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RESEARCH PAPER

Relative impact of mate *versus* pollinator availability on pollen limitation and outcrossing rates in a mass-flowering species

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ABSTRACT

Plant mating systems are driven by several pre-pollination factors, including pollinator availability, mate availability and reproductive traits. We investigated the relative contributions of these factors to pollination and to realized outcrossing rates in the patchily distributed mass-flowering shrub *Rhododendron ferrugineum*. We jointly monitored pollen limitation (comparing seed set from intact and pollen-supplemented flowers), reproductive traits (herkogamy, flower size and autofertility) and mating patterns (progeny array analysis) in 28 natural patches varying in the level of pollinator availability (flower visitation rates) and of mate availability (patch floral display estimated as the total number of inflorescences per patch). Our results showed that patch floral display was the strongest determinant of pollination and of the realized outcrossing rates in this mass-flowering species. We found an increase in pollen limitation and in outcrossing rates with increasing patch floral display. Reproductive traits were not significantly related to patch floral display, while autofertility was negatively correlated to outcrossing rates. These findings suggest that mate limitation, arising from high flower visitation rates in small plant patches, resulted in low pollen limitation and high selfing rates, while pollinator limitation, arising from low flower visitation rates in large plant patches, resulted in higher pollen limitation and outcrossing rates. Pollinator-mediated selfing and geitonogamy likely alleviates pollen limitation in the case of reduced mate availability, while reduced pollinator availability (intraspecific competition for pollinator services) may result in the maintenance of high outcrossing rates despite reduced seed production.

INTRODUCTION

Evidence of a global reduction in pollinator populations and fragmentation of plant populations is increasing worldwide (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Winfree *et al.* 2011). These global changes create an urgent need to understand the relative contributions of pollinator and mate availabilities in affecting pollination and plant reproductive success. Many angiosperms, including self-compatible species, naturally exhibit pollen limitation (Knight *et al.* 2005); however variable pollination environments may exacerbate pollen limitation and impact contemporary mating patterns.

Pollination failure occurs when too few compatible, too many conspecific incompatible or heterospecific pollen grains are deposited on stigmas, and has two main potential causes: pollinator limitation and mate limitation (Ashman *et al.* 2004; Knight *et al.* 2005; Aizen & Harder 2007). Pollinator and mate limitations may be correlated because small and sparse plant populations have a reduced attractiveness for pollinators (Jennersten 1988; Kunin 1997; Dauber *et al.* 2010). Under such conditions, pollen limitation is expected to be negatively related to both flower visitation rate and population size (Rathcke 1983), likely creating an Allee effect in small populations (*i.e.,* a positive relationship between density or population size and any components of fitness; Groom 1998). However, particularly in disturbed environments, pollinator and mate limitations may occur independently and have different ecological and evolutionary consequences on plant mating systems, individual fitness and population demography and survival (Eckert *et al.* 2010; Harder & Aizen 2010). Disentangling the effects of plant *versus* pollinator limitation at local scales is therefore a key step toward an accurate understanding of plant–pollinator interactions, pollination and mating systems in a changing world (Cheptou 2012).

Pollinator limitation can occur independently of mate limitation under several ecological contexts. First, a pollinator community can be altered (reduction in pollinator abundance or diversity) in plant population of any size, density and isolation (Potts *et al.* 2010). Second, the lack of pollinators can affect pollination more strongly than the lack of mates in small populations with low attractiveness (Rathcke 1983). Finally, in large dense populations (Zimmerman 1980; Campbell & Husband 2007; Ward *et al.* 2012) or for mass-flowering species (Harder & Barrett 1996; Snow *et al.* 1996; Johnson *et al.* 2012), pollinator limitation may result from high intraspecific competition for pollinator services. Insect visits per flower are therefore expected to decrease with increasing population size (Rathcke 1983; Schmitt 1983; Schmid-Hempel & Speiser 1988), and pollen limitation would be consequently higher in large populations (Spigler & Chang 2009; Ward *et al.* 2012).

Mate limitation can occur independently of pollinator scarcity in the case, for example, of rare species sharing pollinators with co-flowering and more abundant species, or in the case of a highly attractive species disproportionately visited relative to its abundance in the community (Delmas *et al.* 2014). Under limited mate availability, pollen quality (incompatible pollen), pollen quantity and heterospecific pollen deposition are likely to limit seed production (Brys *et al.* 2004; Duncan *et al.* 2004; Aizen & Harder 2007; Campbell & Husband 2007). By increasing conspecific pollen and decreasing heterospecific pollen in pollinator's and stigmatic pollen loads, pollination is expected to be positively related to plant density, creating the conditions for the Allee effect to occur under limited mate availability (Groom 1998; Cheptou & Avendano 2006).

Autonomous self-pollination in hermaphroditic species ensures seed production in unpredictable pollination environments (Baker 1955; Eckert *et al.* 2006) and may alleviate pollen limitation (Larson & Barrett 2000). Under mate limitation, three selfing modes can theoretically provide reproductive assurance if they contribute to ensuring seed production (Goodwillie *et al.* 2005): (i) autonomous selfing, (ii) pollinator-mediated selfing occurring among flowers within an individual plant – geitonogamy (Harder & Barrett 1996; Eckert 2000; Karron *et al.* 2004), and (iii) pollinator-mediated selfing occurring within a flower – facilitated selfing (Goodwillie *et al.* 2005; Barrett & Case 2006; Vaughton & Ramsey 2010). When pollinator visits are abundant, facilitated selfing may play a key role in reducing mate limitation, particularly in buzz-pollinated species (Buchmann 1983; Larson & Barrett 1999). Although facilitated selfing and geitonogamy require the same flower attributes, such as outcrossing to maximise pollinator attraction, high selfing rates may not always be associated with an increase in the capacity for autonomous selfing (*e.g.*, reduced herkogamy, small flowers). Otherwise, predominant outcrossing can be maintained under pollinator limitation in the case of intraspecific competition for pollinator services, since this context may select for increased attractive structures and floral life span enhancing outcrossing rates (Eckert *et al.* 2010).

In this study, we aimed to infer the relative contribution of pollinator and mate availabilities to pollination and realized outcrossing rates in the light of reproductive trait variability. The species studied, *Rhododendron ferrugineum* (Ericaceae), is characterised by a broad range of patch floral display and abundance and diversity of flower-visiting insects. Insect visitation rates have been shown to decrease with increasing *R. ferrugineum* patch floral display (Delmas *et al.* 2014). High visitation rates in small patches and low visitation rates in large patches resulted in similar levels of pollinator-mediated pollen transfer, which limited the reproductive success by 34% on average (Delmas *et al.* 2014). This measure of pollen transfer limitation (comparing seed set from emasculated and pollen-supplemented flowers) took both outcross and geitonogamous pollen deposition into account. However, the relative contribution of autonomous and facilitated selfing to seed production is still unknown. Moreover, pollination estimates need to be monitored along with progeny array analysis since the proportion of selfed *versus* outcrossed progeny is dependent on the pollination context. Specifically, we first examined the effects of pollinator and mate availabilities (*i.e.*, flower visitation rate and patch floral display) on seed production estimated as an index of pollen limitation. Second, because reproductive traits influence the quantity and quality of pollen deposited independently of pollinator and plant availabilities, we tested whether flower size, herkogamy and autofertility varied according to patch floral display. Finally, we quantified the impact of insect visits, patch floral display and reproductive traits on the realized outcrossing rates of *R. ferrugineum*. We used this approach combining pollination and outcrossing rate estimation in 28 natural *R. ferrugineum* patches varying in patch floral display sizes and in flower visitation rates. This allowed us to address the following questions: (i) is seed production pollen-limited along the patch floral display gradient; if so, (ii) is seed production limited by pollinator availability, mate availability or both; and (iii) what is the relative contribution of pollinator availability, mate availability and floral traits to the realized mating system?

MATERIAL AND METHODS

Study species

Rhododendron ferrugineum L. (Ericaceae) dominates and structures heathland communities that are widely and patchily distributed between 1400 and 2300 m a.s.l., mainly in the Alps, the Pyrenees, in Jura and of the Northern Apennines (Ozenda 1985). It is an evergreen shrub with well-branched trailing stems 0.7–0.8 m in height. This species is protandrous, self-compatible and reproduces both sexually and vegetatively through layering. It has a massive floral display, reaching more than 300 inflorescences per m^2 with 10.8 ± 2.1 tubular flowers per inflorescence (mean ± SD assessed on 120 randomly chosen inflorescences from distinct individuals in the study site). Flowers are bright red nectariferous with poricidal anther dehiscence (Escaravage *et al.* 1997) and present stamen dimorphism, with an inner whorl of five long stamens and an outer whorl of five short stamens (Escaravage *et al.* 2001). Mean (±SD) corolla length and width were, on average, 10.43 ± 0.84 mm and 5.60 ± 0.75 mm, respectively (n = 107 individuals). Mean herkogamy was 0.33 ± 0.22 mm (n = 107 individuals; see below for measurement details). The flowers, each lasting about 10 days, are initiated the year before they open (Escaravage *et al.* 1997). The flowering period of a patch lasts for 20–30 days (June–July). Bumblebees (*Bombus* spp.) and honeybees (*Apis mellifera*) are the most efficient pollinators of *R. ferrugineum* (Escaravage & Wagner 2004). A list of foraging insects visiting *R. ferrugineum* in the study site is presented in Delmas *et al.* (2014).

Study site and patch characteristics

The study was conducted on a 3-km^2 area in the French Central Pyrenees, near the village of Camurac (42°46′31″N, 01°55′45″E). Altitudes ranged from 1550 m to 1750 m a.s.l., where snow cover usually lasts from late October until May. We used the 28 *R. ferrugineum* heathland patches found in this location (all patches were included in the study) because they are characterised by a broad gradient of *R. ferrugineum* floral display. We considered a heathland patch as a visually distinct aggregation of *R. ferrugineum* shrubs separated from another patch by alpine meadow (the distance between one patch and its three nearest patches was, on average, 136 m ranging from 60 m to 351 m, and the maximum distance between two patches across the site was 2 km). In the study site, about 70 co-flowering entomophilous species are associated with *R. ferrugineum* within heathland patches (dominant species were: *Cardamine pratensis*, *Conopodium majus*, *Lotus corniculatus*, *Polygala calcarea*, *Potentilla erecta* and *Ranunculus acris*). Patch structure (*i.e.,* size, *R. ferrugineum* cover and isolation) results from historical burning and livestock grazing practices, decreasing shrub coverage within patches and increasing the distance between patches in the meadow.

The number of *R. ferrugineum* individuals and potential mates ranged from three to visually uncountable individuals in the largest patches. Using an extent genotype dataset of 500 mature individuals (C.E.L. Delmas, unpublished data), we screened for clones, and only found 2.2% of genetically identical individuals (three patches had two genetically identical individuals and one patch had two clonal colonies composed of three and two genetically identical individuals, respectively). We therefore considered each sampled individual as a genet. In this study, we approximated mate availability as the total *R. ferrugineum* floral display per patch. This approximation could turn out to be not totally exact over a restricted part of the floral display gradient, since some large individuals can produce a larger floral display than several smaller individuals. However, the contrasting floral displays considered in the study ensure that the floral display gradient also encompasses a contrasting mate availability gradient. We studied a large number of patches (the experimental unit) in order to sample a broad range of pollination contexts.

To describe *R. ferrugineum* floral display in each patch, we integrated inflorescence density, the cover of the species and the patch area. More specifically, *R. ferrugineum* patch floral display was estimated as the product of (i) the mean density of inflorescences per m² assessed from a 0.25 × 0.25-m plot placed on 20 randomly chosen individuals per patch, and (ii) the total area (m²) covered by *R. ferrugineum* within each patch, estimated from the area occupied by the focal shrub in a 400-m² plot randomly placed at the patch core and the total area of each patch obtained from the geographic coordinates of patch perimeters recorded every 5 m. Within patches, the percentage of *R. ferrugineum* cover ranged from 0.18% to 98%, and total patch area ranged from 0.01 to 15.77 ha (1.73 ± 0.38 ha). Overall, the estimation of *R. ferrugineum* patch floral display ranged from 170 to more than 33×10^6 *R. ferrugineum* inflorescences (mean: 2,529,257).

Flower visitation rate

To test the contribution of insect visits to pollination, reproductive success and mating patterns in *R. ferrugineum*, we randomly selected four individuals per patch, if possible, resulting in a total of 105 individuals (visitation rates were monitored in 27 patches among the 28). We recorded the number of flowers visited by any foraging insects on the selected individuals per patch during two replicate 10-min observation periods (35 h of monitoring overall; in most cases we performed eight observation periods per patch). The observations were conducted between 4 June and 30 June 2009, during the peak of each individual flowering period, between 10:00 and 18:00 h, in sunny and clear conditions that favoured optimal pollinator activity. Days and hours of sampling were randomly applied across individuals for the first sampling round. For the second round, we performed the survey in the morning, if it had been done in the afternoon during the first round and *vice versa*, to incorporate potential temporal differences in pollinator activity. A visitation rate per flower per 10 min was obtained by dividing the number of flowers visited by the total number of open flowers on each individual. Total flower number for each individual was obtained by multiplying the total number of inflorescences by 10.8 ± 2.1 (mean ± SD of flower number per inflorescence assessed on 120 randomly chosen inflorescences across the site).

Overall, Apoidae (mainly *Bombus* spp.) performed 80% of the visits (16% of visits were performed by Diptera and 4% by Lepidoptera). Mean number of visits per *R. ferrugineum* individual was 56.4 ± 5.6, and the mean number of visits per flower (per 10 min) was 0.03 ± 0.012 (all visitors pooled). Mean flower visitation rate significantly increased with decreasing *R. ferrugineum* floral display (all visitors pooled, $P = 0.002$; see Table 2 in Delmas *et al.* 2014). Insect assemblage description, visitation rate per taxon and relationships with patch floral display are fully described in Delmas *et al.* (2014).

Pollen limitation and autofertility

In June 2009, three pollination treatments replicated twice on each target individual (n = 109) were performed to assess whether naturally pollinated flowers (receiving pollen from pollinators and from autonomous selfing) were limited by total pollen receipt and to explore the contribution of autonomous selfing to seed production. Following the methodology proposed in Eckert *et al.* (2010), the treatments were: (i) intact inflorescences left to natural pollination (F_{IN}), (ii) intact inflorescences hand-outcrossed (F_{IX}) and (iii) bagged inflorescences left unmanipulated (F_{IB}). The two inflorescences per treatment were randomly selected on each individual. Five flowers per inflorescence were manipulated and we gently removed the other flowers, as in Escaravage *et al.* (1997). This had no effect on the production of seeds of the remaining flowers (Escaravage 1997). Outcross-pollen supplementations were carried out every other day with pollen collected from a distant individual in the same patch (more than 5 m away). For the F_{IB} treatment, we bagged inflorescences at the bud stage to avoid any effect on pollen deposition. To count seeds, fruits were harvested in August 2009 just before dehiscence to ensure the full development of the seeds. Two fruits per manipulated and control inflorescence were randomly selected, dissected under a stereomicroscope and filled seeds counted. As counting the number of aborted seeds is not possible in *R. ferrugineum* because of their size (<1 mm and non-differentiable from small pieces of the dissected fruit), we quantified the mean number of ovules per ovary per individual by collecting two inflorescence buds per individual before blooming and preserved these in 70%

alcohol. Two flowers from each inflorescence bud were dissected under a stereomicroscope and the ovules counted (there are about 500 ovules per flower). We then calculated the mean seed set per treatment per target individual by dividing the mean number of mature seeds by the mean number of ovules for each of the three treatments.

Pollen limitation was assessed for each individual from the index used by Larson & Barrett (2000) as: $\mathrm{PL} = 1 - F_{IN}/F_{IX}$. This index estimates the proportional increase in seed production *via* supplemental outcross pollination. Negative values, which indicate higher seed production in intact than in pollen-supplemented flowers, were set to zero, as previously applied (Young & Young 1992; Larson & Barrett 2000). Strong negative values are likely caused by manipulation artefacts, such as damage to flowers during pollination, and very small negative values are likely caused by chance, when pollen limitation does not actually occur. The inclusion of negative values did not change the trends found in this study. The contribution of autonomous self-pollination to seed production for each individual was estimated from the index of autofertility (Eckert *et al.* 2010) as: $\mathrm{AF} = F_{IB}/F_{IX}$ which assesses the fraction of maximum seed production potentially achieved *via* autonomous self-pollination. As done for pollen limitation, values higher than one, which indicate higher seed production in bagged than in pollen-supplemented flowers, were set to one. They are likely caused by chance and indicate full ability to autonomously self. The inclusion of values above one did not change the trends found in this study.

Floral traits

To describe floral phenotypes and take into account their contributions to mating patterns, we randomly collected two open-pollinated flowers on each target individual ($n = 109$) at/or near the peak of the 2009 flowering period, and preserved them in 70% alcohol. We measured (i) the length and width of the tubular corolla (to 0.01 mm), (ii) the length of the style (from the base of the ovary to the top of the stigmatic surface), and (iii) the height of the ten anthers (from filament insertion at the base of the ovary to the pore level). To assess the physical separation of anthers from the stigma within an average flower, we subtracted the height of the stigma from the height of each anther (ten anther–stigma distances were calculated). When an anther was below the stigma, we obtained a negative value (approach herkogamy; Webb & Lloyd 1986). Since the autonomous deposition of self-pollen on the stigma can theoretically only be influenced by anthers above or in contact with the stigmatic surface, we did not consider negative values. Therefore, herkogamy – which can be assimilated as the smallest distance between the stigmatic surface and the closest pore of one of the anthers –was assessed through selecting the lowest anther–stigma separation among the positive values (reverse herkogamy).

Mating system estimation

In July 2009, we collected young leaves from the 109 target maternal individuals. Leaf material was conserved in silica gel until DNA extraction. In August 2009, we collected mature fruits from the target maternal individuals for progeny array analyses. We randomly chose about three unmanipulated inflorescences on each individual to obtain open-pollinated seeds (all seeds were pooled). Three maternal individuals were not sampled since their fruits had already cracked open at the time of seed collection. The size of *R. ferrugineum* seeds is 1–2 mm, and we germinated an unknown number of seeds from each maternal plant on moistened filter paper in Petri dishes (relative humidity 100%) in the glasshouse for 1 month (12 h:12 h as light:dark photoperiod, 20 °C and 15 °C for day and night temperatures, respectively). The greenhouse conditions allow minimising inbreeding depression at the germination stage (Cheptou *et al.* 2000) and thus reduced the potential bias in outcrossing rate estimates. Ideally, ten seedlings per family were randomly collected and frozen until DNA extraction (mean 9.6 seedlings per family). Germination rate was high (almost all seeds germinated) but was not monitored exactly as that was not the purpose of this study. Total sample size was 106 families including 1001 progeny for outcrossing rate estimation.

The DNA was extracted from leaves and seedlings with the DNeasy Plant Kit (Qiagen, Courtaboeuf, France) following the manufacturer's protocol. Genotypes of each DNA sample were scored using 12 polymorphic microsatellite loci. Nine of the 12 were developed through pyrosequencing technology (Delmas *et al.* 2011): RF6P2, RF14P3, RF38P1, RF41P1, RF46P2, RF47P1, RF56P1, RF74P1 and RF81P1; two were developed in *Rhododendron metternichii* (Naito *et al.* 1998): RM3D2 and RM2D2; and one in *Rhododendron simsii* (Dendauw *et al.* 2001): AZA-003. Cross-species amplification showed successful amplification and polymorphism for these last three loci on a subset of our DNA samples (data not shown). We amplified the 12 loci in three different PCR sets using fluorescence labelled forward primers. PCR amplifications of seedling and maternal plant samples were prepared using a Tecan EVO200 liquid handling station (Tecan France, Lyon, France) and performed in a 12 µl mix containing 2× Qiagen Multiplex PCR Master Mix, 2.5 pmol of each primer, 4 µl DNA for seedlings (around $1\ \mathrm{ng{\cdot}\mu l^{-1}}$) or 3 µl DNA for adults (around $50\ \mathrm{ng{\cdot}\mu l^{-1}}$), and RNase-free water. All technical details are described in Delmas *et al.* (2011). All these loci were unambiguously sized using an ABI 3730 sequencer (Applied Biosystems, Courtaboeuf, France) with the 1200 LIZ and 400HD ROX GeneScan size standard (for RF loci and RMD/AZA loci, respectively) and GENEMAPPER version 4.0 software. Genotype errors due to stutter bands, allele dropout and null alleles were estimated using the MICRO-CHECKER software (van Oosterhout *et al.* 2004).

Statistical analyses

To describe the production of seeds achieved through the different pollination modes, we compared the seed set of the three pollination treatments using a mixed model with the individual as a random factor to account for non-independence of the three treatments performed on the same individual. A Tukey test was performed on treatment least squares means with the approximation described in Kramer (1956) to adjust for multiple comparisons (Tukey–Kramer test performed with the Proc GLIMMIX).

To test whether flower visitation rate and patch floral display jointly affect pollen limitation, we used a generalised linear model (PROC GLM). To assess the potential contribution of reproductive trait variability to the pollination and realized outcrossing rates along the patch floral display gradient, we tested whether reproductive traits (corolla length and width, herkogamy and autofertility as dependent variables) were affected by patch floral display (independent variable) with a multivariate regression model (PROC REG) that accounts for non-independence of reproductive trait measurements. In the case of varying traits (plastic and/or genetic variation), we expected flower size and herkogamy to be smaller and autofertility to be higher in patches characterised by high pollen limitation and/or high selfing rate.

We used the multilocus mating system program (MLTR; Ritland 2002) to analyse genetic data (genotypes of progeny and maternal parents). We calculated maximum likelihood estimates of single-locus and multilocus outcrossing rates (t_s and t_m, respectively) as well as correlated mating parameters: r_s the correlation of selfing, and r_p the correlation of outcrossed paternity (Ritland 1989). The proportion of full-sibs among outcrossed sibs within progeny arrays is represented by r_p, and a low r_s indicates that the selfing rate does not vary among families within a patch. An estimation of the number of different paternal parents within the progeny can be assessed with $1/r_p$ (Sun & Ritland 1998). Biparental inbreeding (inbreeding as a result of crossing with related individuals; Ritland 1986) was assessed as the difference ($t_m - t_s$). A Newton-Raphson algorithm was used to fit the observed proportions of genotypes descended from a known maternal genotype to the proportions expected under the mixed-mating model (Ritland & Jain 1981; Ritland 1989). Standard errors for each estimate were calculated as the SD of 1000 replicate bootstrap estimates, with the progeny array as the unit of resampling. The program can separately estimate allelic frequencies in the pollen pool and in the ovule pool. As pollen and ovule allele frequencies did not differ significantly, we constrained the equality of frequencies to increase the statistical power of other estimates (Ritland 2002). The analyses were performed with the patch identity as a group factor and mating system estimates (t_s, t_m, r_s *and* r_p) were obtained for each patch (n = 28).

Finally, to test the joint effects of flower visitation rate, patch floral display and reproductive traits on outcrossing rate estimation (t_m, r_p and biparental inbreeding), we used multiple regression linear models (PROC REG) that allow the selection of the best fitting models with the Akaike information criterion (AIC). The model that minimises AIC provides the most reliable fit to the data (Sakomoto *et al.* 1986). We ranked models based on ΔAIC, estimated as the difference between each AIC value and the smallest value. Models with $\Delta AIC \leq 2$ were considered to have substantial support (Burnham & Anderson 2004).

Because the sample size for flower visitation rate was 105 individuals in 27 patches, the final sample size of multiple models that include this variable was 27. Independent variables (flower visitation rate, patch floral display and reproductive traits when appropriate) were simultaneously included and log-transformed so the could be simultaneously analysed in models. The normality of standardised residuals and equality of variance were checked in all models. In addition, we screened for multi-collinearity among the predictor variables of multiple regression models by calculating variance inflation factors (VIF option in PROC REG). Correlations among variables will inflate VIFs and cause a loss of precision in the models. The VIFs for the predictor variables in this study were <2, which indicates that multi-collinearity did not exist in the multiple regression models. Finally, we checked for spatial autocorrelation in the residuals of each model using Moran's I parametric test implemented in the PROC VARIOGRAM in SAS Software (version 9.3; SAS Institute, Cary, NC, USA). Spatial autocorrelation was not detected in any of the models (see Tables 1, 2, 3).

RESULTS

Effects of the pollination context on pollen limitation and reproductive traits

Due to weather, animals or humans causing deterioration of inflorescences, the final data set included seed set from 1073 fruits, allowing pollen limitation estimation for 93 individuals from 28 patches, and autofertility estimation for 89 individuals from 27 patches. Mean seed set differed significantly between treatments ($F_{2,186} = 95.51$, $P < 0.0001$; Fig. 1). Overall, the mean seed set from intact flowers (F_{IN}, mean ± SE = 0.68 ± 0.021) was not significantly different from the mean seed set from cross-pollen supplemented flowers ($F_{IX} = 0.70 \pm 0.024$, $t = -0.78$, $P = 0.717$; Fig. 1). No pollen limitation was detected overall (PL = 0.11 ± 0.016) even though pollen limitation increased significantly with increasing *R. ferrugineum* patch floral display (Table 1, Fig. 2a) and to a lesser extent with increasing flower visitation rate (Table 1). Autonomous self-pollination ($F_{IB} = 0.41 \pm 0.03$) resulted in the production of seeds but was significantly lower than seed set from cross-pollen supplemented flowers ($t = -11.35$, $P < 0.0001$; Fig. 1), and the index of autofertility reached 0.58 ± 0.03 on average. Autofertility (Fig. 2b), as well as corolla length, corolla width and herkogamy, were not significantly related to patch floral display (Table 2), suggesting that the capacity for autonomous self-pollination did not vary along the patch floral display gradient.

Outcrossing rate estimation

Overall, all loci were polymorphic and the number of alleles per locus varied from two to 18. Overall, *R. ferrugineum* showed a mixed mating system with a mean t_m reaching 0.51, ranging from 0.16 ± 0.07 to 0.79 ± 0.3 per patch (Fig. 2c). Complete selfing or complete outcrossing was not recorded. Average biparental inbreeding ($t_m - t_s$) was significantly higher than zero (0.12 ± 0.029; *t*-test, $P = 0.0002$) suggesting that outcross mating occurs between relatives within patches. The over-

Table 1. Generalised linear models of the effect of patch floral display and visitation rate on pollen limitation; n = 27 patches. A Moran's I parametric test was performed on the residuals from the regression model and no significant spatial autocorrelation was detected (Moran's I = 0.039, *P* = 0.261).

source of variation	df	estimate^a	SE	*P*-value
pollen limitation				
intercept	1,24	0.237	0.118	0.057
Rf patch floral display	1,24	0.039	0.011	**0.002**
flower visitation rate	1,24	0.186	0.082	**0.031**

Rf: *Rhododendron ferrugineum*. *P*-values <0.05 are in bold. ^{a}Values are log scale.

Table 2. Multivariate regression of the effects of patch floral display on reproductive traits. N = 27 patches (patches including data for the five variables considered). All reproductive traits were included simultaneously in the multivariate model. A Moran's I parametric test was performed on the residuals from the regression model and no significant spatial autocorrelation was detected (Moran's I = 0.037, *P* = 0.538).

source of variation	df	estimate[a]	SE	*P*-value
reproductive traits				
corolla length				
intercept	1,25	10.23	0.33	<0.0001
Rf patch floral display	1,25	0.04	0.07	0.562
corolla width				
intercept	1,25	5.14	0.34	<0.0001
Rf patch floral display	1,25	0.09	0.07	0.202
herkogamy				
intercept	1,25	0.44	0.08	<0.0001
Rf patch floral display	1,25	−0.19	0.02	0.236
autofertility				
intercept	1,25	0.62	0.10	<0.0001
Rf patch floral display	1,25	−0.008	0.02	0.701

multivariate statistics	df	value	F	*P*-value
Wilks' Lambda	4,22	0.84	1.03	0.412

Rf: *Rhododendron ferrugineum*. [a]Values are log scale.

all proportion of siblings with a common father (r_p) was 0.71 and one to six different paternal parents were found within progeny of individual plants (assessed with $1/r_p$). The correlation of selfing (r_s) was low on average (0.17), ranging from −0.08 ± 0.1 to 0.63 ± 0.4. Only five patches had a value of r_s significantly different from zero (*t*-test, $P < 0.001$). This indicates that the outcrossing rate varies significantly among families within these five patches only.

Effects of visitation rate, patch floral display and reproductive traits on outcrossing rate

Autofertility and patch floral display were the two most important predictors of t_m, as indicated from the best-fitting model with the lowest AIC (Table S1). The four other models selected ($\Delta AIC < 2$) all included autofertility, patch floral display and, respectively, corolla length, herkogamy, corolla width or flower visitation rate (Table S1). As expected, t_m increased significantly with *R. ferrugineum* patch floral display (Table 3, Fig. 2c) and decreased significantly with autofertility (Table 3). Flower visitation rate was the most important predictor of r_p (lowest AIC; Table S2). Thirteen other models were selected ($\Delta AIC < 2$), most of them included floral traits along with flower visitation rate (Table S2). We found that r_p decreased significantly with increasing flower visitation rate (Table 3). Biparental inbreeding was not significantly affected by patch floral display (Table 3), which was the only factor selected in the best-fitting model (Table S3).

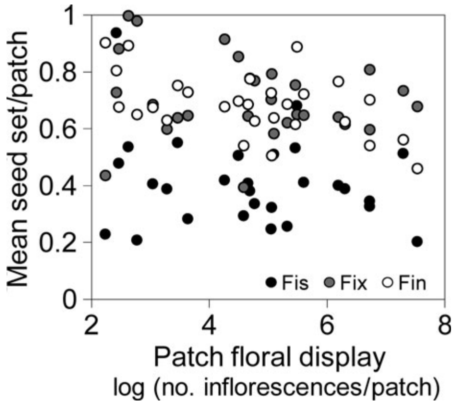

Fig. 1. Mean seed set per patch related to patch floral display (estimation of the total number of inflorescences per patch, log-transformed). F_{IN} = seed set from intact flowers; F_{IX} = seed set from pollen supplemented flowers; F_{IB} = seed set from intact bagged flowers.

DISCUSSION

We studied the relative contribution of insect visits, patch floral display and reproductive traits to the pollination and realized outcrossing rates in a patchily distributed mass-flowering species. We showed that patch floral display (as a proxy for mate availability) and the ability to autonomously self were the key factors driving the realized outcrossing rates of this species.

Table 3. Best predictors of mating patterns based on multiple regressions with the Akaike information criterion (AIC) to select models. Sample sizes are 27 patches for multilocus outcrossing rate (t_m), 27 patches for the correlation of paternity (r_p) and 28 patches for biparental inbreeding. A Moran's I parametric test was performed on the residuals from the full regression models and no significant spatial autocorrelation was detected.

					full model statistics			
source of variation	df	estimate[a]	SE	*P*-value	R^2	AIC	SSE	Moran's I
t_m								
intercept	1,24	0.64	0.17	0.001	0.36	−90.57	0.63	−0.04 $^{P = 0.58}$
Rf patch floral display	1,24	**0.05**	**0.21**	**0.037**				
autofertility	1,24	**−0.61**	**0.02**	**0.008**				
r_p								
intercept	1,25	−0.80	0.63	0.22	0.18	−46.49	3.73	−0.04 $^{P = 0.45}$
flower visitation rate	1,25	**−0.87**	**0.38**	**0.03**				
biparental inbreeding								
intercept	1,26	0.22	0.097	0.03	0.04	−93.51	0.61	−0.04 $^{P = 0.41}$
Rf patch floral display	1,26	−0.02	0.020	0.33				

Rf: *Rhododendron ferrugineum*. *P*-values <0.05 are in bold type. [a]Values are log scale.

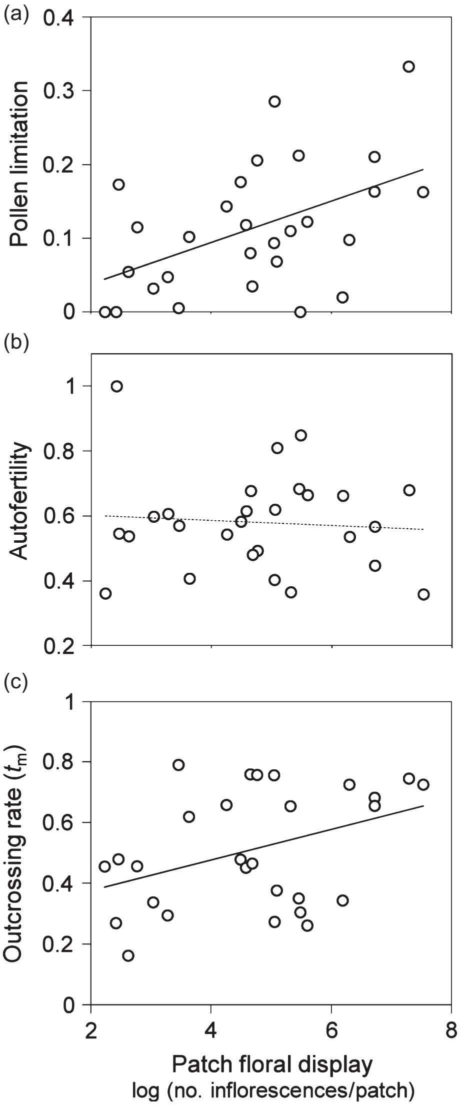

Fig. 2. Effects of *R. ferrugineum* patch floral display (estimation of the total number of inflorescences per patch, log-transformed) on (a) pollen limitation (intact/pollen-supplemented flowers; $y = 0.028x–0.0181$), (b) autofertility (bagged/pollen-supplemented flowers; $y = -0.0078x + 0.617$), and (c) multilocus outcrossing rate ($y = 0.0504x + 0.2749$). Full lines represent significant simple linear regressions. Mean pollen limitation and autofertility per patch were estimated as the average of individual estimates from the same patch. See Table 1, 2 and 3 for full statistics.

In particular, our results demonstrated an increase in pollen limitation and outcrossing rates (t_m) with increasing patch floral display. These findings suggest that the pollination and realized outcrossing rates in this species are driven by pollinator limitation (due to intraspecific competition for pollinator services) in large patches and to mate limitation (arising from the association of higher flower visitation rates and smaller patch floral display) in small patches.

Drivers of pollen limitation

In small populations, pollination is usually positively related to flower availability, providing the conditions necessary for the Allee effect to occur (Cheptou & Avendano 2006; Eckert *et al.* 2010). Our study showed that pollen limitation was mainly affected by patch floral display in this focal mass-flowering species. In contrast to our expectations that pollen limitation should decrease with increasing floral availability (Waites & Agren 2004; Ward & Johnson 2005), we found the highest rate of pollen limitation in large patches. Overall, these findings indicate that individuals from the smallest patches were less prone to pollination failure than individuals from the largest ones. Pollination was mainly driven by high intraspecific competition for pollinator services, since higher pollen limitation in the largest patches resulted from pollinator dilution among the multitude of flowers, as described in Delmas *et al.* (2014). Intraspecific competition for pollinator services has been shown in other systems, such as Mediterranean myrtle (González-Varo *et al.* 2009), tropical coffee agroforestry systems (Veddeler *et al.* 2006) and invasive milkweeds (Ward *et al.* 2012), but the relationship between insect visits and plant population attributes or plant reproductive success is expected to depend on the type of pollinators involved (Aizen & Feinsinger 1994; Garibaldi *et al.* 2013).

In a joint study (Delmas *et al.* 2014), we showed that pollinator-mediated pollen transfer, measured through flower emasculation, was similar along the patch floral display gradient, and reached 70% of optimal seed production on average. Since the capacity for autonomous selfing (autofertility, flower size, herkogamy) does not vary with patch floral display, the lower pollen limitation found in small patches resulted from facilitated selfing. In the Pyrenees, *R. ferrugineum* is mainly pollinated by *Bombus soroeensis* (C.E.L. Delmas, unpublished data), which exhibits 'buzz-pollination' behaviour. Buzz pollination likely enhances intrafloral self-pollen deposition for species with poricidal anthers (Buchmann 1983; Larson & Barrett 1999). Facilitated selfing is therefore a key mechanism in enhancing seed production in small patches of *R. ferrugineum.* The slightly positive relationship between pollen limitation and visits was surprising as flower visitation rate was higher in small sparse patches (Delmas *et al.* 2014) where pollen limitation was lower. The quantity of pollen deposited is expected to increase, with increasing visitation rates likely reducing pollen limitation (Fernández *et al.* 2012). However, at high visitation rates, insects may compete for rewards and visit surrounding species, thus carrying small amounts of conspecific pollen and high proportions of heterospecific pollen leading to higher pollen limitation (Knight 2003; Duncan *et al.* 2004; Jakobsson *et al.* 2009). In particular, this occurs when pollinator abundance decreases more slowly than floral availability in patchily distributed populations, as observed in *R. ferrugineum* (Delmas *et al.* 2014). However, here, this trend is more likely due to the variability in both pollen limitation and visitation rate and relies on a few patches in which we found low pollen limitation and low visitation rates.

Joint effects of the pollination context and reproductive traits on mating patterns

We found a mixed mating strategy in *R. ferrugineum* ($0.2 < t_m < 0.8$; *sensu* Goodwillie *et al.* 2005) and variable outcrossing rates among patches, even at a small spatial scale. Moreover, autofertility was highly variable and much lower than that occurring in *R. ferrugineum* populations in the Alps

(AF = 0.92; Escaravage *et al.* 1997). Accordingly, herkogamy was higher in the Pyrenees than in the Alps (0.3 cm *versus* 0.1 cm on average). This suggests that the ability of *R. ferrugineum* for autonomous selfing is variable both at local and species' range scales. In the study site, variable autofertility could result from variable dichogamy (the separation of the presentation of pollen and stigmas in time; Lloyd & Webb 1986).

In hermaphroditic self-compatible plants, t_m depends on factors affecting the relative amount of self *versus* outcross pollen received by stigmas and post-pollination processes (Holsinger 1991; Lloyd & Schoen 1992). The effects of insect visits, mate availability and variation in reproductive traits have been mainly considered separately (*e.g.,* Karron *et al.* 1995, 1997, 2004, 2006). However, all these factors may affect the quantity and quality of pollen deposited. Considering pre-pollination factors only, we showed that patch floral display (as a proxy of mate availability) and the ability to autonomously self (autofertility) were the key factors driving realized outcrossing in this mass-flowering species. We found that t_m increased with increasing *R. ferrugineum* patch floral display and was about 30% higher in large patches. This indicates that the higher mate availability in large patches favoured outcrossed pollen transfer. Additionally, t_m was negatively correlated to autofertility while flower visitation rates and floral traits were not found to be important drivers of the realized outcrossing rates. The pollination context has been proved to be a strong determinant of the realized mating system in some species (Routley *et al.* 1999; Franceschinelli & Bawa 2000), while relationships between floral traits and mating systems have been demonstrated in others (Elle & Hare 2002; Takebayashi *et al.* 2006; Herlihy & Eckert 2007). Both facilitated selfing and geitonogamy could contribute to a large proportion of selfed seeds (Porcher & Lande 2005; Vaughton *et al.* 2008; Vaughton & Ramsey 2010) and thus play a key role in maintaining a mixed mating system and alleviating pollen limitation in small sparse patches that were sufficiently visited by foraging insects. Facilitated selfing and geitonogamy were expected to be higher in small than in large patches because of higher flower visitation rates in the former. These two selfing modes imply the maintenance of traits favouring pollinator attraction in small patches. Therefore, the similar flower phenotypes (floral traits and self-fertility) recorded in large and small patches could result from similar selective pressures for pollinator attraction, from gene exchanges between patches (García-Ramos & Kirkpatrick 1997) or from a lack of selection on these traits.

In clonal long-lived species such as *R. ferrugineum* (Escaravage *et al.* 1998; Pornon *et al.* 2000), vegetative propagation may affect outcrossing rates and population persistence may be little dependent on seed recruitment (Eriksson 1989). However, repeated seedling recruitment has apparently occurred even in populations characterised by high clonality (Escaravage *et al.* 1998; Pornon *et al.* 2000). Here, large patches, in which high clonality and low seedling recruitment are expected, had the lowest selfing rates. In addition, most of the *R. ferrugineum* shrubs in the study site are genetically distinct individuals, as the number of clones is extremely low for this clonal species (five clone colonies of two to three ramets found among 500 individuals, Delmas C.E.L. unpublished data). Clonality is therefore not a driving factor of realized outcrossing rates.

Despite variable outcrossing rates, the correlation of paternity (r_p) indicated a small number of father-sired seeds both in small sparse and in large dense patches (mean 1.8 fathers per progeny array). Yet, values of r_p have been shown to decrease with increasing conspecific density (Smouse & Sork 2004; Mimura *et al.* 2009). In addition, we found that r_p slightly decreased with increasing visit number, suggesting that higher visitation rates may result in a higher diversity of pollen donors (Dudash & Ritland 1991). Whatever the pollination environment (insect visits or floral display), biparental inbreeding indicated that mating events occurred partly between relatives. Pollinator foraging behaviour and small seed dispersal have been shown to be responsible for mating among near-neighbour individuals, likely resulting in population substructure (Levin 1988; Heywood 1991). Indeed, pollen dispersal has been shown to be restricted to small distances in *R. ferrugineum* (Escaravage & Wagner 2004).

CONCLUSIONS

We showed increased pollen limitation of seed production and increased outcrossing rates in large plant patches. We hypothesise that in large patches, intraspecific competition for pollinator services and high mate availability lead, respectively, to higher pollen limitation and higher outcrossing rates. Throughout the continuum of patch floral display, *R. ferrugineum* benefit from traits involved in pollinator attraction (*e.g.,* mass-flowering habit, buzz pollination). Altogether, the higher visitation rate, facilitated selfing and geitonogamy could be efficient in alleviating pollen limitation arising from mate limitation in small populations but could induce high selfing rates. Finally, the strength of lifetime inbreeding depression needs to be assessed because the evolutionary outcomes of selfing in small patches depend on the fitness of inbred individuals, and because high lifetime inbreeding depression has been observed in long-lived species (Morgan *et al.* 1997).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Models of multilocus outcrossing rate (t_m) with patch floral display, visitation rate, autofertility, herkogamy, corolla width and length as predictors. Models are ordered from best to worst according to the Akaike information criterion and only models with ΔAIC < 2 are shown.

Table S2. Models of correlation of paternity (r_p) with patch floral display, visitation rate, autofertility, herkogamy, corolla width and length as predictors. Models are ordered from best to worst according to the Akaike information criterion and only models with ΔAIC < 2 are shown.

Table S3. Models of biparental inbreeding (BI) with patch floral display, visitation rate, autofertility, herkogamy, corolla width and length as predictors. Models are ordered from best to worst according to the Akaike information criterion and only models with ΔAIC < 2 are shown.

REFERENCES

Aizen M.A., Feinsinger P. (1994) Habitat fragmentation, native insect pollinators, and feral honey bees in Argentine “Chaco Serrado”. *Ecological Applications*, **4**, 378–392.

Aizen M.A., Harder L.D. (2007) Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. *Ecology*, **88**, 271–281.

Ashman T.L., Knight T.M., Steets J.A., Amarasekare P., Burd M., Campbell D.R., Dudash M.R., Mazer S.J., Johnston M., Mitchell R.J., Morgan M.T., Wilson W.G. (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology*, **85**, 2408–2421.

Baker H.G. (1955) Self-compatibility and establishment after ‘long distance’ dispersal. *Evolution*, **9**, 347–349.

Barrett S.C.H., Case A.L. (2006) The ecology and evolution of gender strategies in plants: the example of Australian *Wurmbea* (Colchicaceae). *Australian Journal of Botany*, **54**, 417–433.

Biesmeijer J.C., Roberts S.P.M., Reemer M., Ohlemuller R., Edwards M., Peeters T., Schaffers A.P., Potts S.G., Kleukers R., Thomas C.D., Settele J., Kunin W.E. (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, **313**, 351–354.

Brys R., Jacquemyn H., Endels P., van Rossum F., Hermy M., Triest L., De Bruyn L., Blust G.D.E. (2004) Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. *Journal of Ecology*, **92**, 5–14.

Buchmann S.L. (1983) Buzz pollination in angiosperms. In: Jones C.E., Little J.R. (Eds), *Handbook of experimental pollination biology*. Van Nostrand Reinhold, New York, USA, pp 73–113.

Burnham K.P., Anderson D.R. (2004) Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods & Research*, **33**, 261–304.

Campbell L.G., Husband B.C. (2007) Small populations are mate-poor but pollinator-rich in a rare, self-incompatible plant, *Hymenoxys herbacea* (Asteraceae). *New Phytologist*, **174**, 915–925.

Cheptou P.O. (2012) Clarifying Baker’s law. *Annals of Botany*, **109**, 633–641.

Cheptou P.O., Avendano L.G. (2006) Pollination processes and the Allee effect in highly fragmented populations: consequences for the mating system in urban environments. *New Phytologist*, **172**, 774–783.

Cheptou P.O., Imbert E., Lepart J., Escarre J. (2000) Effects of competition on lifetime estimates of inbreeding depression in the outcrossing plant *Crepis sancta* (Asteraceae). *Journal of Evolutionary Biology*, **13**, 522–531.

Dauber J., Biesmeijer J.C., Gabriel D., Kunin W.E., Lamborn E., Meyer B., Nielsen A., Potts S.G., Roberts S.P.M., Sõber V., Settele J., Steffan-Dewenter I., Stout J.C., Teder T., Tscheulin T., Vivarelli D., Petanidou T. (2010) Effects of patch size and density on flower visitation and seed set of wild plants: a pan-European approach. *Journal of Ecology*, **98**, 188–196.

Delmas C.E.L., Escaravage N., Pornon A. (2014) Massive floral display affects insect visits but not pollinator-mediated pollen transfer in *Rhododendron ferrugineum*. *Plant Biology*, **16**, 234–243.

Delmas C.E.L., Lhuiller E., Pornon A., Escaravage N. (2011) Isolation and characterization of microsatellite loci in *Rhododendron ferrugineum* (Ericaceae) using pyrosequencing technology. *American Journal of Botany*, **98**, e120–e122.

Dendauw J., De Riek J., Arens P., Van Bockstaele E., Vosman B., De Loose M. (2001) Development of sequenced tagged microsatellite site (STMS) markers in azaleae. *Acta Horticulturae*, **546**, 193–197.

Dudash M.R., Ritland K. (1991) Multiple paternity and self-fertilization in relation to floral age in *Mimulus guttatus* (Scrophulariaceae). *American Journal of Botany*, **78**, 1746–1753.

Duncan D.H., Nicotra A.B., Wood J.T., Cunningham S.A. (2004) Plant isolation reduces outcross pollen receipt in a partially self-compatible herb. *Journal of Ecology*, **92**, 977–985.

Eckert C.G. (2000) Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology*, **81**, 532–542.

Eckert C.G., Kalisz S., Geber M.A., Sargent R., Elle E., Cheptou P.O., Goodwillie C., Johnston M.O., Kelly J.K., Moeller D.A., Porcher E., Ree R.H., Vallejo-Marin M., Winn A.A. (2010) Plant mating systems in a changing world. *Trends in Ecology & Evolution*, **25**, 35–43.

Eckert C.G., Samis K.E., Dart S. (2006) Reproductive assurance and the evolution of uniparental reproduction in flowering plants. In: Harder L.D., Barrett S.C.H. (Eds), *Ecology and evolution of flowers*. Oxford University Press, Oxford, UK, pp 183–203.

Elle E., Hare J.D. (2002) Environmentally induced variation in floral traits affects the mating system in *Datura wrightii*. *Functional Ecology*, **16**, 79–88.

Eriksson O. (1989) Seedling dynamics and life histories in clonal plants. *Oikos*, **55**, 231–238.

Escaravage N. (1997) *Système de reproduction et stratégie de colonisation de Rhododendron ferrugineum L. (Ericaceae) (étage subalpin; Alpes du Nord)*. PhD Thesis, University of Grenoble, France.

Escaravage N., Flubacker E., Pornon A., Doche B., Till-Bottraud I. (2001) Stamen dimorphism in *Rhododendron ferrugineum* (Ericaceae): development and function. *American Journal of Botany*, **88**, 68–75.

Escaravage N., Pornon A., Doche B., Till-Bottraud I. (1997) Breeding system in an alpine species: *Rhododendron ferrugineum* L (Ericaceae) in the French northern Alps. *Canadian Journal of Botany*, **75**, 736–743.

Escaravage N., Questiau S., Pornon A., Doche B., Taberlet P. (1998) Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology*, **7**, 975–982.

Escaravage N., Wagner J. (2004) Pollination effectiveness and pollen dispersal in a *Rhododendron ferrugineum* (Ericaceae) population. *Plant Biology*, **6**, 606–615.

Fernández J.D., Bosch J., Nieto-Ariza B., Gómez J.M. (2012) Pollen limitation in a narrow endemic plant: geographical variation and driving factors. *Oecologia*, **170**, 421–431.

Franceschinelli E.V., Bawa K.S. (2000) The effect of ecological factors on the mating system of a South American shrub species (*Helicteres brevispira*). *Heredity*, **84**, 116–123.

García-Ramos G., Kirkpatrick M. (1997) Genetic models of adaptation and gene flow in peripheral populations. *Evolution*, **51**, 21–28.

Garibaldi L.A., Steffan-Dewenter I., Winfree R., Aizen M.A., Bommarco R., Cunningham S.A., Kremen C., Carvalheiro L.G., Harder L.D., Afik O., Bartomeus I., Benjamin F., Boreux V., Cariveau D., Chacoff N.P., Dudenhöffer J.H., Freitas B.M., Ghazoul J., Greenleaf S., Hipólito J., Holzschuh A., Howlett B., Isaacs R., Javorek S.K., Kennedy C.M., Krewenka K.M., Krishnan S., Mandelik Y., Mayfield M.M., Motzke I., Munyuli T., Nault B.A., Otieno M., Petersen J., Pisanty G., Potts S.G., Rader R., Ricketts T.H., Rundlöf M., Seymour C.L., Schüepp C., Szentgyörgyi H., Taki H., Tscharntke T., Vergara C.V., Viana B.F., Wanger T.C., Westphal C., Williams N., Klein A.M. (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*, **339**, 1608–1611.

González-Varo J.P., Arroyo J., Aparicio A. (2009) Effects of fragmentation on pollinator assemblage, pollen limitation and seed production of Mediterranean myrtle (*Myrtus communis*). *Biological Conservation*, **142**, 1058–1065.

Goodwillie C., Kalisz S., Eckert C.G. (2005) The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution and Systematics*, **36**, 47–79.

Groom M.J. (1998) Allee effects limit population viability of an annual plant. *The American Naturalist*, **151**, 487–496.

Harder L.D., Aizen M.A. (2010) Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**, 529–543.

Harder L.D., Barrett S.C.H. (1996) Pollen dispersal and mating patterns in animal-pollinated plants. In: Lloyd D.G., Barrett S.C.H. (Eds), *Floral biology: studies on floral evolution in animal pollinated plants*. Chapman & Hall, New York, USA, pp 140–190.

Herlihy C.R., Eckert C.G. (2007) Evolutionary analysis of a key floral trait in *Aquilegia canadensis* (Ranunculaceae): genetic variation in herkogamy and its effect on the mating system. *Evolution*, **61**, 1661–1674.

Heywood J.S. (1991) Spatial analysis of genetic variation in plant populations. *Annual Review of Ecology and Systematics*, **22**, 335–355.

Holsinger K.E. (1991) Mass-action models of plant mating systems: the evolutionary stability of mixed mating systems. *The American Naturalist*, **138**, 606–622.

Jakobsson A., Lazaro A., Totland O. (2009) Relationships between the floral neighborhood and individual pollen limitation in two self-incompatible herbs. *Oecologia*, **160**, 707–719.

Jennersten O. (1988) Pollination in *Dianthus deltoides* (Caryophyllaceae): effects of habitat fragmentation on visitation and seed set. *Conservation Biology*, **2**, 359–366.

Johnson S.D., Hollens H., Kuhlmann M. (2012) Competition *versus* facilitation: conspecific effects on pollinator visitation and seed set in the iris *Lapeirousia oreogena*. *Oikos*, **121**, 545–550.

Karron J.D., Jackson R.T., Thumser N.N., Schlicht S.L. (1997) Outcrossing rates of individual *Mimulus ringens* genets are correlated with anther–stigma separation. *Heredity*, **79**, 365–370.

Karron J.D., Mitchell R.J., Bell J.M. (2006) Multiple pollinator visits to *Mimulus ringens* (Phrymaceae) flowers increase mate number and seed set within fruits. *American Journal of Botany*, **93**, 1306–1312.

Karron J.D., Mitchell R.J., Holmquist K.G., Bell J.M., Funk B. (2004) The influence of floral display size on selfing rates in *Mimulus ringens*. *Heredity*, **92**, 242–248.

Karron J.D., Thumser N.N., Tucker R., Hessenauer A.J. (1995) The influence of population-density on outcrossing rates in *Mimulus ringens*. *Heredity*, **75**, 175–180.

Knight T.M. (2003) Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*. *Oecologia*, **137**, 557–563.

Knight T.M., Steets J.A., Vamosi J.C., Mazer S.J., Burd M., Campbell D.R., Dudash M.R., Johnston M.O., Mitchell R.J., Ashman T.L. (2005) Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology, Evolution and Systematics*, **36**, 467–497.

Kramer C.Y. (1956) Extension of multiple range tests to group means with unequal numbers of replications. *Biometrics*, **12**, 307–310.

Kunin W.E. (1997) Population size and density effects in pollination: pollinator foraging and plant reproductive success in experimental arrays of *Brassica kaber*. *Journal of Ecology*, **85**, 225–234.

Larson B.M.H., Barrett S.C.H. (1999) The ecology of pollen limitation in buzz-pollinated *Rhexia virginica* (Melastomataceae). *Journal of Ecology*, **87**, 371–381.

Larson B.M.H., Barrett S.C.H. (2000) A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society*, **69**, 503–520.

Levin D.A. (1988) Local differentiation and the breeding structure of plant populations. In: Gottlieb L.D., Jain S.K. (Eds), *Plant evolutionary biology*. Chapman & Hall, New York, USA, pp 305–329.

Lloyd D.G., Schoen D.J. (1992) Self- and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences*, **153**, 358–369.

Lloyd D.G., Webb C.J. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand Journal of Botany*, **24**, 135–162.

Mimura M., Barbour R.C., Potts B.M., Vaillancourt R.E., Watanabe K.N. (2009) Comparison of contemporary mating patterns in continuous and fragmented *Eucalyptus globulus* native forests. *Molecular Ecology*, **18**, 4180–4192.

Morgan M.T., Schoen D.J., Bataillon T.M. (1997) The evolution of self-fertilization in perennials. *The American Naturalist*, **150**, 618–638.

Naito K., Isagi Y., Nakagoshi N. (1998) Isolation and characterization of microsatellites of *Rhododendron metternichii* Sieb. et Zucc. var. *hondoense* Nakai. *Molecular Ecology*, **7**, 927–928.

Ozenda P. (1985) *La végétation de la chaine alpine dans l'espace montagnard européen*. Masson, Paris, France, pp 344.

Porcher E., Lande R. (2005) The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *Journal of Evolutionary Biology*, **18**, 497–508.

Pornon A., Escaravage N., Thomas P., Taberlet P. (2000) Dynamics of genotypic structure in clonal *Rhododendron ferrugineum* (Ericaceae) populations. *Molecular Ecology*, **9**, 1099–1111.

Potts S.G., Biesmeijer J.C., Kremen C., Neumann P., Schweiger O., Kunin W.E. (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, **25**, 345–353.

Rathcke B.J. (1983) Competition and facilitation among plants for pollination. In: Real L. (Ed), *Pollination biology*. Academic Press, New York USA, pp 305–329.

Ritland K. (1986) Joint maximum likelihood estimation of genetic and mating structure using open-pollinated progenies. *Biometrics*, **42**, 25–43.

Ritland K. (1989) Correlated matings in the partial selfer *Mimulus guttatus*. *Evolution*, **43**, 848–859.

Ritland K. (2002) Extensions of models for the estimation of mating systems using n independent loci. *Heredity*, **88**, 221–228.

Ritland K., Jain S. (1981) A model for the estimation of outcrossing rate and gene frequencies using n independent loci. *Heredity*, **47**, 35–52.

Routley M.B., Mavraganis K., Eckert C.G. (1999) Effect of population size on the mating system in a self-compatible, autogamous plant, *Aquilegia canadensis* (Ranunculaceae). *Heredity*, **82**, 518–528.

Sakomoto Y., Ishiguo M., Kitagawa G. (1986) *Akaike information criterion statistics*. K.T.K. Scientific, Tokyo, Japan.

Schmid-Hempel P., Speiser B. (1988) Effects of inflorescence size on pollination in *Epilobium angustifolium*. *Oikos*, **53**, 98–104.

Schmitt J. (1983) Density-dependent pollinator foraging, flowering phenology, and temporal pollen dispersal patterns in *Linanthus bicolor*. *Evolution*, **37**, 1247–1257.

Smouse R.E., Sork V.L. (2004) Measuring pollen flow in forest trees: an exposition of alternative approaches. *Forest Ecology and Management*, **197**, 21–38.

Snow A.A., Spira T.P., Simpson R., Klips R.A. (1996) The ecology of geitonogamous pollination. In: Lloyd D.G., Barrett S.C.H. (Eds), *Floral biology: studies on floral evolution in animal-pollinated plants*. Chapman & Hall New York, USA, pp 191–216.

Spigler R.B., Chang S.M. (2009) Pollen limitation and reproduction varies with population size in experimental populations of *Sabatia angularis* (Gentianaceae). *Botany*, **87**, 330–338.

Sun M., Ritland K. (1998) Mating system of yellow starthistle (*Centaurea solstitialis*), a successful colonizer in North America. *Heredity*, **80**, 225–232.

Takebayashi N., Wolf D.E., Delph L.F. (2006) Effect of variation in herkogamy on outcrossing within a population of *Gilia achilleifolia*. *Heredity*, **96**, 159–165.

van Oosterhout C., Hutchinson W.F., Wills D.P.M., Shipley P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Vaughton G., Ramsey M. (2010) Pollinator-mediated selfing erodes the flexibility of the best-of-both-worlds mating strategy in *Bulbine vagans*. *Functional Ecology*, **24**, 374–382.

Vaughton G., Ramsey M., Simpson I. (2008) Does selfing provide reproductive assurance in the perennial herb *Bulbine vagans* (Asphodelaceae)? *Oikos*, **117**, 390–398.

Veddeler D., Klein A.-M., Tscharntke T. (2006) Contrasting responses of bee communities to coffee flowering at different spatial scales. *Oikos*, **112**, 594–601.

Waites A.R., Agren J. (2004) Pollinator visitation, stigmatic pollen loads and among-population variation in seed set in *Lythrum salicaria*. *Journal of Ecology*, **92**, 512–526.

Ward M., Johnson S., Zalucki M. (2012) When bigger is not better: intraspecific competition for pollination increases with population size in invasive milkweeds. *Oecologia*, **171**, 1–9.

Ward M., Johnson S.D. (2005) Pollen limitation and demographic structure in small fragmented populations of *Brunsvigia radulosa* (Amaryllidaceae). *Oikos*, **108**, 253–262.

Webb C.J., Lloyd D.G. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany*, **24**, 163–178.

Winfree R., Bartomeus I., Cariveau D.P. (2011) Native pollinators in anthropogenic habitats. *Annual Review of Ecology, Evolution, and Systematics*, **42**, 1.

Young H.J., Young T.P. (1992) Alternative outcomes of natural and experimental high pollen loads. *Ecology*, **73**, 639–647.

Zimmerman M. (1980) Reproduction in *Polemonium*: competition for pollinators. *Ecology*, **61**, 497–501.