Genetic structure and post-pollination selection in biennal plants

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Chapter 5

Cryptic self-incompatibility in *Echium vulgare* (Boraginaceae)

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ABSTRACT

The concept of cryptic self-incompatibility (CSI) is appealing to many researchers, although it is still unclear whether or not it is a common phenomenon. We studied CSI in *Echium vulgare*, which shows low selfing rates in the field despite being self-compatible. Twenty genotypes, combined in 10 pairs were used for 3 pollination treatments: self-pollination, outcrossing (reciprocal cross within each pair) and pollination with mix pollen from both donors. A sample of 10 seeds per plant from the mix pollination treatment was genotyped with microsatellite loci. No effects of selection against selfing overall 20 genotypes were found although for 2 genotypes we found significant CSI. We detected maternal and paternal effects on pollen tube growth and maternal effects on pollen germination. However, there were no significant differences in pollen germination and growth between self and outcross pollen averaged overall 20 genotypes. Pollen tube growth and germination in the two genotypes that showed CSI were not different from that in plants that did not show CSI. Therefore, we found no evidence that CSI in *E. vulgare* is due to pre-zygotic mechanisms.

INTRODUCTION

Inbreeding depression is believed to select for adaptations that reduce self-fertilisation like allelic self-incompatibility, and temporal and spatial separation of anthers and stigmas. However, the adaptive value of such selection mechanisms diminishes when available outcross pollen is limiting seed set. Therefore, a combination of inbreeding depression and pollen limitation should lead to the evolution of mating strategies that allow for flexible adjustment of the level of selfing. One such mechanism is cryptic self-incompatibility (CSI, Bateman, 1956): self-pollination results in full seed set when only self pollen is available but the success of self-pollen is strongly reduced when it competes with outcross pollen. Therefore, by definition, CSI can only be shown if results from single donor and mixed pollinations are compared.

Since Bateman's study (1956), the concept of CSI received a lot of attention and is still proposed as a possible explanation for low selfing rates in fully self-compatible plants (Galloway et al., 2003; Hammerli and Reusch, 2003). Apparently, the idea is very appealing although, as we will show further, there is little reason to assume that CSI is a mechanism widespread among the plant species.

Traditionally, CSI has been tested by applying equal proportions of self and outcross pollen and performing paternity analysis of offspring. Results were then compared with results from single donor experiments or as a null hypothesis equal success of self-pollen and outcross pollen was assumed. Over-representation of outcrossed offspring resulting from mixed pollination with this method has been found in 4 studies (Bateman, 1956; Bowman, 1987; Jones, 1994; Weller and Ornduff, 1977). Five studies did not find such over-representation (Baker and Shore, 1995; Johnston, 1993; Montalvo, 1992; Pound et al., 2003; Travers and Mazer, 2000) and 2 studies found it only for a part of the maternal genotypes used in pollinations (Rigney et al., 1993; Sork and Schemske, 1992)

Pollen tube growth observations were also used as a method of detecting CSI. Lower success of self-pollinations can be caused by slower pollen tube growth or more

frequent attrition of self-pollen tubes compared to outcross ones. Such differences in pollen performance were detected in 5 species (Aizen et al., 1990; Cruzan, 1989; Eckert and Allen, 1997; Hessing, 1989; Weller and Ornduff, 1989), in 3 other species no such differences were found (Casper, 1985; Fenster and Sork, 1988; Ortega-Olivencia et al., 1998) and in one species different results were observed depending on maternal genotype (Snow and Spira, 1991)

The experimental design in some studies that report CSI can be questioned because of three reasons. Firstly, the number of genotypes examined was very low (1-3 genotypes) in some studies (e.g., Aizen et al., 1990; Bateman, 1956; Sork and Schemske, 1992). Such a small sample may not be representative for the population, because individuals may differ with the intensity of CSI. Secondly, Travers and Mazer (2000) pointed out that the use of morphological markers for paternity analysis limits the possibility to draw conclusions on CSI in the species because only recessive homozygotes are used as pollen recipients (e.g. Bowman, 1987; Jones, 1994 used this method). Under-representation of selfed offspring may then be a result of selection against pollen baring recessive alleles and the results can not be generalised to the whole species. Thirdly, the examples from heterostylous species (e.g. *Decodon verticillatus* - Eckert and Allen, 1997; *Amsinckia grandiflora* - Weller and Ornduff, 1989) should not be considered together with non-heterostylous ones. In heterostylous species, the flower morphs may differ in e.g. pollen size, papillae length on the stigmatic surface (Richards, 1997) and therefore, self(illegitimate)-pollen may have more disadvantages than only a slower growth of pollen tubes compared to outcross pollen.

To sum up, there is a need to prove CSI using many genotypes of non-heterostylous species and paternity analysis done with molecular markers independent of morphological characters. In this study, we describe effects of CSI in non-heterostylous *Echium vulgare.* We performed single and mixed pollen donor pollinations in combination with the paternity analysis. We used 20 genotypes for the crosses and for the paternity analysis we used microsatellite loci. The aim of this study is to answer the following questions: 1) Is there a selection against selfing in *E. vulgare*? 2) If so, is this due to CSI? 3) Does pollen germination or pollen tube growth cause CSI? 4) Does CSI improve offspring quality measured as seed mass and seed germination?

MATERIALS AND METHODS

Study species

Echium vulgare (viper's bugloss) is a self-compatible, hermaphroditic biennial, pollinated by bumblebees. Spontaneous selfing in this species is rare, but bumblebees can cause self-pollination while moving from one flower to another within the plant (geitonogamy). Experiments in which flowers of *E. vulgare* were hand pollinated with either self or outcross pollen showed that averaged over 10 genotypes, self-pollination results in as many seeds as outcrossing, although some genotypes produced more seeds after outcrossing and others after selfing (Melser et al., 1997). Rademaker et al. (1999) predicted the selfing rates for *E. vulgare* in the field based on pollen dynamics and pollinator behaviour. In their model they assumed that self and outcross pollen have the

same siring success, and that the selfing rate depends only on the proportion of the self-pollen on the stigma. Model predictions were compared with selfing rates measured by means of RAPDs. The measured selfing rates ranged from 0 to 30 % what is only half or less of the theoretical prediction. One possible explanation for this discrepancy can be the occurrence of cryptic self-incompatibility. We expect that selection for CSI may operate in natural populations of *E. vulgare* because it is favoured by the fact that inbreeding depression affects survival and reproductive success (Melser, 2001, chapter 7; Melser et al., 1999).

Collecting and growing the plants.

In April 2002, large rosette plants that were likely to flower the same year were collected in the dune area of Meijndel. The original location of each plant was marked on a map. The plants were potted in 4.5 l pots filled with dune sand and then placed in an experimental garden. On May 12, they were sprayed with insecticide Decis® (1 ml in 1 l water) and the next day they were transported to a climate room (humidity 70%, 16 h light 20^{o}C, 8 h dark 15^{o}C from 11pm till 7 am). Spraying with Decis® was repeated twice within two weeks. From the end of May onwards, each plant was provided with a small amount (25ml per week) of nutrient solution (Steiner, 1986). This amount doubled in July because the plants showed symptoms of nutrient deficiency. We gave the plants minimum amounts of nutrients to stimulate abortion rates as high as in the natural environment. In order to prevent unplanned pollinations, we sprayed plants to remove infections of aphids and gall mites (spraying once with 1% Savona, once with 0.5 g/l Pirimor and twice with 0.045% Torque L.). The spraying did not have any visible effects on the condition of the plants. First flowers opened in the beginning of June. The pollinations were applied from July 1, onwards.

Choosing pairs of plants before pollination.

We screened the microsatellite patterns in 30 flowering plants in order to combine at least 20 of them in pairs in such a way that two plants from each pair do not share any alleles in at least one microsatellite locus. The advantage of such an experimental design is that the paternity analysis of seeds from the mixed pollination treatment for each pair can be done with only one microsatellite locus. We extracted DNA from flowering plants and genotyped it with 6 microsatellite loci, five of which (*E3-40, E3-46, E3-56, E3-84, E3-91*) were already characterised by Korbecka et al. (2003). The PCR for the sixth locus: *E2-64* was carried out with the same concentration of reagents and PCR programme but the locus specific annealing temperature in the first 20 cycles was 60^{o}C. The sequences of the primers of locus *E2-64* were:

-forward primer GGAGCTGTGAAGCCAATGAG
-reverse primer ATTTTGCGAACAAGCGGTAG

The forward primer was labelled with Tamra label. This locus contains GA repeats and the PCRs with the designed primers result in fragments of length: 120-150 bp.

After successful genotyping we not only combined 20 flowering plants in pairs but we also calculated multilocus heterozygosity for every experimental plant. We measured the physical distance in the field between the plants from each pair. This distance varied between 40 – 675 meters. We decided to take 10 seeds after mixed pollination from every plant for paternity analysis. Although, this number is rather low, our preference was to genotype a small number of offspring from each of many

genotypes rather than a large number of offspring from each of a few genotypes. We made this choice because (sub)populations may differ in the intensity of CSI, as suggested by Travers and Mazer (2000).

Pollinations

Flowers with a receptive stigma were marked on each plant. Receptive stigmas are characterised by longer styles than anthers and spreading of the stygmatic lobes. All the pollinated flowers were in the middle of the cymes. Flowers were randomly assigned to one of 3 treatments:

- self-pollination (with pollen from another flower of the same plant),
- outcrossing (with pollen from the other plant in a pair),
- mixed pollination (pollen from both plants mixed together and applied to both plants).

After applying pollen to a flower, its sepals were marked with coloured paint coding for one of the three pollination treatments, and the position of the flower (number of stem and number of a cyme) and the time of pollination were noted to identify the pollinated flower. A toothpick with the end covered with parafilm was used for applying pollen. In single donor pollinations the pollen was applied straight from the flowers. In the mix pollination treatment we first mixed the pollen from two plants together on a glass plate. Anthers were taken apart with a toothpick to release pollen. We used five flowers from each plant to make a mix. One anther from each of these five flowers was preserved for later pollen counting, to estimate how many pollen grains each plant contributed to the mixture. Pollinations were divided over 2-7 sessions for each pair of plants. Number of flowers used for each pollination type varied among plants. On average, 36.6 flowers (min.: 16) were used for single donor pollinations and 33.2 flowers (min.: 15) for mix pollinations.

Because pollination techniques differed between single donor and mix pollinations we compared the number of pollen tubes that started to germinate inside the stigma within the first 5 hours after pollination for both pollination types. Average number of pollen tubes for single donor pollination was 5.98 and for mix pollination - 5.49. This difference was not significant (paired samples t-test: $t = -0.854$, $df = 19$, $p = 0.404$), therefore we conclude that the pollination techniques for both pollination types were equally effective.

Seeds were harvested after they ripened. All seeds from single pollen donor pollinations and a sample of 10 seeds per plant from mixed pollinations were weighed. Seeds from mixed pollination were germinated and used for paternity analysis.

Pollen counting

The five anthers from each plant that were preserved for pollen counting were put together in an eppendorff in 1 ml of 95% ethanol and stored at 4^{o}C. One day before counting, eppendorffs were kept in a sonication bath for 1 minute to disrupt the walls of the anthers and free the pollen. Then the ethanol was evaporated slowly at 25-30^{o}C and the pollen was re-suspended by vortexing in 0.1 ml of mixture consisting of 20% glycerol, 20% sucrose and 60% water. Then a drop of the pollen suspension was applied to a counting chamber and the number of pollen grains was counted under the light microscope in a grid with a volume of 25×10^{-5} mm^3. Counts for each eppendorff were replicated four times. Average number of viable pollen grains per grid varied

widely among genotypes (range: 42.0-262.5, average: 153.53). The reliability of the pollen counting with this method was demonstrated by the average coefficient of variation for 4 replicate counts per eppendorff that equalled 12.81% (range: 0.92 and 37.36 %). Collapsed pollen grains were not counted as Melser et al. (1997) showed that they are not viable. Average counts of viable pollen were used to calculate a proportion of self-pollen in the mix.

Pollen tube growth measurement

On average, a sample of 4 styles per plant from each pollination treatment was preserved for later measurement of pollen tube growth. The stigmas from these flowers were fixed in a mix of ethanol and acetic acid (4:1) five hours after pollination. The fixation lasted 1 hour and was followed by transfer of the stigmas into 70% ethanol for storage and later pollen tube. Then the stigmas were stained in aniline blue and the length of the pollen tube was measured under a fluorescent microscope according to Martin (1959). We couldn't measure the length of the pollen tubes that grew below half of the length of the style because they were difficult to distinguish from structural parts of the stigma. Instead, we counted the number of pollen tubes that started to germinate from the surface of the stigma (this number is later used as a measure of pollen germination) and the number of pollen tubes at 0.65 mm from the tip of stigmatic lobe. We used the ratio of these two counts as a measure of pollen tube growth rate (pollen tube number ratio). We tested for paternal and maternal effects on pollen germination and pollen tube growth. To test for paternal effects, we correlated the success of the pollen of each plant when used as self-pollen vs. when used as outcross pollen. Then, to test for the maternal effects, we correlated the success of self-pollen and outcross pollen applied to the same plant.

We assume that we pollinated the flowers with sufficient amounts of pollen for the two reasons: 1) we weren't able to distinguish and count individual pollen grains on the surface of the majority of the stigmas, because the density was too high. 2) the seed set in this experiment (Fig. 1) was equal or higher than seed set of plants in the field for which Klinkhamer *et al.* (1994) have shown that they are not pollen limited. In their field experiment the number of seeds per flower varied from 1.50-1.75 in the beginning of the flowering season to 0.45-0.55 at the end of the flowering season.

Only few styles (6 out of 266 observed styles) that didn't have any pollen tubes and didn't have any pollen on the surface of stigma were probably unripe and were excluded from analysis.

Germination of seeds and paternity analysis

In December 2002, we germinated the seeds for paternity analysis. The 200 seeds were weighed and placed on wet filter paper. Within one week 60% of the seeds germinated. The next 4 days the germination rate didn't increase anymore. We recorded which seeds germinated spontaneously and we added 3 ml of giberelic acid solution (1 mg/ml, Sigma) per Petri dish to stimulate germination of the remaining seeds. This treatment increased the germination rate to 86%. Seeds that didn't germinate were pilled from the seed coat and frozen in –80^{o}C for latter DNA extraction. Two seeds appeared to be empty and four seeds were lost during germination or pilling. The seedlings were grown for one moth and then frozen as well. One seedling died and no material was recovered for DNA extraction. The DNA extraction was carried out as

described by Korbecka et al. (2003). We did only one PCR per seedling with the selected locus. The genotyping of seedlings was done in two separate labs. Therefore, for half of the seedlings PCR conditions were exactly the same as the parental genotypes. For the other half we used a different concentration and type of Taq polymerase (0.4 units per reaction, Bioline), a different PCR machine (PTC-100 programmable thermocycler, MJ Research) and sequencer (capillary sequencer ABI 310). We took 3 samples per locus for analysis in both labs and found the same results.

Statistical analysis

We used t-test (SPSS v. 10.0) for pairwise effects of: self-pollination vs. outcrossing and single donor vs. mix pollinations for seed set, seed mass, pollen tube number ratio and pollen germination. The differences between paired variables were normally distributed (one sample Kolmogorov-Smirnov test).

Differences between the number of selfed and outcrossed offspring from the mix pollinations were tested over all 20 genotypes using χ^2 test. We tested against a 50:50 expected ratio because every plant was used as pollen donor as pollen recipient. χ^2 tests for each of the maternal genotype individually were done using the expected ratio based on the proportion of self and outcross pollen in the mix. To account for the number of comparisons, significance levels were adjusted using improved Bonferroni correction (Hochberg, 1988).

RESULTS

Is there selection against selfing in E. vulgare?

Averaged over all 20 maternal plants, selfing resulted in 1.91 seeds per flower (SD = 0.64) and outcrossing in 2.04 seeds per flower (SD = 0.60) after single donor pollinations (Paired samples t-test: $t = -1.171$, $df = 19$, $p = 0.256$). Some plants have a higher number of seeds per flower after selfing, others after outcrossing, but this was significant only for two plants (Fig. 1).

Among 192 seeds sampled from the mixed pollination treatment, 88 were selfed and 104 were outcrossed ($\chi^2 = 1.33$, $df = 1$, $p = 0.248$, one-sided test). Therefore, averaged over all 20 genotypes we didn't find significant selection against selfing. However, for two maternal plants the observed number of selfed offspring was significantly lower than expected (Fig. 2, plants no.: 65 and 14). After improved Bonferroni correction the difference only remains significant for plant 65 ($p = 0.00024$, $\alpha = 0.0025$, $p < \alpha$)

Seventeen out of all 20 plants used in the experiment (including plant 14) were heterozygous in all 6 microsatellite loci. Plant 65 had the lowest heterozygosity of all plants (it was homozygous in 2 loci). No statistics can be calculated to link the heterozygosity to the selection against selfing, because the latter was detected only for two plants.

Is the selection against selfing due to CSI?

To test whether selection against selfing was stronger after mix than after single donor pollination, we used the results on seed set after self-pollination and outcrossing to

calculate the expected number of selfed seeds after mixed pollination. The expected number of selfed offspring was 4.42 and 5.60 respectively for plant 65 and 14. For both plants, the observed number of selfed seeds after mixed pollination was significantly lower than expected (plant 65: $\chi^2 = 7.92$, $p = 0.0029$; plant 14: $\chi^2 = 7.36$, $p = 0.0077$). Therefore, selection against selfing that was detected in 2 out of 20 maternal plants can be described as CSI.

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Figure 1 Seed set after single donor pollinations: number of seeds per flower (+SE) in 20 experimental plants after selfing and outcrossing.
*****- significant difference in seed set between the pollination treatments (two sample Mann-Whitney test, $p<0.05$)

Figure 2 Results of paternity analysis of seeds from the mix donor pollination. The observed number of selfed offspring is determined by paternity analysis. The expected number of selfed seeds is calculated on bases of the proportion of self-pollen in the pollen mixture applied, multiplied by the number of seeds genotyped. The number of seeds genotyped equals 10, except plants no.: 9, 14, 26, 73, 77 which had 9 seeds genotyped and plant no.: 28 which had 7 seeds genotyped.
*-observed number of selfed offspring is significantly lower than expected
($\chi^2 = 5.998$, $df = 1$, $p = 0.014$),
**- observed number of selfed offspring is significantly lower than expected ($\chi^2 = 12.99$, $df = 1$, $p < 0.001$), the difference remains significant after improved Bonferroni correction.

Fig. 1 Seed set after single donor pollinations

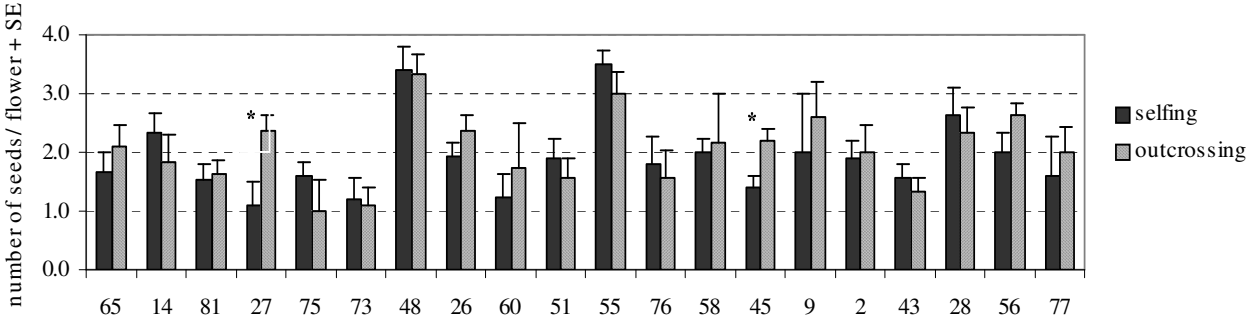
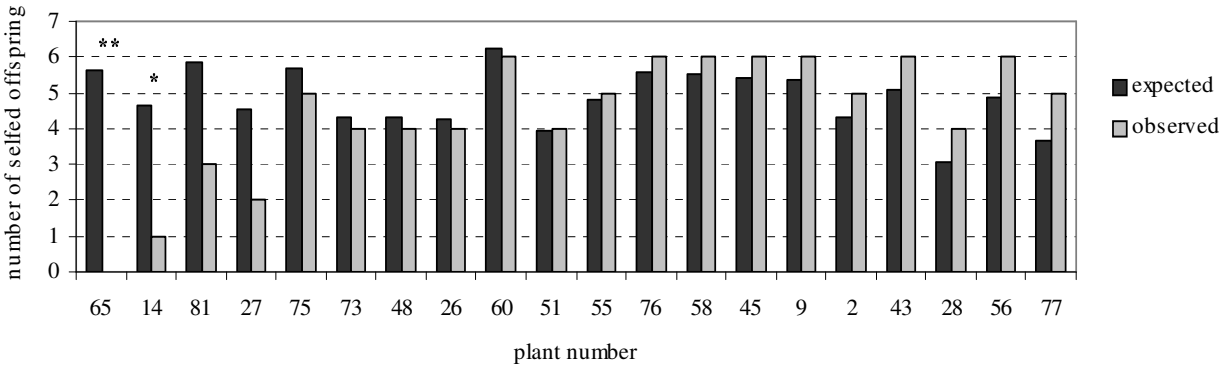


Fig. 2 Results of paternity analysis of seeds from mix donor pollination



Is the mechanism of CSI pre-zygotic?

We found maternal effects on pollen germination and pollen tube growth and paternal effects on pollen tube growth (Fig. 3). Over all 20 genotypes, there was no difference in pollen germination between self and outcross pollen (paired samples t-test: t = 0.749, df = 19, p = 0.463) and no difference in pollen tube growth (paired samples t-test: t = -0.921, df = 19, p = 0.369). On the basis of pollen tube growth data for the two plants showing CSI (Fig. 3B), we conclude that the mechanism of CSI is not likely to be pre-zygotic. If the difference in pollen tube growth was to explain the proportion of selfed offspring after mix pollination, we would expect for the two plants over-representation rather then under-representation of selfed offspring.

Is the quality of selfed-offspring lower?

Seed masses of selfed and outcrossed seeds from single donor pollinations did not differ significantly for 20 maternal plants (paired samples t-test: t = -0.405, df = 19, p = 0.690). Average seed mass of selfed seeds was 3.46 mg (SD = 0.47) and average mass of outcrossed seeds: 3.50 mg (SD = 0.44)

Selfed and outcrossed seeds from mix pollination did not differ in seed mass either (paired samples t-test: t = 0.209, df = 18, p = 0.837). Average seed mass of selfed seeds from this pollination treatment was 3.45 mg (SD = 0.63) and average seed mass of outcross seed was 3.44 mg (SD = 0.64). However, selfed seeds from mix pollinations had a significantly lower germination rate compared to outcrossed seeds (selfed seeds: 52.3 %, outcrossed seeds: 68.3 %, $\chi^2 = 5.1241$, df = 1, p = 0.0236).

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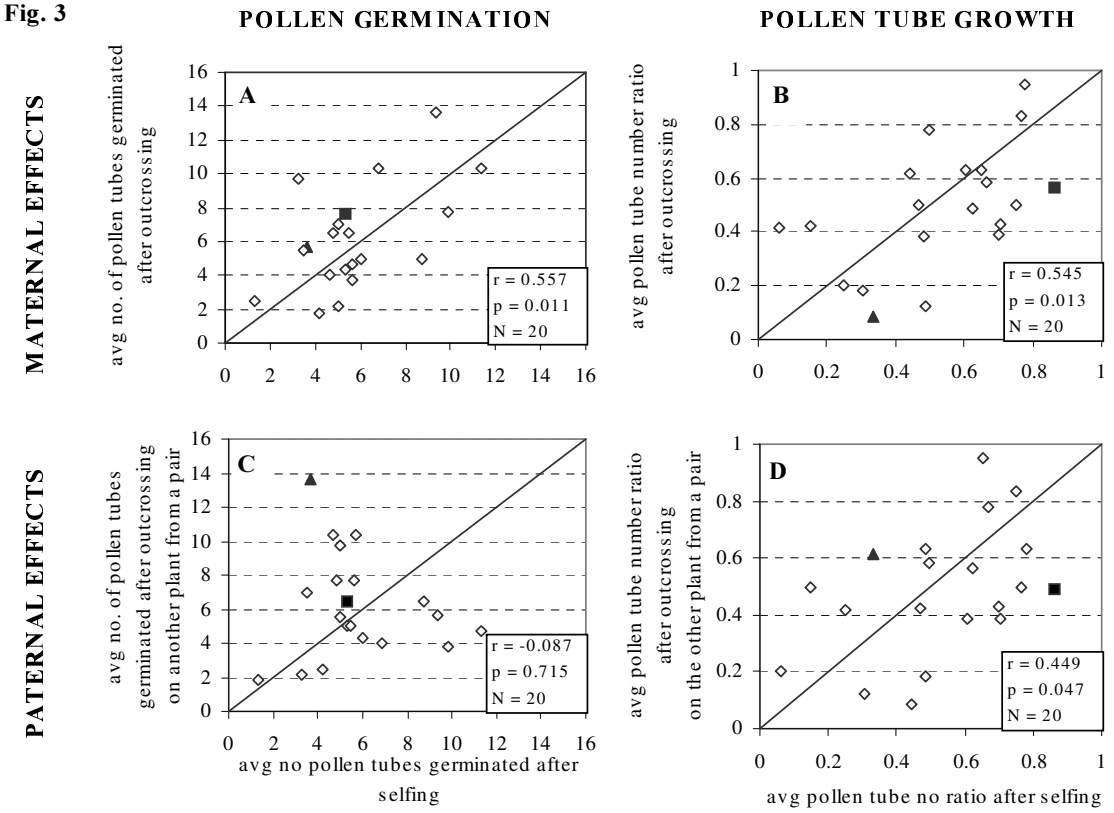
Figure 3 Maternal and paternal effects in pollen germination and pollen tube growth.
A: The relationship between the number germinated pollen tubes on stigmas of the same mother plant after self-pollination and outcrossing.
B: The relationship between the pollen tube number ratio of the same mother plant after self-pollination and outcrossing.
C: The relationship between the number germinated pollen tubes on stigmas after self-pollination and outcrossing with pollen from the same donor.
D: The relationship between the pollen tube number ratio after selfing and outcrossing with pollen from the same donor.
Pollen germination was measured as number of pollen tubes that germinated within 5 hours after pollination.
Pollen tube growth was measured as the ratio of number of pollen tubes that reached the distance 0.65-mm within 5 hours after pollination divided by the total number of germinated pollen tubes within this time.
In all four graphs we indicated data for two plants showing CSI: plant 14 was marked as ▲ and plant 65 as ■. To aid a visual comparison of success of self-pollen vs. outcross pollen we drew the lines y = x.

Fig. 3


POLLEN GERMINATION
POLLEN TUBE GROWTH
MATERNAL EFFECTS
PATERNAL EFFECTS
A
B
C
D
avg no. of pollen tubes germinated after outcrossing
avg pollen tube number ratio after outcrossing
avg no. of pollen tubes germinated after outcrossing on another plant from a pair
avg pollen tube number ratio after outcrossing on the other plant from a pair
avg no pollen tubes germinated after selfing
avg pollen tube no ratio after selfing
r = 0.557
p = 0.011
N = 20
r = 0.545
p = 0.013
N = 20
r = -0.087
p = 0.715
N = 20
r = 0.449
p = 0.047
N = 20

DISCUSSION

Our results show that CSI occurs in *E. vulgare*. However, not in all genotypes. We detected CSI in 2 out of 20 plants. Our experiment was designed to detect general patterns of selection against selfing over all 20 genotypes and we used a low number of seed for paternity analysis for each genotype. Therefore, the power of the χ^2 tests per genotype was rather low and this may have led to an underestimation of the number of genotypes where CSI may play a role. A visual examination of Fig.1 and 2 shows however, that it can be expected (even with higher numbers) only in 3 genotypes at most. We therefore do not think that CSI can explain low selfing rates found in the field.

The 20 genotypes were sampled over a large area and may come from different subpopulations. In a study of the genetic structure in one *E. vulgare* population, we detected isolation by distance within an area 6 x 20 meters. Plants growing with 1-2 meters are related to each other (chapter 4 in this thesis). Therefore, it is likely that experimental plants come from different subpopulations, which may differ in genetic load and outcross-pollen availability. It is possible that CSI differs among subpopulations and locally occurs at high frequencies. At least three studies of CSI are in line with our results. Travers and Mazer (2000) applied equal proportions of selfed and outcrossed pollen trying to reproduce results of Bowman (1987) in *Clarkia unguiculata*. They used allozymes instead of morphological characters for paternity analysis. In contrast to Bowman's report (1987), they couldn't find any evidence for CSI. One of their explanation was that populations differ in the intensity of CSI and they may have selected the population where pollen limitation doesn't play a role or that the plants do not have a genetic load high enough to select against selfing. In another CSI study, Rigney et al. (1993) performed pollinations in *Erythronium grandiflorum* using self and outcrossed pollen mixed in equal proportions. Then they sampled one fruit per plant and found a bimodal distribution of the percentage of outcrossed seeds per fruit. Twenty-two % of fruits had only selfed seeds and 46% fruits only outcrossed ones. These results suggest that selection against selfing is not equal for all the plants from the same population. In the third study, Snow and Spira (1991) showed in *Hibiscus moscheutos* that relative pollen tube growth rate correlates with the proportion of seeds sired by a certain pollen donor. However, out of 16 crosses, in 5 self-pollen grew slower than outcross pollen, in 4 it grew faster and in 7 cases the difference was not significant. This clearly indicates that intensity of CSI may differ among individuals. These differences may coincide with differences in genetic load. Therefore, we performed both single and mix donor pollinations to test if in individual plants the selection against selfing was stronger after mixed pollination. In some CSI studies, this comparison is not made or the same average inbreeding depression is assumed for all experimental plants. These methodological flaws may be a reason why in some cases CSI was not found (Johnston, 1993).

The mechanism of CSI

We weren't able to determine whether CSI in *E. vulgare* occurs in the pre- or post-zygotic stage. We didn't find any significant differences in the performance of self and outcross pollen in the styles preserved five hours after pollination. However, to rule out completely the possibility that CSI is due to pre fertilisation selection against selfing,

we would have to conduct a more detailed observation of pollen tube growth until fertilisation takes place. In some species, self-incompatibility reactions are visible only when self-pollen tubes enter the ovules (Waser and Prince 1991). Although the majority of studies attribute CSI to slower pollen tube growth of the self-pollen compared to outcross pollen (Eckert and Allen, 1997; e.g. Weller and Ornduff, 1977), a post-zygotic mechanism is also possible. In conditions where embryos have to compete for limited resources provided by the mother, selective abortion of selfed embryos can lower the percentage of selfed progeny. Moreover, a combination of pre-and post-zygotic mechanisms leading to CSI may occur, since both mechanisms have been detected in *E. grandiflorum* (Cruzan, 1989; Rigney, 1995). In *E. vulgare* evidence for selective embryo abortion was found by Melser (2001, chapter 7), who showed that offspring produced in a period with high embryo abortion survives as seedlings compared to offspring from a period with low embryo abortion

The importance of CSI

Traditionally, the importance of CSI has been emphasised under conditions of pollen limitation, because if no outcross pollen is available, bad selfed offspring is better than none. However, even if pollen is not limited, CSI may have an advantage over SI. If selection eliminates pollen and/or embryos with deleterious mutations, such selection may not be detected after single donor self-pollination if it doesn't affect seed numbers. However, when self-pollen or selfed embryos have to cope with better competitors (outcross pollen/outcross embryos), selection against selfing is stronger and it is detected because it most likely reduces the number of selfed offspring. If, either by chance or because the maternal plant has a low genetic load, a selfed offspring has good quality it can still win competition. Such selfed offspring has the advantage of passing two copies of the genome to the next generation (with SI such advantage would be lost). We expect CSI to differ among individuals depending on their genetic load. *E. vulgare* supports this explanation, because there is no pollen limitation in the dune population (Klinkhamer et al., 1994) and, as shown above, CSI is present only in few genotypes.

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APPENDIX

Is the inheritance in *E. vulgare* disomic or tetrasomic?

Paternity analysis of crosses between pairs of plants where one of the parents had 4 different alleles have shown that tetrasomic inheritance is likely to occur in *E. vulgare*. In the tables below you can see that allel a (177 bp) in locus *E3-40* is inherited by the

offspring in combination with every other allel out of the 3 alleles present in the parent 77 (Tab.1) or parent 51 (Tab. 2). Moreover, one seed inherited double copy of one of the 4 alleles present in the parent (seed no 7, Tab. 2), suggesting that double reduction took place (Darlington, 1929; Mather, 1936; Winton and Haldane, 1931).

Tab. 1
Alleles detected in microsatellite locus *E3-40* in outcrossed offspring coming from a pollination with a mixture of pollen from plants 77 and 9. Plant 77 had alleles a, b, c, f and plant 9 - alleles d, e.

		Allele lengths (bp) and names (letter)					
		177	**180**	**186**	190	192	**195**
	seed no.	**a**	**b**	**c**	d	e	**f**
outcrossed seeds harvested on plant 77	1	**a**			d	e	**f**
	2	**a**				e	**f**
	3		**b**	**c**	d	e	
	4	**a**	**b**		d	e	
outcrossed seeds harvested on plant 9	5		**b**	**c**	d	e	
	6	**a**		**c**		e	
	7	**a**	**b**		d	e	

Tab.2
Alleles detected in microsatellite locus *E3-40* in outcrossed offspring coming from a pollination with a mixture of pollen from plants 51 and 81. Plant 51 had alleles a, b, e, f and plant 81 - alleles c, d.

		Allele lengths (bp) and names (letter)					
		177	**180**	186	190	**192**	**195**
	seed no.	**a**	**b**	c	d	**e**	**f**
outcrossed seeds harvested on plant 81	1		**b**	c		**e**	
	2		**b**	c	d		**f**
	3	**a**	**b**		d		
	4		**b**	c	d		**f**
	5		**b**	c		**e**	
	6	**a**		c	d		**f**
	7		**b**	c			
outcrossed seeds harvested on plant 51	8	**a**		c	d	**e**	
	9				d	**e**	**f**
	10	**a**	**b**	c	d		
	11		**b**	c	d	**e**	
	12	**a**		c	d		**f**
	13		**b**	c	d		**f**

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