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Within/between population crosses reveal genetic basis for siring success in *Silene latifolia* (Caryophyllaceae)

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Abstract

Divergence at reproductive traits can generate barriers among populations, and may result from several mechanisms, including drift, local selection and co-adaptation between the sexes. Intersexual co-adaptation can arise through sexually antagonistic co-evolution, a timely hypothesis addressed in animals but, to our knowledge, not yet in flowering plants. We investigated whether male and female population of origin affected pollen competition success, offspring fitness and sex ratio in crosses within/between six genetically differentiated populations of the white campion, *Silene latifolia*. Each female was crossed with pollen from one focus male from the same population, and pollen from two focus males from two distinct populations, both as single-donor and two-donor crosses against a fixed tester male with a 2-h interpollination interval ($n = 288$ crosses). We analysed paternity with microsatellite DNA. Male populations of origin significantly differed for siring success and *in vitro* pollen germination rates. *In vitro* pollen germination rate was heritable. Siring success also depended on sex ratio in the female family of origin, but only in between-population crosses. In some female populations, two-donor crosses produced less female-biased sex ratios compared with single-donor crosses, yet in other female populations the reverse was true. Offspring sex ratio varied with donor number, depending on the female population. Within/between population crosses did not differ significantly in seed set or offspring fitness, nor were siring success and offspring fitness significantly correlated. Altogether this suggests reproductive divergence for traits affecting pollen competition in *S. latifolia*.

Introduction

Spatial and temporal variations in biotic and abiotic factors may influence trait evolution and lead to local adaptation (Kawecki & Ebert, 2004). Divergence among allopatric populations can lead to speciation, whereby divergence at reproductive traits is particularly important in generating barriers among extant populations (Parker & Partridge, 1998; Howard & Berlocher, 1998; Kirkpatrick & Ravigne, 2002; Knight & Turner, 2004; Salzburger *et al.*, 2006). Furthermore, several processes including antagonistic co-evolution between the sexes can lead to reproductive divergence and local co-adaptation of males and females in traits affecting post-mating success (Holland & Rice, 1998; Andres & Arnqvist, 2001; Knowles & Markow, 2001; Rowe *et al.*, 2003). To our knowledge, this timely idea has yet to be investigated for male and female traits affecting post-pollination success in plants (Arnqvist & Rowe, 2005).

In plants, competition among different pollen donors may frequently occur, even when some flowers are pollen limited (e.g. Bernasconi *et al.*, 2006), due to multiple pollinator visitation, pollen carryover and floral structures (syncarpy, Armbruster *et al.*, 2002) that impose a common

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race of pollen tubes towards the ovules. The fertilization success of each competing pollen donor may depend on both the male and female traits expressed in the sporophyte or in gametophyte (pollen tube and embryo sac). Indeed, fertilization success may depend on male traits affecting pollen competitive ability (Snow & Spira, 1991; Snow *et al.*, 2000; Hedhly *et al.*, 2005) and female traits influencing pollen germination on the stigma, pollen tube growth through the style, fertilization, seed and fruit abortion (Marshall, 1988, 1991; Snow & Spira, 1991; Baker & Shore, 1995; Cruzan & Barrett, 1996; Krauss, 2000; Lankinen *et al.*, 2006). Male and female optima over these traits and over the outcome of fertilization may differ, analogous to sexual conflict in animals (Chapman *et al.*, 1995; Rice, 1996; Rice & Holland, 1997). Whereas each male (pollen donor) will increase its fitness by maximizing its share of paternity, females (pollen recipients) may benefit by increasing diversity of pollen and sires to avoid inbreeding, genetic incompatibility and selfish genes (Willson & Burley, 1983; Delph & Havens, 1998; Baker & Shore, 1995; Bernasconi *et al.*, 2004). Indeed, pollination with large and diverse pollen loads can increase female fitness through higher offspring number and quality (Schlichting *et al.*, 1987, 1990; Winsor *et al.*, 1987; Karron & Marshall, 1990; Snow, 1990; Mitchell, 1997; Niesenbaum, 1999; Paschke et al., 2002; Bernasconi *et al.*, 2003; Bernasconi, 2003; Armbruster & Rogers, 2004; Herrero, 2003; Vergnerie, 2006; Young & Young, 1992).

We investigated genetic variation among native populations of the white campion, *Silene latifolia* for male reproductive success under conditions of pollen competition. We addressed whether male and/or female population of origin influence paternity in within- and between-population crosses. Effects of male genotype (population of origin) are expected if populations differ, e.g. due to locally variable selection on traits affecting siring success (for instance, through variation in the intensity of pollen competition) or drift (e.g. at the Y chromosome). Effects of male × female genotype are expected under local co-adaptation between the sexes, either as a result of sexual conflict (Rowe *et al.*, 2003; Arnqvist & Rowe, 2005), or if co-adaptation occurs for 'female preference' (Andres & Arnqvist, 2001). Recent studies indicate that sexual conflict does not necessarily result in males from foreign populations always obtaining highest paternity success, but that different scenarios are possible (reviewed in Arnqvist & Rowe, 2005). Although between-population crosses often reveal significant interactions between the sexes (e.g. Andres & Arnqvist, 2001), they cannot on their own conclusively demonstrate sexually antagonistic coevolution. Instead, the functional analysis of single traits and their consequences for male, female and offspring fitness must be taken into account. Whereas antagonistic effects should balance within populations, they might become apparent in between-population crosses (Brandvain & Haig, 2005). We therefore also investigated whether paternity success of focus males correlated with female or offspring fitness, to examine whether variation in pollen competitive ability is associated with fitness costs (a specific prediction of sexual conflict, see Pizzari & Snook, 2003) or benefits to females. The white campion is a suitable study organism because it has separate sexes and does not reproduce clonally, and thus depends entirely on sexual reproduction for its fitness. Moreover, multiple within-fruit paternity is common in natural populations (S. Teixeira & G. Bernasconi, unpublished manuscript). Frequent pollen competition generates selection for male traits increasing pollen competitive ability, and strengthens the potential for conflict (Brandvain & Haig, 2005).

Specifically, we used a design involving within- and between-population crosses in two blocks of three populations each (Fig. 1). These populations are geographically separated (Table 1) and a microsatellite DNA analysis revealed significant genetic divergence (Jolivet & Bernasconi, 2007). Furthermore, European *S. latifolia* populations show strong genetic structure for variation at the Y chromosome (Ironside & Filatov, 2005). We reared an F1 generation under standardized conditions to reduce the effect of maternal environmental conditions and thus to better isolate genetic differences among populations. With these F1 plants, we conducted two-donor crosses on the same female plant with pollen from within the same population of origin of the female, and pollen from two males from two distinct populations, using a fixed tester male as a competitor. Additionally, we conducted control crosses with pollen from each male as a single donor. This control allows testing the effect of providing pollen competition on seed set, offspring sex ratio and offspring fitness, and to correlate performance as single donor (e.g. seed set) with performance in competition (e.g. paternity). A total of 288 crosses were conducted. We assessed paternity in the two-donor crosses using molecular markers, examined potential correlates of siring success (pollen germination *in vitro*, the number of pollen grains per anther, sex ratios of the paternal and maternal family of origin, seed set in single-donor crosses) and measured female and offspring fitness, including sons' *in vitro* pollen germination rates. This design addresses the following questions: (i) is there genetic variation among male and female populations (and families) of origin for traits affecting siring success? Can we identify such traits through a correlation analysis of pollen, male and female traits with siring success? (ii) Is there an interaction between male and female genotype (population of origin) in determining paternity, as might be expected under local co-adaptation? Is the direction of such an interaction indicating that pollen from the 'home' population is more or less successful than 'away' pollen? (iii) Do male and female populations of origin affect offspring fitness, and does this point at inbreeding or outbreeding depression? (iv) Is *in vitro* pollen germination heritable? and (v) Do the number of donors

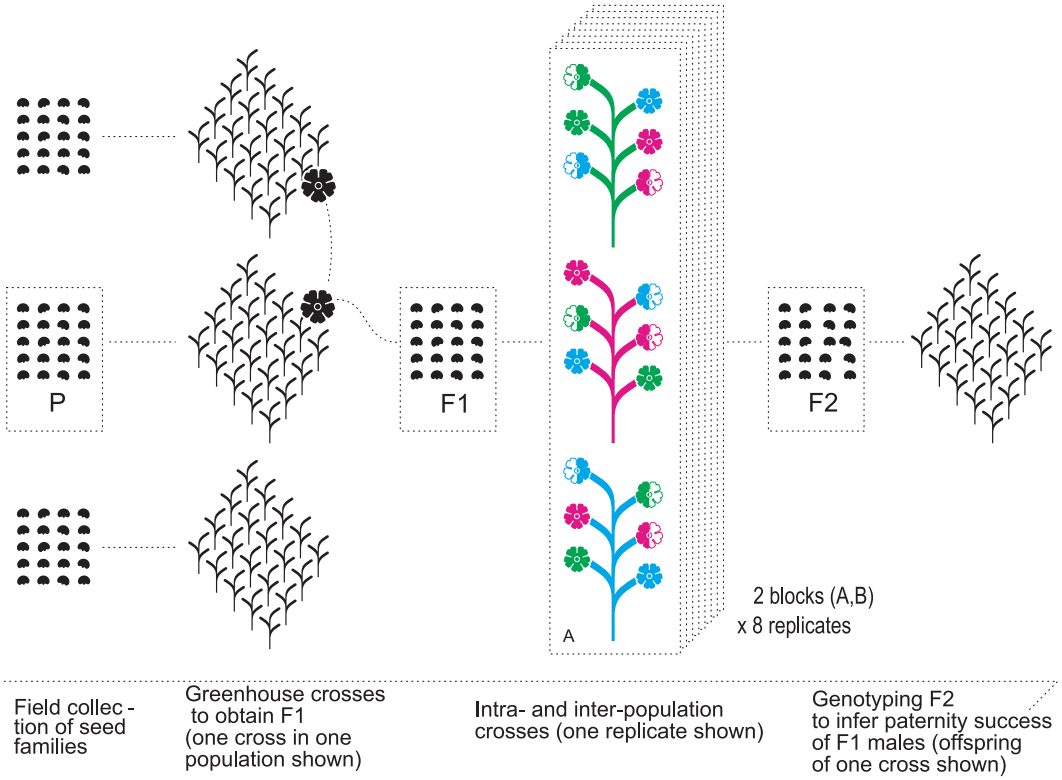

Fig. 1 Design for intra- and interpopulation crosses in *Silene latifolia*. Crosses were conducted on F1 plants. Parents (P) were field collected as seeds (from 15 females per population), raised (20 seeds per fruit) in the greenhouse and crossed within populations between families (shown only for one population) to obtain an F1 generation free of maternal environmental effects. The six populations were assigned to two different blocks (three populations per block). In each block, F1 females were crossed with one male from a different family but the same population, and two males from the other two different populations (shown as different colours). Each replicate thus consisted of three females (from three different populations) and three males (from three different populations); per block there were eight such replicates. Pollinations for each focus male were conducted both as single-donor pollinations and as competitive pollinations against a fixed tester male. In total, we conducted 288 crosses [(three males × three females × two levels of pollen competition) per replicate × eight replicates per block × two blocks]. Twenty seeds per fruit from competitive pollinations were raised for paternity analysis (in four replicates per block and a total of 1440 offspring genotyped). We determined seed family sex ratio (F1, F2) and offspring fitness components (F2: age at first flowering; for a subset, size at first flowering) of 20 offspring per fruit.

(single- vs. two-donor crosses) and among-male variation in male siring success influence female fitness, offspring sex ratio or offspring fitness?

Materials and methods

Study species

The white campion *S. latifolia* (Poiret) (= *Silene alba* (Miller) Krause = *Melandrium album* (Miller) Garcke; Caryophyllaceae) is a dioecious short-lived perennial plant native in Europe. *Silene latifolia* is patchily distributed along disturbed roadside and agricultural habitats, on calcareous and sandy soils (Baker, 1947). It occurs throughout Europe and the Mediterranean region (Baker, 1947). Morphological and molecular variations suggest that *S. latifolia* found refuge in northern Africa during the last glaciations from where it colonized its current range with the spread of agriculture (Mastenbroek & Vanbrederode, 1986; Vellekoop *et al.*, 1996). Within the European range, populations are genetically and phenotypically differentiated and exhibit very high variation (Wolfe *et al.*, 2004; Ironside & Filatov, 2005; Jolivet & Bernasconi, 2007). *Silene latifolia* was introduced in North America in the mid-1800s where it has become invasive (McNeill, 1977; Wolfe, 2002). The plant emerges in early spring, flowers from May to October and overwinters as a rosette. It builds two to five, and up to 40 fruits per season, with 48–359 seeds per fruit (Baker, 1947). Sex determination is chromosomal; males are the heterogametic sex. *Silene latifolia* is animal pollinated, mainly by nocturnal moths (Shykoff & Bucheli, 1995; Young, 2002).

Table 1 (a) Geographic origin of *Silene latifolia* populations sampled for intra- and interpopulation crosses to assess effect of male and female population of origin on paternity success and offspring fitness. Size = number of flowering individuals at sampling date. (b) Sample sizes for crosses performed for two blocks of three populations each. In each block and male–female combination, for fitness measures there were eight replicates (each replicate using plants from different families, total $n = 288$ crosses); genetic analysis of paternity in two-donor crosses was conducted for four replicates (total $n = 72$ crosses, 1440 offspring genotyped); offspring sex ratios were determined for the offspring analysed for paternity and within-population, single-donor crosses of the same focus males (total $n = 96$ crosses). Tester competitors for two-donor crosses all originated from an independent population (AL).

Code	Locality	Country	Coordinates	Size	
(a)					
CT	Cottendart	CH	46°58′30″N 6°50′50″E	1000	Block 1
HO	Millingerwaard	NL	51°52′45″N 6°00′55″E	2000	Block 1
PA	Gagny	F	48°53′11″N 2°32′36″E	400	Block 1
SC	Sesto Calende	I	45°44′08″N 8°37′00″E	> 100	Block 2
VN	Village-Neuf	F	47°36′25″N 7°33′31″E	80	Block 2
GR	Saint-Martin d'Uriage	F	45°09′51″N 5°51′34″E	60	Block 2
AL	Göttingen	D	51°33′20″N 9°58′01″E	24	Tester males (competitors)

	Single-donor crosses						Two-donor crosses						
	Female origin							Female origin					
Focus male origin	CT	HO	PA	SC	VN	GR	Competitor origin	CT	HO	PA	SC	VN	GR
(b)													
CT	8	8	8				AL	8	8	8			
HO	8	8	8				AL	8	8	8			
PA	8	8	8				AL	8	8	8			
SC				8	8	8	AL				8	8	8
VN				8	8	8	AL				8	8	8
GR				8	8	8	AL				8	8	8

Plant collection and rearing

We sampled single fruits from distinct female plants in geographically separated populations across Europe (Table 1). The rationale behind sampling geographically distant populations is that crosses between genetically diverged populations might reveal male–female co-adaptation at a local scale. We collected fruits from plants which were at least 2 m apart. In three small populations (GR, VN and AL), we sampled all flowering females. To avoid or reduce the effects of maternal environmental variation on the traits measured, and thus better isolate genetic variation, we conducted our main experiment with F1 plants (Fig. 1). These F1 plants were obtained by crossing plants which were reared from the field-collected seeds (P generation) in the greenhouse. We crossed one female from each field-collected seed family (a seed family describes all the seeds in one fruit) with a male from the same population, but from a different family to avoid inbreeding. Plants (P) for crosses were obtained by germinating 20 seeds from each of 15 field-collected fruits per population in six populations (SC, GR, VN, CT, PA and HO) and from 11 field-collected fruits in population AL. Seeds from the same field-collected fruit were germinated together in Petri dishes on cotton wool and filter paper and watered with 10^{-3} mol L^{-1} Gibberellic acid (GR, VN, SC and AL) in a germination cabinet (21 °C, RH = 80%, 16 h light). For the CT, PA and HO populations, we germinated the seeds in Jiffy peat pellets (Jiffy7, 703) in the greenhouse (see below). After 15 days, we placed the seedlings in 10-cm pots containing a 3 : 1 mixture of soil/sand (Tref BF4 from GVZ-Bolltec Zurich, Switzerland; 0.5- to 3-mm sand). When the seedlings flowered, we recorded the gender to estimate the sex ratio in the parental family of origin. Crosses were conducted using sets of three populations (blocks, see below). Plants were reared in two distinct greenhouses: GR, VN, SC and AL (23 ± 1 °C/19 ± 1 °C day/night, RH = 30 ± 5%, artificial light for 16 h) and CT, PA, HO (23 ± 2 °C/18 ± 1 °C day/night, RH = 55 ± 10%, artificial light for 16 h, plus natural light; lamps EYE Clean-Ace 6500 K, 400 W; Iwasaki Electronics Co., Tokyo, Japan). Plants were placed at random positions within the greenhouse. The block factor (germination and greenhouse conditions) was accounted for in the analysis and in the subsequent design.

Intra- and interpopulation crosses

We conducted within- and between-population crosses among three populations in each of two blocks. This provides replication at the level of population, while keeping the required number of crosses (male–female combinations) feasible. Populations were randomly assigned to blocks. We chose eight F1 seed families in each of the six populations SC, GR, VN, CT, PA and HO

(total of 48 families); the population AL was used for tester males. For each F1 family, we germinated 20 seeds in Jiffy peat pellets, reared the plants in the greenhouse, and recorded the proportion of seeds that successfully germinated within 36 days (germination rate), time to germination for 20 seeds, the proportion of germinated seedlings that flowered within 112 days (flowering rate) and their age (in days) at first flowering, as well as the sex ratio of the ensuing F1 plants. For one block (populations CT, PA and HO), we also measured size at first flowering (number of leaves, length of the longest leaf, height of the stem and number of stems). As potential correlates of paternity success of pollen donors (see below), we measured *in vitro* pollen germination rate and the number of pollen grains per anther in one male (focus males) of each F1 family.

For intra- and interpopulation crosses, we chose one male and one female in each F1 family for a total of 48 individuals in each sex, using restricted randomization (i.e. blind selection and assignment of males and females based on a list of plants that were bolting). Three weeks before starting crosses, we repotted all females (ø18 cm pots) and all males (ø13 cm pots). Separately for each block, we randomly assigned each individual to a replicate consisting of three females and threes focus males plus a tester male (from AL population; Fig. 1). In total, there were 16 such replicates, eight in each block. Each individual in each replicate was from a different F1 family. On each female, we conducted six different crosses: each focus male was tested in single- and two-donor pollinations against a fixed competitor (the tester male); three focus males were used (one 'local' male and two 'foreign' males from two different populations, Fig. 1). Thus, there were a total of 18 crosses in each replicate ($= 3$ males $\times$ 3 females $\times$ 2 levels of pollen competition), eight replicates per block and two blocks, giving a grand total of 288 crosses. The total pollen load was the same in single- and two-donor crosses (four anthers). In the latter, we applied two anthers from the focus male and after 2 h we applied two anthers from the tester male. Two-donor pollinations allowed us to test whether paternity success of focus males under conditions of pollen competition is influenced by the population of origin of the male or of the female. Single-donor pollinations were conducted to test whether allowing or preventing competition among pollen donors affects seed set or seed mass. In two-donor pollinations, we used a fixed 2-h interval to allow post-pollination senescence in the stigma to start. A design with an interpollination interval allows for differences among males in potential correlates of siring success to be expressed; this includes differences in pollen germination, pollen tube growth and post-pollination of stigmatic wilting. Post-pollination wilting of the stigma is a candidate mechanism of male–female interactions affecting paternity: pollen donors may induce wilting of the female receptive structures to prevent later-arriving pollen from fertilizing ovules, whereas pollen recipients may resist manipulation (Lankinen *et al.*, 2006). After 2 h, the stigma starts to wilt and pollen has grown about 20% of the style length, hence fertilization has not yet taken place (A. Burkhardt and G. Bernasconi, unpublished results). We bagged all pollinated flowers and collected the seeds when the fruit opened. We estimated seed set as the total seed mass and recorded the mass of 10 seeds per fruit. From this, we estimated seed number as $= 10$(total seed mass/mass of 10 seeds).

In vitro pollen germination and pollen counts per anther

Males may vary in their pollen germination ability or in the number of pollen grains per anther, and this may influence paternity success independently of the interaction with the female. Other traits (e.g. *in vivo* pollen tube growth rates) may additionally play a role, yet we chose *in vitro* pollen germination and pollen count per anther, because these traits can be measured without confounding by female influence. To assess *in vitro* pollen germination, we prepared the medium from a stock solution (1 g L^{-1} boric acid, 3 g L^{-1} calcium nitrate, 2 g L^{-1} magnesium sulphate and 1 g L^{-1} potassium nitrate, stored at 4 °C). One day before pollen sampling, we mixed 10 g of sucrose, 10 mL of stock solution and deionized water for a total volume of 100 mL, then added 0.5 g of agar and heated gently until the solution was limpid. The medium was poured in ø35-mm Petri dishes and allowed to solidify. Each Petri dish was sealed to prevent contamination. We rubbed three dehiscent anthers from each male against the medium, placing the pollen of the three anthers in separate areas. Samples were placed for 2–3 h at 27 °C and stored at −20 °C before counts. Pollen samples from all focus males (and from all sons respectively) were taken on a single day, to avoid confounding by environmental or time factors. For each anther, we counted (10×5 magnification) how many pollen grains out of 100 had germinated, as indicated by a visible pollen tube. We examined 100 grains for each of the three anthers and used the average to obtain one measure per male. To investigate heritability of *in vitro* pollen germination rate, we examined father/son regression, by including only data from the sons of two-donor crosses, which were sired by the focus male, as indicated by their multilocus microsatellite DNA genotype. This yielded 27 and 29 cases per block respectively.

To estimate the number of pollen grains per anther, we stored two dehiscent anthers from unopened flowers in 1 mL of 70% ethanol at 4 °C. Before analysis, we evaporated the ethanol (65 °C). We controlled that all anthers were open, added 1 mL of deionized water, and placed the vial for 10 min in an ultrasound bath. We vortexed and immediately collected 400 μL of the pollen grain suspension to which we added 5 mL of CASYton

(Schaerfe System, Reuligen, Germany). The samples were analysed with a CASY cell counter (Model TT; Schaerfe Systam, Reuligen, Germany), counting particles of ø30–45 μm. We calculated pollen grains per anther, as the number of pollen grains in the sample × 16.875 (dilution factor).

Paternity assignment and measurement of offspring fitness

We assigned paternity using a microsatellite DNA analysis of each potential parent and 20 seedlings (F2) from two-donor crosses, in four replicates per block for a total of 1440 seeds, i.e. 48 F2 families issued from interpopulation crosses and 24 F2 from intrapopulation crosses for a total of 72 F2 families. Due to germination or amplification failures, the final sample for paternity included 1123 offspring, i.e. an average of 16 offspring per cross. We extracted DNA from leaves (kept at −20 °C) using the Macherey–Nagel Nucleospin Plant Kit (Macherey-Nagel GmbH and Co., Düren, Germany). The extracted DNA was run in 0.8% agarose gels to optimize dilutions for a final concentration of 10 ng μL^{-1}. We amplified two microsatellite primer loci (SL1 and SL6) as a duplex, as described in Teixeira & Bernasconi (2007). These two loci are highly polymorphic (Jolivet & Bernasconi, 2007; Teixeira & Bernasconi, 2007); sequences have been deposited in GenBank (accessions DQ469337–DQ469344). Assigning paternity was facilitated as we knew the genotypes of the maternal plant and both putative fathers. Polymerase chain reaction (PCR) was performed using 1× Quiagen Multiplex PCR master Mix, 2 μM of each primer and 10 ng of genomic DNA. The PCR amplification was conducted in a Biometra thermocycler (15 min at 95 °C; 30 cycles composed of 30 s denaturation at 94 °C, for 90 s at $T_m = 60$ °C and 60 s elongation at 72 °C, followed by a final elongation step of 30 min at 60 °C). Fragments were analysed on an ABI 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA) with internal size standard Genescan 350 and scored with GeneMapper v3.7 (Applied Biosystems). We assigned paternity by comparing offspring genotype to those of the mother and the two potential fathers.

For offspring for which paternity was assigned, we assessed fitness components (germination rate and time, flowering rate and age at first flowering, offspring sex ratio in each family and *in vitro* pollen germination rate of one son per family) to address potential in/outbreeding depression. For block 2, we also measured size at first flowering (number of leaves and length of the longest leaf). For analysis, we used family means.

Data were analysed using R 2.0.0 (Ihaka & Gentleman, 1996; R Development Core Team, 2004), GENSTAT 9.1 (1995; Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK) and graphs were produced with SPSS 14.0 for Windows.

Results

Male and female effects on paternity shares

The focus male sired overall a proportion of 0.75 ± 0.24 of the offspring (0.79 ± 0.21 in within-population crosses, 0.73 ± 0.25 in between-population crosses, Fig. 2). As pollen of the focus male was applied 2 h before the pollen of the competing male (tester male), this either indicates first-male advantage in *S. latifolia* or consistently low performance of the (second) tester males from AL [Wilcoxon one-sample signed-rank test against the null hypothesis of equal paternity, significant nonequal paternity in males from populations SC ($V = 66$, $P = 0.004$); VN ($V = 66$, $P = 0.004$), CT ($V = 78$, $P = 0.0005$) and HO ($V = 65$, $P = 0.005$); no significant deviation from equal paternity for males from populations PA ($V = 51$, $P = 0.12$) and GR ($V = 40$ $P = 0.56$)].

Male population of origin and male family within populations both had a significant effect on paternity shares (Table 2a). As we used F1 males for crosses, this indicates genetic variation among males for siring success (Fig. 2a). Paternity shares were not significantly influenced by either female population of origin, or by the interaction between male and female population of origin. The specific contrast 'home vs. away' comparing the success of males from the own vs. a foreign population did not reveal any significant overall difference in performance of males: in some male–female combinations, local males achieved highest paternity, whereas in other combinations foreign males had highest success (Fig. 2a). Of the covariables initially entered in the model (listed in Table 2 caption; see also Table 3), male populations differed significantly for *in vitro* pollen germination (univariate ANOVA, $F_{5,155} = 17.6$, $P < 0.001$) and sibling sex ratio in the male family of origin ($F_{5,168} = 6.15$, $P < 0.001$), but neither significantly so in pollen per anther ($F_{5,154} = 1.44$, $P = 0.21$) nor for seed set in single-donor pollinations ($F_{5,66} = 1.24$, $P = 0.30$). Of these covariables, none significantly explained variation in siring success. The only significant effect was given by the additional covariable, expressing a trait of the female: the female sibling sex ratio in between-population crosses. That is, the sex ratio in the female family of origin was strongly significantly associated with the paternity success of the focus male, when male and female stemmed from different populations. The direction of this effect was such that the paternity success of the (foreign) focus male was higher, the more female-biased the sibling sex ratio of the female was.

Effects of male and female population of origin and pollen competition on seed set

Total seed mass (a measure of seed set) was significantly influenced by the population of origin of the female

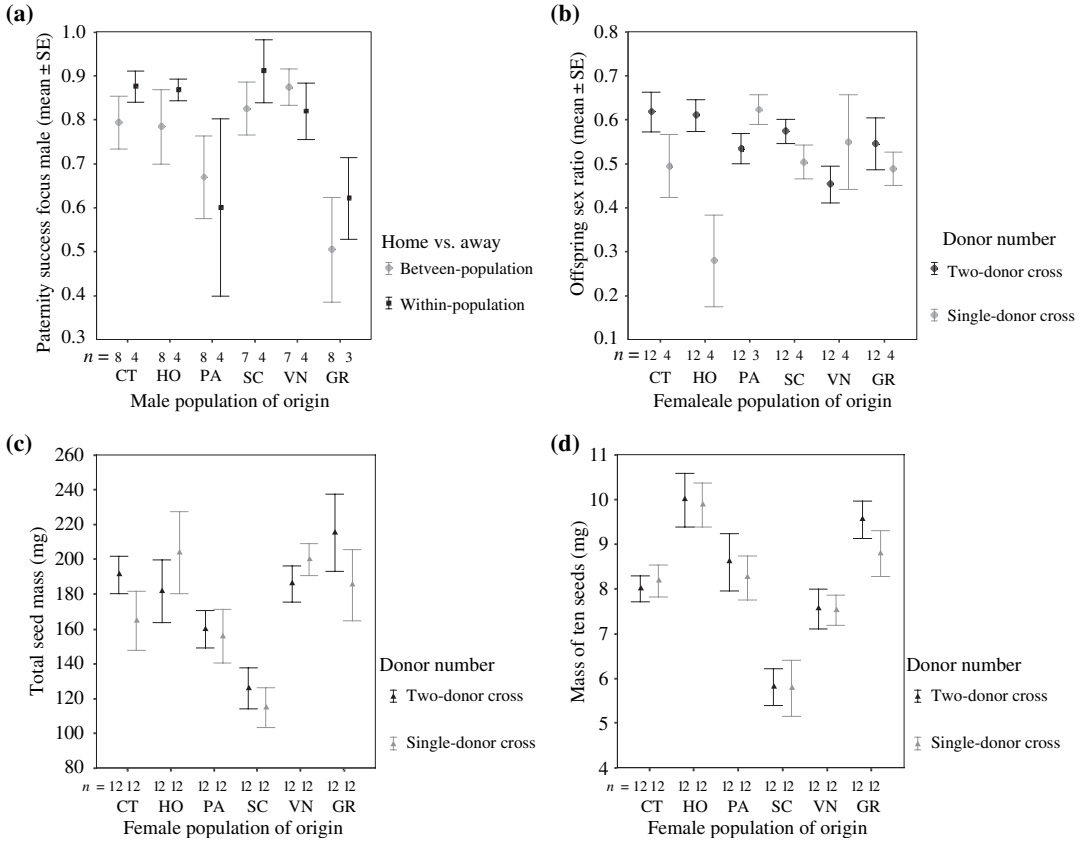

Fig. 2 Observed means (±SE) for (a) paternity success achieved by focus males from different populations in within- vs. between population crosses in *Silene latifolia*; (b) offspring sex ratios (proportion females), (c) total seed mass (mg) and (d) mass of 10 seeds (mg) produced by females from different populations in single- and two-donor crosses. Paternity and sex ratios were determined for 20 offspring per cross; N = number of crosses.

(Fig. 2c). However, neither male population of origin, nor its interaction with female population influenced total seed mass (Table 2b). Also, there was no overall higher or lower total seed mass in within vs. between crosses (Table 2b, 'home vs. away contrast', $P = 0.528$). Additionally, both female and male family within populations explained variation in seed set. Moreover, depending on female family, providing conditions of pollen competition affected seed set, as indicated by a significant interaction between donor number and female family. Similar variation among maternal populations was obtained for individual seed mass (as estimated by weighing 10 seeds, Fig. 2d).

Offspring fitness

We tested in block 2 (SC, VN and GR) whether males obtaining high paternity shares impose a cost on female by lowering offspring fitness. There was no significant correlation between paternity share of the focus male and offspring fitness (time to germination, age at first flowering, germination rate, flowering rate, pollen germination of sons, ANCOVA, all $P > 0.1$), nor between paternity share of the focus male and total seed mass, mass of 10 seeds or seed number when its pollen was applied in single-donor crosses (ANCOVA: all $P > 0.2$).

We tested whether male and female populations of origin influenced offspring fitness, and whether plants issued from between- and within-populations crosses exhibited inbreeding or outbreeding depression. Germination rate of the seeds was strongly influenced by the population of origin of the female parent ($F_{4,54} = 3.52$, $P = 0.013$), but not by the population of origin of the male parent ($F_{4,54} = 2.01$, $P = 0.11$) nor their interaction ($P = 0.73$). Flowering rates (the number of germinated offspring that flowered within 90 days from germination) differed significantly when comparing within- and between-population crosses, but there was a low variation in this variable (Table 4). Time until germination, age at first flowering, size at first flowering for both genders separately were not significantly influenced by the interaction between male and female population of origin nor by the type of cross (between/within

Table 2 Accumulated analysis of variance for male and female population of origin, their interaction, donor number (single- vs. two-donor cross, where applicable), male and female family and significant covariables on (a) paternity success of the focus male, (b) total seed mass and (c) offspring sex ratios following experimental intra- and interpopulation crosses in the white campion, *Silene latifolia*.

Source of variation	den.	d.f.	ss	ms	F_{obs}	$P(F > \|F_{obs}\|)$
(a) Paternity success of focus male						
Block	I	1	0.0020	0.0020	0.07	0.795
Male population of origin	G	4	0.9780	0.2445	3.61	0.025
Female population of origin	H	4	0.1384	0.0346	1.18	0.373
Home vs. away population	I	1	0.0232	0.0232	0.81	0.379
Female × male population	I	7	0.1295	0.0185	0.65	0.714
Female sibling sex ratio (away)	H	1	0.4409	0.4409	15.02	0.003
Male family	I	18	1.2201	0.0678	2.36	0.030
Female family	I	11	0.3229	0.0294	1.02	0.460
Residual		21	0.6019	0.0287		
Total		68	3.8568	0.0567		
(b) Total seed mass						
Block	K	1	887	887	0.47	0.497
Donor number	J	1	1262	1262	0.31	0.582
Male population	H	4	11 033	2758	0.50	0.733
Female population	I	4	10 8566	27 141	5.30	0.011
Home vs. away population	K	1	763	763	0.40	0.528
Donor number × female population	J	5	13 383	2677	0.67	0.654
Female × male population	K	7	18 018	2574	1.36	0.237
Male family	K	18	98 551	5475	2.88	< 0.001
Female family	K	12	61 421	5118	2.70	0.005
Donor number × female family	K	18	72 390	4022	2.12	0.013
Residual		72	13 6709	1899		
Total		143	522 981	3657		
(c) Offspring sex ratios						
Block	M	1	0.0385	0.0385	3.04	*0.095*
Donor number	L	1	0.0614	0.0614	2.18	0.159
Male population of origin	J	4	0.0200	0.0050	0.22	0.923
Female population of origin	K	4	0.0709	0.0177	0.91	0.490
Home vs. away population	M	1	0.0051	0.0051	0.40	0.533
Donor number × female population	L	5	0.4794	0.0959	3.40	0.028
Female × male population	M	7	0.0759	0.0108	0.86	0.554
Pollen germination	J	1	0.0655	0.0655	2.88	0.110
Pollen germination, quadratic term	J	1	0.0966	0.0966	4.25	*0.057*
Male family	M	15	0.3413	0.0228	1.80	0.103
Female family	M	12	0.2341	0.0195	1.54	0.183
Donor number × female family	M	16	0.4506	0.0282	2.23	0.041
Residual	M	22	0.2783	0.0127		
Total		90	2.2172	0.0246		

The following covariables were examined in initial models: pollen count and *in vitro* pollen germination of focus males, seed set of focus males in single-donor cross, sibling sex ratio of male and female. Residual diagnostics (procedure RCHECK in GenStat) revealed that an analysis of untransformed values fulfilled the assumptions of homoscedasticity and normality. den. = denominator for appropriate *F*-test.

populations). From these results, with the exception of a significant effect for flowering rates, we cannot find any strong evidence for either inbreeding or outbreeding depression.

Offspring sex ratios

Offspring sex ratios were not significantly affected by either male or female population of origin, or by their interaction (Table 2b). Also, male and female sibling sex ratios (initially entered in the model) did not significantly explain variation in offspring sex ratios. However, there was a significant interaction between the number of donors (single- vs. two-donor cross) and female population of origin ($F_{5,16} = 3.4$, $P = 0.028$), indicating that the effect of donor number varied with female population of origin (Fig. 2b): following pollen competition (two-donor crosses), in four populations more females were produced, and in two populations fewer females than following single-donor crosses on the same maternal plant. Moreover, there was also a significant interaction between donor

Table 3 Traits of focus males: *in vitro* pollen germination (% germinated pollen grains out of 100 examined), pollen number per anther and sex ratio (proportion females) in the male family of origin, by population of origin of the male in *Silene latifolia*.

Male population of origin	Male sibling sex ratio	*In vitro* pollen germination	Pollen per anther
SC	0.50 ± 0.10	37.8 ± 6.1	1542 ± 982
VN	0.64 ± 0.09	35.7 ± 12.7	1911 ± 446
GR	0.61 ± 0.06	22.2 ± 12.0	2078 ± 1917
CT	0.46 ± 0.11	17.0 ± 10.6	2234 ± 1814
HO	0.54 ± 0.18	40.9 ± 14.9	2211 ± 1118
PA	0.58 ± 0.16	26.3 ± 18.1	1825 ± 297

number and female family of origin, again indicating at the within-population scale that providing or not conditions of pollen competition affected offspring sex ratios, however, depending on the female genotype. Finally, there was a trend for offspring sex ratios to vary quadratically with the father's *in vitro* pollen germination rate.

Heritability of *in vitro* pollen germination rates

An ANCOVA testing for the correlation of father pollen germination rate on son pollen germination rate, while initially accounting for male population of origin and female population of origin and their interactions, revealed that only female populations of origin significantly differed with respect to son pollen germination rate ($F_{5,41} = 7.23$, $P < 0.001$), whereas across all male populations there was a positive, significant correlation between father and son pollen germination rates ($F_{1,41} = 13.77$, $P < 0.001$; Fig. 3). Overall, the estimated slope coefficient for father/son regression was 0.364 ± 0.096 ($\beta \pm$ SE of estimate). This strongly suggests that pollen germination rate is heritable, and that the maternal parent also affects this trait.

Discussion

Our study reveals a significant genetic variation among male populations and families in paternity success,

Table 4 Offspring fitness and phenotype following intra- and interpopulation crosses in *Silene latifolia*: seed germination rate, number of individuals that flowered out of the individuals that had germinated, age (days) and size at first flowering (number of leaves) by offspring gender.

	Seed germination rate			Flowering rate		
Female/male	SC	VN	GR	SC	VN	GR
SC	0.95 ± 0.06	0.91 ± 0.09	0.88 ± 0.17	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
VN	0.95 ± 0.04	0.78 ± 0.18	0.61 ± 0.30	1.00 ± 0.00	1.00 ± 0.00	0.98 ± 0.05
GR	0.90 ± 0.11	0.78 ± 0.33	0.78 ± 0.18	1.00 ± 0.00	0.97 ± 0.06	1.00 ± 0.00
	CT	HO	PA	CT	HO	PA
CT	0.85 ± 0.06	0.94 ± 0.08	0.83 ± 0.18	0.90 ± 0.16	1.00 ± 0.00	1.00 ± 0.00
HO	0.90 ± 0.20	0.98 ± 0.05	0.85 ± 0.23	1.00 ± 0.00	0.99 ± 0.03	1.00 ± 0.00
PA	0.75 ± 0.00	0.95 ± 0.07	0.58 ± 0.35	0.98 ± 0.03	0.99 ± 0.02	1.00 ± 0.00
	Age at first flowering (daughters)			Size at first flowering (daughters)		
	SC	VN	GR	SC	VN	GR
SC	30.1 ± 1.6	37.3 ± 5.3	31.7 ± 0.8	–	–	–
VN	32.5 ± 4.2	36.9 ± 2.2	33.8 ± 3.2	–	–	–
GR	32.4 ± 1.9	35.4 ± 1.8	32.8 ± 2.7	–	–	–
	CT	HO	PA	CT	HO	PA
CT	27.3 ± 0.7	28.6 ± 0.8	28.4 ± 2.4	73.0 ± 10.7	83.5 ± 8.1	81.3 ± 8.5
HO	30.0 ± 1.1	32.9 ± 1.4	30.8 ± 1.4	76.9 ± 6.5	80.3 ± 12.7	85.1 ± 6.9
PA	30.5 ± 2.0	32.1 ± 1.8	29.0 ± 2.9	79.0 ± 7.4	87.6 ± 6.4	78.1 ± 14.7
	Age at first flowering (sons)			Size at first flowering (sons)		
	SC	VN	GR	SC	VN	GR
SC	33.0 ± 2.4	36.8 ± 2.2	34.6 ± 2.5	–	–	–
VN	34.9 ± 3.1	37.7 ± 1.3	33.9 ± 2.3	–	–	–
GR	32.0 ± 1.5	35.1 ± 3.4	32.9 ± 4.8	–	–	–
	CT	HO	PA	CT	HO	PA
CT	28.6 ± 0.7	33.6 ± 2.7	31.6 ± 1.0	117.2 ± 9.1	128.6 ± 11.9	112.6 ± 12.4
HO	31.4 ± 1.5	34.3 ± 1.7	34.4 ± 2.3	106.5 ± 19.0	113.1 ± 2.1	105.5 ± 15.3
PA	32.8 ± 1.2	34.4 ± 3.3	33.3 ± 2.1	122.7 ± 13.3	112.9 ± 27.2	95.0 ± 16.4

Values are the mean of treatments ± SD.

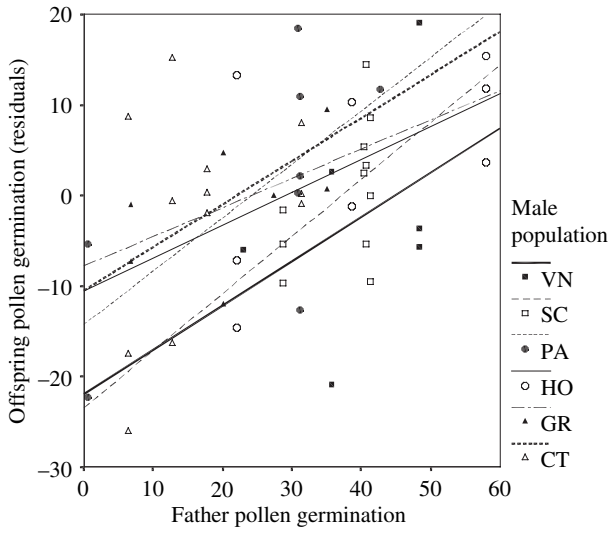

Fig. 3 Father–son regression for *in vitro* pollen germination rate (%). The *y*-axis gives the unstandardized residuals of pollen germination rate of sons after accounting for variation among population of origin of the female (mother).

a correlation of female sibling sex ratio with paternity in between-population crosses, heritable variation for *in vitro* pollen germination rates, and significant interactions between female genotype and donor number in affecting offspring sex ratio and seed set.

Genetic variation among populations for siring success at pollen competition

Male population of origin and male family significantly influenced siring success of focus males against a fixed competitor. As we took care to use F1 males in crosses to avoid confounding by maternal environmental effects, this indicates that male success at pollen competition is nonrandom with respect to genetic variation among males, thus suggesting that in this species, heritable traits exist, which can affect pollen competitive ability (e.g. as in maize, Arthur *et al.*, 2003) or post-pollination events. Several studies have found that individual donors within plant populations differ in seed siring success (e.g. Mitchell & Marshall, 1995; reviewed in Bernasconi, 2003); however, our study also indicates genetic variation between populations, and thus divergence in traits that affect post-pollination fertilization success. Divergence in these traits may result from several processes, including local selection for pollen competitive ability, but also drift, including for Y chromosome variation (Lawson Handley *et al.*, 2006). Divergence among extant populations in *S. latifolia* has also been found for quantitative traits (e.g. life-history traits, Jolivet & Bernasconi, 2007) and sexually dimorphic traits (Delph *et al.*, 2002). An interesting future direction would be to apply QTL mapping techniques to identify factors affecting this variation, and to include information from population parameters such as plant densities and pollinator abundances. As we assessed paternity using DNA from seedlings, differences in paternity shares may also result from differential mortality (including seed abortion) from zygote to seedling (Gilchrist & Partridge, 1997). However, a lack of a paternal population effect on seed germination supports the interpretation that variation in siring success among populations of origin of the pollen donor cannot be ascribed to differential mortality or seed failures.

An analysis of paternity also shows that the focus male, whose pollen was applied 2 h before the competitor's pollen, obtained on average more than 50% paternity, indicating first-male advantage. Alternatively, this may be due to a low performance of pollen from the AL (tester) population, which was also applied second. However, the latter seems unlikely, as in a separate study we found that first-male advantage in *S. latifolia* further increases with longer interpollination intervals (A. Burkhardt & G. Bernasconi, unpublished results). First-male advantage has been reported in other species (*Hibiscus moscheutos* Snow *et al.*, 2000; *Persoonia mollis*, Krauss, 2000), which is consistent with the idea that rapid pollen germination and pollen tube growth, as well as additional mechanisms resulting from variation in the timing of pollen deposition (layering of pollen on the stigma, 'head-start' effects), are likely to play a major role in determining siring success in flowering plants. First-male advantage may also be proximately mediated by post-pollination wilting of the stigmatic surfaces, and may therefore also depend on the female response (Lankinen *et al.*, 2006). It would thus be interesting in future studies to investigate whether male and female genotypes affect pollen precedence and how rapidly it increases with interpollination interval.

There was no consistent pattern for either local (home) or foreign (away) male advantage. Local males sired a higher share of offspring than foreign males against a fixed competitor in four of six possible combinations, with no overall significant effect. This suggests that the degree of reproductive divergence among populations varies for different pairs of populations, including the population of the tester male. The combinations where local males were more successful may reflect greater degree of reproductive isolation between pairs of populations. In *Turnera ulmifolia*, in a study competing self-pollen against pollen from within- vs. between-populations, self-pollen had a strong advantage against pollen from foreign populations, and the extent of this advantage was correlated with increasing morphological divergence between populations (Baker & Shore, 1995), suggesting reproductive isolation through pollen competition in that species. Moreover, the outcome of pollen competition may not only depend on interactions of the focus male with the female, but also on interactions between competing males. We used a fixed tester male, which was always from the same independent foreign population, but in future studies it would be interesting

to explore variation in this third component by including also crosses against foreign vs. local competitors.

Among the tested covariables, the sex ratio in the female family of origin was strongly significantly associated with the paternity success of the focus male, when male and female stemmed from different populations. This suggests a specific influence of female genotype on paternity, if sibling sex ratio is associated with heritable traits, which may affect pollen performance (or compatibility) during pollen/style interactions, at fertilization, or paternal/maternal interactions during seed development (Waser & Price, 1991; Huck *et al.*, 2003; Hedhly *et al.*, 2005; Glaettli *et al.*, 2006; see also below). None of the other covariables, including pollen germination rate *in vitro*, pollen count per anther, seed set of focus males in single-donor cross, and male sibling sex ratio significantly explained variation in paternity success. In our crosses, we used a constant pollen load of four anthers. Our estimates of pollen grain number per anther indicate that on average we deposited around 6000 pollen grains on each flower. Although the exact number of pollen grains from each donor may have varied, our pollen loads provided conditions of intense pollen competition because in *S. latifolia*, female flowers contain 200–350 ovules (leading to a pollen/ovule ratio of approximately 20–30). In *Raphanus sativus*, experimentally varying pollen load size did not affect the proportion of seeds fathered by different donors, i.e. the relative siring success of different donors was constant across pollen load sizes (Marshall *et al.*, 2000). Thus, further studies are needed to determine which forces shape local selection for pollen competitive ability, and to what extent divergence is due to drift or population history, or selection on sporophytic traits (e.g. drought resistance, or attractiveness to pollinators; Shykoff & Bucheli, 1995; Delph *et al.*, 2005) which may lead to correlated responses in gametophytic traits.

Female and offspring fitness in relation to pollen competition, male siring success and parental population of origin

Seed set was significantly affected by the female's, but not the male's, population of origin. As maternal plants were a greenhouse-reared F1 generation, this suggests genetic variation among populations in this trait. Moreover, depending on female family, providing conditions of pollen competition affected seed set. As total pollen load was constant, this comparison refers directly to the effect of having higher genetic diversity (two donors) in the pollen load. However, in both treatments the pollen load exceeded the number of available ovules (see above), and effects may be stronger for lower pollen loads, or a larger range of donor numbers (Paschke *et al.*, 2002; Bernasconi *et al.*, 2003; Vergnerie, 2006). Indeed, in natural populations seeds within fruits are sired on average by around four, and up to nine, different donors (S. Teixeira & G. Bernasconi, unpublished manuscript).

There was no significant correlation between paternity share of the focus male and offspring fitness and total seed mass. A negative correlation might be expected under sexually antagonistic coevolution, because males are selected to evolve traits that increase siring success even at a cost to female fitness (Pizzari & Snook, 2003; Arnqvist & Rowe, 2005). However, lack of evidence does not demonstrate lack of effect, and possibly under natural conditions (e.g. variable pollen loads and stochastic pollinator visitation patterns) such costs to female and offspring fitness may become apparent. In particular, we do not know how pollen loads used in our experimental crosses compare with those naturally deposited on plants. Indeed, *S. latifolia* populations can be small and isolated, reducing the chances of obtaining pollen (Richards, 2000). One potential source of conflict, which should be investigated in future studies by experimentally varying pollen loads, is post-pollination reduction of female receptivity, which may lead to pollen limitation and thus reduce female fitness while ensuring high paternity for the male (Lankinen *et al.*, 2006).

Our experiment did not reveal either out- or inbreeding depression. There was no significant difference when comparing within-/between-population crosses for most offspring fitness traits, except for offspring flowering rates. This variable only varied slightly, so that single observations may have had a strong influence, and therefore we are cautious in deriving conclusions from this effect. Germination rate varied with female population of origin, which may reflect different genetic loads among populations (Willi & Fischer, 2005). Our study populations were relatively large and had a high molecular variation (Jolivet & Bernasconi, 2007), thus inbreeding depression might be apparent only in offspring of closely related individuals, in less benign conditions, or in populations with lower genetic variability (Richards, 2000). Indeed, pollen competition can also provide conditions for inbreeding avoidance (Waser & Price, 1993; Souto *et al.*, 2002; Bernasconi *et al.*, 2004; Glaettli *et al.*, 2006). Studies in other species found evidence for optimal crossing distances (Waser & Price, 1989; Waser *et al.*, 2000). Interestingly, offspring sex ratios varied with the number of donors (i.e. single- vs. two-donor crosses); however, this effect depended on female population of origin. Similarly, a previous study found that males with extreme sex ratio phenotype (producing 80–100% female progeny) had reduced siring ability in pollen competition (Taylor *et al.*, 1999). Our results indicate that maternally inherited determinants interact with donor number in determining offspring sex ratio.

Heritability of *in vitro* pollen germination rate

We found evidence that *in vitro* pollen germination rate is heritable in *S. latifolia*, as indicated by a significant positive father-son regression. This correlation was

estimated for offspring arisen in two-donor crosses (but including only sons sired by the focus male, as indicated by paternity analysis). As we first reared all plants for one generation in the greenhouse (see Fig. 1), all parents had experienced standardized conditions, thus reducing or eliminating parental (paternal or maternal) environmental effects. In *Viola tricolor*, *in vitro* pollen growth rate was heritable, but in addition also significantly correlated with siring success (Skogsmyr & Lankinen, 2000). In *Betula pendula*, siring success was consistent among recipient plants and positively correlated with both *in vivo* and *in vitro* pollen tube growth rate (Pasonen *et al.*, 1999). Because of the overlap in gene expression between the gametophytic and sporophytic phases in plants, pollen performance may correlate with sporophytic traits. As a result, pollen competition may result in offspring of higher quality (Bernasconi *et al.*, 2004), or higher pollen competitive ability (Bernasconi & Keller, 2001). However, we do not know whether in *S. latifolia* *in vitro* pollen germination rates correlate with pollen germination and pollen tube growth rate *in vivo*. For instance, in *Prunus avium*, *in vitro* pollen germination rates were not always a good predictor of *in vivo* pollen germination (Hedhly *et al.*, 2005). Indeed, the female sporophytic tissue may also influence both pollen germination (e.g. inhibition of pollen germination through pH-dependent stigma proteins, Ganeshaiah & Shaanker, 1988) and pollen tube growth, a heterotrophic process that requires at least partly resources from the style. Interestingly, female population of origin also significantly affected pollen germination rate of sons. This suggests maternally inherited determinants of pollen competitive ability, if pollen germination under some circumstances (e.g. simultaneous arrival of pollen of different donors on the stigma) influences paternity success. Importantly, a significant maternal influence implies that variation in pollen germination rate is not merely due to degeneration and drift at the Y chromosome.

In conclusion, we find significant among-population genetic variation for siring success at pollen competition. In particular, the male population of origin had a strong effect on paternity shares of the two competing males, with female effects being apparent only as a correlation with female sibling sex ratio in between-population crosses. The intensity of pollen competition and the costs/benefits of attracting pollinators in this species are likely to depend also on additional factors, including: (i) biotic interactions, e.g. with the seed predator *Hadena bicruris*, and the pathogenic fungus *Microbotryum violaceum* (Biere *et al.*, 2002); and (ii) variation in population characteristics, pollinator abundance and patterns of flowering phenology of males and females. Thus, in future studies it would be interesting to address whether among-population variation in the prevalence of interspecific antagonists and in pollen competition intensity constrain or promote reproductive divergence among populations via sex-specific traits affecting fertilization success and post-pollination selection.

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