation,  $\alpha$  and  $\beta$  are the parameters to be fitted,  $r = (x^2 + y^2)^{1/2}$  is the distance crossed and  $\Gamma$  is the Gamma function. The mean distance crossed by pollen (or dye) grains is given by  $\delta_k = \alpha[\Gamma(3/\beta)/\Gamma(2/\beta)]$  (Hardy et al. 2004; Oddou-Muratorio and Klein 2008), so that the kernel can be fully characterized by  $\alpha$  and  $\beta$  or, more conveniently, by  $\delta_k$  and  $\beta$ .  $\delta_k$  may differ from the mean distance of observed (dye or effective pollen) dispersal events, as it represents a potential dispersal distribution of pollen, before fecundation, whereas the true realized distribution depends on this kernel as well as on the opportunities for fecundation according to the dispersal distance crossed (thus on the spatial distribution of potential mates) and on the particular sample available.  $\beta$  determines the shape of the distribution: it is fat-tailed (leptokurtic distribution) when  $\beta < 1$ , thin-tailed when  $\beta > 1$ , and the kernel reduces to a bivariate exponential distribution when  $\beta = 1$ , and to the bivariate normal distribution when  $\beta = 2$ .

A maximum likelihood ( $L$ ) approach was used to fit the parameters  $\delta_k$  and  $\beta$  on the observed pollen dispersal events identified by paternity analysis. The general principle is to compute the likelihood of the data (the observed dispersal events) as a product of the probabilities that these events occur under the dispersal model, and to adjust the  $\alpha$  and  $\beta$  model parameters to reach the highest likelihood. This approach was adapted for fluorescent dye dispersal events, assuming that the observed fraction of stigmas with dye per recipient individual must be proportional to the dispersal

kernel centred on the dye source and hence decays proportionally to  $\exp[-(r/\alpha)^\beta]$  where  $r$  is the distance from the dye source. For each recipient individual  $k$ , we computed  $O_k$ , the observed proportion of stigmas with dye divided by the summation of the observed proportions over all recipient individuals. The corresponding expected value for a recipient individual  $k$  at a distance  $r_k$  from the dye source under the dispersal kernel is given by  $E_k = \exp[-(r_k/\alpha)^\beta] / \sum_i \exp[-(r_i/\alpha)^\beta]$ , with the summation being over all ( $i$ ) recipient individuals. A maximum  $L$  estimate for dye dispersal events was approximated by a  $\chi^2$  test (Sokal and Rohlf 2000), finding the  $\alpha$  and  $\beta$  parameter values minimizing the quantity  $\sum_k [(O_k - E_k)^2 / E_k]$  using Excel Solver.

To check the consistency between dye and paternity methods, confidence intervals (CI) of estimated parameters were needed, but they could not be obtained from our maximum  $L$  approach. So we used simulations as in Hardy et al. (2004) to assess CI of pollen dispersal  $\delta_k$  and  $\beta$  parameters. Knowing the positions of potential fathers and of sampled seeds in our real dataset, we simulated mating events generating the set of sampled seeds as independent realizations of the pollen dispersal process for given  $\delta_k$  and  $\beta$  parameters. For each of 200 simulations we assessed the curve representing the cumulated distribution of dispersal distances and eventually the mean curve over all replicates. The area separating the cumulated curve of the true pollen dispersal events and the mean curve obtained by simulation (between-curve area for observed data) indicates whether the model fits the data well (small area) or not (large area). The between-curve area was also computed for each simulation with respect to the mean curve to establish the frequency distribution of the between-curve area. The data were considered as consistent with the  $\delta_k$  and  $\beta$  parameters whenever the observed between-curve area belonged to the 95 or 80% lowest values of the distribution. In this way, a wide range of  $\alpha$  and  $\beta$  values were tested for consistency to delineate the range of parameter values compatible with the paternity data at 95 or 80% confidence. If the  $\alpha$  and  $\beta$  (and thus  $\delta_k$ ) parameters calculated for dye dispersal fell within this range, we assumed that they were consistent with the pollen dispersal kernel derived from the paternity analysis.

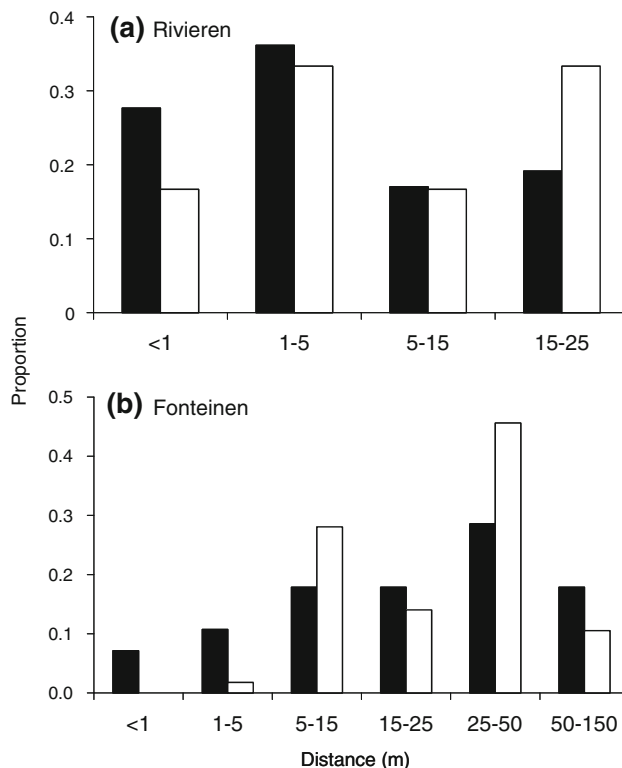
## Results

### Within-population realized pollen dispersal assessed using paternity analysis

In Rivieren, CERVUS identified a potential (most likely) father for 47 of 50 offspring, and when tested at relaxed confidence (80%), assigned a single father to each of 22 of the 50 offspring (sampled seedlings). No pollen donor could be assigned for three offspring (data insufficient for

discriminating among several potential fathers compatible with the offspring as the genotypes corresponded to common alleles). Of the 33 potential pollen donors, 24 were identified to have sired progeny, among which 15 (62.5%) sired one offspring and nine sired two to five offspring. The distance between the maternal plant and the assigned pollen donor ranged from 0.2 to 22.9 m, with a mean of 6.9 m (Table 1). Most of the assigned fathers were located  $<5$  m from the maternal plant (for 64.8% = 30 of 47 offspring with a most likely pollen donor; Fig. 1a). Similar results were found at relaxed (80%) confidence (results not shown).

In Fonteinen, CERVUS assigned a single father to each of 28 of the 50 (56.0%) offspring at relaxed confidence. No father could be assigned to 22 offspring (44.0%), because of insufficient data for discriminating among several compatible fathers. Of the 58 flowering adults present in the population, 18 were assigned as pollen donors; 72.2% of them (13 of 18) sired one offspring and 27.8% (five of 18) sired three offspring. The distance between the maternal plant and the assigned pollen donor ranged from 0.2 to 136.3 m, with a mean of 32.4 m (Table 1). Most of the pollen flow events (for 82.1% = 23 of 28 offspring)



**Fig. 1** Frequency distribution of distances of realized pollen dispersal inferred from paternity assignment (black bars most-likely fathers) and of dye dispersal (white bars). **a** Rivieren (offspring number = 47, dye recipient individuals = 12). **b** Fonteinen (offspring number = 28, dye recipient individuals = 57)

**Fig. 2** Distribution of dye deposition (fraction stigmas with dye) as a function of the distance to dye source. **a** Rivieren [ $y = 0.709 - 0.329 \times \log(x)$ ,  $R = -0.498$ ,  $P = 0.022$ ]; **b** Fonteinen [ $y = 1.678 - 0.691 \times \log(x)$ ,  $R = -0.562$ ,  $P < 0.001$ ]; **c** Meerveld A [ $y = 0.923 - 0.447 \times \log(x)$ ,  $R = -0.590$ ,  $P < 0.001$ ]; **d** Meerveld B [ $y = 0.802 - 0.276 \times \log(x)$ ,  $R = -0.445$ ,  $P < 0.001$ ]; **e** Rood Klooster [ $y = 0.743 - 0.365 \times \log(x)$ ,  $R = -0.750$ ,  $P < 0.001$ ]. **a** Differs from **b–e** in distance range

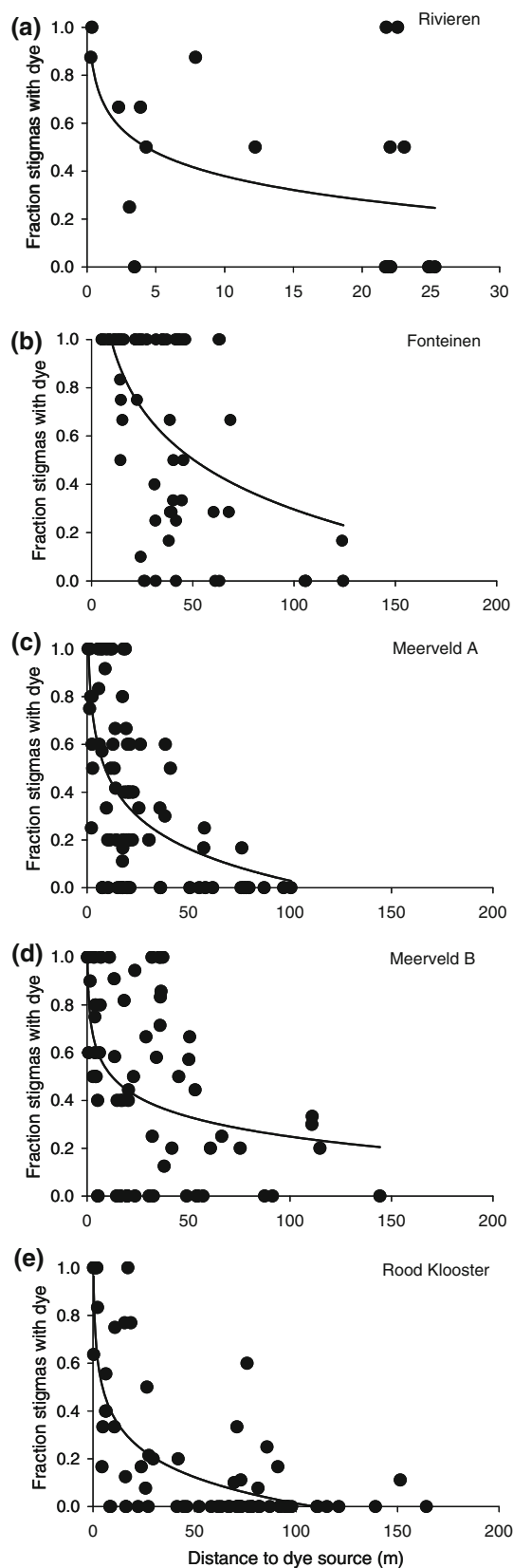
were located at less than 50 m (distance between assigned fathers and maternal plants; Fig. 1b).

### Dye dispersal patterns

In the five studied populations, 44.3–83.8% of the sampled recipient individuals showed dye deposition (Table 1). For these recipient individuals, the fraction of stigmas with dye ranged 8–100%. Except in Meerveld A and B, the distance ranges of dye transfers corresponded to the potential distances to dye source, and so covered the whole population area (Table 1; Fig. 2). In Rivieren, the mean distance of dye transfer was 10.3 m and 50% of the dye transfers occurred within 5 m (Fig. 1a). In Fonteinen, the mean distance of dye transfer was 31.7 m and 89.5% of the dye transfers occurred within 50 m (Fig. 1b). In Meerveld A and B and Rood Klooster, 94.8, 81.6 and 74.2% of the dye transfers occurred within 50 m, respectively. The regression analyses showed a significantly negative relationship between the fraction of stigmas with dye and the distance to dye source for the five populations ( $R$  ranging from  $-0.445$  to  $-0.750$ ,  $P < 0.05$ ; Fig. 2). Testing for differences in within-population (for the same range of distances, i.e. up to 110 m) dye dispersal patterns between pairs of populations using tests of homogeneity of slopes showed no significant differences after Bonferroni corrections (interaction between population and distance to dye source:  $F_{(1,142)} = 0.86-3.27$  and  $F_{(1,126)} = 1.71-5.65$ ,  $P > 0.008$ ).

### Comparison between observed realized pollen and dye dispersal events

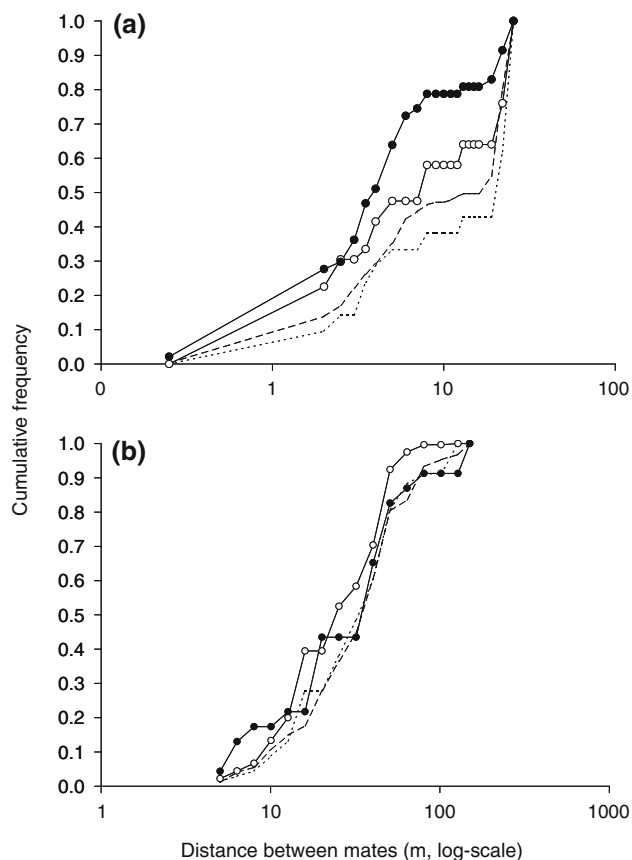
When comparing observed effective pollen and dye dispersal distance distributions with their respective expected distribution under random mating, it appeared that both realized pollen and dye dispersal distances were limited relative to random matings in Rivieren and Fonteinen populations [Fig. 4; for Rivieren the curves based on the assignment data at relaxed (80%) confidence were found to be identical to these for most likely fathers; results not shown]. In both populations, the distance distributions expected under random mating for dye and paternity methods were similar (Fig. 3), indicating that the two



observed dispersal cumulative frequencies curves could be compared for difference in shape. The apparent difference around 5–10 m between dye and pollen curves in Rivieren (Fig. 3a) could be explained by the lack of dye data for the distance classes between 5 and 20 m (only two individuals; Fig. 2a). No significant difference (K–S test:  $P > 0.10$ ) in curve shape was found between the distributions of dye dispersal and realized pollen flow based on paternity analysis for both populations. The gap found between pollen and dye dispersal curves in Rivieren (Fig. 3a) was similar for the observed and random mating distributions.

#### Modelling realized pollen and dye dispersal

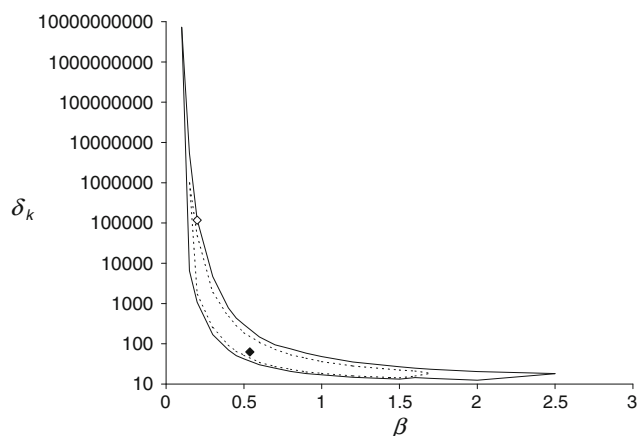
In Rivieren, the best-fitting parameter values of the pollen dispersal model with respect to the paternity analysis (most likely fathers) were  $\alpha = 4.50$  and  $\beta = 0.53$  ( $L = -134.6$ ; mean distance travelled by pollen grains,  $\delta_k = 63$  m),



**Fig. 3** Cumulative frequency distributions of dye (white dots) and realized pollen (black dots) dispersal distances in **a** Rivieren (most-likely fathers) and **b** Fonteinen. The distance between mates corresponds to source–recipient and paternal–maternal plant for dye and realized pollen dispersal, respectively. The broken lines represent the distance distribution expected under random mating for dye (narrow dashes) and effective pollen (wide dashes). All cumulative frequencies are given for the same distance ranges

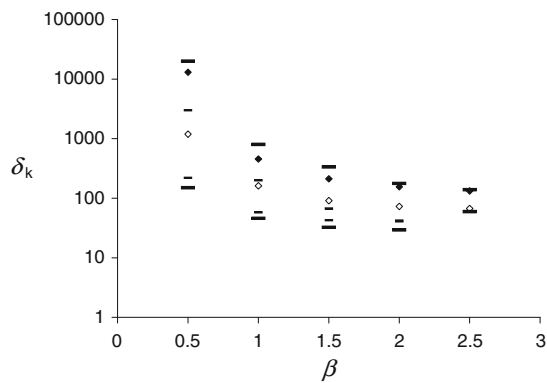
indicating a leptokurtic distribution. As these values fell within the 80% confidence area assessed by simulations (Fig. 4), the model can be considered consistent with the data. However, the wide range of  $\beta$  and  $\delta_k$  parameter values covered by the 95 and 80% confidence levels highlight the limited precision achieved on each parameter. Low precision on each parameter occurred because data could be consistent both with low  $\beta$  and high  $\delta_k$  and with high  $\beta$  and low  $\delta_k$  (Fig. 4). The best-fitting  $\alpha$  and  $\beta$  values with respect to dye dispersal data ( $\alpha = 0.49$ ,  $\beta = 0.20$ ,  $\delta_k = 117,718$  m,  $\chi^2 = 0.85$ ) fell within the range of the 95% confidence area assessed by simulations (Fig. 4), so that they could be considered as in agreement with the paternity data. Similar patterns were found for the paternity results at relaxed (80%) confidence (results not shown).

In Fonteinen, the log-likelihood values for paternity data calculated for a wide range of  $\beta$  values (0.4–3.0) were very similar ( $L$  varying from  $-83.2$  to  $-83.8$ ), so that it was not possible to obtain best-fitting parameter values defining the shape of the pollen dispersal kernel. Therefore 95 and 80% CI and best-fitting  $\alpha$  values for dye and paternity data were inferred for fixed values of  $\beta$  (0.5, 1.0, 1.5, 2.0, and 2.5), and used to calculate  $\delta_k$ . Consistency between the dye and paternity methods could be assumed if both observed  $\delta_k$  values fall within the range of 95 or 80% CI obtained by simulations using the paternity results. All the  $\delta_k$  values (for  $\beta = 0.5$ –2.5) obtained for dye dispersal (1,189–67 m) and realized pollen flow (13,037–132 m) fell within the range of the 95 or 80% CI obtained by the simulations (Fig. 5), indicating consistency between the two methods. Here again, the available data provided limited precision on the parameters of the dispersal distribution models.



**Fig. 4** Ranges of  $\beta$  (shape of the pollen dispersal model) and  $\delta_k$  (mean distance travelled by pollen grains under the model) that fit the paternity inference data in Rivieren, at a 95% (plain line) and 80% (broken line) confidence level (obtained by simulating mating events in the population). Best-fitting parameter values for dye and realized pollen dispersal are shown by the white and black diamond, respectively





**Fig. 5** Best-fitting values of  $\delta_k$  (mean distance travelled by pollen grains under the model) for dye (white diamonds) and realized pollen dispersal (black diamonds) at fixed  $\beta$  values (shape of the pollen dispersal model) in Fonteinen. The 95% (wide dashes) and 80% (narrow dashes, not calculated for  $\beta = 2.5$ ) confidence levels were obtained by simulating mating events in the population (based on paternity assignment data)

## Discussion

### Fluorescent dye dispersal as an estimate of realized pollen dispersal

Two methods have been used to investigate contemporary pollen dispersal patterns of isolated urban populations of the insect-pollinated forest herb *P. elatior*, i.e. fluorescent dye particles as pollen analogues estimating potential pollen dispersal and paternity assignment quantifying realized pollen dispersal. They yield consistent results in the two populations, because (i) both methods detect distance-limited dispersal compared to random mating, (ii) both methods lead to similar cumulative dispersal distance curves in situations where cumulative distributions expected under random mating are also similar, and (iii) best-fitting parameters of a dispersal distribution model with respect to dye dispersal data are within the ranges of parameter values consistent with realized pollen dispersal data. The dye dispersal patterns observed within the larger study populations were similar to those observed in a small population (Fonteinen) for the same range of distances to dye source, suggesting that dye dispersal may be representative of realized pollen dispersal in larger populations too.

Congruence between fluorescent dye dispersal and realized pollen dispersal suggests that pollen grains and dye particles deposited on anthers are transported by insects in similar ways. Campbell (1991) found discrepancies between dye dispersal and gene flow quantified by paternity analysis in *I. aggregata*. In this case, realized pollen dispersal distances exceeded dye dispersal, suggesting that post-pollination events (selection against pollen of genetically related neighbours or biparental

inbreeding depression) may have occurred. Little is known about post-pollination in *P. elatior*. For instance, it is unclear whether the deposition of many illegitimate (incompatible) pollen grains on the stigmas, as reported by Schou (1983), might physically interfere with compatible pollen grains through stigma clogging (McKenna 1992). Small-scale biparental inbreeding between relatives clumped as a result of restricted seed dispersal (Van Rossum and Triest 2006b) might reduce short-distance pollination success through increased fruit abortion (Van Rossum et al. 2002). However, the good correspondence found between potential pollen dispersal inferred by dye dispersal and realized pollen dispersal for *P. elatior* populations suggests that these factors are negligible or have little influence on pollen flow patterns. The main factor determining realized pollen dispersal patterns appears therefore to be pollen movement from the donor to recipient stigmas.

However, our results also indicate that the estimation of the dispersal distribution model parameters suffers low precision. The advantage of estimating the parameters of a dispersal kernel is that it gives the potential dispersal ability independently of the spatial configuration of the studied populations. The mean dispersal distance of the kernel,  $\delta_k$ , should predict the mean realized dispersal distance in an unbounded high-density population where individuals are randomly distributed. The actual mean distance of observed dispersal events is typically less than  $\delta_k$  because the studied populations extend over a limited area and the experimental setup cannot detect long-distance dispersal events. Here, confidence intervals on  $\delta_k$  cover several orders of magnitude. Limited precision may result from the limited number of examined progeny for paternity analyses. Nevertheless, similar lack of precision was observed previously with larger sample size (Hardy et al. 2004) and results also from the inverse correlation between the inferred level of kurtosis ( $\beta$  parameter) and mean dispersal distance ( $\delta_k$  parameter). Indeed, fat-tailed dispersal models (long mean distance) or thin-tailed dispersal models (short mean distance) may fit paternity data equally well. Consequently, unless much larger sample sizes are available, it may be hard to detect subtle differences in dispersal patterns between dye and pollen. For instance, as paternity analysis concerns the whole flowering season and dye dispersal a few days at the flowering peak, there might be slight differences in pollination and dispersal patterns related to floral phenology and pollinator guild composition and abundance changing through the season (Handel 1983; Kwak et al. 1998; Kitamoto et al. 2006). The studied populations were assumed to be spatially isolated and there is some indication that gene flow might be very restricted between *P. elatior* populations within the same urban forest fragments (Stiers 2005; Van Rossum 2008). However,

infrequent long-distance pollen movements, including immigrant pollen flow, may be difficult to detect (and so possible differences between dye and pollen at long distances) with both methods (Campbell 1991; Karron et al. 1995; Gaudeul and Till-Bottraud 2004), unless large samplings (and several highly polymorphic markers to achieve high exclusion probabilities) are considered (e.g. Marshall et al. 1998).

#### Pollen dispersal patterns

Pollen dispersal within all the studied *P. elatior* populations shows a decay with respect to spatial distance, with most pollen (dye) deposited at short distances and a few grains moving longer distances. More than 80% of the pollen dispersal events (and dye transfers) occur within 50 m for Fontainen; values of dye transfers within 50 m are similar for the three larger populations (74–95%). This is consistent with results reported from studies of spatial genetic structure based on spatial autocorrelation analyses, which indicated isolation by distance and relatively small neighbourhood sizes ( $N_b$  33–58) as a result of restricted seed dispersal, but also to a lesser extent of restricted pollen dispersal (Van Rossum and Triest 2006b; Jacquemyn et al. 2009; Stiers et al., unpublished results). Similar pollen or dye dispersal patterns, i.e. with most depositions at relatively short distances (<50–100 m), were reported for other insect-pollinated plant species, e.g. for the forest congeners *Primula sieboldii* (Kitamoto et al. 2006) and *P. vulgaris* in the agricultural landscape (Van Geert et al. 2010), and for grassland or meadow herbs, such as *Centaurea corymbosa* (Hardy et al. 2004), *Scabiosa columbaria* (Kwak et al. 1998), and *Silene alba* (Richards et al. 1999). Distance-limited pollen dispersal can be expected in insect-pollinated plant species because of: (1) pollinator behaviour and movement, resulting in deposition of most of the pollen grains on the first recipient individuals visited after the “source” plant and in loss of pollen by active grooming and by visiting flowers of other species; and (2) passive loss of pollen during flight (Campbell 1985; Inouye et al. 1994; Cresswell et al. 1995; Rademaker et al. 1997; Cresswell and Hoyle 2006).

There is also evidence of dye deposition at longer distances, up to 151 m in Rood Klooster. A study of dye dispersal patterns among urban populations of *P. elatior* connected by wooded linear elements indicated long-distance events of dye dispersal (up to 300 m) (Van Rossum and Triest 2006a). However, no dye transfer was detected when populations were 700 m apart (Stiers 2005). Large pollinators, such as large bees and bumblebees, especially queens, which are the main pollinators of *P. elatior*, can forage and therefore carry pollen or dye over long distances, from hundreds of metres up to more than 2 km

(e.g. Walther-Hellwig and Frankl 2000; Gathmann and Tschamtko 2002; Knight et al. 2005; Greenleaf et al. 2007; Van Rossum 2010). Secondary transfer, i.e. the remobilisation of particles deposited on a recipient flower by a second pollinator, and their subsequent re-deposition on stigmas of other individuals might also contribute to increase pollen dispersal distances (Thomson and Eisenhart 2003; Cresswell and Hoyle 2006).

#### Conclusion

Although subtle difference in dispersal patterns between dyes and pollen may have remained undetected in our study, the fluorescent dye method can be considered as a relatively good estimator to evaluate the distribution of distances of realized pollen dispersal for self-incompatible forest herbs, such as *P. elatior*, mainly pollinated by Hymenoptera, at least within the investigated distance range. It may therefore provide the opportunity to investigate pollen dispersal patterns in larger populations (several hundred individuals) and in connected patches of small populations, where it may be difficult or too costly to apply paternity analyses.

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