

## An experimental study of the S-Allee effect in the self-incompatible plant *Biscutella neustriaca*

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**Abstract** Homomorphic self-incompatibility (SI) evolved in many plant families to enforce selfing avoidance, and is controlled by a single multiallelic locus (the S-locus). In a fragmented landscape, strong variation in population size and in local density is expected to cause strong variation in allelic diversity at the S-locus, which could generate an Allee effect on female reproductive success by constraining compatible pollen availability. In this experimental study, we aimed at detecting this SI-specific Allee effect (or S-Allee effect) in the endangered species *Biscutella neustriaca*. We demonstrated the occurrence of a SI mating system in the species and determined compatibility relationships among genotypes through a large set of controlled pollinations. For the experiment, we chose three different pollen receptor genotypes, each compatible with respectively 100, 75 and 25% of four other genotypes, which constituted the pollen sources. We placed different ramets of each receptor at different distances from the pollen sources to control for pollen limitation due to low local density, and we measured the seed set on each receptor plant three times consecutively. Analyses performed with generalized linear mixed models showed that both the distance to the pollen

sources and the mate availability due to SI had a significant effect on seed set, with a strong reduction observed when mate availability was limited to 25%. Our results suggest that pollen limitation due to a restriction in compatible mate availability could occur in small or scattered populations exhibiting low allelic diversity at the S-locus.

**Keywords** Allee effect · Pollen limitation · Mate availability · Self-incompatibility · Generalized linear mixed model · Brassicaceae

### Introduction

In many hermaphrodite plant species, self-fertilization is avoided through genetic systems known as self-incompatibility (SI) systems. These SI systems are of particular interest in the context of conservation genetics because of their complex influence on population viability (Castric and Vekemans 2004; Busch and Schoen 2008). On the one hand, SI systems may contribute to minimizing the deleterious effects of inbreeding in small populations through selfing avoidance, and through a restriction in mating with close relatives. The importance of these effects should be tempered, however, as theoretical studies suggest that the sheltering of deleterious alleles linked to the locus controlling SI may strengthen inbreeding depression in small populations (Glémin et al. 2001), and that the restriction in biparental inbreeding due to SI may be only minor (Cartwright 2009). On the other hand, SI systems may limit the success of outcrossing pollination in small populations by reducing the availability of adequate (i.e. compatible) mates (Byers and Meagher 1992; Vekemans et al. 1998). Hence, SI systems may cause a “mate-finding Allee effect” (Gascoigne et al. 2009), a process generating a positive relationship

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between reproductive success and population size because of an increase in access to mates in larger populations.

Two types of SI systems are known to occur: heteromorphic SI, where incompatibility groups are associated with differences in flower morphology; and homomorphic SI, where such morphological differences are lacking. In species with heteromorphic self-incompatibility with either two (distyly) or three (tristyly) cross-compatible morphs, strong biases in morph frequencies in small populations resulting from genetic drift were often reported (Barrett and Husband 1997; Eckert et al. 1996). These biases would cause a reduction in the availability of compatible pollen in some morphs and indeed several studies showed that these resulted in a decrease in maternal reproductive success (Ågren 1996; Brys et al. 2007; Brys et al. 2008; Waites and Ågren 2004). Unlike heteromorphic SI, the widespread homomorphic SI systems (Igic et al. 2008) are characterized generally by more than three different mating types, typically of the order of 10–30 within natural populations (Lawrence 2000; Castric and Vekemans 2004). Mate specificities are defined by multiple alleles at two coadapted linked genes constituting the self-incompatibility locus (S-locus), and coding for proteins involved in molecular recognition between pistil and pollen, respectively (Takayama and Isogai 2005). In these species, fertilization is allowed only when pollen grain factors are not recognised by S-locus proteins expressed in the pistil, i.e. between individuals sharing and expressing no common specificity at the S-locus. The high levels of allelic diversity at the S-locus commonly observed within populations were found to be due to the action of strong frequency-dependent selection on pollination success (Wright 1939). However, genetic drift in small isolated populations would cause a substantial decrease in the number of co-segregating allelic types (Busch and Schoen 2008; Schierup et al. 1997; Yokoyama and Hetherington 1982), and theory predicts that this may lead to a critical decrease in the availability of compatible mates (Byers and Meagher 1992; Vekemans et al. 1998). More recently, it was realized that this phenomenon may generate a positive relationship between population size and maternal fitness, corresponding thus to a mate-finding Allee effect (Busch and Schoen 2008; Levin et al. 2009), coined the “S-Allee effect” by Wagenius et al. (2007).

Several empirical studies detected a significant positive relationship between mate availability, defined as the proportion of cross-pollinations within population that are compatible, and population size in species with homomorphic SI (e.g. Reinartz and Les 1994; Campbell and Husband 2007; Fischer et al. 2003; Glémin et al. 2008; Pickup and Young 2008; Wagenius et al. 2007; Willi et al. 2005). This was generally assumed to be due to a reduction in S-locus allelic diversity in small populations, although only in one case the actual number of S-alleles was estimated

(Glémin et al. 2008). An extreme case of absence of compatible mates in a critically endangered clonal species was reported by DeMauro (1993), who suggested that genetic drift and clonal reproduction lead to fixation of a single mating type at the S-locus. In some empirical studies, the mean maternal reproductive success, estimated as a mean seed set, was also found to be significantly positively related to population size, in agreement with, but not demonstrating the S-Allee effect hypothesis (Fischer et al. 2003; Glémin et al. 2008; Willi et al. 2005). In a field experiment, Elam et al. (2007) tested the effect of population size and mean genetic relatedness within population on maternal fitness measured as mean fruit- or seed set. They found that maternal fitness was significantly related to population size for each of three categories of mean genetic relatedness, thus demonstrating an Allee effect. However, they could not distinguish whether this effect was due to shared S-alleles between individuals (the S-Allee effect) or to biparental inbreeding. We suggest that the two main limitations of previous experimental and empirical studies aiming at detecting an S-Allee effect are: (1) a lack of direct information on the number and distribution of S-locus genotypes within population (with the exception of Glémin et al. 2008); and (2) a lack of quantitative assessment of other processes that would generate a mate-finding Allee effect.

An important phenomenon that contributes to mate-finding Allee effect is the efficiency of pollination service (Ashman et al. 2004). Indeed, the amount of pollen transferred among individuals often depends on population size and density because in animal-pollinated plants small patches or populations are less attractive to pollinators and thus receive fewer pollinator visits than large ones, and because at low density plants may receive relatively more heterospecific pollen grains than at high density (Antonovics and Levin 1980; Ashman et al. 2004). Surveys of the literature on the efficiency of pollination service concluded that most plant species exhibit pollen-limited fecundity (Knight et al. 2005; Larson and Barrett 2000). Hence, studies aiming at testing the S-Allee effect should disentangle the factors associated to overall pollen transfer, related to the efficiency of pollination, and those associated to the proportion of compatible pollen in the pollen load (Campbell and Husband 2007).

In this experimental study, we aimed at testing the effect of a reduction in mate availability on seed production due to a SI mating system. Plants used in the experiment belonged to the endangered species *Biscutella neustriaca* (Brassicaceae), which was considered as self-incompatible (F. Hendoux, personal observations). Using a large set of controlled pollinations, we first demonstrated the occurrence of a SI system in this species. Then we performed an experiment allowing us to distinguish between the effects of overall pollination efficiency (by controlling spatial distance among mates) and of compatible mate availability on maternal reproductive

success. Our specific aims were (1) to determine if maternal reproductive success depends on spatial distance from pollen donors; (2) to assess the respective effects of overall pollination efficiency and individual genetic effects (including S-locus genotype effects) on maternal reproductive success by comparing different individuals under identical spatial arrangements; and (3) to test the effect of SI in pollen limitation by comparing individuals with varying proportion of compatible mates. We also used progeny analyses with microsatellite markers to confirm the compatibility categories inferred from cross-pollinations.

## Methods

### The species

*Biscutella neustriaca* Bonnet is a perennial, herbaceous, xerophilic plant species belonging to Brassicaceae. It is an outcrossing species, presumably self-incompatible (F. Hendoux, personal observations), that is pollinated by generalist insect pollinators (*Episyrphus*, *Bombus*, Lepidoptera and Coleoptera, B. Valentin and C. Blondel, personal communications; J.-B. Leducq, personal observations). Limited vegetative propagation by rhizomes does occur. Flowers are carried by floral stems with indefinite terminal growth, and contain two ovules (Fig. S1). When both ovules are fertilized, a full silicule develops with two flat ovoid lodges, each containing one seed. Some fruits contain only one lodge (and thus carry only one seed). *B. neustriaca* is a narrowly-distributed declining species restricted to the Seine Valley in the vicinity of Rouen (Eure, France). Populations are located on calcareous cliffs and slopes surrounding the river bank. The species is protected through national and international treaties (Directive 92/43/EEC -Conservation of natural habitats and of wild fauna and flora—Annex II; Bern convention on the conservation of European wildlife and natural habitats—Appendix I). It suffers from vegetation closing due to abandonment of sheep grazing practices, and from extension of housing areas, and is the subject of conservation management under the European Life Programme (LIFE06 NAT/F/000137- Rescue of *Viola hispidula* and *Biscutella neustriaca* on the Seine Valley). In 2008, a maximum of about 3000 individuals were estimated to occur in seven disconnected populations.

### Sampling and controlled cross-pollinations

The plant material used in the experimental setup was sampled from a collection of plants maintained in the Conservatoire Botanique National de Bailleul (CBNBL, Bailleul, North of France) that were originally obtained from seeds collected in two natural populations. These

populations were located respectively in Romilly-sur-Andelle (Bn1c: 49°19'03" N, 1°14'33" E) and Amfreville-sous-les-monts (Bn4a: 49°17'48" N, 1°15'55" E).

In order to check the occurrence of a SI system in this species and to determine the S-locus phenotype of individuals to use for the experiment, controlled cross-pollinations were realized in 2007 among 21 individuals in a full diallel setup. Before pollination, a freshly-opened flower was chosen on the top of the flowering stem of the maternal plant. The pedicel of the flower was labelled with the name of the pollen donor and the date of the cross. An entire 2–3 days-old flower with opened stamens was taken from the pollen donor with forceps previously cleaned with 70% ethanol, and the stamens were rubbed on the stigma of the maternal plant. Each pair of individuals was hand-pollinated at least five times in each direction. Also, ten negative controls (unpollinated flowers) and five enforced self-pollinations were performed on each plant to check for the strength of self-incompatibility. Pollinations were performed in a greenhouse at 20–22°C and plants were stored in an insect-proof cage in order to prevent pollen contamination.

Cross-compatibility between two individuals was assessed by measuring fruit-width 7 days after pollination, using a digital caliper. Fruits with a width higher than the cut-off value 2.91 mm were considered as resulting from a “successful” pollination. This method is fast and reliable and avoids problems related to loss of labels during fruit maturation, when handling large numbers of pollinations simultaneously. The cut-off value was determined from a preliminary survey in which we compared the dynamics of pistil/fruit growth across 15 days after 39 enforced self-pollinations and 130 cross-pollinations (Fig. S2). Selfed pistils did not show any growth and were characterized by a mean width of  $1.21 \text{ mm} \pm 0.22$  (S.D.) mm. Cross-pollinated pistils showed a marked bimodal distribution at each recorded date (3, 5, 7, 9, 11, 13 and 15 days) with one mode centred around  $1.49 \pm 0.84$  mm, and the other mode increasing and reaching a plateau at  $8.74 \text{ mm} \pm 1.73$  mm after 7 days. The former category of cross-pollinations was considered as “unsuccessful” whereas the latter was considered as “successful”. By fitting a normal distribution to the “unsuccessful” cross-pollination results, we computed that for a P-value of 0.001, the maximum fruit width at day 7 that could be considered as unsuccessful was 2.91 mm, our cut-off value. In interpreting results from the diallel setup, we considered a pair of individuals as “compatible” in a given direction when “successful” pollination occurred in at least 30% of the replicates.

### Experimental design

The experiment was conducted in spring 2008 at the CBNBL, which is located more than 250 km away from

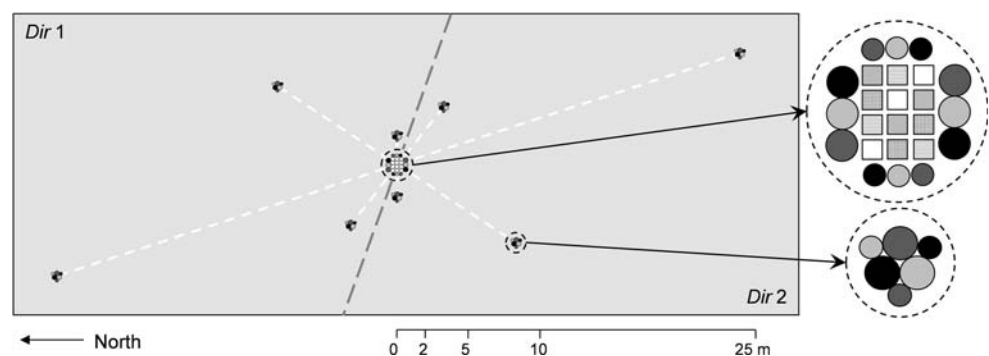
natural populations of *B. neustriaca*. The experimental garden, a flat hayfield, measured 30 × 60 m. In order to test the effect of compatible pollen availability on maternal reproductive success, the experimental design contained two groups of plants: the pollen donor plants (D) and the maternal plants or pollen receptors (R). Based on the results from controlled pollinations, we chose four individuals for the D group and three individuals in the R group in such a way to get three categories of compatibility between D and R, and strict incompatibility among individuals of the R group: category (individual genotype) R1 was compatible with all four pollen donors; category R2 was compatible with three out of four donors; and category R3 was compatible with a single pollen donor. In a greenhouse maintained at 20°C, we grew three ramets of each D individual and twenty ramets of each R individual, obtained after plant cutting. In order to check for a possible effect of plant and inflorescence size on seed set (the ratio of number of seeds produced per ovule produced), ten ramets of each R1, R2 and R3 were grown in large pots (diameter 17 cm) and ten in small pots (diameter 11 cm). This difference in pot size was sufficient to allow optimal vegetative and reproductive development for plants in large pots, and constrained development in small pots. Plants were then transferred at 0–5°C during 3 months and then again at 20°C during 1 month, to induce a synchronous flowering among all individuals. Pollen donors were grouped together at the centre of the field and randomly arranged (Fig. 1). Pollen receptors were placed in ten identical groups, each composed of one small and one large ramet of each of R1, R2 and R3. Two receptor groups were placed at each of five different distances from the centre of the field (respectively at 0, 2, 5, 10 and 25 meters from the pollen donors). At each distance class, the two receptor groups were placed at opposite sides (roughly North vs South) on a line crossing the centre of the field. Different orientations of these lines were chosen for each pair of receptor groups, in order to avoid potential restrictions in pollinator movement that could occur in a one-dimensional setup (Fig. 1).

### Seed set measurement

The experiment started when all plants were able to produce flowers. To avoid bias from fertilizations that occurred prior to the experiment, old flowers were removed from plants initially. Then, all flowering stems of the receptor plants were marked under their youngest unmanipulated flower, using a coloured paint mark. After 10 days, we measured the seed set, i.e. the proportion of ovules developing into seeds, from the last paint mark to the top of the stem at the last wilted flower, over which a new mark was painted. We also counted the number of floral stems for each individual. Measurements were repeated three times consecutively, each separated by 10 days.

To measure seed set, we counted the total number of flowers produced by each individual (noted  $P_i$  for individual  $i$ ) and determined whether each flower had been successfully fertilized according to the criterion used in the cross-pollination experiment (i.e. pistil/fruit width larger than 2.91 mm). Then the successfully fertilized flowers were classified as fruits with a single or two fertilized ovules, based on whether they presented visually a single or two lodges, respectively, and we counted the total number of fertilized ovules for each individual (noted  $F_i$ ). Seed set was defined for each replicate receptor individual  $i$  as  $SS_i = F_i/P_i$ . In order to control for maternal effects on ovule production in pollen receptors, we used positive controls during the experiment: we isolated a ramet of each receptor individual in a cage placed on the edge of the experimental field; every 2 days we performed manual pollinations with a brush on fresh flowers of each individual with a bulk of compatible pollen. However, for unknown reasons these enforced pollinations were largely inefficient as compared to open pollination in the experiment, so we did not get proper positive controls. Hence, we carried out a second experiment in May 2009 in an experimental garden located near the greenhouse on the University campus. We placed two ramets of each of the three receptors used in the experiment at the center of a circle drawn with seven different individuals that were

**Fig. 1** Spatial arrangement of plants in the experimental field. Squares represent ramets of each of four pollen donors located at the centre of the field. Filled circles represent ramets of each of the three pollen receptors placed at different distances from pollen donors (respectively at distances 0, 2, 5, 10 and 25 m)



fully compatible with all receptors, based on the cross-pollination results. We measured seed set for each individual after two weeks, according to the same procedure as outlined above.

### Molecular analysis on progeny

In order to confirm the compatibility patterns between pollen donors and receptors inferred by enforced cross-pollinations, we performed a paternity analysis on some of the progeny obtained in the experiment. A number of seeds collected in the experiment were sown at 22°C in the greenhouse and seedlings were allowed to grow until sufficient leaf material was available. We obtained leaf material from each pollen donor, each pollen receptor, and from 88 seedlings: 41 from individual R1, 34 from R2 and 13 from R3. DNA was extracted from 10 to 15 mg of dried leaf material using the extraction kit NucleoSpin® 96 Plant from Macherey–Nagel®.

We genotyped all individuals for nine microsatellite loci, using original specific primers developed in our lab (Leducq et al., in preparation). Forward primers (F) were labelled with either Applied Biosystems® FAM or VIC dyes (Table S1). Several loci were amplified simultaneously using a multiplex PCR procedure (Table S1). For each multiplex PCR, the reaction mixture (10 µl) contained 20 ng DNA, 5 µl Qiagen® Multiplex 2×, 0.0075 µl of each forward primer, 0.0375 µl of each reverse primer, 0.2 µl Applied Biosystems® FAM or VIC dyes and QSP 10 µl of water. Amplifications were performed on Mastercycler® EpGradient S Eppendorf® thermocycler with the following conditions: 15 min at 94°C, 5 cycles of 45 s at 95°C, then 5 min respectively at 68, 66, 64, 62 and 60°C, 1 min at 72°C, 5 cycles of 45 s at 95°C, then 1 min respectively at 58, 56, 54, 52 and 50°C, 1 min at 72°C, 27 cycles of 45 s at 95°C, 30 s at 47°C, 1 min at 72°C and one cycle of 7 min at 72°C. After amplification, PCR multiplex products were loaded two by two (multiplex 1 with multiplex 3, and multiplex 2 with multiplex 4) on a 16-capillary ABI 3130 sequencer. Each sample contained 1 µl of each PCR multiplex product, 9.7 µl of formamide and 0.3 µl of size marker 500Liz Applied Biosystems®. Genotypes were determined with the software GENEMAPPER™ v3.7 (Applied Biosystems®).

We performed a paternity assignment analysis on multilocus genotypes of 88 seedlings from all receptor plants using the maximum-likelihood method implemented in Cervus 3.0 (Marshall et al. 1998), considering both pollen receptors and donors as candidate fathers. Assignment was considered unambiguous if the likelihood of the most likely father was significantly higher than that of the second most likely father. Significance was determined by simulations of 10,000 multilocus genotypes based on a level of

confidence of 95%, allowing for 1% of genotyping error in likelihood calculations.

### Seed set statistical analysis

A generalized linear mixed model (GLMM) was used to identify the factors that determine the maternal reproductive success, which was estimated by the seed set ( $SS_r$ ) on each ramet  $r$  at three different dates. In short, GLMM is an extension to the generalized linear model in which the linear predictor contains both random and fixed effects. In our GLMM analysis, each outcome of the response variable,  $SS$ , was assumed to be generated from a binomial distribution, with a logit link with the random and fixed effects. The mean,  $\mu$ , of the distribution depends on the random and fixed effects  $X$ , through

$$E(SS) = \mu = g^{-1}(X\beta),$$

where  $E(SS)$  is the expected value of  $SS$ ;  $X\beta$  is the linear predictor, a linear combination of the random and fixed variables with unknown parameters  $\beta$ , and  $g$  is the logit link function (Zuur et al. 2009). The unknown parameters,  $\beta$ , were estimated with maximum likelihood techniques. The following fixed effects were considered: distance separating a pollen receptor from pollen donors ( $D_x$ ); individual genotype effects of pollen receptors R1, R2 and R3 ( $R_i$ ); direction with respect to the location of the pollen donors (North vs South; noted  $Dir$ ); pot size ( $S$ ); number of floral stems ( $H$ ); and number of flowers produced by a pollen receptor in the current time period ( $Ft$ ). We also considered two random effects: the effect of the replicate receptor ramet, including an interaction term with the number of stems produced ( $Z_{ram}$ ), and the effect of the date of measurement, including an interaction term with the distance to pollen donors ( $Z_{date}$ ). We determined the best model by a two steps backward selection procedure described in Zuur et al. (2009), based on Akaike's information criterion (AIC) and likelihood ratio tests when the models compared were nested: the first step aims at finding the optimal structure of the random component; the second determines the optimal structure of the fixed component. Computations were performed using the *lme4* package (Bates and Maechler 2009) in R (R Development Core Team 2009). We checked for collinearity between all fixed variables and used only non-collinear variables: the Pearson correlation coefficient was computed between all explanatory variables and we removed a variable when it was higher (resp. lower) than 0.5 (resp.  $-0.5$ ) as suggested in Zuur et al. (2009). We found that the size of the pot and the number of floral stems were correlated (more stems were produced in large pots than in small pots) and we thus decided to remove the variable pot size from the GLMM analysis. All other factors were

non-collinear among each other and were thus considered in the analysis.

**Results**

**Controlled pollinations and choice of pollen donors and receptors**

In our preliminary survey aiming at comparing the dynamics of pistil/fruit growth after self- and cross-pollinations, no fruit growth was observed for self-pollinations and for a substantial proportion (81 over 130 = 62.3%) of cross-pollinations (Fig. S2). These observations suggested that *B. neustriaca* has a functional SI system. This was largely confirmed by results from the full set of controlled pollinations performed according to a diallel setup (21 by 21 individuals). First of all, the outcome from negative controls (unmanipulated flowers) and from self-pollinations were all unsuccessful (respectively, first column and diagonal in Fig. 2). Also, among the 406 pairwise combinations tested for compatibility, 134 (33%) could be considered as incompatible (grey cells in Fig. 2). Among the 21 individuals tested, female mate availability, defined as the

proportion of compatible pollen donors for a given maternal individual, ranged from 0.51 to 0.85. The outcome of the cross-pollinations were congruent with at least eight distinct incompatibility groups (framed cells in Fig. 2), i.e. groups of individuals showing full incompatibility with other members of the group but showing compatibility with most individuals of the other groups (taking into account the fact that an individual may belong to two groups if it expresses two co-dominant S-alleles). For instance, individuals 1–3 were found to be incompatible with each other, but compatible with most other individuals in the sample (with some exceptions that we consider here as false negatives because they are not shared by all individuals of this group), and were thus considered as belonging to incompatibility group I. In two cases (dotted-framed cells in Fig. 2) we observed non-reciprocal patterns of compatibility suggesting the occurrence of dominance relationships among S-alleles, which is characteristic of sporophytic self-incompatibility (SSI), i.e. a type of SI system characterized by the fact that the pollen phenotype is determined by the diploid genotype at the S-locus of the pollen parent. Firstly, the pistils of individual 4 could not be fertilized by pollen from individuals 1 to 3, whereas pollen from individual 4 was successful in fertilizing pistils from these individuals. In addition, individual 4

	Paternal plant																					Inferred incomp. group		
	Negative controls	1	2	(D1) 3	(R2) 4	5	(R1) 6	(R3) 7	(D3) 8	(D4) 9	(D2) 10	11	12	13	14	15	16	17	18	19	20		21	
1	0.00	0.00	0.00	0.00	1.00	1.00	0.60	0.60	0.50	1.00	0.50	1.00	0.80	0.70	0.70	0.40	1.00	0.80	0.90	0.80	1.00	0.90	I	
2	0.00	0.00	0.00	0.00	0.80	–	1.00	1.00	1.00	1.00	1.00	0.60	1.00	0.80	1.00	1.00	1.00	–	1.00	0.80	1.00	1.00	I	
3	0.00	0.00	0.00	0.00	0.80	0.40	0.20	1.00	1.00	0.60	0.70	0.00	1.00	0.00	0.80	0.20	1.00	1.00	0.40	0.40	0.60	1.00	I	
4 (R2)	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	1.00	0.80	1.00	1.00	1.00	0.60	0.80	1.00	1.00	–	1.00	0.80	1.00	0.80	I / II	
5	0.00	0.80	–	0.60	0.00	0.00	0.00	0.00	1.00	–	1.00	1.00	1.00	0.60	1.00	0.80	–	1.00	0.00	1.00	1.00	1.00	II	
6 (R1)	0.00	0.60	1.00	1.00	0.00	0.00	0.00	0.00	0.80	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	0.20	1.00	1.00	1.00	II / VII	
7 (R3)	0.00	0.80	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.60	0.60	0.80	0.60	1.00	–	1.00	0.00	0.80	1.00	II / III	
8	0.00	0.80	0.60	0.40	1.00	0.80	0.60	0.00	0.00	0.00	0.00	1.00	1.00	0.00	1.00	0.60	0.80	0.40	1.00	0.20	0.40	1.00	III	
9	0.00	0.80	0.80	0.60	1.00	0.60	1.00	0.00	0.00	0.00	0.00	0.60	0.80	1.00	1.00	0.60	1.00	–	0.60	0.00	0.80	0.80	III	
10	0.00	1.00	1.00	0.80	0.80	0.80	1.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.70	1.00	1.00	0.10	1.00	0.77	1.00	III / IV	
11	0.00	1.00	0.40	1.00	1.00	0.60	0.80	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.30	1.00	1.00	1.00	0.00	1.00	1.00	0.90	0.00	IV / VI
12	0.00	1.00	0.20	0.60	0.60	0.80	0.40	1.00	0.80	0.80	0.10	0.14	0.00	0.10	0.00	0.00	0.00	0.60	1.00	1.00	0.60	0.20	0.00	IV / V
13	0.00	1.00	1.00	1.00	0.80	0.80	1.00	1.00	1.00	1.00	1.00	0.80	0.10	0.00	0.10	0.00	0.10	0.00	1.00	0.80	1.00	1.00	1.00	V
14	0.00	0.70	0.80	1.00	1.00	0.80	0.40	1.00	1.00	1.00	0.60	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.50	1.00	1.00	V
15	0.00	1.00	0.80	0.60	1.00	1.00	0.80	1.00	1.00	0.80	1.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.80	V
16	0.00	0.40	1.00	0.40	1.00	–	1.00	1.00	0.60	1.00	0.20	0.60	0.00	0.00	0.00	0.00	0.00	–	0.40	0.60	0.00	1.00	0.00	V
17	0.00	0.20	1.00	0.20	–	0.80	–	–	0.20	–	0.30	0.00	1.00	0.60	0.40	0.80	1.00	0.00	0.60	0.30	0.30	0.40	0.00	VI
18	0.00	0.80	0.80	0.80	0.60	1.00	0.00	1.00	1.00	1.00	0.80	1.00	0.80	0.40	1.00	0.80	0.60	0.00	0.00	0.60	1.00	1.00	1.00	VII
19	0.00	1.00	0.75	0.40	1.00	0.80	0.60	1.00	0.40	0.80	0.20	0.78	1.00	0.00	1.00	0.80	1.00	0.67	1.00	0.00	0.00	0.80	0.00	(III) / VIII
20	0.00	1.00	1.00	1.00	1.00	1.00	1.00	0.80	0.80	1.00	0.80	1.00	0.80	0.50	0.80	1.00	0.80	0.80	0.80	0.00	0.00	1.00	1.00	VIII
21	0.00	0.80	0.80	0.20	1.00	1.00	0.40	1.00	1.00	1.00	0.60	0.60	0.60	0.20	0.80	0.80	1.00	0.40	0.80	0.80	0.80	0.20	0.00	unknown
Inferred incomp. group		I	I	I	(I)/II	II	II/VII	II/III	III	III	III/IV	IV/VI	IV/V	V	V	V	V	VI	VII	III/VIII	VIII	unknown		

**Fig. 2** Results of controlled pollinations given as the proportion of successful pollinations based on at least 5 replicates per combination. Pairs of individuals were considered as incompatible (grey cells) when less than 30% of the replicate pollinations were successful. Values for self pollinations are indicated along the diagonal and values for negative controls (10 unpollinated flowers per individual) are indicated in the first column. Eight incompatibility groups (roman

numerals and framed cells) were identified. Dotted cells indicate when incompatible crosses are not reciprocal but are consistent over all individuals of the same incompatibility group (the incompatibility group that is not expressed either in pollen or pistil is then parenthesized) Bold values indicated results for pollinations realized between individuals chosen for the experiment. Unrealized crosses are indicated by horizontal bars

was found to be incompatible through pollen and pistils with individuals 5–7 belonging to incompatibility group II. This suggests that individual 4 is heterozygous for alleles corresponding to incompatibility groups I and II, with both alleles expressed co-dominantly in pistils but not in anthers (where only allele of group II is expressed). Secondly, individual 19 was found to express alleles of groups III and VIII in pollen, but only allele of group VIII in pistils.

For the experiment, we chose seven plants within three incompatibility groups with clearly defined compatibility patterns (groups I, II and III, Fig. 2). The four pollen donors were chosen as follows: D1 belonged to group I; D2, D3 and D4 belonged to group III. Pollen receptor R1 was chosen within group II so as to be compatible with all donors. R2 belonged to group I and thus was compatible only with individuals D2, D3 and D4. R3 belonged to group III and thus was compatible only with D1. Hence, R1, R2 and R3 were respectively compatible with 100, 75 and 25% of pollen donors. Moreover, all receptor individuals were expressing the S-allele of group II and thus were cross-incompatible.

**Paternity analysis**

All individuals used in the experiment had distinct multilocus genotypes with a number of allelic differences between pairs of genotypes ranging from 7 to 12 over a total of 18 gene copies (at 9 microsatellite loci). The results from paternity analysis on a sample of 88 offspring produced in the experiment supported the compatibility relationships obtained by cross-pollinations. Overall, 73 seedlings (83%) were assigned to a father at a confidence level of 95% (Table 1). Assigned fathers belonged to the pollen donor group in all cases but one (a single offspring from R1 was assigned to R2), confirming strong incompatibility among pollen receptors. According to expectation, we found D1 as the only effective father in R3 progeny, whereas it did not sire any R2 progeny; R2 progeny was sired by D2, D3 and D4; and R1 progeny was sired by all pollen donors.

**Factors influencing variation in seed set**

According to the two steps procedure of model selection in the GLMM analysis, random effects (replicate ramets and dates) were first tested. Most of the deviance was found to be explained by these random effects (null deviance = 13553 vs residual deviance for a model with random effects only = 967.4; compare the last two rows in Table 2). Moreover, comparisons between the best model (AIC = 886.5) that includes both sources of random effects with models lacking either the replicate ramets effect ( $Z_{ram}$ , AIC = 1242) or the dates effect ( $Z_{date}$ , AIC = 1111) showed a large significant increase in AIC values, indicating that both random effects were important and that the ramets effect was the strongest. For ramets as a random effect, we detected an additive effect of the factor (with variance  $\sigma^2 = 2.81$ ), indicating that seed set is highly variable among replicate ramets, as well as a weak but significant interaction with the number of stems produced (with variance  $\sigma^2 = 0.07$ ). A high negative correlation between the additive and interaction effects was also found ( $-0.92$ ), indicating that replicate ramets with high average seed set showed a strong negative relationship between seed set and number of stems produced. For dates as a random effect, we detected an additive effect of the factor (with variance  $\sigma^2 = 0.13$ ) as well as a significant interaction with the distance from pollen donors (with variance  $\sigma^2 = 0.02$ ).

Analysis of the fixed effects showed that three factors were significantly influencing the seed set results, namely distance from pollen donors ( $D_x$ ), genotype of the pollen receptor ( $R_i$ ), and number of flowers produced ( $Fl$ ; Table 2). Moreover, a single interaction term between fixed effects was significant, namely the interaction between pollen receptor genotype and number of flowers ( $R_i:Fl$ ). Removing any of these factors lead to an increase in AIC and a significant log-likelihood ratio test, when compared to the best model. In contrast, when compared to the best model (AIC = 886.5), neither the addition of the direction with respect to the location of pollen donors ( $Dir$ , AIC = 887.6), nor the number of stems produced ( $H$ , AIC = 888.4) did

**Table 1** Paternity assignment using microsatellite markers

Mother	Candidate father							No father assigned	Total progeny
	R1 [II]	R2 [(I)/II]	R3 [II/III]	D1 [I]	D2 [III/IV]	D3 [III]	D4 [III]		
R1 [II]	0	1	0	3	17	7	7	6	41
R2 [I/II]	0	0	0	0	4	4	21	5	34
R3 [II/III]	0	0	0	9	0	0	0	4	13

The values reported are the number of seedlings of a given mother assigned to each candidate father (considering both pollen receptors and donors), as determined by the maximum-likelihood method of Marshall et al. (1998). The incompatibility phenotype of each individual is indicated in brackets

**Table 2** Results of the model selection procedure in the GLMM analysis of the seed set data

Model	AIC (d. f.)	Residual deviance
<b>Best model</b>		
$I + D_x + R_i*Fl + Z_{ram} + Z_{date} + \varepsilon$	886.5 (16)	854.5
<b>Random effects</b>		
$I + D_x + R_i*Fl + Z_{date} + \varepsilon$	1242 (13)	1216
Additive term in $Z_{date}$ only	1345 (11)	1323
Interaction term in $Z_{date}$ only	1431 (11)	1409
$I + D_x + R_i*Fl + Z_{ram} + \varepsilon$	1111 (13)	1085
Additive term in $Z_{ram}$ only	1268 (11)	1246
Interaction term in $Z_{ram}$ only	1283 (11)	1261
<b>Fixed effects (in addition to <math>I + Z_{ram} + Z_{date}</math>)</b>		
$D_x + R_i*Fl + Dir$	887.6 (17)	853.6
$D_x + R_i*Fl + H$	888.4 (17)	854.4
$D_x*Fl + R$	897.6 (18)	861.6
$D_x + R_i + Fl$	901.7 (14)	873.7
$R_i*Fl$	902.0 (12)	878.0
$D_x*R_i + Fl$	908.8 (22)	864.8
$R_i + D$	932.4 (13)	906.4
$D_x + Fl$	939.4 (12)	915.4
$Fl$	950.7 (8)	934.7
$R_i$	952.0 (9)	934.0
$D_x$	970.1 (11)	948.1
No fixed effects	981.4 (7)	967.4
Null deviance	(177)	13553

For each model tested, estimates are given for the AIC statistic, the number of degrees of freedom (d.f.), and the residual deviance of the model. Models are identified with a symbolic description of the linear predictor using the following fixed effects: intercept ( $I$ ), distance from pollen donors ( $D_x$ ), genotype of the pollen receptor ( $R_i$ ), number of flowers produced ( $Fl$ ), number of floral stems ( $H$ ), and direction ( $Dir$ ). Random effects are noted  $Z_{ram}$  for replicate ramets, including an interaction term with the number of stems,  $Z_{date}$  for dates, including an interaction term with distance to pollen donors, and  $\varepsilon$  stands for the residuals. Symbols separating factors indicate whether their effects are additive (+), in interaction (:), or both (\*)

improve significantly the quality of the prediction. Estimates and significance of the  $\beta$  parameters of the best fit model are given in Table 3. Negative estimates of  $\beta$  indicate that the factor considered has a negative effect on seed set. For the distance from pollen donors effect, significantly lower seed set results were observed with increasing distances (Fig. 3), although the seed sets at 0 and 2 m. were not found to be significantly different ( $P$ -value = 0.246). For the pollen receptor genotype effect, a significantly higher seed set was observed for R1 ( $P = 0.002$ ) and significantly lower seed set for R3 ( $P < 0.001$ ), as compared to R2 (Fig. 3). Hence, the seed set was positively correlated to the proportion of compatible pollen donors (100% for R1, 75% for R2, and 25% for R3). The number of flowers produced by a pollen

**Table 3** Estimates and significance of the fixed effect  $\beta$  parameters of the best fit model in the GLMM analysis of the seed set data

Effect	$\beta$ parameter estimate (S.E.)	z-value	$P$ value
<b>Intercept</b>	<b>0.919 (0.342)</b>	<b>2.688</b>	<b>0.007</b>
$D_2$	-0.392 (0.338)	-1.159	0.246
$D_5$	<b>-1.607 (0.346)</b>	<b>-4.655</b>	<b>&lt;0.001</b>
$D_{10}$	<b>-2.792 (0.409)</b>	<b>-6.83</b>	<b>&lt;0.001</b>
$D_{25}$	<b>-4.051 (0.69)</b>	<b>-5.872</b>	<b>&lt;0.001</b>
$R_1$	<b>0.854 (0.275)</b>	<b>3.112</b>	<b>0.002</b>
$R_3$	<b>-1.950 (0.277)</b>	<b>-7.035</b>	<b>&lt;0.001</b>
$Fl$	<b>-0.001 (5.10<sup>-4</sup>)</b>	<b>-2.281</b>	<b>0.022</b>
$R_1:Fl$	2.4.10 <sup>-4</sup> (6.8.10 <sup>-4</sup> )	0.352	0.724
$R_3:Fl$	<b>-0.002 (6.0.10<sup>-4</sup>)</b>	<b>-3.956</b>	<b>&lt;0.001</b>

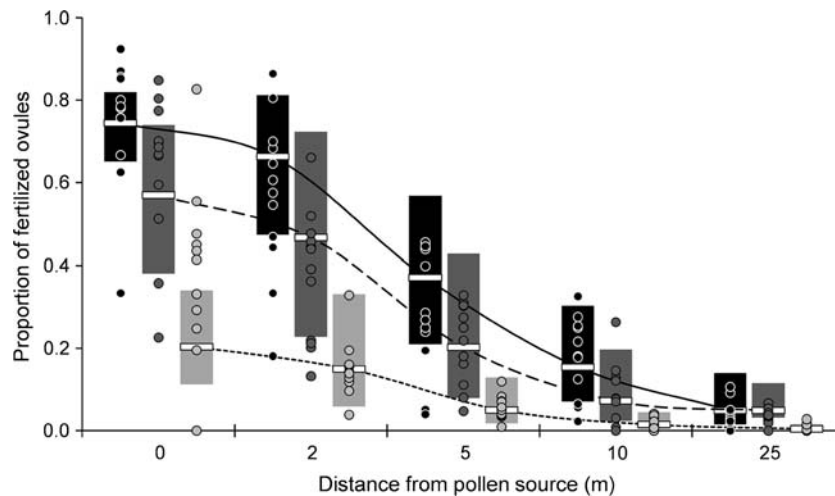
The following statistics are given: estimates and standard errors (SE) of the  $\beta$  parameters; z-value and  $P$ -value of the z-test. Variables of the model: intercept ( $I$ ), distance from pollen sources ( $D_x$ ), pollen receptor genotype ( $R_i$ ), number of flowers produced ( $Fl$ ). The symbol “:” is used to indicate the interaction between two effects. Significant effects are given in bold

receptor had a small direct negative effect on the seed set ( $P = 0.022$ ). The observed significant interaction between pollen receptor genotype and number of flowers was essentially due to a decrease in seed set in replicate ramets of genotype R3 when the number of flowers they produced increased. It is noteworthy that the inclusion of a distance by receptor genotype interaction term did not improve the prediction (Table 2). This suggests that the negative effect of distance to pollen donors on seed set was comparable in each receptor genotype.

Pollen donors produced roughly similar numbers of flowers (Table S2) so that relative mate availabilities of the different pollen receptor genotypes estimated from the number of flowers produced by their compatible donors (100% for R1; 74–87% for R2; and 13–26% for R3) were similar to the expected mate availabilities based on the proportion of compatible pollen donors (respectively, 100, 75, and 25%). One can note, however, that at the second date the relative mate availability for genotype R3 was about two times lower than expected (0.13 vs 0.25) because of a lower flower production from its single compatible pollen donor D1, which would increase the magnitude of the pollen limitation effect for this genotype.

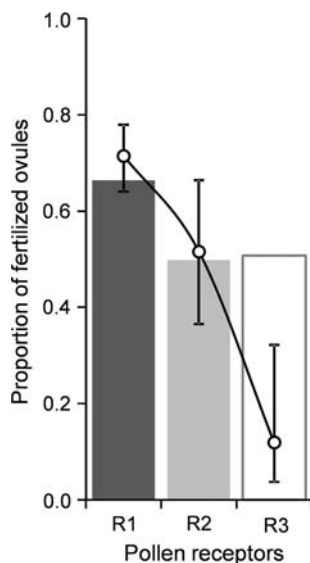
The positive control results obtained in 2009 under conditions of high mate availability for all receptor genotypes were compared to results from the experiment at a distance of 0 m. from pollen donors, i.e. when removing the effect of distance to pollen donors (Fig. 4). We found no difference in seed set between positive controls and experimental results for genotypes R1 and R2 (mean seed set respectively of 0.66 and 0.49 in control). This suggests that the significantly lower seed set observed in R2 as





**Fig. 3** Proportion of fertilized ovules plotted against distance from pollen sources, measured for each 4 ramets, dates and directions together, for three pollen receptors R1 (black circles), R2 (dark grey circles) and R3 (grey circles). Proportion of fertilized ovules expected

compared to R1 in the experiment may be due at least in part to genetic differences in female fertility between the two genotypes. In contrast, the seed set of the positive control in R3 (mean seed set = 0.49) was found to be significantly higher than the experimental result under strongly reduced mate availability. Hence, it appears that the significantly lower seed set observed in the experiment in R3 as compared to R1 and R2 was caused by pollen limitation associated to the S-Allee effect.



**Fig. 4** Proportion of fertilized ovules (seed set) estimated in two ramets of each pollen receptor genotype (R1 in dark grey, R2 in grey, R3 in white) placed in conditions of high compatible pollen availability. The continuous line represents the expectation and standard error of the proportion of fertilized ovules at distance 0 m. from the pollen sources, under the best GLMM model obtained in our analysis

under the best GLMM for each pollen receptor according to distance from pollen sources (R1: continuous line, R2: broken line, R3: dotted line). Standard errors of the GLMM predictions are represented by bars in black (R1), dark grey (R2) and grey (R3)

**Discussion**

Self-incompatibility in *B. neustriaca*

We demonstrated the occurrence of a SI system in *B. neustriaca*. Firstly, no seeds were produced under open-pollination in an insect-free greenhouse, or under forced self-pollinations. Secondly, under forced cross-pollination, all individuals were shown to be able to produce seeds but a fraction (about one-third) of pairwise combinations of individuals was consistently associated to fertilization failure, and could be classified as incompatible. The patterns of cross-compatibility were consistent with at least eight incompatibility groups, putatively corresponding to eight functional alleles at the S-locus. Most incompatible combinations were reciprocal, i.e. when ovules from individual *i* could not be fertilized by the pollen of individual *j*, pollination of *j* by *i* also failed. However, in two cases, non-reciprocal cross-incompatibilities were found, suggesting that the corresponding S-alleles were not expressed either in the pollen or in the pistil when occurring together with some other S-alleles. This result indicates that dominance relationships occur among S-alleles, which is characteristic of sporophytic self-incompatibility (SSI), as is the case in other Brassicaceae species (Laurens et al. 2008; Ockendon 1975; Prigoda et al. 2005).

Experimental evidence for an S-Allee effect

Our experiment allowed disentangling pollen limitation effects (i.e. reduction in seed set due to limited availability of compatible pollen) caused either by an overall limitation in pollen transfer due to spatial isolation, or by a limitation

in the occurrence of compatible mates due to SI. Evidence for an overall limitation in pollen transfer was demonstrated by a significant decrease in seed set of the pollen receptors with distance from pollen sources. Such a decrease in female reproductive success was effective when the distance between receptors and donors was higher than two meters, whereas the seed set was close to zero for the largest distance (25 m). Evidence for an effect of the SI system is suggested by the observed significant relationship between seed set of a pollen receptor and the proportion of pollen donors compatible to its S-locus genotype. Indeed, significant differences in seed set were observed among pollen receptor individuals (R1 compatible with all four pollen donors; R2 compatible with 75% of pollen donors; and R3 compatible with 25% of the pollen donors only; results confirmed by paternity analysis): R1 had a slightly higher seed set than R2, while both R1 and R2 had a seed set about three times higher than R3 (Fig. 3, Table 3). Although consistent with the expectations based on the S-Allee hypothesis, these results could also reflect some inherent differences in reproductive success among genotypes. A proper way to test this would be to repeat the experiment with independent genotypes possessing similar properties of compatibility. Unfortunately, our experiment did not include such independent replicates. However, we obtained results from positive controls, i.e. estimates of seed set in the same genotypes under conditions of maximal mate availability (100%). These results indeed revealed differences in reproductive success among genotypes and suggested that the reduction in seed set in the experiment for R2 as compared to R1 could be due entirely to such differences, whereas the large reduction for R3 as compared to R1 and R2 could not. Hence the experimental evidence for a role of SI in reducing reproductive success seems at least robust in the case of the R3 receptor genotype, which was subject to a strong reduction in mate availability.

Our results revealed significant effects of spatial pollen limitation, and of compatible mate availability due to SI, but they did not reveal a significant interaction term between these two effects, indicating that they acted additively and did not compensate for each other. A strikingly similar result was obtained by Elam et al. (2007) in an experimental study on the invasive Californian wild radish. Their experiment allowed them to test the effect on fruit set of local density (by comparing isolated patches of size ranging from 2 to 20 individuals) and of genetic relatedness among individuals (by comparing patches with either full-siblings, half-siblings, or unrelated individuals) as a proxy for compatible mate availability, as well as the interaction between these two effects. They found that both effects were significant, with smaller fruit set in smaller patches and in patches with higher genetic relatedness, but

they did not find a significant interaction term. Although the two experiments are different, and the composition of the patches in terms of S-locus genotypes was not determined in the wild radish experiment, it is interesting to observe that in both cases the factors influencing pollen limitation, i.e. local density and compatible mate availability acted additively instead of synergistically. This suggests that in empirical studies within natural populations, the two effects could be separated and quantified independently.

Altogether, our results suggest that a reduction in mate availability by a factor of four or higher (the percentage of flowers produced by pollen donors compatible with R3 ranged from 13 to 26%, Table S2) is likely to cause a substantial reduction in seed set in *B. neustriaca*, at least under the pollination conditions prevailing at the time and location of the experiment. Strictly speaking, the reduction in mate availability for receptor R3 in this experiment was even stronger if we consider incompatible pollen grains landing on pistils of R3, which were produced by surrounding receptor plants of genotypes R1, R2 and R3. Hence, it may be that a very high reduction in mate availability is necessary to influence patterns of seed set in this species.

Replicates of this experiment using other genotypes with similar properties of compatibility would be necessary to confirm these results, and it would also be meaningful to set up this experiment under similar environmental conditions as those prevailing in the field. We suggest that experiments like this one could be done in collaboration with conservation practitioners, at the occasion of a procedure of reintroduction of the species in sites where it is now absent.

#### Investigating the S-Allee effect within natural populations

Up to now, estimates of compatible mate availability within natural populations have remained scarce because of technical difficulties in genotyping the S-locus and, for SSI, the need to estimate patterns of dominance relationships among all S-alleles (Castric and Vekemans 2004; Billiard et al. 2007). Hence, most investigations aiming at evaluating the impact of SI on pollen limitation estimated mate availability from the mean compatibility rate of controlled crosses realized between individuals randomly sampled from natural populations (Reinartz and Les 1994; Fischer et al. 2003; Willi et al. 2005; Pickup and Young 2008, Campbell and Husband 2007, Wagenius et al. 2007). Although some of these studies found a significant positive relationship between mean seed set and population size, they could not distinguish between effects of overall pollination efficiency, inbreeding depression and compatible

mate availability due to the S-locus. In two studies, however, average mate availability (computed as the percentage of compatible pollen donors for each plant in the sample) could be determined based on S-locus genotypes. In *Brassica insularis*, a species with a SSI system, Glémin et al. (2008) obtained values of the average mate availability within four populations, ranging from 60% (in a population with 15 S-alleles co-segregating) to 19% (with 5 S-alleles). They also showed that patterns of variation in average seed set across populations were at least partially related to estimates of average mate availability, suggesting that values of mate availability below 25% do have an effect on female reproductive success in this species, as was observed in our experimental study. In wild pear (*Pyrus pyraster*), a species with a gametophytic SI system, i.e. where pollen phenotype is determined by the haploid genotype at the S-locus, samples from each of 15 populations were genotyped at the S-locus (Holderegger et al. 2008). Despite the small population sizes ( $N$  ranging from 8 to 88 individuals), the number of S-alleles maintained within populations was relatively high (9–21 S-alleles) and estimates of average mate availability ranged from 92.9 to 100% based on S-locus genotypes. Such high values are typically expected for gametophytic SI systems (Vekemans et al. 1998) and strongly suggest that S-Allee effects do not occur in wild pear. However Holderegger et al. (2008) pointed out that such estimates of average population mate availability are based on the assumption of random mating within populations, and they suggested that empirical studies of the S-Allee effect should instead aim at estimating realized mate availability at the individual level. Moreover, because local density could affect reproductive success independently from population size, these two factors should be considered separately (Kirchner et al. 2005). Hence, future studies on the S-Allee effect within natural populations should take into account realized patterns of pollen transfer, and patterns of genetic structure at the S-locus, as well as levels of inbreeding depression in order to assess the effect of the SI system independently from other factors affecting reproductive success.

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