What influences pollen dispersal in the clonal distylous *Menyanthes trifoliata* (Menyanthaceae)?

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Background and aims – Habitat fragmentation may compromise population survival and decrease the ability to face environmental changes. Small and isolated populations in particular may suffer from disrupted pollination processes. However, a species' response to fragmentation may vary according to life history traits. Understanding how pollen dispersal is shaped by population and life history traits such as mating system and clonal growth is therefore essential for assessing species' response to fragmentation.

Methods – We investigated pollen dispersal using fluorescent dyes in nine Belgian populations in two regions of the distylous insect-pollinated clonal herb *Menyanthes trifoliata*. We examined whether overall and legitimate (i.e. between-morph) dye dispersal patterns differed between morphs and regions, and whether they were influenced by population traits (size, area, plant density, morph ratio, co-flowering species) and individual plant traits (number of flowers).

Key Results – No evidence of dye transfer between adjacent populations (distant from 1.3 to 11.8 km) was found, suggesting no or very restricted between-population pollen flow. Within-population dye dispersal followed a decaying, usually leptokurtic, distribution, with most dye deposition at short distances (80% at less than 1.9–49.1 m), and a maximum distance of 336.6 m. Pins and thrums as recipients or donors did not differ in dye dispersal. Legitimate dye dispersal was not less successful than overall dispersal despite spatial clumping of the morphs. The distribution was similar or simply shifted to longer distances. The proportion of dyed stigmas was positively related to flowering population size and within-population heterospecific floristic richness, and to the number of flowers when considering legitimate dye deposition at distances < 32 m.

Conclusions – Our study highlights the effects of the spatial distance to dye source, population size and co-flowering floral resources, i.e. population traits related to attractiveness to pollinators, on pollen dispersal patterns.

Key words – Clonal growth, distyly, fluorescent dye, heterospecific floristic richness, insect pollination, legitimate pollen, *Menyanthes trifoliata*, pollen dispersal, population size.

INTRODUCTION

The fragmentation of wild habitats constitutes one main cause of plant diversity erosion. The resulting small and isolated remnant plant populations may suffer from disruption of pollination processes and gene flow, and demographic and reproductive failure, resulting in genetic erosion and increasing inbreeding depression. These processes may compromise population survival and decrease plant species ability to face environmental changes (Wilcock & Neiland 2002, Ooster-

meijer et al. 2003, Aguilar et al. 2008, Angeloni et al. 2011). However, a species' response to habitat fragmentation and hence the management measures necessary to ensure population viability may vary according to life history traits, such as the mating system, gene dispersal ability, life form and vegetative growth ability (Charpentier 2001, Ghazoul 2005, Leimu et al. 2006). For instance, insect-pollinated plant species are particularly sensitive to habitat fragmentation, especially when they have a self-incompatible mating system, as they require cross-pollination to produce seeds. So they

depend on the availability of compatible mates, which is often reduced in small populations (e.g. Waites & Ågren 2004, Aguilar et al. 2006).

Successful seed set in animal-pollinated species also relies on the pollinator services (e.g. Ashman et al. 2004, Ghazoul 2005). The pollinator guilds themselves can be negatively impacted by habitat fragmentation, resulting in a decline in abundance and diversity (Harris & Johnson 2004, Goulson et al. 2008, Winfree et al. 2011, Mayer et al. 2012a). They may also change foraging behaviour, e.g. spending more time in large than in small fragments (Kwak et al. 1998, Cresswell & Osborne 2004). As pollen dispersal is often directly related to pollinator activities (Young et al. 2007, Mayer et al. 2012b), pollination processes may be disrupted in small isolated populations of self-incompatible plant species, reducing seed set (Wilcock & Neiland 2002, Aguilar et al. 2006).

Some self-incompatible plant species also show flower heteromorphism, such as heterostyly. Heterostyly is characterized by the co-occurrence of two (distyly) or three (tristyly) genetically inherited floral morphs (for distylous species: long-styled and short-styled, also called pin and thrum, respectively). A supergene complex controls the stamen-style compatibility reaction and floral polymorphism (Ganders 1979, Lewis & Jones 1992). The morphs show differences in style length and anther position (reciprocal herkogamy), but also in pollen grain number and size. These traits favour the deposition of compatible pollen grains on stigmas and thus promote intermorph pollination, i.e. disassortative mating, and increase male fitness (e.g. Ganders 1979, Barrett 1992, Hodgins & Barrett 2008, Baena-Díaz et al. 2012). However, when individuals of the same morph are incompatible, this system reduces the number of possible mates (by half or two-thirds), which may lead to a lack of compatible pollen in small populations, especially in case of skewed morph ratios, and so reinforces the negative effects of habitat fragmentation (e.g. Kéry et al. 2003, Brys et al. 2004, 2007). Moreover, the greater accessibility of pin stigmas and thrum anthers to insects may lead to asymmetric pollen transfers, with pin plants being more efficient pollen recipients, so receiving more pollen transfers, and thrum plants rather functioning as pollen donors (Husband & Barrett 1992, Matsumura & Washitani 2002, Cawoy et al. 2006, Baena-Díaz et al. 2012, but see Massinga et al. 2005), especially under pollination limitation (González et al. 2005, Van Rossum et al. 2006). Pollen dispersal patterns might also be affected by other morph-specific differences that may make them differentially attractive for pollinators, such as pollen and nectar quantity (e.g. Ganders 1979, McCall 1996, Ornelas et al. 2004, Cawoy et al. 2006).

Clonal propagation in heterostylous species, especially when growth is extensive and intermingling restricted (phalanx type), may structure the population into patches of incompatible mates (morphs). This may in turn interfere with legitimate (between-morph) pollen dispersal due to pollinator foraging behaviour (Nishihiro & Washitani 1998, Charpentier 2001, Wang et al. 2005). Indeed, pollinators often fly short distances between flowers, and the distribution of pollen dispersal is often leptokurtic, meaning that most pollen is deposited at short distances (e.g. Austerlitz et al.

2004, Hardy et al. 2004, Van Rossum 2009, 2010). In case of a clumped distribution of the morphs, pollen deposition at short distances is expected to lead to illegitimate pollination, as neighbouring plants likely belong to the same morph, except at the borders of patches of different morphs (Nishihiro & Washitani 1998, Charpentier 2001, Wang et al. 2005). Consequently legitimate pollen dispersal, compared to overall (i.e. potential, without considering morph type) or illegitimate pollen dispersal, may consist of fewer events, only occurring at longer distances, and therefore might strongly rely on pollinator flight abilities (Scobie & Wilcock 2009).

Even if the distance between donor and recipient plants often appears as the main factor influencing pollen dispersal for insect-pollinated species, pollen deposition patterns within and between populations may also be affected by other population traits, such as population size, flowering plant density and heterospecific floristic richness, and by individual plant traits, such as the number of flowers or nectar/pollen reward (e.g. Thomson & Plowright 1980, Kwak et al. 1998, Cresswell & Osborne 2004, Ghazoul 2005, Van Rossum 2009, 2010, Van Rossum & Triest 2010, Briscoe Runquist 2012). These factors may determine population attractiveness for pollinators, and influence the intensity of plant visitation. For instance, we may expect fewer visits of small isolated and/or sparse populations, as they may be less visible or insufficiently rewarding (e.g. Cresswell et al. 2002, Van Rossum & Triest 2010). The presence of other floral resources may facilitate pollination, but may also represent competition for pollinator services and lead to pollen loss on heterospecific flowers, especially for species with a generalist pollination system (Ghazoul 2005, 2006, Flanagan et al. 2011). Understanding how pollen dispersal is shaped by such population traits that interact with each other and by life history traits such as the mating system and clonal growth is thus essential for assessing species response to fragmentation. However, to our knowledge, knowledge on whether pollen dispersal patterns might differ between morphs and whether legitimate pollen dispersal differs from overall (potential) pollen dispersal still remains limited (Matsumura & Washitani 2002, Adler & Irwin 2006, Ishihama et al. 2006, Van Rossum & Triest 2006, Baena-Díaz et al. 2012).

We investigated pollen dispersal patterns in fragmented populations of *Menyanthes trifoliata* L., a distylous self-incompatible insect-pollinated, clonal herb occurring in wetlands (Hewett 1964, Nic Lughadha & Parnell 1989). In Belgium, this species is rare and declining despite the protection of its biotopes, and shows a highly fragmented distribution (Saintenoy-Simon et al. 2006), with small populations incurring genetic erosion (J. Raabová et al., unpubl. res.). Results on spatial genetic structure using microsatellites have indicated that our study populations of *M. trifoliata* are characterized by a spatial clumping of the clones of the same genet, and so of the morphs, as a result of extensive clonal growth by creeping rhizomes, with little intermingling of the clones and skewed morph ratios. Clones may extend up to 16 m length (J. Raabová et al., unpubl. res.). We used fluorescent powdered dyes as pollen analogues to examine the spatial pattern of overall and legitimate pollen dispersal within Belgian populations of varying size. Dye dispersal may be considered as a reliable estimator of potential or realized pollen

dispersal for insect-pollinated species (Waser 1988, Rademaker et al. 1997, Van Rossum et al. 2011, but see Thomson et al. 1986, Campbell 1991). This method is convenient when it appears difficult to perform paternity analyses using molecular markers, e.g. in case of large populations or when seed germination rates are too low under controlled conditions to obtain enough seedlings, which had been the case for *M. trifoliata*. We addressed the following questions: (1) What is the pattern of overall and legitimate pollen (dye) dispersal within and between populations? (2) Do pollen dispersal patterns differ between morphs (as dye recipients and donors) and populations? (3) Do the following factors influence pollen dispersal: population traits (population size and area, flowering plant density, morph ratio, co-flowering floral resources) and number of flowers? We discuss the implications of the results for pollen dispersal patterns of clonal distylous species in fragmented habitats.

MATERIALS AND METHODS

The species

Menyanthes trifoliata (Menyanthaceae) is a long-lived perennial aquatic herb that occurs in pioneer stages of bogs, fens and shallow waters. It has a circumboreal distribution extending south to 40°N (Hewett 1964). The species is distylous, with pin (long-styled) and thrum (short-styled) morphs. The morphs show reciprocal herkogamy and differ in pollen grain size (pin:thrum ratio = 0.87) and amount (minimum 2000 and 960 grains per anther for pin and thrum, respectively) (Nic Lughadha & Parnell 1989). Hand-pollinations have demonstrated that distyly is strictly self-incompatible, as no seeds were obtained from intra-morph pollination (Leprince 2010). In May-June, the plant produces racemose inflorescences with 6–35 whitish flowers, with five stamens (electronic appendix 1). The flowers are feebly protogynous and remain open for 2–3 days. The single-styled ovaries are overtopped by a bilobed stigma. Nectar is secreted at the base of the ovary (Knuth 1909, Hewett 1964). The flowers are insect-pollinated, mainly by Hymenoptera (especially Apidae), Diptera and Lepidoptera (Knuth 1909, Nic Lughadha & Parnell 1989, Thompson et al. 1998). In the study sites the following visitors were frequently observed: bumblebees (mainly *Bombus pratorum*, as well as *B. pascuorum* and *B. terrestris* sensu lato), syrphid flies (*Helophilus pendulus, Eristalis pertinax*), and *Empis tessellata* (Empididae). Lepidoptera were also observed, e.g. *Carterocephalus palaemon* (Hesperiidae) and *Pieris napi* (Pieridae) (G. Hans, pers. obs.).

Studied populations

We investigated pollen dispersal in nine populations (named A to I) occurring in fens and bogs in two Belgian regions (Plateau des Tailles and Lorraine), about 60 km apart (table 1). Three additional populations found in these regions had inaccessible plants (water too deep) or too few flowers to carry out the experiment. Bogs and fens in these regions currently consist of a network of fragmented patches, but two centuries ago they consisted of large continuous open areas, used for peat extraction and agro-pastoral activities.

Table 1 – Population details of nine populations of *Menyanthes trifoliata* from two regions (Plateau des Tailles and Lorraine). Geographic coordinates (latitude, longitude), population size (number of flowering ramets, N), population area (in m²), flowering plant density (number of flowering ramets m⁻²), proportion of pin flowering ramets (Pin prop.), heterospecific richness and Shannon-Wiener diversity index (within population and total) and mean number of flowers per ramet. * also including 7.4% of homostylous ramets.

Population	Latitude (N)	Longitude (E)	N	Population area (m^{-2})	Flowering plant density (m^{-2})	Pin proportion	Heterosp. richness Within	Heterosp. richness Extended	Diversity index Within	Diversity index Extended	Number of flowers
Plateau des Tailles											
A Grande Fange	50°14'37"	05°46'46"	>1500	16666	0.9	0.37	13	13	2.51	2.51	17.9
B Massotais	50°13'58"	05°45'20"	480	7860	0.1	0.70	4	5	1.39	1.48	13.5
C Fange de Pouhon	50°14'33"	05°41'57"	357	40	8.9	0.52	6	6	1.79	1.79	12.9
D Wé de Pourceaux*	50°14'37"	05°44'49"	135	230	0.6	0.67	0	3	0.00	1.08	14.5
Lorraine											
E Sampont	49°40'36"	05°41'58"	600	5953	0.1	0.46	6	6	1.70	1.70	19.2
F Heinsch	49°41'45"	05°43'45"	400	2578	0.2	0.29	3	7	1.05	1.89	17.1
G Stockem	49°40'29"	05°46'17"	225	228	1.0	0.32	3	15	1.01	2.60	21.4
H Grendel	49°45'14"	05°49'11"	128	664	0.2	0.65	3	3	1.01	1.01	18.5
I Plate dessous-les-Monts	49°42'00"	05°32'12"	52	67	0.8	0.66	7	7	1.89	1.89	20.4

These extensive practices have since been abandoned, leading to forest recolonization of these wetlands. Belgian wetlands have also incurred area losses due to fragmentation, by plantations of spruce and by urbanization, with a speeding up of these processes after World War II (Parent 1973, Verté 2007, Cristofoli et al. 2010). The geographical distance between populations ranged from 1.3 to 11.8 km between neighbouring populations and to 21.2 km in maximal distance. Ramet population size (called hereafter "population size"), corresponding to the number of flowering ramets (one ramet corresponding to one inflorescence; electronic appendix 1), ranged from 52 to >1500. Ramet and genet population sizes were found to be significantly positively correlated (J. Raabová et al., unpubl. res.). Population area (m²), i.e. the surface covered by the patches of *M. trifoliata*, was estimated for each population based on plant mapping using a GPS (Garmin Oregon 400t, Southampton, UK). Flowering plant density was calculated as the number of flowering ramets divided by the population area (for population A, this was done on the central part of the population where the dye dispersal study was realized). Morph type was noted for all flowering ramets in small populations and for up to 500 randomly chosen flowering ramets in the larger ones. The number of other co-flowering insect-pollinated species (= heterospecific richness), which might (partly) share insect visitors with *M. trifoliata*, such as *Caltha palustris*, *Cardamine amara*, *C. pratensis*, *Cirsium palustre*, *Lychnis flos-cuculi*, *Valeriana dioica*, and *Vaccinium uliginosum* (e.g. Knuth 1908, 1909; pers. obs.), was counted within the populations. Each species' cover was estimated following an abundance scale (1: < 10%, 2: 10–40%, 3: > 40% cover) and used to calculate the Shannon-Wiener diversity index. These measures were extended to the surrounding vegetation (on 10 m border) outside the population itself (= extended heterospecific richness and Shannon-Wiener diversity), as some important floral resources for insects (e.g. *Cytisus scoparius*) were also observed at the vicinity of the populations.

Estimating pollen dispersal using fluorescent dye

Four colours (orange, yellow, pink and blue) of fluorescent dye (RADGLO® Series R and PS, Radiant Color Corp., Belgium) were used as pollen analogues to estimate overall and legitimate pollen dispersal. Previous studies showed no difference in dispersal patterns among the used dye colours (e.g. Van Rossum 2009, 2010, Van Rossum & Triest 2010). The experiment was conducted at the peak of flowering, during 3-day periods of dry, relatively sunny, non-windy weather, in May 2009. A different colour was used for each population within each region, except for populations H and I, which are 21.2 km apart. On day 1 and 2, dyes (one single colour per population) were applied once early in the morning with wooden toothpicks to dehiscing anthers of thirty recently opened flowers (4–10 ramets) of the same morph within an area of ~1 m² (= dye source ramets; thrum: populations A, E–G, I; pin: B–D, H). The dye source location was chosen so as to allow a wide range of potential distances between the dye donors and recipient flowering ramets (inflorescences), i.e. within the central third of the population, or at one side of the population for the small populations (C, D, H and I). On day 3, stigmas that had been receptive during the experi-

mental period were harvested from 3–7 flowers on 21–50 recipient ramets per population (table 2). In all populations the recipient ramets were randomly sampled along a transect that usually covered the whole population area (some populations being patchy). The dye source and recipient ramets were mapped using a GPS, a 50 m tape measure and a laser distance meter (Leica Disto A5, Heerbrugg, Switzerland). The stigmas (two lobes per flower) were embedded in a semi-permanent mount of glycerine jelly on a microscope slide (Van Rossum 2010).

In total, 1634 stigmas collected from 393 flowering ramets were examined for dye particles at 100x magnification under a fluorescence microscope. Presence or absence of fluorescent dye particles was recorded on each stigma. For each recipient ramet, the proportion of stigmas with dye deposition was calculated (proportion of dyed stigmas = number of stigmas with dye divided by the total number of collected stigmas). This proportion can be considered as an indicator of pollinator activity (Van Geert et al. 2010). For each recipient ramet (inflorescence), the number of flowers was counted and ranged from 8 to 33 (mean per population given in table 1). The potential distances (range and mean) from dye source to recipient ramets within each population are given in table 2. These mean values were calculated for (i) overall dispersal, i.e. for all (pin and thrum) ramets and (ii) legitimate dispersal, i.e. for the ramets of the morph that was not used as dye source (pin in A, E–G, and I and thrum in B–D and H).

Data analysis

The proportion of dyed stigmas was logit-transformed (log $[(y + \varepsilon)/(1 - y + \varepsilon)]$), with ε the minimum non-zero proportion y; Warton & Hui 2011). The other variables were transformed (natural log or Box Cox) when necessary to achieve normality and homoscedasticity. Depending on whether the relationship between the variables was linear or exponential, the analyses were based on a standard linear model (identity link function, significance determined using a F test) or on a generalized linear model (GLM, power link function and significance determined using a likelihood ratio chi-square test). All analyses were performed using STATISTICA version 10 (Statsoft 2010).

Overall and between-morph dye dispersal patterns – As no dye transfers were observed between populations (see below), the analyses only concerned within-population dispersal patterns. The overall dye dispersal pattern over the whole population area and whether the pin recipient ramets showed higher dye transfers than thrum recipient ramets were investigated for each population separately using an analysis of covariance (ANCOVA, table 3), which tested the relationship between the mean proportion of dyed stigmas for each recipient ramet (dependent variable), and the distance to dye source and the number of flowers (independent variables), with morph type of the recipient ramets as grouping variable. The interaction between the two continuous variables and the grouping variable was also tested. The shape of overall dye dispersal distribution was described for each population using the best-fitting parameter β of the dye dispersal kernel, an exponential power function characterizing dye dispersal,

Table 2 – Overall and legitimate dye dispersal results within nine populations of *Menyanthes trifoliata* from two regions (Plateau des Tailles and Lorraine).
Number of sampled recipient ramets (*n*), potential distance to dye source in m (mean with ranges), effective distance of dye transfers in m (mean with ranges and for 80% of the dye transfers), proportion of recipient ramets showing dye and mean proportion of dyed stigmas (with ranges), and best fitting β parameter value of the dye dispersal kernel. The proportion of dyed stigmas is the number of stigmas with dye divided by the total number of collected stigmas.

	Population	*n*	Distance to dye source (m)	Distance of dye transfers (m)	80% dye transfers(m)	Ramets with dye	Proportion dyed stigmas	β
	Overall dye dispersal							
	Plateau des Tailles							
A	Grande Fange	50	29.9 (0.3–68.4)	29.4 (0.3–68.4)	49.1	0.98	0.68 (0.00–1.00)	1.24
B	Massotais	40	47.5 (0.5–154.4)	45.7 (0.5–154.4)	42.4	0.88	0.65 (0.00–1.00)	0.30
C	Fange de Pouhon	43	18.1 (0.2–35.0)	18.1 (0.2–35.0)	32.7	1.00	0.98 (0.50–1.00)	-
D	Wé des Pourceaux	41	4.9 (0.1–11.2)	4.9 (0.1–11.2)	8.9	1.00	0.97 (0.25–1.00)	1.56
	Lorraine							
E	Sampont	50	124.7 (0.6–366.6)	53.3 (0.6–366.6)	39.7	0.72	0.49 (0.00–1.00)	0.55
F	Heinsch	48	23.3 (0.5–51.4)	22.7 (0.5–51.4)	46.1	0.96	0.88 (0.00–1.00)	0.64
G	Stockem	50	6.3 (0.2–26.8)	6.3 (0.2–26.8)	4.4	1.00	0.90 (0.10–1.00)	1.17
H	Grendel	50	16.5 (0.2–29.8)	17.0 (0.6–29.8)	25.2	0.86	0.52 (0.00–1.00)	1.34
I	Plate dessous-les-Monts	21	11.4 (1.4–20.2)	4.9 (1.4–15.5)	1.9	0.29	0.13 (0.00–0.83)	0.61
	Legitimate dye dispersal							
	Plateau des Tailles							
A	Grande Fange	17	20.5 (3.6–57.8)	20.5 (3.6–57.8)	41.9	1.00	0.81 (0.25–1.00)	
B	Massotais	19	75.1 (6.9–154.4)	84.9 (6.9–154.4)	151.5	0.79	0.46 (0.00–1.00)	
C	Fange de Pouhon	22	32.5 (28.4–35.0)	32.5 (28.4–35.0)	34.5	1.00	0.96 (0.50–1.00)	
D	Wé des Pourceaux	13	8.0 (2.6–11.2)	8.0 (2.6–11.2)	10.1	1.00	0.96 (0.83–1.00)	
	Lorraine							
E	Sampont	23	117.2 (2.3–366.6)	51.5 (2.3–366.6)	54.9	0.74	0.44 (0.07–1.00)	
F	Heinsch	12	29.4 (4.7–51.4)	29.4 (4.7–51.4)	49.3	1.00	0.90 (0.70–1.00)	
G	Stockem	9	14.6 (1.4–26.8)	14.6 (1.4–26.8)	15.9	1.00	0.71 (0.13–1.00)	
H	Grendel	17	25.2 (17.4–29.8)	25.2 (17.4–29.8)	27.7	0.82	0.41 (0.00–0.90)	
I	Plate dessous-les-Monts	13	13.5 (4.3–20.2)	10.9 (6.3–15.5)	15.5	0.15	0.04 (0.00–0.33)	

with the mean proportion of dyed stigmas used to fit the function (for more details, see Hardy et al. 2004, Van Rossum et al. 2011). The dispersal kernel is fat-tailed (leptokurtic distribution) when $\beta < 1$, and thin-tailed when $\beta > 1$ (e.g. Hardy et al. 2004).

Legitimate dye dispersal patterns – Due to the clumping of the morphs and biased morph ratios (J. Raabová et al., unpubl. res.), it was difficult to directly compare illegitimate and legitimate dye transfers. Therefore we examined whether overall (for all recipient ramets) and legitimate (i.e. from the dye source morph to the opposite recipient morph) dye dispersal patterns might differ from each other at the popu-

lation level and for each recipient population separately. At the population level, Wilcoxon matched pairs tests (*n* = nine populations) were performed on the proportion of recipient ramets showing dye deposition and the distance for 80% of the dye transfers. For each population separately, we performed two analyses: (i) we tested whether overall and legitimate dye dispersal distributions might differ in general shape using a Kolmogorov-Smirnov (K-S) tests for goodness of fit (for this purpose the distances were not grouped into classes; Sokal & Rohlf 2000; see Van Rossum et al. 2011 for more details). The variables tested were the effective distance of dye transfers, but also the potential distance to dye

Table 3 – ANCOVA of the relationships between mean proportion of dyed stigmas and distance to dye source, the number of flowers per ramet and morph type of the recipient ramets, for nine populations of *Menyanthes trifoliata*.
Based on a linear model for populations A, E and H (between brackets: results for the whole model, with R = multiple regression coefficient) and on a GLM (power link function and likelihood ratio χ^2 test) for populations B–D, F, G and I. Only the effects with $P < 0.05$ are shown.

Variable	Estimate	df	F or χ^2	P
A ($R = 0.733$, $F_{(5,44)} = 10.22$, $P < 0.001$)				
Distance to dye source	-0.688	1,44	27.05	< 0.001
B				
Distance to dye source	-0.193	1,34	10.29	0.001
C				
Number of flowers	0.043	1,37	8.12	0.004
Morph x number of flowers	-0.043	1,37	8.12	0.004
D				
Distance to dye source	-0.046	1,35	6.14	0.013
E ($R = 0.849$, $F_{(5,44)} = 22.72$, $P = 0.001$)				
Distance to dye source	-0.833	1,44	84.00	< 0.001
F				
Distance to dye source	-0.015	1,42	4.67	0.031
G				
Distance to dye source	-0.773	1,44	13.58	0.001
Number of flowers	0.035	1,44	5.15	0.023
H ($R = 0.260$, $F_{(5,44)} = 0.64$, $P = 0.673$)				
I				
Distance to dye source	-0.041	1,15	7.27	0.007
Morph type	2.693	1,15	11.77	< 0.001
Morph x distance to dye source	-0.058	1,15	12.85	< 0.001
Morph x number of flowers	-0.095	1,15	9.31	0.002

source to test whether plant distribution might affect the observed dye dispersal patterns; (ii) we tested for differences in mean proportion of dyed stigmas using an ANCOVA (also testing for homogeneity of slopes), with the distance to dye source as independent variable and dispersal type (overall or legitimate) as grouping variable. The analyses were carried out on the whole data sets as well as on a data set restricted to the effective (successful) dye transfers in populations A, B, E, F and H (too small sample size for population I and all recipient individuals showing dye deposition in populations C, D and G).

Effect of population traits, region and morph type of the dye source on dye dispersal patterns – We examined whether dye dispersal patterns might be affected by popula-

tion traits and whether thrum dye source ramets were better dye donors than pin ones. We also tested whether regions differed in dye dispersal patterns, which we did not expect. First, Spearman correlation coefficients (r_s) were calculated between the proportion of recipient ramets showing dye deposition and the population variables (population size and area, flowering plant density, pin proportion, within-population and extended heterospecific richness and Shannon-Wiener diversity index). For these population variables differences between regions were tested by Mann-Whitney U-tests. Second, we used a mixed model nested ANCOVA to investigate whether overall or legitimate dye deposition may differ between regions or the morph types of the dye source (pin or thrum), taking individual plant traits (distance to dye source and the number of flowers) into account (table 4). To

Table 4 – Nested mixed model ANCOVA of the effects of region and / or dye source morph type on mean proportion of dyed stigmas for overall and legitimate dye dispersal, for three distance ranges (up to 52, 32 and 12 m), with distance to dye source and the number of flowers as covariates.

| Variable	β	df	F	P
Distance up to 52 m				
Overall dye dispersal				
Region		1,2	0.44	0.575
Population		2,145	4.91	0.009
Distance to dye source	-0.610	1,145	92.56	< 0.001
Number of flowers	0.058	1,145	0.67	0.413
Legitimate dye dispersal				
Region		1,2	0.53	0.540
Population		2,48	7.05	0.002
Distance to dye source	-0.571	1,48	26.83	< 0.001
Number of flowers	-0.024	1,48	0.38	0.845
Distance up to 32 m				
Overall dye dispersal				
Region		1,5	1.44	0.281
Population		5,219	16.62	< 0.001
Distance to dye source	-0.345	1,219	42.25	< 0.001
Number of flowers	0.026	1,219	0.21	0.651
Dye source morph type		1,5	0.06	0.820
Population		5,219	18.88	< 0.001
Distance to dye source	-0.345	1,219	42.25	< 0.001
Number of flowers	0.026	1,219	0.21	0.651
Legitimate dye dispersal				
Region		1,5	2.60	0.161
Population		5,58	8.95	< 0.001
Distance to dye source	-0.465	1,58	11.58	0.001
Number of flowers	0.254	1,58	4.22	0.044
Dye source morph type		1,5	0.47	0.522
Number of flowers		5,58	11.89	< 0.001
Distance to dye source	-0.465	1,58	11.58	0.001
Number of flowers	0.254	1,58	4.22	0.044
Distance up to 12 m				
Overall dye dispersal				
Region		1,7	1.76	0.225
Population		7,179	34.81	< 0.001
Distance to dye source	-0.105	1,179	4.80	0.030
Number of flowers	-0.086	1,179	2.40	0.122
Dye source morph type		1,7	0.94	0.768
Population		7,179	37.55	< 0.001
Distance to dye source	-0.105	1,179	4.80	0.030
Number of flowers	-0.086	1,179	2.40	0.122
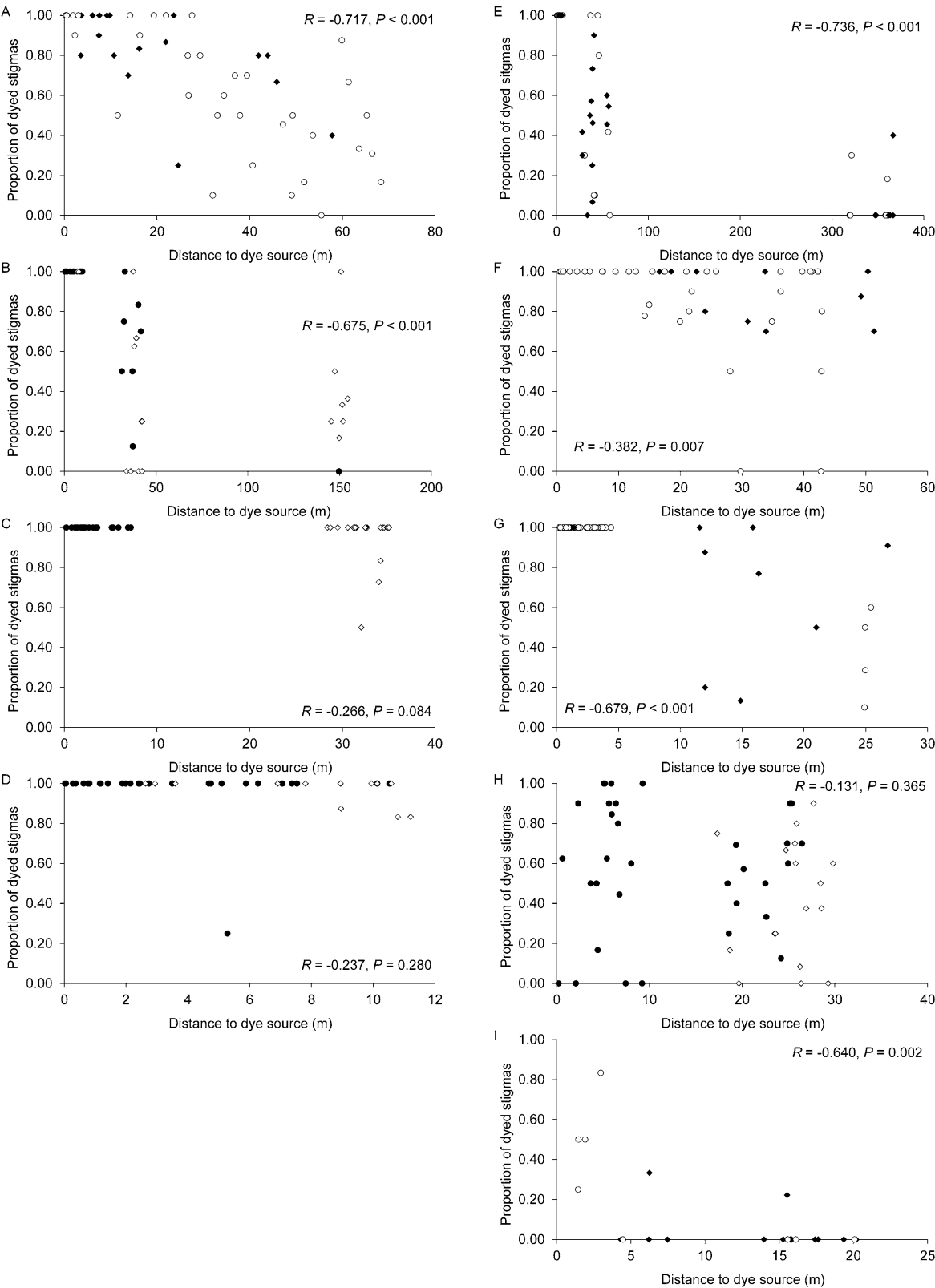
Figure 1 – Distribution of dye deposition (mean proportion of dyed stigmas) as a function of the distance to dye source. A–I: populations A to I. Legitimate dye deposition events are indicated by diamond symbols. Morph type: pin (black), thrum (white). *R*: univariate (linear or exponential) regression coefficient for the overall dye dispersal (variables logit-, log- or Box Cox transformed when necessary).

this end, the analyses were carried out for the same range of distance to dye source, for three maximal distances, so that we could compare populations for distance ranges as large as possible as well as comprising (almost) all populations: up to 52 m (large populations only: A, B, E, F), 32 m (all populations except D and I that have very small areas), 12 m (all populations). The analyses were not performed for the legitimate deposition at 12 m because of too small samples for most populations, and a dye source morph type effect was not tested at 52 m because there was only one population with pin as dye source. Population (nested in region or in dye source morph type, which were entered as fixed effects) was entered as a random effect, and the distance to dye source and the number of flowers per ramet as covariables. The degrees of freedom of the error terms were adjusted for statistical dependence using the Satterthwaite method. Finally, multiple regression analyses with a forward stepwise selection procedure were carried out for the three distance ranges to test the relationships between the mean proportion of dyed stigmas per recipient ramet and population traits (population size and area, flowering plant density, pin proportion, within-population and extended heterospecific richness and Shannon-Wiener diversity index), with population and individual variables (distance to dye source and the number of flowers) as covariates (effect of population covariates tested against the variation among populations). When the analysis revealed a significant effect, Tukey HSD tests or univariate regression analyses (on mean values for independent population variables) were performed.

RESULTS

Overall and between-morph dye dispersal patterns

No dye transfers were observed between populations as no other dye colour than the within-population dye source colour was found on the recipient stigmas. The overall proportion of recipient ramets showing dye deposition within populations varied from 0.29 to 1.00 (table 2). For these ramets showing dye deposition, the proportion of dyed stigmas ranged from 0.07 to 1.00 (mean: 0.13–0.98). The distance to dye source for effective within-population dye transfers varied from 0.1 to 366.6 m, and so usually covered the sampling area, with 80% of the dye transfers occurring at less than 1.9 to 49.1 m (table 2). The ANCOVA based on linear or GLM models (table 3, fig. 1, electronic appendix 2) revealed a significantly ($P < 0.05$) negative relationship between mean proportion of dyed stigmas per recipient ramet and the distance to dye source for all populations, except for populations C and H. A significantly positive relationship between mean proportion of dyed stigmas and the number of flowers per ramet was only found for populations C and G (table 3). A recipient morph type effect and a significant interaction between morph type and distance to dye source were only found in population I, and there was a significant interaction between morph and the number of flowers for populations C and I (table 3). Populations C and I showed a difference in morph ramet distributions, with only one morph type located at the shortest distances to the dye source (fig. 1). The best-fitting β parameter describing the shape of the distribution curves ranged from 0.30 to 1.56 (table 2). No describing

curve based on the dispersal kernel model could be obtained for population C.

Legitimate dye dispersal patterns

When considering dye dispersal leading to legitimate (intermorph) deposition events only (table 2), the proportion of recipient ramets showing dye deposition did not significantly differ from overall dye deposition (Wilcoxon matched pairs test $Z = 0.73$, $P = 0.463$), but 80% of the legitimate dye transfers occurred at significantly ($Z = 2.07$, $P = 0.038$) longer distances than the overall ones, i.e. 10.1 to 151.5 m compared to 1.9 to 49.1 m. For populations C, G and H, the distribution of the potential distance to dye source and of the effective distance of dye transfers shifted to higher distance values for legitimate dispersal compared to overall dye dispersal as shown by a significant difference (K-S test: $P < 0.05$) in curve shape (table 2). No significant difference in curve shape was detected for the other populations. The test of homogeneity of slopes (based on linear or GLM models) showed no significant difference ($P > 0.10$) between overall and legitimate mean proportion of dyed stigmas ($F_{1,63} = 0.56$ for population H; $\chi^2 = 0.01$–1.38 for A–G) and for the interaction dispersal type x distance to dye source ($F_{1,63} = 0.37$ for population H; $\chi^2 = 0.01$–0.98 for G), except for population I where a trend for higher overall (0.13) than legitimate (0.04) mean proportion of dyed stigmas was found ($\chi^2 = 3.42$, $P = 0.064$). When only considering the effective (successful) dye transfers (for populations A, B, E, F and H), the tests of homogeneity of slopes showed similar results (not shown).

Effect of population traits, region and morph type of the dye source

The proportion of recipient ramets showing dye deposition was not correlated to any of the population variables ($r_s = -0.407$–0.576, $P > 0.05$). No difference between regions was found for the proportion of recipient ramets showing dye deposition and for the population variables (Mann-Whitney U-tests, $P > 0.05$). The mixed model nested ANCOVA revealed no significant ($P > 0.05$) difference in mean overall and legitimate proportion of dyed stigmas per recipient ramet between regions and between dye source morph types whatever the distance range, i.e. up to 52, 32 or 12 m (table 4). Mean proportion of dyed stigmas and the distance to dye source were negatively related in all analyses ($\beta = -0.610$–-0.105, $P \leq 0.030$). The number of flowers had a positive effect on mean legitimate proportion of dyed stigmas per recipient ramet ($\beta = 0.254$, $P = 0.044$), only at intermediate distances (up to 32 m).

Significant ($P < 0.05$) multiple regression models investigating the effects of population traits were found for the three distance ranges, with mean proportion of dyed stigmas per recipient ramet being significantly negatively related to the distance to dye source, for overall dye dispersal ($\beta = -0.610$, -0.333, $P < 0.001$ and $\beta = -0.187$, $P = 0.004$, for distance ranges up to 52, 32 and 12 m, respectively) as well as for legitimate dye dispersal ($\beta = -0.556$ and -0.465, $P < 0.001$ and $P = 0.001$, for distance ranges up to 52 and 32 m, respectively). Mean legitimate proportion of dyed stigmas increased with the number of flowers for a distance range up

to 32 m ($\beta = 0.254$, $P = 0.044$). For a distance range up to 52 m, a significantly negative effect of heterospecific richness on overall mean proportion of dyed stigmas was found ($\beta = -1.430$, $P = 0.007$; fig. 2), with population F showing a significantly higher mean proportion of dyed stigmas than the three other large populations (Tukey HSD tests, $P < 0.05$) (table 1). When distances ranged up to 32 m, there was a significantly positive (quadratic in the univariate analysis) relationship between mean overall proportion of dyed stigmas and heterospecific richness within populations ($\beta = 0.231$, $P < 0.001$; fig. 3A). At short distances (up to 12 m), overall mean proportion of dyed stigmas was positively related to population size ($\beta = 0.428$, $P < 0.001$, quadratic in the univariate analysis: fig. 3B). There was no significant correlation between population size and heterospecific richness within population ($r_s = 0.383$, $P > 0.05$). No other variable ($P > 0.05$) was selected in the forward stepwise selection procedure.

DISCUSSION

Between-population dye dispersal patterns

All dye particles on stigmas originated from the population under investigation, thus, no dye transfer between adjacent populations (distant from 1.3 to 11.8 km) could be proven. This suggests that beyond 1.3 km, populations might be spatially isolated from each other, or at least gene flow might be very restricted. A similar pattern has been found in the same regions for the bumble bee-pollinated *Comarum palustre* (Mayer et al. 2012b) and *Vaccinium uliginosum* (Brédat 2010). One has to admit that infrequent long-distance dispersal events between populations may be difficult to detect using the dye method (Campbell 1991, Gaudeul & Till-Bottraud 2004, Van Rossum et al. 2011, but see Van Geert et al. 2010 and Van Rossum 2010 who detected between-population long-distance dye deposition up to 1.0–2.6 km).

Within-population dye dispersal patterns

The main factor that influenced the amount of dye deposition on stigmas within the studied *M. trifoliata* populations was the distance to the dye source. Dye dispersal usually showed an exponentially decreasing, often leptokurtic (best-fitting β parameter of the dispersal kernel < 1) distribution with respect to spatial distance. Most dye particles were deposited at short distances (80% at less than max. 49.1 m) and long-distance events were rarer, occurring up to 366.6 m. Such a leptokurtic or exponential decay curve shape is a common pattern for pollen dispersal (e.g. Cresswell et al. 1995, Gaudeul & Till-Bottraud 2004, Scobie & Wilcock 2009, Van Rossum 2009, 2010, Van Geert et al. 2010). Similar distance ranges of within-population dye or pollen deposition were found for other bee-pollinated plant species (Kwak et al. 1998), e.g. for the distylous *Primula elatior* (Van Rossum et al. 2011, Van Rossum & Triest 2012) and *Vaccinium oxycoccos* (Van Rossum et al. 2013). However, even if fluorescent dye usually provides a reliable analogue for pollen movement, especially by bees (Waser 1988, Dudash 1991, Rademaker et al. 1997, Van Rossum et al. 2011), mirroring pollinator activity (Mayer et al. 2012b), dye particles may be smaller and less

sticky than pollen grains and may be transported less far or farther than pollen and/or in different amounts, and may under- or overestimate pollen dispersal (Thomson et al. 1986, Campbell 1991, Adler & Irwin 2006), so that whether dye dispersal accurately reflects pollen dispersal in *M. trifoliata* still needs to be verified.

Morph-specific differences in dye dispersal patterns

Morph-specific differences in floral characteristics (e.g. reciprocal herkogamy, nectar production, pollen grain size and quantity) may lead to asymmetrical pollen transfers with preferential pollen recipient and donor or morph-differential attractiveness to pollinators, which might further affect pollen dispersal patterns (e.g. Husband & Barrett 1992, Matsumura & Washitani 2002, Ornelas et al. 2004, Massinga et al. 2005, Cawoy et al. 2006, Baena-Díaz et al. 2012). No differences in dye dispersal patterns between morphs were observed for the study populations of *M. trifoliata*, neither when the morphs were recipient individuals nor when they acted as dye donors. This suggests that there might be no preferential visitation of the morphs by the pollinators, and that style length and anther position, do not influence dye deposition. This is consistent with the findings for other distylous self-incompatible species, such as *Jasminum fruticans* (Thompson 2001) and *Gelsemium sempervirens* (Adler & Irwin 2006), but contrasted with *Fagopyrum esculentum* (Cawoy et al. 2006), for which thrum flowers were preferentially visited by honeybees.

Legitimate dye dispersal patterns

The spatial clumping of the morphs, combined with pollinator foraging behavior leading to decaying pollen dispersal distributions, might interfere with legitimate pollen dispersal (Charpentier 2001, Wang et al. 2005, Scobie & Wilcock 2009). We might expect an increase in illegitimate unsuccessful pollination at short distances, except at the edge of patches, and legitimate pollen delivery at longer distances, therefore relying on pollinator flying abilities (Wang et al.
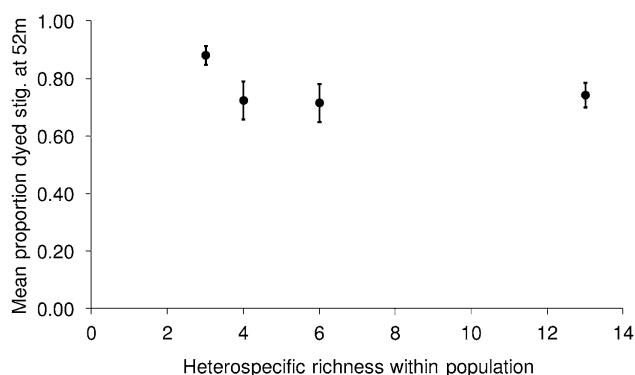

Figure 2 – Distribution of mean proportion of dyed stigmas (± SE) at 52 m (populations A, B, E, F) as a function of the species richness within site.

2005, Scobie & Wilcock 2009). Our present findings indicate that legitimate dye dispersal in *M. trifoliata* populations is not less successful than overall dye dispersal for the same distance ranges. The legitimate dye deposition distribution was similar or showed a shift to higher distance values, likely as a result of the spatial distribution of the morphs and so of the legitimate recipient ramets, but covered the whole population area. This indicates that the within-population distances we investigated ranged within pollinator flying abilities for foraging. However, a trend for lower legitimate proportion of dyed stigmas was observed in population I, which was the only population with a very small number of flowering ramets (52 in 2009). This pattern certainly deserves to be further looked into, with additional very small populations.

Effect of individual and population plant traits and region on dye dispersal patterns

The number of flowers is an individual plant trait that has been reported to positively influence visitation rates, and therefore, to promote pollen transfer, as a larger number of flowers represent a higher, more attractive, resource for insects (Dudash 1991, Vaughton & Ramsey 1998, Makino et al. 2007). The present study on *M. trifoliata* showed a positive relationship between the number of flowers and mean

Figure 3 – Relationship between: A, species richness within site and mean proportion of dyed stigmas (± SE) at 32 m (seven populations, overall dye dispersal); and B, ramet population size and mean proportion of dyed stigmas (± SE) at 12 m (nine populations, overall dye dispersal). R^2 = quadratic coefficient of determination of the univariate regression model. Mean proportion of dyed stigmas was logit-transformed.

proportion of dyed stigmas in two populations, and a significantly higher proportion of dyed stigmas when the number of flowers on the flowering ramet increased, when considering dye deposition at intermediate distance (< 32 m).

Population traits (e.g. population size, flowering plant density, available floral resources) may also affect within-population pollen dispersal patterns through changing population attractiveness and pollinator behaviour (e.g. Kwak et al. 1998, Ghazoul 2005). Our study of *M. trifoliata* showed that dye deposition was positively related to ramet population size and heterospecific richness within the population, while no significant relationship, however based on only nine populations, was found with any other variables tested, such as flowering plant density and population area. This indicates that populations when very small and/or very poor in co-flowering species may be less attractive or insufficiently rewarding for pollinators compared to large populations, leading to lower visitation and pollen deposition, whereas pollinators may spend more time in large populations (Cresswell et al. 2002, Cresswell & Osborne 2004, Waites & Ågren 2004, Van Rossum & Triest 2010). However, the relationship is quadratic, due to the fact that in one very large population, also rich in other floral resources we observed lower dye deposition on the stigmas, suggesting that in this case there may be competition with other species for pollinators or *M. trifoliata* pollen wastage on other co-flowering species (Ghazoul 2006, Flanagan et al. 2011). Other population traits, not considered here, such as varying pollinator abundance and guilds over the flowering season, might also affect dye dispersal (Aizen 2001, Hayter & Cresswell 2006). These aspects certainly merit further investigation, e.g. by adding very small and very large populations to the study, by characterizing pollinator guilds, and by examining the resource quality of the other co-flowering species for insects compared to *M. trifoliata* in relation to pollinator constancy.

Implications for conservation

Maintaining or restoring gene flow through pollen dispersal often appears as a key factor for the long-term persistence of populations in fragmented habitats (e.g. Kwak et al. 1998, Oostermeijer et al. 2003, Menz et al. 2011). In both regions, fen and bog populations appear isolated from each other, suggesting that restoring connectivity through extending habitat surface (e.g. fen and bog restoration after cutting of spruce plantation), biological corridors or stepping-stone populations might be needed (Van Geert et al. 2010, Van Rossum & Triest 2012). Pollen dispersal may also be shaped by the interaction between population traits (e.g. size, density) and species life-history traits such as the mating system and vegetative growth ability (e.g. Charpentier 2001, Ashman et al. 2004, Ghazoul 2005, Van Rossum & Triest 2010). Our findings highlight the potential role of the spatial distance, population size and co-flowering floral resources on pollen dispersal patterns. These factors may affect population attractiveness to pollinators but also pollinator behaviour (Kwak et al. 1998, Cresswell & Osborne 2004, Ghazoul 2006). Legitimate pollen dispersal may occur despite the patchy structure of the morphs, provided that distances between morphs remain within pollinator foraging ranges. However, other stud-
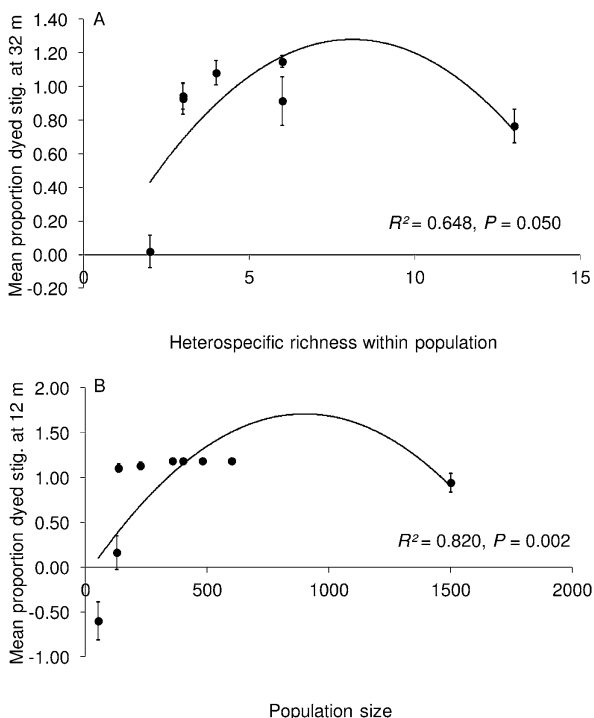
ies reported that illegitimate pollination due to the spatial clumping of the morphs of *M. trifoliata* might also result in compatible pollen wastage and clogging of the stigmas with illegitimate pollen (Nic Lughadha & Parnell 1989, Thompson et al. 1998). Whether it may reduce seed set as found in other insect-pollinated heterostylous species (Nishihiro & Washitani 1998, Wang et al. 2005, Ishihama et al. 2006, Scobie & Wilcock 2009), and affect the long-term viability of the populations, still needs to be evaluated.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of: (1) photograph of one flowering ramet (inflorescence) in bud stage and of flowers of *Menyanthes trifoliata*; and (2) ANCOVA of the relationships between mean proportion of dyed stigmas and distance to dye source, the number of flowers per ramet and morph type of the recipient ramets.

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