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Genetic structure and post-pollination selection in biennial plants

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**Genetic structure and post-pollination selection
in biennial plants**

Korbecka, Grażyna

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Genetic structure and post-pollination selection in biennial plants

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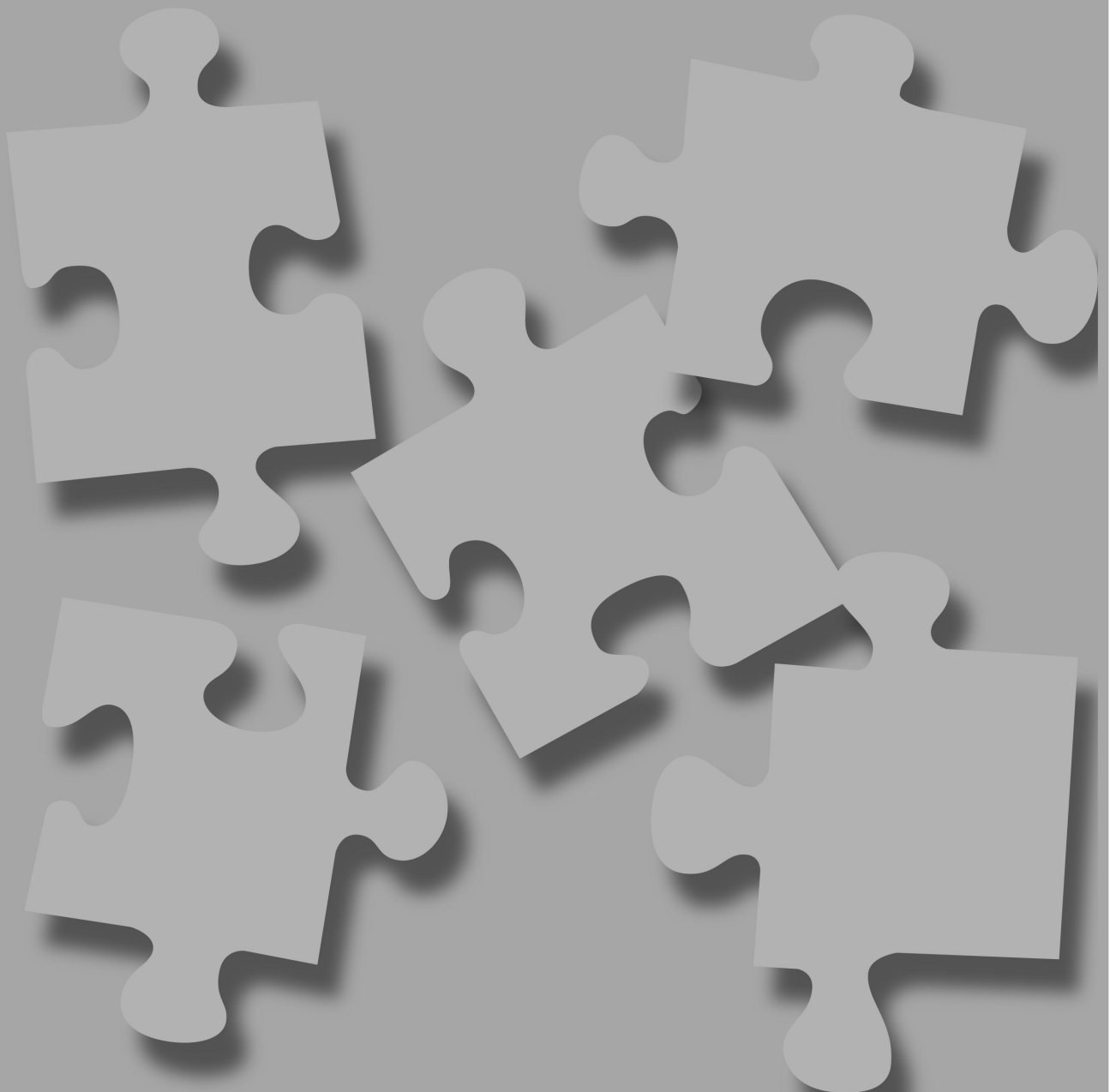
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Chapter 1

General introduction



The main purpose of reproduction in all organisms is to ensure that an individual transfers its genes to the next generation. The reproductive success of an individual has two important components: the number of offspring and the quality. The number of offspring may be directly related to the amount of energy obtained by a parent. In contrast, the quality of an individual results from both the amount of energy or resources invested in it and its genetic constitution. The genetic constitution is shaped by a number of processes acting during or after mating. These processes account for sexual selection and selective embryo abortion.

In 1871, Darwin introduced the concept of sexual selection to explain the presence of characters that increase the probability of mating and getting offspring but not necessarily to increase the individual's survival. He considered mainly secondary sexual characters and behaviors. Since Darwin countless studies on animals have presented such a role for male ornaments, songs and scents attracting mates and for courtship behaviors. However, it became also clear that post-copulatory mechanisms may influence the reproductive success of an individual as well. When a female mates with two or more males, sperm from different males may compete in female reproductive tracts for fertilization (Parker, 1970). The female can also influence the paternity of her offspring. Mechanisms of post-copulatory female choice (so called *cryptic female choice*) include for example: changes in rates of oviposition, timing of ovulation, sperm dumping, digesting of sperm and selective storage of sperm (Birkhead and Møller, 1998; Eberhard, 1996).

Apart from sexual selection, post-fertilisation processes may influence genetic constitution of offspring in animals. Developing embryos may be aborted depending on their genotype. Such a selective embryo abortion has been described for example in mammals, where an embryo may be rejected by the mother just before its implantation (Haig, 1993).

Bateman (1948) used the term *sexual selection* for the first time in reference to plants. Since then it appears in plant breeding literature, although still less frequently compared to animal studies (Willson, 1994). Plants are unable to search for their mates or attract them in any way. They depend in their mating success on biotic or abiotic pollinating agents. However, as I will describe later, a number of morphological adaptations can modulate pollen transfer. After pollination, a number of processes can influence the paternity of seeds. These processes are analogous to sperm competition and cryptic female choice known in animals. Selective embryo abortion can also play a role in plants. Below, I will describe how sexual selection acts in plants during and after mating. I will also write about a potential for selective embryo abortion to act in plants.

Mating in plants

Plants can not choose their mates directly. However, they may have many adaptations that lead to an increase of pollen export or variation of pollen that lands on the surface of a stigma. Such adaptations are: prolonged receptivity of the stigma and characters increasing the number of pollinators visits like high nectar production rate and attractive floral display (Stephenson and Bertin, 1983).

Almost seventy five percent of the flowering plant species are hermaphroditic (Charnov, 1982, p. 254). Therefore, mate choice in self-compatible plants often involves the avoidance of self-pollination. Various morphological and physiological

features like: position of the stigma (herkogamy) and timing of its receptivity (protandry, protogyny) can minimize self-pollination within the same flower (autogamy). However, there may be still a lot of self-pollination, which can not be prevented, as pollinators tend to visit neighboring flowers on the same genet (geitonogamy). As a result self-compatible plants may experience a considerable degree of inbreeding depression. Apart from autogamy and geitonogamy, crosses between related individuals (biparental inbreeding) may increase inbreeding. Such crosses can be common in populations with a genetic structure because pollinators tend to visit neighboring plants. The level of biparental inbreeding depends on the genetic structure of the population.

Many studies have detected a genetic structure in plant populations (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004). However, they usually aimed at estimating the dispersal distances of the species, and they frequently include juveniles into data sets. To my knowledge, there are only few recent studies with the main objective to estimate biparental inbreeding (Griffin and Eckert, 2003).

Post-pollination selection

Because opportunities to choose mates prior to pollination are limited, post-pollination mate choice is essential for sexual selection in plants. Selection at this stage operates directly on the gametes. Bernasconi *et al.* (2004) emphasized in their review that this is in contrast to animals, where post-copulatory mechanisms discriminate among available ejaculates rather than sperms. Intra-ejaculate competition among individual sperms can not be strong because of limited gene expression at this stage.

Pollen, unlike sperm, does not move in a fluid. After a pollen grain lands on a stigma, its vegetative cell elongates producing a pollen tube, which grows into a structure of the style and delivers a generative cell to the ovule. The complexity of this process, direct interaction with the female tissues and also high levels of gene expression in pollen (Becker *et al.*, 2003) suggest that there is ample opportunity for sexual selection to act at this stage. Gametophytic self-incompatibility is a clear example of such selection favoring outbreeding (Richards, 1997).

However, sexual selection may discriminate also among compatible pollen that is genetically heterogenous. The term *pollen competition* has been widely used in pollination studies to describe differential fertilization success of such pollen.

One experimental approach to test whether pollen competition takes place is to apply different pollen loads and measure offspring quality. It is assumed that higher pollen loads lead to stronger selection. Therefore, offspring resulting from such a pollination treatment will be more vigorous. This prediction was confirmed by many studies that detected enhanced germination, vegetative growth or/and reproductive performance among plants grown from the seeds sired in higher pollen loads treatment (e. g. Bjorkman, 1995; Quesada *et al.*, 1996; Richardson and Stephenson, 1992).

Other studies use a more direct approach in which single donor pollinations with pollen from different donors are followed by measurement of the pollen tube growth. Often such measurements show high variation in pollen performance among donors (e. g. Bjorkman *et al.*, 1995; Sari-Gorla *et al.*, 1995) and pollen tube growth correlates with siring success (Pasonen *et al.*, 1999; Snow and Spira, 1991)

Pollen competition is believed to be responsible for cryptic self-incompatibility (CSI): a slow growing self-pollen may fertilize all ovules in single donor pollinations,

but when it is applied in a mixture with a faster growing outcross pollen its fertilization success is greatly reduced. Bateman (1956) reported CSI for the first time in *Cherianthus cheiri* – a species producing full seed set when pure self-pollen is applied. He performed two mix pollination treatments on one yellow flowered individual: in the first treatment he used a mixture of self-pollen and pollen from a red flowered donor, in the second treatment – a mixture of outcross pollen from two donors: yellow and red flowered. The proportion of red flowered offspring equaled 92.2% and 22.7% in the first and second treatment respectively. This result suggests a strong disadvantage of self-pollen in *C. cheiri*.

Since Bateman, CSI has been studied by means of pollen tube growth measurements or/and paternity analysis following mixed pollinations. So far, most of the evidence for CSI comes from studies on heterostylous species. In these species self-pollination is a kind of illegitimate pollination and self-pollen may have more disadvantages than only a slower growth. Other CSI studies showed a number of other methodological flaws like low number of genotypes or using morphological characters for paternity analysis (see introduction to chapter 5). Therefore, I concluded that there is a need for a study of CSI in non-heterostylous species, using many genotypes and selectively neutral markers for paternity analysis.

Embryo abortion in plants

After fertilization, embryos can be selectively aborted depending on their genotype. This is however rather difficult to prove in plants. First one has to prove that ovules that did not develop into seeds were indeed fertilized, embryos were potentially viable and moreover, that the observed abortion was selective. A common observation in plants is that they produce more ovules than seeds (Bawa and Webb, 1984; Stephenson, 1981). Seed-ovule ratios equal on average 85% for annuals and 50% for perennials (Wiens, 1984). However, low fruit or seed production can not be attributed directly to embryo abortion, because not necessarily all of the ovules are fertilised in the field. Burd (1994) reviewed pollen limitation in field studies by comparing fruit or seed production after natural and after hand pollination with outcrossed pollen. He assumed that after hand pollination excess of pollen were applied and thus seed-set and fruit-set were not pollen limited. Burd (1994) reported that 62% out of 258 analysed species was significantly pollen limited in the field. On basis of data provided in the same paper, I made a frequency distribution of percent fruit set for the species for which pollen limitation was not found (fruit set is defined as fraction of flowers setting fruits). This distribution indicates a great potential for embryo abortion (Fig. 1a). One should keep in mind, however, that fruit abortion may be related to seed number rather than seed quality or genetic constitution, even when pollen is not limiting seed production. Although, pollen is not limiting fruit production, still 77 out of 90 species have a fruit set lower than 90% in the field (Fig. 1a). A part of this abortion under natural conditions can be explained by a post fertilisation self-incompatibility mechanism (Seavey and Bawa, 1986). However, after pollination with outcross pollen still only 23 out of 90 species have a fruit set higher than 90% (Fig. 1b).

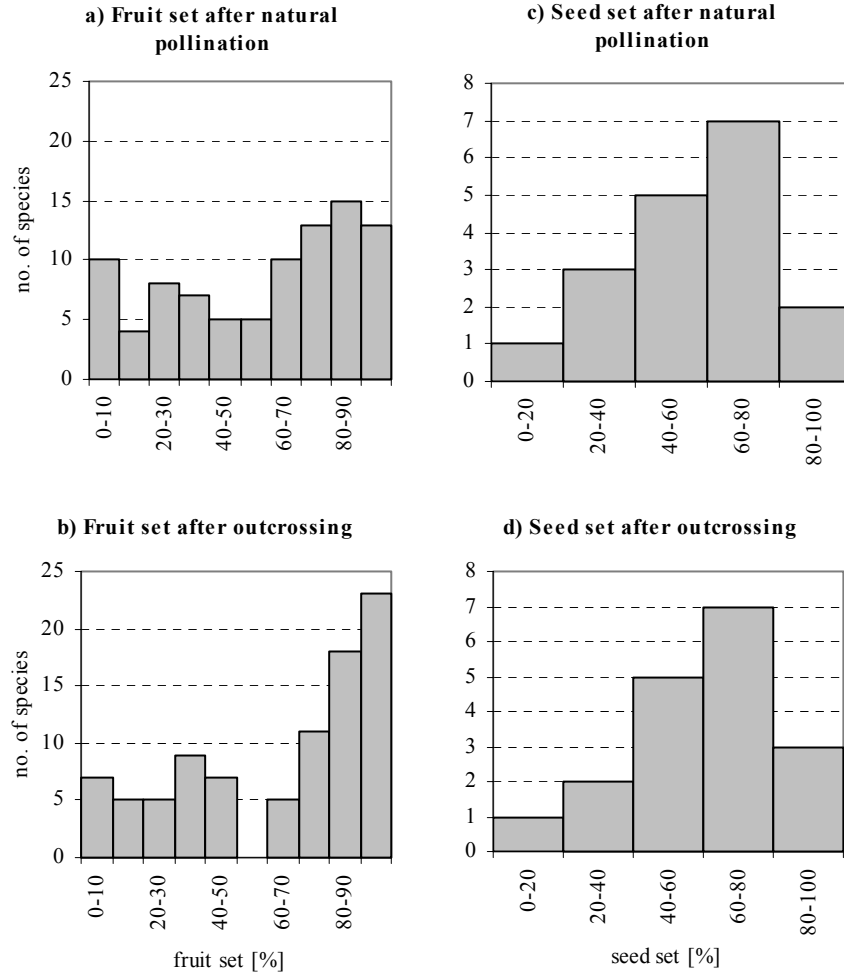


Fig. 1

Fruit set (fig. 1a and 1b) and seed set (fig. 1c and 1d) distributions after natural pollination (fig. 1a and 1c) and outcrossing by hand pollination (fig. 1b and 1d) for plant species that do not show pollen limitation according to Burd (1994). If data were available for more than one population an average was calculated for those populations that did not show pollen limitation. Fruit set was analysed for 90 species from 39 families, and seed set for 18 species from 10 families. Two species (*Erythronium propullans* and *Igna quaternata*) that did not have any fruit set after outcross pollination were excluded from the analysis.

An even better indication for embryo abortion is provided by a similar frequency distribution made for the percentage seed set (% of ovules that develops into seeds). Burd (1994) provides information about seed set in only 28 species, out of which 18 did not show pollen limitation. Figure 1c shows the frequency distribution of seed set for those 18 species after natural pollination. Only for 2 of them seed set exceeds 80% in the field. After outcrossing only three species have such a high seed set (Fig. 1d). Thus, even if pollen is not limiting seed production, many ovules do not mature seeds, indicating that many embryos are aborted.

Selective embryo abortion

There are three hypotheses explaining this apparent "overproduction" of ovules in plants. First, in hermaphroditic plants the production of "excess" flowers can result from selection on optimal division of resources into male and female function. Flowers that do not contribute to seed production may disperse pollen and contribute to siring seeds (e. g. Sutherland and Delph, 1984). Second, it has been suggested that in variable environments it is impossible for the plant to predict how much of the resources will be available during seed maturation. The overproduction of zygotes gives an opportunity of adjusting the seed set to the number that is optimal in a certain reproduction period. Such a strategy is called bet-hedging (Kozłowski and Stearns, 1989; Lloyd, 1980). Third, embryo abortion may serve to increase female fitness by providing the possibility to select for superior offspring and avoid investment of resources in seeds that produce offspring with a low fitness later in life (Willson and Burley, 1983).

I will focus on the third hypothesis, as a part of sexual selection. The selective embryo abortion (SEA) hypothesis received a lot of attention in 1980s (Casper, 1988; Stephenson, 1981; Willson and Burley, 1983). However, experiments designed to test it on the level of the phenotype were not able to discriminate between artifacts resulting from a treatment and effect of SEA (e. g. Casper, 1988).

Thesis outline

The experimental part of my thesis is based on two biennial species, tetraploid *E. vulgare* and diploid *C. officinale*. I start my thesis by characterizing microsatellites for both species (chapter 2 and 3). This characteristic includes a description of PCR conditions and a test for polymorphism of developed microsatellite loci.

In chapter 4, I used the developed microsatellites to test for a genetic structure in natural populations of *E. vulgare* and *C. officinale*. Using two species in such a test gives an opportunity for an interesting comparison. I expected to find a stronger genetic structure in *C. officinale* because of a lower ploidy level and higher selfing rate. Apart from testing for a genetic structure, I estimate the amount of biparental inbreeding in the populations of both species.

In chapter 5, I deal with aspects of post-pollination selection in *E. vulgare* only. Since, selfing rates in this species are much lower than predicted from the plant size and pollen dynamics, I tested for cryptic self-incompatibility.

In the following chapter 6, I study the potential for SEA by collecting literature data on genetic maps of plants. This chapter was greatly inspired by previous work of Melser (2001) who used phenotypic approach to study SEA in *E. vulgare* and *C. officinale*. Since the phenotypic approach has proven to have many limitations, I searched for an alternative way to test this hypothesis – on the level of a genotype.

SEA, like every selection, should show up in segregating progeny of one cross as a departure from Mendelian segregation. I assumed that if there is a considerable level of selective embryo abortion in plants—many genetic maps of plants should report non-Mendelian segregation of molecular markers. Therefore in chapter 6, I review genetic maps of plants to estimate the level of non-Mendelian segregation. I also propose an experimental design that will allow for attributing the detected non-Mendelian segregation to embryo abortion.

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Chapter 2

Characterization of six microsatellite loci in *Echium vulgare* (Boraginaceae)



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Characterization of six microsatellite loci in *Echium vulgare* (Boraginaceae).
Molecular Ecology Notes 3: 274-276

ABSTRACT

Echium vulgare is a tetraploid plant with a very low selfing rate in the field. We suspect that cryptic self-incompatibility plays a role in this species. In order to show lower success of self pollen/selfed embryos, after pollination with a mixture of self and outcross pollen, a paternity analysis has to be done. For the purpose of such analysis we developed microsatellites in *E. vulgare*. In this article, we report on six microsatellite loci which are easy to score, polymorphic, with number of alleles per locus ranging from two to eight and, therefore, suitable for paternity analysis.

* * *

Echium vulgare is a tetraploid ($2n=4x=32$), hermaphroditic species pollinated by bumblebees. It produces one to 20 flowering stems, each with hundreds of flowers. *E. vulgare* is self-compatible and bumblebees can cause self pollination by moving from one flower to another within the same plant. Using RAPD's, Rademaker *et al.* (1999) found that selfing rates of *E. vulgare* in the field vary between 0 and 30 % which is only half or less of the theoretical prediction based on bumblebee behavior and pollen dynamics. On average, single pollen donor pollinations with self pollen resulted in the production of as many seeds as outcrossing (Melser *et al.* 1997). Why then are so few selfed seeds produced in the field? A logical explanation would be that self pollen or selfed embryos lose competition when a flower is pollinated by both self and outcross pollen. This is called *cryptic self-incompatibility* because the lower success of self pollen/selfed embryos can be detected only when mixture of self and outcross pollen is applied. A paternity analysis of seeds, following mixed pollination is essential for testing the hypothesis about the presence of cryptic self-incompatibility.

Microsatellites are the best markers for paternity analysis since they are codominant, highly variable and often allow for distinguishing among individuals from the same population. The aim of this study was to develop microsatellite primers suitable for such an analysis in *Echium vulgare*. To our knowledge, this article will be the first report on microsatellite loci in the family Boraginaceae.

Enrichment was done separately for dinucleotide (GA and CA), trinucleotide (AAG and ATG) and tetranucleotide (GATC and GATA) repeats following the procedure described in Hale *et al.* (2001). Enriched DNA was ligated into BAP (dephosphorylated) BamHI digested "ready-to-go" PUC18 vector (Pharmacia) and cloned using JM 109 competent cells (Promega). Positive colonies with inserts were sequenced using ABI Prism Big Dye Terminator (version 1.0) cycle sequencing ready reaction kits (Applied Biosystems) following manufacturers recommended conditions and detected using an automated sequencer ABI 377 (Applied Biosystems). Thirty-six primer pairs were designed using PRIMER 3 program available on the web (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi>). Nineteen primers gave PCR product of expected size for five tested individuals, subsequently these primers were tested for polymorphism using dCTPs labeled with rhodamine dye: R110 (Applied Biosystems). PCRs were carried out in a volume 10 μ L, containing 1 ng DNA, 10 mM Tris HCl, 50 mM KCl, 2.0 mM MgCl₂, 0.1 mM each of the dNTPs, 0.2 μ M [R110] dCTPs, 4 pmol of each primer, 1 μ g bovine serum albumin (BSA) and 0.4 U *Taq* DNA

polymerase (Amersham Pharmacia). All PCRs were carried out in a T₃ thermocycler (Biometra). PCR fragments were detected on the ABI 377 along with an internal size standard ROX-500 and analysed using GENESCAN[®] software (Applied Biosystems). Out of these microsatellites: four microsatellites were not polymorphic, three most probably have null alleles, and for the remaining 12 were ordered fluorescently labeled forward primers. In this article we describe microsatellites with the most clear and easy to interpret patterns.

We tested the developed microsatellites on leaf material from 30 flowering plants of *E. vulgare* collected in the dune area of Meijendel (near The Hague, the Netherlands). The biggest distance between two collected plants was approximately 675 meters. DNA was extracted from 0.1 g of fresh or frozen (−80°C) plant material with a Nucleon Phytopure extraction kit (Amersham). Extraction was followed by PEG precipitation to remove polysaccharides. To 500 µL of DNA in sterile water 250 µL of PEG solution (40% PEG-8000, 30 mM MgCl₂) was added. After incubation for 30 min at room temperature and centrifuging at 13 000 rpm the pellet was washed twice with 70% cold ethanol and resuspended in 100 µL of 0.1TE buffer (10mM Tris, 0.1 mM EDTA).

The PCRs were carried out in a volume 10 µL, containing 0.1-1 ng DNA, 1 µL 10x concentrated PCR buffer supplied with the *Taq* polymerase (containing Tris Cl, KCl, (NH₄)₂SO₄, and 15 mM MgCl₂), 0.1 mM each of the dNTPs, 4 pmol of each primer, 0.5 mM MgCl₂ additional, 1 µg BSA and 0.2 U *Taq* DNA polymerase (Qiagen). The forward primers of microsatellites were fluorescently labeled with one of fluorescent dyes: Fam, Joe, Tamra. Final MgCl₂ concentration was 2.0 mM. We used a low template concentration because the reaction with 10 ng of DNA not always gives a PCR product. Moreover, adding BSA improves PCR. This suggests that there are inhibitory compounds present in DNA extract. The seedlings seem to contain more of these compounds than the flowering plants. For that reason, we used 1 ng of DNA from flowering plants and 0.1 ng of DNA from seedlings in the PCR. After denaturation for 2 min at 95°C, PCR's were performed for 20 cycles under the following conditions: 15s at 95 °C, 15s at annealing temperature (see table 1), 15s at 72 °C, then for 10 cycles under the same conditions but with the annealing temperature lowered by 4 °C and finally there was an extension step of 30 min at 72 °C. PCR program for locus E2-11 differed only with a doubled time (30s) for annealing. Labelled PCR products were detected on the ABI 377 using an internal size standard ROX 500 and analysed using GENESCAN[®] software (Applied Biosystems).

All the presented microsatellites were highly polymorphic within the population of Meijendel and therefore suitable for paternity analysis. We designed a pollination experiment in which 22 out of the 30 collected plants were combined in pairs of plants that do not share any allele at least in one out of 6 tested microsatellites. Such an experimental design will allow for very rapid paternity analysis of offspring from pollinations with a mixed self and outcross pollen. Allele frequencies in this tetraploid species are not easy measured, as we are not able to determine if a peak was the product of one or more copies of an allele. As a result, no information on expected heterozygosity is available.

Table 1 Characteristics of microsatellite loci in *Echium vulgare*.

Name*	Primer sequence (5'→3')	Repeat	T _a (°C)	Allele size range (bp)	No. of alleles	H _o	GenBank Accession number
<i>E2-11</i>	F: CCAACCATTTCATCCAAC R: AGTCTTGCCATTCGATGACC	CTCTCAT	58	242-249	2	0.97	AY185304
<i>E3-40</i>	F: CCATTGTTTCACCCGCTAAT R: CCACAGAAGGGGAGTTTGAG	TCA	58	177 - 195	7	1.00	AY185305
<i>E3-46</i>	F: GGGGCTAACTGAATGCAGAA R: CCTCCCATATCCGTTGTCAT	CA	60	220 - 234	6	0.97	AY185306
<i>E3-56</i>	F: GCTAAGAAAGCGTTGGCAAG R: GATCAAGACGCAAGCGAGTA	CAT	61	260 - 285	5	0.96	AY185309
<i>E3-84</i>	F: CCCCAGTGCAATGAGATAG R: GGAATGGAGCCTAGTGCTTG	GAT	62	293– 305	5	0.83	AY185307
<i>E3-91</i>	F: AAGAGCAATCCAGCCTTTGA R: GATGTTGTCTGCCCAAATCA	GAT	61	169 - 196	8	0.97	AY185308

*E2, sequenced clone originates from enrichment for dinucleotides; and E3, for tri nucleotides. The following number is a number of a sequenced clone.

T_a, locus specific annealing temperature; H_o, observed heterozygosity.

Forward primers were labeled. For locus *E3-40* – Fam label was used, for *E3-84* – Tamra label and for the rest of loci – Joe label.

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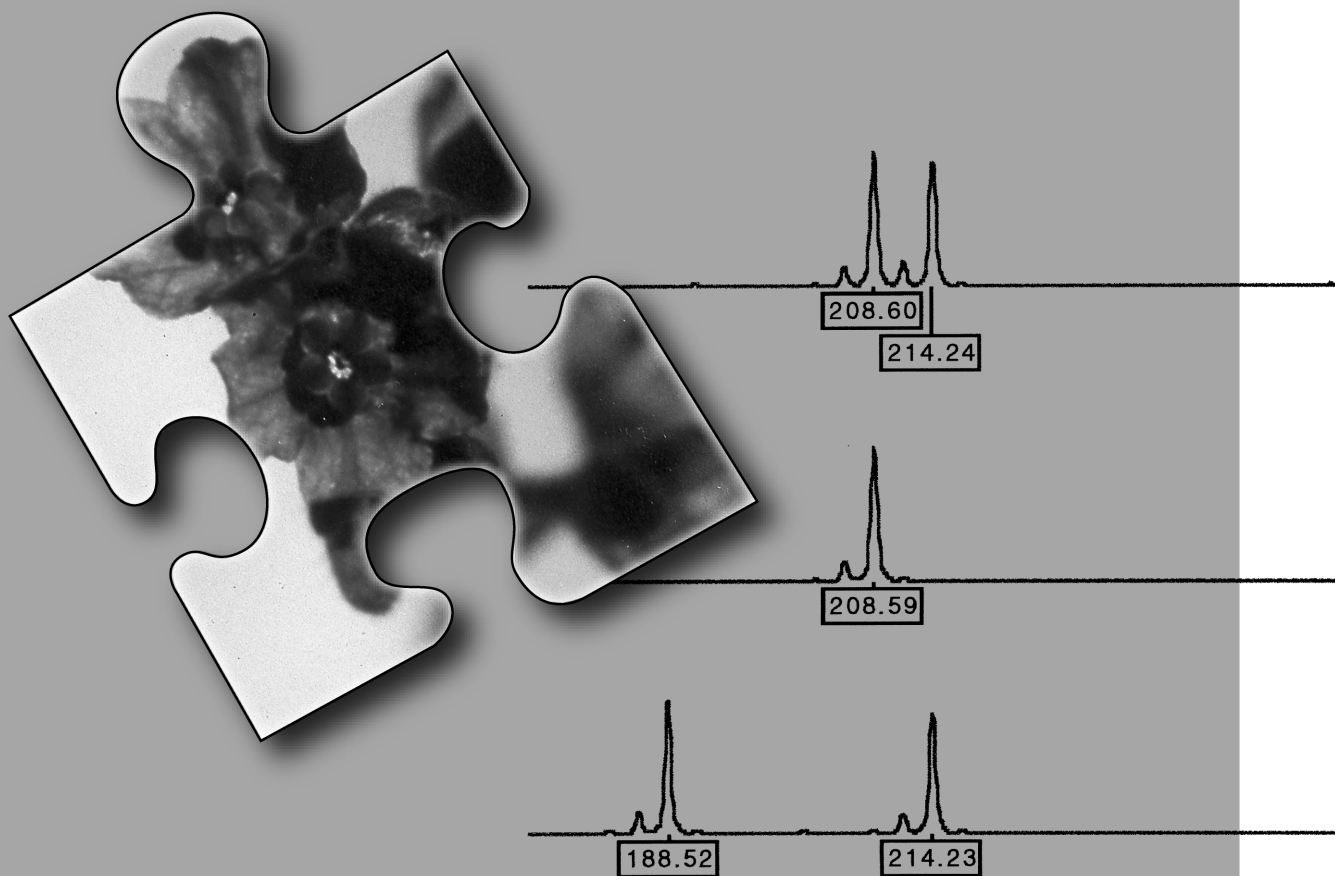
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Chapter 3

Characterization of nine microsatellite loci in *Cynoglossum officinale* (Boraginaceae)



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ABSTRACT

Cynoglossum officinale is a biennial plant pollinated by bumblebees. We developed microsatellite loci in order to study the population genetic structure and effects of inbreeding in this species. In this paper, we describe nine polymorphic microsatellites for *C. officinale*. Between two and four alleles per locus were observed in a sample of 20 individuals from one population. Multiplexing allowed the seven most useful loci to be genotyped using three PCR reactions.

* * *

Inbreeding depression is of great interest to both evolutionary biologists and conservation ecologists. Studies of inbreeding depression in plants often only concentrate on the selfing rate, while crosses between related individuals can also intensify inbreeding. Such crosses take place when the pollinators visit neighboring plants and there is a genetic structure in the population (the neighboring plants are related). We intend to study fine scale genetic structure of a population of *Cynoglossum officinale*, a diploid, biennial plant, pollinated by bumblebees. Although, seeds of this species have a clear adaptation to dispersal via animals, large mammals are absent in the studied dune area. Therefore, we suspect that dispersal by gravity plays an important role and we expect to find genetic structure in the population. In this paper, we describe microsatellite loci developed to test this prediction.

Genomic DNA of one individual from the dune area of Meijndel (near The Hague, the Netherlands) was enriched separately for dinucleotide (GA and CA) and trinucleotide (AAG and ATG) repeats, following the procedure described in Hale *et al.* (2001). Enriched DNA was ligated into BAP (dephosphorylated) *Bam*HI digested "ready-to-go" pUC18 vector (Pharmacia) and cloned using JM 109 competent cells (Promega). The plasmid DNA from bacterial colonies were sequenced using ABI Prism Big Dye Terminator (version 1.0) cycle sequencing ready reaction kits (Applied Biosystems) following manufacturer's recommendations and detected using a capillary sequencer ABI 310 (Applied Biosystems). Twenty-two primer pairs were designed using PRIMER 3 program (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi>). PCRs were carried out in a volume 10 µL, containing 5 ng DNA, PCR buffer (16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, 0.01% Tween-20), 2.0 mM MgCl₂, 0.2 mM each of the dNTPs, 2 pmol of each primer and 0.5 U *Taq* DNA polymerase (Bioline). All PCRs were performed using a PTC-100 programmable thermocycler (MJ Research). After denaturation for 12 min at 95°C, PCR's were performed for 20 cycles under the following conditions: 15s at 95 °C, 15s at 55 °C, 15s at 72 °C, then for 10 cycles: 15s at 89 °C, 15 s at 50 °C and 15 s at 72 °C and a final extension of 30 min at 72 °C. Out of twenty-two primers, 17 primers gave PCR product of the expected size for five individuals, when tested on 2% Metaphor-agarose gels (FMC BioProducts). We judged polymorphism based on these gels and ordered fluorescently labelled forward primers for 4 loci. The other 13 loci were further tested for polymorphism using fluorescent dCTPs (FdCTPs), giving us five more polymorphic loci. We used FdCTPs labelled with dyes R110 and R6G (Perkin Elmer) at a concentration of 0.5 µM in the PCRs. The labelled PCR fragments were detected on an

ABI 310 using an internal size standard ROX-500 and analysed using GENESCAN[®] software (Applied Biosystems). In this paper, we characterise all nine loci, although in routine analysis we only use the seven most polymorphic ones (indicated with a fluorescent label in Table 1)

We tested the microsatellites on leaf material from 20 *C. officinale* plants collected from one population in the dune area of Meijendel. The largest distance between two collected plants was 41 meters. The leaves were dried in silica gel and stored at -20°C . Approximately 1 cm^2 of each leaf was homogenized in 1.3mL of 2x CTAB extraction buffer (1% PVP 40, 0.5% v/v β mercapto-ethanol). The CTAB extraction protocol was adapted for smaller quantities after Doyle and Doyle (1987). After extraction DNA was resuspended in 100 μL of TE buffer (10mM Tris, 1 mM EDTA).

In routine analysis, PCRs and detection were carried out as described above, but we used forward primers fluorescently labeled with 6-FAM, JOE or TAMRA. Six primer pairs were combined in two multiplex sets (Table 1) using the same PCR program and annealing temperature as described above. PCR for locus *C2-42* was carried out separately with 30 sec annealing to increase the intensity of the signal. This locus with 4 alleles: 110, 112, 116 and 124 bp, shows a clear decrease of peak height with increasing size.

We performed a test for Hardy-Weinberg equilibrium for all loci using a program called ARLEQUIN (Schneider *et al.* 2000) and found a significant deviation for two loci: *C2-42* and *C3-79* (Table 1), which showed lower observed than expected heterozygosity. Most other loci also showed a lower than expected heterozygosity, albeit non-significant. We did not find any homozygotes for null alleles among 20 individuals. Moreover, in further 80 individuals tested for 7 loci with fluorescently labeled primers (Table 1) we did not find such homozygotes either. Therefore, we conclude that selfing or mating with related individuals is responsible for lowering heterozygosity. We performed tests for linkage disequilibrium using above-mentioned program ARLEQUIN. No linkage disequilibrium was observed in the population.

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Table 1 Characteristics of microsatellite loci in *Cynoglossum officinale*.

Name †	Primer sequence (5'→3')	Label	Repeat	Size of the cloned allele (bp)	Allele size range in a screened population (bp)	No. of alleles	H _e	H _o
<i>C2-19</i> ¹	F: CTCCGGTGGTGGTCTTC R: TCCAGGTTAAGAACCAAGC	JOE	(GA) ₂₆	138	115-131	3	0.56	0.40
<i>C2-42</i>	F: TCAAACACGTGAGAAAATATAGAA R: TGATTCCAATCAATCTTCGTTTT	6-FAM	(GA) ₁₂	116	110-124	4	0.50	0.40*
<i>C2-43</i> ²	F: ACCCCCTTCTCCAGTT R: GGAATAGCAGACCATGTCC	TAMRA	(CT) ₇ (CA) ₁₀	133	128-136	3	0.52	0.35
<i>C2-45</i>	F: TGATGATATTTTCAACCCCTATCTCAT R: AGCTCAGCAGATATCCAAACGA		(CT) ₆ CG(CT) ₉	128	128-140	2	0.35	0.37
<i>C2-62</i> ¹	F: CCTGTCATACCCGAACTCG R: AGTAGGGAATTGGGCTTTGG	6-FAM	(CT) ₁₂	169	167-171	3	0.43	0.40
<i>C2-72</i> ²	F: GAAATTGAGGAAGGAGATGACG R: GATCATGTGGGGGAATCATAA	JOE	(GA) ₁₅ C(GA) ₂	102	91-101	4	0.60	0.45
<i>C3-30</i>	F: GCTTGCAACAAGCAGACAAC R: TTGTGTCACATTGCTGTCG		(CAT) ₉	150	137-147	2	0.23	0.20
<i>C3-41</i> ¹	F: GTGCAAGGTGCAGGGTAAG R: TGTCATAGGCTCTGCTCTTCTCC	TAMRA	(GAT) ₇	134	133-136	2	0.27	0.15
<i>C3-79</i> ²	F: GCACCAGGTTCTGTGTAGT R: GCTTTTGGCTGAGCTGTTT	JOE	(GAA) ₇ ... (GAA) ₁₆ ... (GAT) ₆	220	188-214	4	0.61	0.30**

†C2 means that the sequenced clone originates from enrichment for dinucleotides, and C3 - for trinucleotides, the following number is a number of a sequenced clone and the number in superscript (1 or 2) is the same for those primers, which were taken together for the same multiplex PCR; H_e, expected heterozygosity; H_o, observed heterozygosity; *, ** statistically significant deviation from Hardy-Weinberg equilibrium (P<0.05 or P<0.01, respectively).
GenBank accession numbers between AY434455 and AY434463 for the described microsatellite loci, in order presented in this table.

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Chapter 4

Fine-scale genetic structure in *Echium vulgare* and *Cynoglossum officinale*



Korbecka G., Klinkhamer P.G.L., Vrieling K. and K. Wolff

ABSTRACT

The presence of a genetic structure in plant populations can lead to an increase of inbreeding. Pollinators tend to visit neighboring plants, causing crosses among related individuals (biparental inbreeding). We tested for fine-scale genetic structure in two species, pollinated by bumblebees, *Echium vulgare* and *Cynoglossum officinale* in order to estimate the amount of biparental inbreeding, using 7 polymorphic microsatellite loci per species. The slope of the regression line between pair-wise kinship coefficients and $\ln$ of physical distance was significantly negative for *E. vulgare* but not for *C. officinale*. Average kinship coefficients per distance class were significantly higher than zero for both species only in the first distance interval (including distances up to 1.48 meters for *E. vulgare* and up to 6.49 meters for *C. officinale*). This suggests a genetic structure at a very small scale, probably due to leptocurtosis of gene dispersal curves. The genetic structure of both species appeared to be very weak compared to data published for 17 herbaceous species with similar types of pollen and seed dispersal. The estimated amount of biparental inbreeding does not exceed 2 % for *E. vulgare* and *C. officinale*. We conclude, therefore, that the population genetic structure does not intensify inbreeding in the studied species.

INTRODUCTION

It is common among plant species that gene flow is restricted to dispersal of pollen and seeds. As these are often restricted to a limited area, more related plants tend to grow next to each other and a genetic structure is formed within populations (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004 and references there in). The presence of such a structure may have many consequences. Firstly, the adaptive value of various traits may depend on it. If neighboring plants are close relatives, then a strategy, which is ‘better for the neighbors’ but worse for the focus individual may still be favored by selection if it increases inclusive fitness (Hamilton, 1964). For example, one can expect that the direction of selection on plant responses to intraspecific competition (e.g. allelopathy, root competition, overshadowing of the neighboring plants) depends on inclusive fitness. Similarly, resistance to herbivores can be considered. The production of high levels of chemical defenses may be profitable not only for the individual in focus, but also for the neighboring plants, if herbivores consider a group of plants rather than a single plant as one foraging patch. For some traits, it is relevant if a genetic structure is present in a particular life-stage. Klinkhamer et al. (2001) found that neighbors of flowering plants with high nectar production rate received more bumblebees’ visits, irrespective of how much nectar they produced themselves.

Strategies of which the fitness consequences depend on inclusive fitness are not the only consequence of genetic structuring of the populations. Genetic structure among flowering plants may also result in intensified inbreeding. Pollinators like bumblebees often visit neighboring plants, as this optimizes their nectar uptake rate (Heinrich, 1979). In genetically structured populations, such a visitation pattern leads to crosses among related individuals. Even with low selfing due to autogamy and geitonogamy, the proportion of inbreeding that comes from crosses among related individuals (biparental inbreeding) may be substantial. Therefore, the association between

population structure and inbreeding becomes very relevant in species, which are predominantly outcrossing and suffering from inbreeding depression.

Many studies on genetic structure within populations included both juvenile and reproducing individuals, although there may be considerable differences across life stages (e. g. Parker et al., 2001). The genetic structure may decay as plants get older due to the thinning process or it may get stronger if a directional selection operates locally (Chung et al., 2003; Ueno et al., 2002). Therefore, if the genetic structure is studied in relation to biparental inbreeding only flowering plants should be included.

In this paper we test for the presence of a genetic structure in the flowering stage of two species of the Boraginaceae: *Echium vulgare* and *Cynoglossum officinale*, which are both self-compatible, monocarpic biennials pollinated by bumblebees. Inbreeding depression affects survival of rosette plants in both species (see chapter 6 in Melser, 2001). Moreover, in *E. vulgare* inbreeding depression has been detected during reproduction of the offspring. Plants derived from self-pollination have lower seed production and lower siring success compared to outcrossed plants (Melser et al., 1999).

In the studied dune area, both species disperse seeds mainly through gravity. As a consequence, groups of seedlings germinating are observed in a direct neighborhood of places where flowering plants stood the season before, suggesting that these are at least half sibs and a genetic structure is likely to exist in the field populations. The two species differ in flower structure and development. In *E. vulgare* autogamy is prevented by a spatial separation of anthers and stigma and protandry, which is not the case for *C. officinale*, where anthers and stigma are located closely together. Therefore, the latter species is expected to have more inbreeding. Preliminary measurements of selfing rates reported by Rademaker et al. (1999) and Vrieling et al. (1999) support this expectation. In *E. vulgare* the percentage of selfed offspring per mother varies between 0 and 33% (average: 12.5%), while in *C. officinale* it varied between 0 and 70% (average: 32.2%).

Species with higher inbreeding levels are more likely to form a genetic structure in a population (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004). Therefore, we expect to find a stronger population structure in *C. officinale* compared to *E. vulgare*.

MATERIALS AND METHODS

Species description

E. vulgare is a tetraploid species: $2n = 4x = 32$ (Gadella and Kliphuis, 1963; Litardiere, 1943). The inheritance is probably tetrasomic in this species (see appendix to chapter 5). Every plant produces 1-10 flowering stems each with up to 50 cymes and each cyme carries up to 20 flowers. Mean seed weight equals 2.7 mg with a mean length of 2.5 mm (van Breemen, 1984). Seeds disperse by gravity, although secondary dispersal by wind or transport with dried flowers in the fur and feathers of animals is possible. Seeds covered by sand remain viable many years and disturbance of the soil usually increases the number of germinating seedlings of *E. vulgare* (van Breemen, 1984).

C. officinale is a diploid plant: $2n = 2x = 24$ (Gadella and Kliphuis, 1963; Luque and Valdes, 1986). Every plant produces 1-3 flowering stems each with up to 25 cymes and each cyme carries up to 20 flowers. Seeds weigh on average 20 mg and are on

average 6 mm long (van Breemen, 1984). The seed is covered with hooked spines, which enable them to stick to the fur of animals resulting in dispersal over longer distances. In areas grazed by cattle such dispersal plays a significant role. However, in our study area the only largest herbivores are rabbits, which are believed to disperse only a small fraction of *C. officinale* seeds (Rademaker and de Jong, 1999). The majority of the seeds fall next to the mother plants and germinate within 1-2 years after maturation (Boorman and Fuller, 1984; van Breemen, 1984).

In our study areas, both species are predominantly visited by bumblebees (e.g. *Bombus pascuorum* S., *B. terrestris* L., *B. hypnorum* L., *B. pratorum* L.) (Rademaker, 1998)

Study sites

In spring 2001, we selected an *E. vulgare* population in the dune area of Meijendel (near The Hague, The Netherlands, 52°8'N, 4°20'E). This population was located within a rectangular area of 6 x 20 meters and was partly sheltered from the wind by shrubs of sea buckthorn (*Hippophae rhamnoides*). There were 115 flowering plants in the population and 50 of them were randomly chosen, numbered and mapped (fig. 1). We collected a sample of seeds and a leaf for DNA extraction at the peak of flowering.

A *C. officinale* population was sampled in the same dune area in 2003. The population grew in an understorey of a thicket. The predominant tree species in the thicket was *Crataegus monogyna* with a small percentage of poplar trees (*Populus nigra*, *P. alba*) and *Sorbus aucuparia*. Smaller scrubs in the ticket consisted mainly of *Ligustrum vulgare*. The understorey was covered by mosses (~90% of a surface) with nettles (*Urtica dioica*) locally occurring at high density. In 2003, there were 288 flowering plants in the selected area of 40 x 45 meters. We numbered and mapped all the plants and sampled a leaf to dry in silica gel for DNA analysis. We randomly chose 103 plants for DNA extraction (fig. 2). After flowering, the plants were sampled together with their seeds. Twelve flowering plants did not set any full seeds.

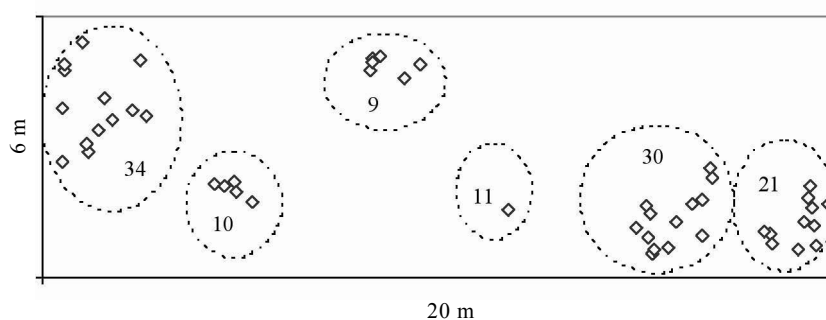


Fig. 1
Map of 49 flowering *E. vulgare* plants sampled for analysis of the population structure. A number next to each group of plants indicates how many flowering plants there were in total in each group. In total there were 115 flowering plants.

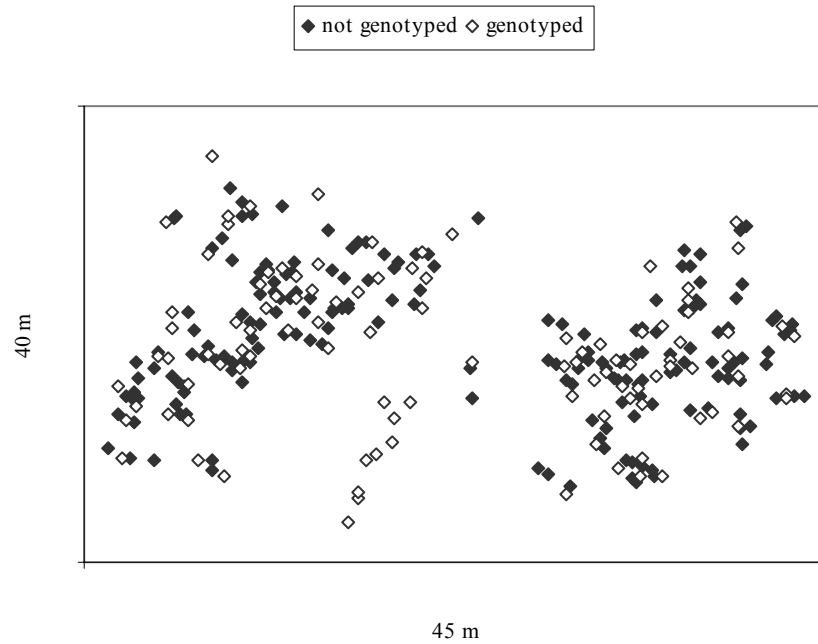


Fig. 2

Map of 288 flowering plants in *C. officinale* population. Open diamonds indicate plants genotyped and included into the analysis of a genetic structure.

Seed germination

We germinated the seeds from the selected flowering plants in order to have enough material for DNA extraction. In *E. vulgare* this germination was a part of a larger experiment, where 20 seeds were germinated from every flowering plant. The seeds were randomized and put on a thin layer of wet sand in replica plates. Then the plates were sealed with parafilm and placed in a climate room (day: 16h, 20°C; night: 8h, 15°C; 70% humidity). The germination percentage per mother equaled on average 89.3% (SE=1.53). We did not include non-germinating seeds into the paternity analysis. However, differential survival can not strongly bias the results. In chapter 5, we have shown that selfed seeds have only 16% lower germination compared to outcrossed seeds.

In *C. officinale*, we germinated only 1 seed per plant (91 seeds in total). The seeds were placed on wet filter paper in replica plates for 24 hours in the same climate room conditions as seeds of *E. vulgare*. The seed coat was removed from the seeds in order to monitor germination. Plates were monitored every day. After about a week one green cotyledone was taken for extraction. Seven seeds/seedlings that were infected by bacteria or fungi or/and did not show a proper germination were frozen at -20°C and successfully genotyped later.

Microsatellite analysis in E. vulgare

The fifty selected plants and two seedlings per flowering plant were genotyped with 7 microsatellite loci. DNA extraction, PCR conditions and characterization of six microsatellite loci followed Korbecka et al.(2003). The 7th locus *E2-83* contained a dinucleotide repeat (GA) and was amplified using forward primer AACCCGACACA-TCCAGCTAC and reverse primer TGGGCCTTATGTAAGTAGTGCT yielding fragments between 180 and 212 base pairs. The forward primer was labeled with a TAMRA label. Locus specific annealing temperature for *E2-83* was 60°C in all 30 cycles.

In the majority of the cases, we were not able to determine the exact genotypes of these tetraploid plants because of a poor correlation between strength of signal and the number of copies of alleles. Therefore, we scored the microsatellites in a dominant fashion noting only the presence of alleles in individuals, without a number of copies per allele.

PCR for 6 loci, apart from locus *E2-83*, were done twice for flowering plants to test repeatability. Out of 300 PCRs, 7 failed in the first round, 5 of them failed again in the 2nd round. The five failed PCRs were from the same flowering plant, which was excluded from analysis together with its two seedlings. All PCRs that were successful twice gave the identical microsatellite pattern.

In order to get a reliable estimate of selfing rate, twenty seedlings were excluded from analysis because their PCRs failed for 4 or more loci. For the final analysis 49 flowering plants and 78 seedlings were used.

Microsatellite analysis in C. officinale

We genotyped the 103 flowering plants and 91 seedlings with 7 microsatellite loci: *C2-19*, *C2-42*, *C2-43*, *C2-62*, *C2-72*, *C3-41* and *C3-79*. DNA extraction and PCR conditions followed Korbecka and Wolff (2004). Multiplexing allowed performing only 3 PCRs per individual to amplify all 7 loci. All 309 PCR for flowering plants and 273 PCRs for seedlings were successfully amplified.

We did not repeat PCRs because the microsatellites appeared to be very reliable and easy to score (Korbecka – unpublished data). Heterozygotes gave equally strong signals from both alleles, apart from locus *C2-42* where the signal intensity appeared to be negatively correlated with allele size.

Test for Hardy-Weinberg (HW) equilibrium

We tested for HW equilibrium in order to support our results on genetic structure. If there is a genetic structure, both nearest-neighbor pollination and selfing will lead to a departure from HW equilibrium. In *C. officinale*, we tested for HW equilibrium using a program ARLEQUIN (Schneider et al., 2000). For *E. vulgare* this analysis could not be done because we did not know the exact genotypes.

Selfing rate

Direct estimate in E. vulgare and C. officinale

We screened the seedlings for the presence of alleles that were not detected in the mother. If we found such alleles in at least one locus, the seedling was classified as outcrossed. This method overestimates the selfing rate because we are not able to detect outcrossing if the pollen has only alleles that are present also in the maternal plant.

However, we assume that this overestimation is minimal as we use 7 microsatellite loci for each species and most of these loci were very polymorphic (Tab. 1 and 2). Population selfing rate in *E. vulgare* was estimated based on offspring from 32 mothers with 2 seeds genotyped and 14 mothers with 1 seed. Three mothers had all seeds excluded from analysis due to too many failed PCR's. In *C. officinale* we used all the 91 seedlings.

Indirect estimate in *C. officinale*

In *C. officinale* we calculated the selfing rate indirectly based on the inbreeding coefficient $s = 2F / (1 + F)$ (Hartl and Clark, 1989), where s is selfing rate (indirect estimate). The inbreeding coefficients for each locus based on observed (H_{obs}) and expected (H_{exp}) heterozygosities was calculated according to the following formula: $F = 1 - H_{obs} / H_{exp}$ (Hartl and Clark, 1989). Then, we used averaged value of F to calculate the indirect estimate of selfing rate. Both self-pollination and biparental inbreeding will influence this estimate. By comparing the direct and indirect estimates of selfing rates we can get an indication of biparental inbreeding.

Genetic structure analysis

We tested genetic structure in both species using the program SPAGED1 (Hardy and Vekemans, 2002). In *E. vulgare*, data for individuals with two or three alleles in a certain locus were encoded as 'incomplete genotypes' with 2 or 1 unknown alleles respectively. The percentage of 'incomplete genotypes' for the parents varied between 67 and 90% depending on the locus. The frequencies of both alleles in an individual with two known alleles are assumed by SPAGED1 to be equal 0.5. A consequence of this way of encoding data is an inaccurate calculation of allele frequencies. The frequencies of common alleles will be underestimated and the frequencies of rare alleles - overestimated. However, on average, it does not bias the estimation of kinship coefficients.

We ran an analysis of genetic structure defining the number of distance classes, in such a way that each class had the same sample size (the same number of pair wise distances). We performed analysis with 6-10 distance classes, but we present correlograms based on analysis with 7 distance classes as a compromise between sample size per class and the physical distance covered per each class. We calculated pairwise kinship coefficients according to Loiselle et al. (1995). The significance of average kinship coefficients ($\hat{F}$) in every distance class was tested using permutation tests (one-sided test: $H_0: \hat{F} = 0$; $H_1: \hat{F} > 0$; 1000 permutations). We regressed the kinship coefficients against the natural logarithm of physical distance (Vekemans and Hardy, 2004). Permutation tests were used to test if the slope of these regression lines ($\hat{b}_F$) were significantly negative, as expected if isolation by distance occurs. These tests were also one-sided ($H_0: \hat{b}_F = 0$; $H_1: \hat{b}_F < 0$; 1000 permutations).

Vekemans and Hardy (2004) suggested that neither the values of kinship coefficients nor the slope of regression line should be used to compare genetic structure in different species because these values are arbitrary and depend on the sampling scheme. They proposed Sp statistics as an objective measure of a genetic structure for interspecific comparisons. Sp can be interpreted as a reciprocal of neighborhood size. Therefore, a low Sp value means that the neighborhood size is large and genetic

structure is weak. We calculated this statistics using a formula including the ploidy level ($k = 2$ for diploids, $k = 4$ for tetraploids) (pers.comm. – Hardy):

$$Sp = k / 2 * (-\hat{b}_F / (1 - \hat{F}_1)), \text{ with } \hat{F}_1 \text{ is the average kinship coefficient in the first}$$

distance interval. The calculated Sp values for *C. officinale* and *E. vulgare* were compared with data presented by Vekemans and Hardy (2004). We chose the 17 herbaceous species that were both animal pollinated and dispersing seeds by gravity for this comparison.

In order to estimate the amount of biparental inbreeding we have to know the frequency distribution of pollen dispersal distances within the population. Such data were not available, we used therefore the approach proposed by Vekemans and Hardy (2004): we will assume that pollen dispersal is restricted to the first distance class. Then the maximum estimate of biparental inbreeding is equal to the kinship coefficient in the first distance class.

RESULTS

Microsatellite analysis

The microsatellite loci used in this study were more variable in *E. vulgare* than in *C. officinale* (Tab. 1 and 2). The average number of alleles per locus equalled 5.7 (40/7) and 3.4 (24/7) in the studied species, respectively.

HW equilibrium

In *C. officinale*, the observed heterozygosities in all seven loci were lower than expected on the basis of non-random mating among the flowering plants. A significant deviation from HW equilibrium was found in 5 loci (Tab. 2). The average inbreeding coefficient (F) equals 0.226.

Selfing rate

In *E. vulgare*, the population selfing rate equals 5.43 % ($N = 46$ flowering plants). In *C. officinale* the population selfing rate equalled: 35.16% ($N = 91$ flowering plants). The indirect estimate of selfing rate in *C. officinale* based on inbreeding coefficient gave a very similar estimate 36.87%. The indirect estimate of selfing rate is not much higher than the direct one suggesting that biparental inbreeding is rare.

Tab. 1

Alleles and their approximated frequencies detected in 7 microsatellite loci in a population of 49 flowering *E. vulgare* plants. The allele frequencies were calculated by SPAGEDL.

Locus	Number of alleles	Above: allele lengths (bp) Below: approx. allele frequencies									
<i>E3-46</i>	7	220	222	224	226	228	230	234			
		0.23	0.27	0.12	0.03	0.16	0.06	0.12			
<i>E3-40</i>	6	178	181	187	190	193	196				
		0.15	0.11	0.31	0.10	0.30	0.03				
<i>E2-11</i>	2	242	249								
		0.55	0.45								
<i>E3-84</i>	5	294	297	300	303	306					
		0.02	0.01	0.43	0.45	0.09					
<i>E2-83</i>	10	180	190	198	200	202	204	206	208	210	212
		0.14	0.05	0.13	0.07	0.01	0.19	0.05	0.18	0.13	0.04
<i>E3-91</i>	6	169	181	184	187	193	196				
		0.26	0.13	0.06	0.08	0.35	0.12				
<i>E3-56</i>	4	268	269	271	286						
		0.39	0.26	0.23	0.13						

Tab. 2

Alleles, their frequencies and hererozygosities of 7 microsatellite loci in a population of 103 flowering *C. officinale* plants.

Locus	H _{obs}	H _{exp}	F	Number of alleles	Above: allele lengths (bp) Below: allele frequencies			
<i>C2-72</i>	0.40	0.56**	0.29	4	91	97	99	101
					0.38	0.07	0.54	0.01
<i>C3-79</i>	0.41	0.59**	0.31	5	188	191	208	214
					0.06	0.05	0.53	0.35
<i>C2-43</i>	0.41	0.50*	0.19	3	128	130	136	
					0.18	0.15	0.67	
<i>C2-19</i>	0.39	0.54*	0.27	3	115	117	131	
					0.03	0.49	0.48	
<i>C2-62</i>	0.32	0.39	0.17	3	167	169	171	
					0.24	0.01	0.75	
<i>C3-41</i>	0.17	0.19	0.14	2	133	136		
					0.90	0.10		
<i>C2-42</i>	0.41	0.51*	0.20	4	110	112	116	124
					0.65	0.04	0.04	0.27

H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity; F inbreeding coefficient

* statistically significant deviation from Hardy-Weinberg equilibrium (P<0.05)

** statistically significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction (P<0.0071).

Genetic structure

1. Regression analysis

In *E. vulgare*, the slope of the regression between kinship coefficients and the natural logarithm of physical distance was significantly lower than zero, indicating the presence of a weak genetic structure ($y = -0.0039 x + 0.0091$; $r^2 = 0.0049$; $N = 1176$; permutation test: $P = 0.023$; Fig.3). Such a significant genetic structure was not detected in *C. officinale* ($y = -0.0053 x + 0.0131$; $r^2 = 0.0003$; $N = 5253$; permutation test: $P = 0.101$; Fig. 3).

2. Permutation tests for average kinship coefficients per distance class.

In an analysis dividing data into 7 distance intervals for both species, the average kinship coefficients in the first distance class ($\hat{F}_1$) equaled 0.0169 and 0.0145 for *C.*

officinale and *E. vulgare* respectively and they were significantly higher than zero (permutation tests, $P < 0.05$). The first distance class in this analysis with 7 classes included pairwise distances between plants up to 1.48 m and 6.49 meters for *E. vulgare* and *C. officinale*, respectively. The average kinship coefficient was consistently higher than zero in the first distance classes if analysis was done with 6-9 distance classes for *E. vulgare*, and with 6-8 classes for *C. officinale*.

3. Biparental inbreeding

Assuming that pollen dispersal is limited to the first distance class, we conclude that biparental inbreeding equals 1.69% and 1.45% for *C. officinale* and *E. vulgare*, respectively.

4. Sp statistics

Sp values equal 0.0054 and 0.0079 for *C. officinale* and *E. vulgare*, respectively.

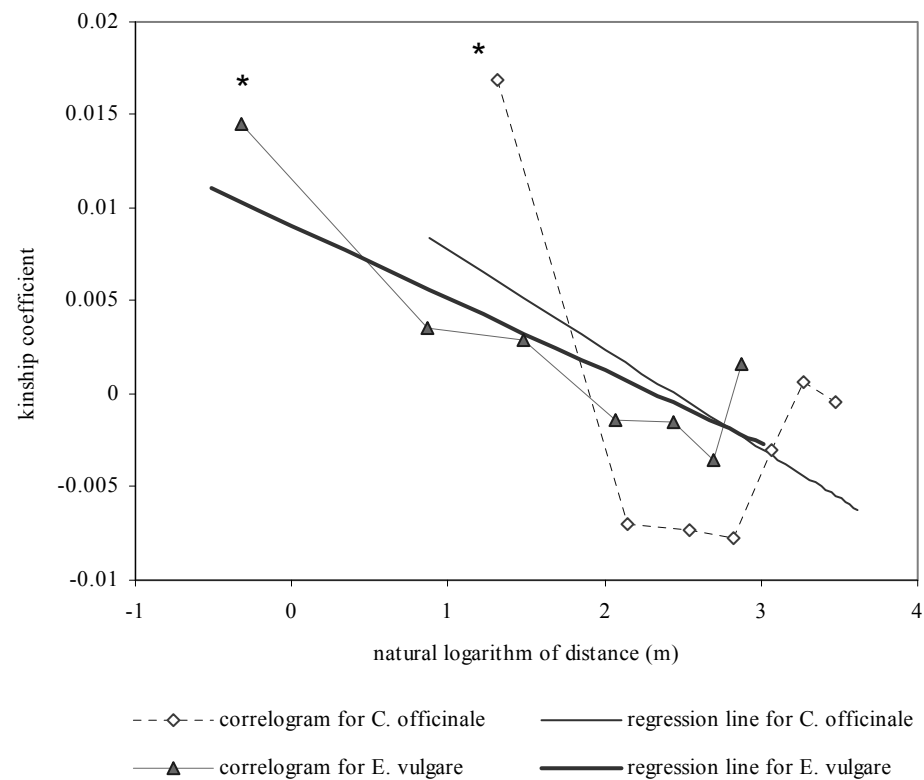


Fig. 3
Correlograms (average kinship coefficients per distance class plotted against a mean natural logarithm of distance in a class) and regression lines (between pairwise kinship coefficients and natural logarithm of distance) for *E. vulgare* and *C. officinale*

* -average kinship coefficient significantly higher than zero (permutation test, $P < 0.05$)

DISCUSSION

Biparental inbreeding

We detected a low level of biparental inbreeding (<2%) which may be an over estimate because we assumed that the pollen dispersal is restricted to the first distance class. This means that crosses among related individuals are rare and do not contribute to the inbreeding in *E. vulgare* and *C. officinale* in the field.

High levels of biparental inbreeding are more likely to be detected in species with higher selfing rates. The reason for it is that these species are more likely to form a strong genetic structure (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004). One of the few studies presenting experimental measurement of biparental inbreeding has reported a level of biparental inbreeding as high as 30% in *Aquilegia canadensis* (a perennial with, on average, 78 % selfing in the field, (Griffin and Eckert, 2003). In another study, Kelly and Willis(2002) found little or no biparental inbreeding in two populations of *Mimulus guttatus*. However, previous report on a genetic structure in this species have shown that the neighboring plants are not related (Sweigart et al., 1999). The experimental design used by Kelly and Willis (2002) and Griffin and Eckert (2003) is based on comparing the levels of apparent selfing in two groups of plants. The first group includes plants randomly transplanted within the population and the second (control) group includes plants only dug out and planted back in the places where they grew originally. This design allows for a more accurate estimation of the amount of biparental inbreeding and certainly more studies using this method are desirable.

Comparison of the genetic structure among the species

According to data reported by Vekemans and Hardy (2004), *Sp* values for the 17 herbaceous species, that were both animal pollinated and dispersing seeds by gravity, varies between 0.00471 for self-incompatible *Arabidopsis halleri* and 0.26316 for *Phaseolus lunatus* (a predominantly selfing plant), with a mean at 0.04328. A comparison of these *Sp* values to the values calculated for our two study species (0.0054 for *C. officinale* and 0.0079 for *E. vulgare*) confirms that the detected genetic structure in populations of flowering plants of these species is very weak. Interestingly, the genetic structure in *C. officinale* is weaker than in *E. vulgare*, which is contrary to our expectation. We can explain this only by more effective seed dispersal in *C. officinale*.

Why is the genetic structure in E. vulgare and C. officinale so weak?

In the analysis of the genetic structure in *E. vulgare* and *C. officinale*, the permutation tests show that the kinship coefficients are significantly higher than zero only for plants in the smallest distance class. This may indicate that there is a genetic structure at a very small scale (up to 1.48 meters for *E. vulgare* and up to 6.49 meters for *C. officinale*). This kind of structure may arise when the gene dispersal curve is leptocurtic, which means that there are essentially two kinds of dispersal: short and long distance dispersal. This explanation is likely for our study species. For example, seeds of *C. officinale* disperse by gravity within a distance up to 1.4 meters from a maternal plant (Boorman and Fuller, 1984). A part of the seeds may disperse secondarily to large distances by means of animals or run-off water. A similar line of reasoning may be used

for pollen dispersal. For example, Richards (1997) described that species with flowers pollinated by animals like bees or butterflies often have leptocurtic pollen dispersal curves due to clumped distribution of the flowering plants, presence of plant patches with various amount of reward and pollinator preferences for more rewarding patches. Pollinator movements within a patch would lead to short distance pollen dispersal and movements among patches – long distance dispersal.

The weak genetic structure in both species may also be a result of a thinning process. High mortality of seedlings and young rosettes has been recorded in *E. vulgare* and *C. officinale* (Jong and Klinkhamer, 1988; Klemow and Raynal, 1985). Therefore, we can not exclude that a genetic structure is more prominent in younger life stages.

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Chapter 5

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



Korbecka G. and P.G.L. Klinkhamer

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 bearing 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°C, 8 h dark 15°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°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°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°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 $25 \times 10^{-5} \text{ mm}^3$. 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 gibberelic 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°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 month 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.

Next page:

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.

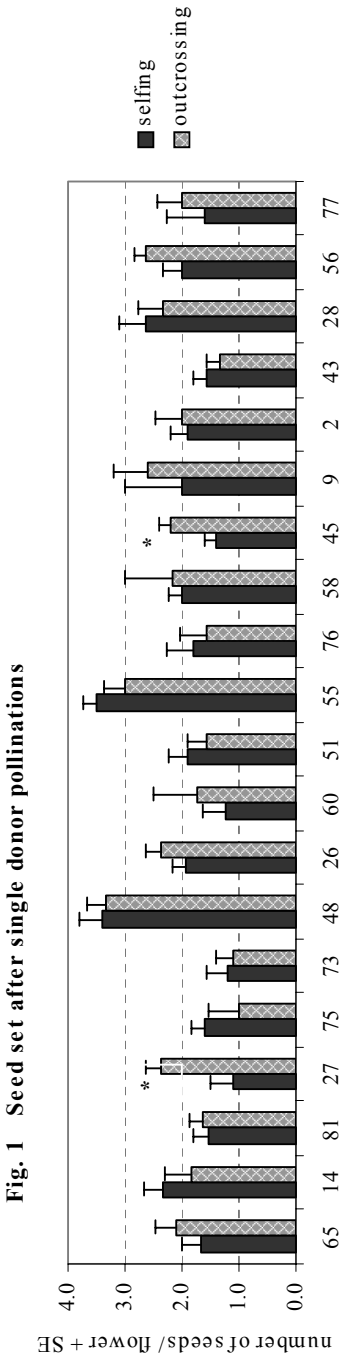
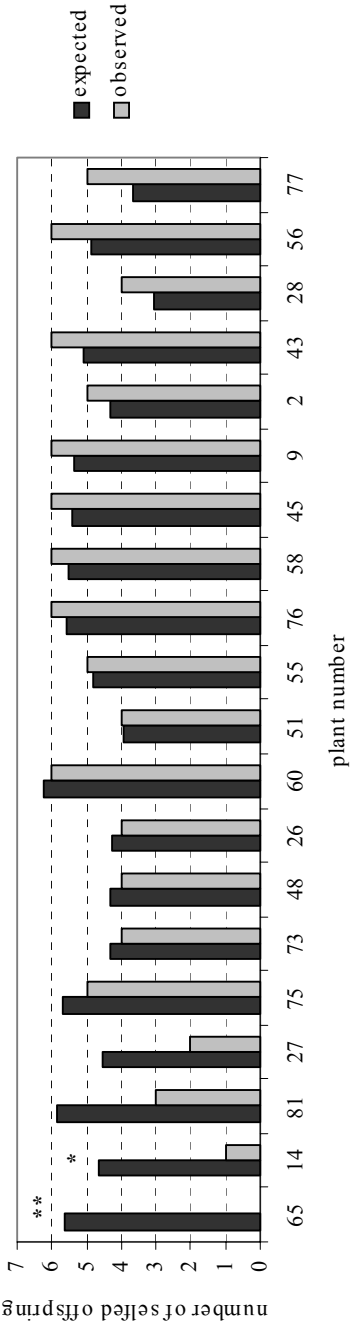


Fig. 2 Results of paternity analysis of seeds from mix donor pollination



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 than 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 %, $\chi^2 = 5.1241$, $df = 1$, $p = 0.0236$).

Next page:

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.

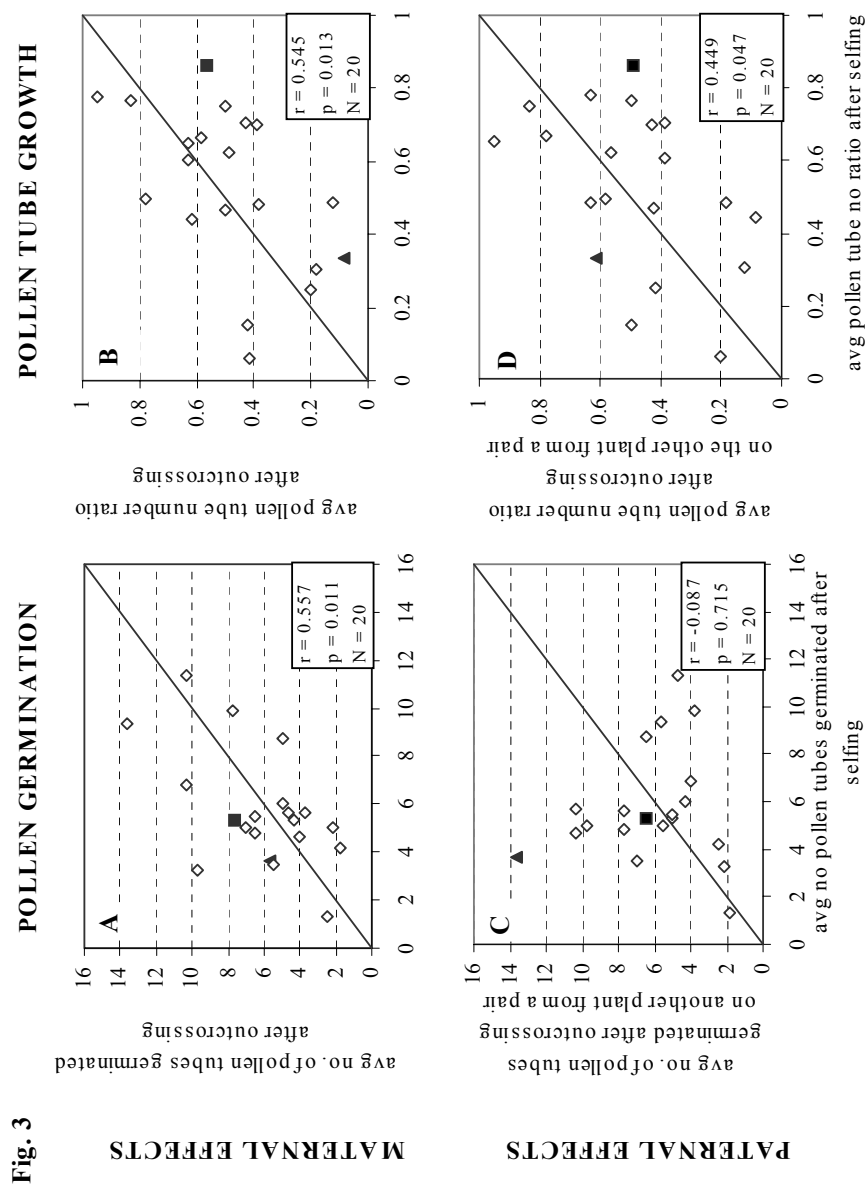
C: The relationship between the number germinated pollen tubes on stigmas after self-pollination and outcrossing with pollen from the same donor.

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 $y = x$.



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 allele a (177 bp) in locus *E3-40* is inherited by the

offspring in combination with every other allele 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

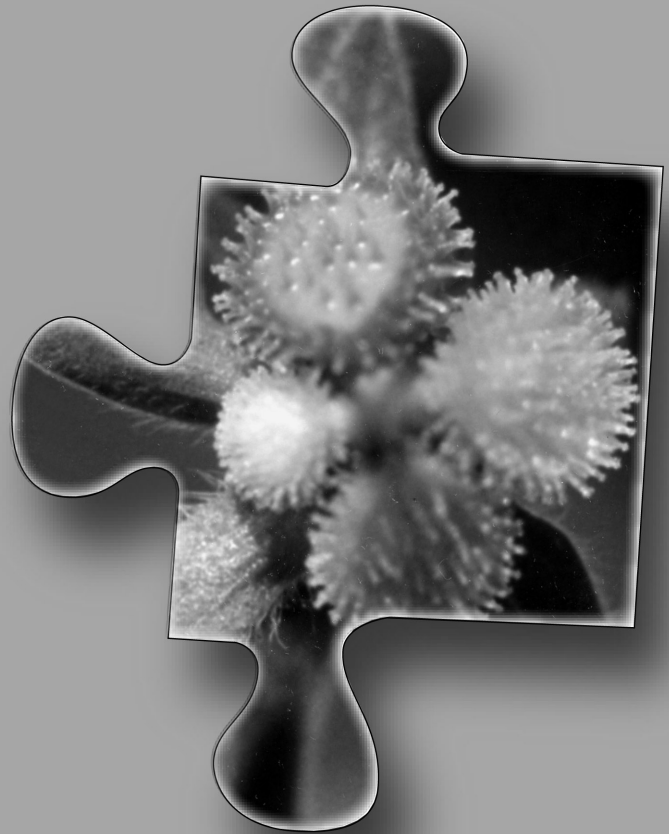
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Chapter 6

Selective embryo abortion hypothesis revisited - a molecular approach



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ABSTRACT

Many plant species abort a large fraction of their embryos. It has often been suggested that embryos of genotypes that would perform worse later in life are preferentially aborted. Such selective embryo abortion would lead to investment of resources only in the offspring with the highest potential fitness. Many studies have shown that otherwise viable embryos are aborted. However, only few manipulative studies have indeed shown a correlation between the level of abortion and offspring quality and these studies have been challenged for their experimental design. Molecular techniques open new opportunities to study selective embryo abortion. Non-random abortion at the level of molecular markers can be observed as deviation from Mendelian segregation: over- or under-representation of markers in the offspring. Subsequently, the over- or under-represented markers can be related to offspring quality later in life. We reviewed the literature on the genetic maps of intraspecific crosses of wild plant species and the selection of cultivated species. The level of non-Mendelian segregation we found in these maps is high. On average, 11.5 % of the tested markers in the genetic maps of wild species and 14.6 % - in the cultivated ones, show a departure from Mendelian segregation. From six studies, providing sufficient data, it was calculated that in 68% of loci segregating in non-Mendelian fashion post-fertilisation selection is involved. We propose that the deviation from Mendelian segregation can be partly explained by selective embryo abortion. We describe an experimental design that allows for attributing selective embryo abortion to the non-Mendelian segregation that is found in a genetic map.

INTRODUCTION

Selective embryo abortion (SEA) is the phenomenon that some genotypes are aborted more frequently than others. The Selective Embryo Abortion hypothesis proposes that the fitness of a female plant can be increased by the selective abortion of genotypes with a potential low quality later in life such that an increase in the level of abortion leads to an increase in offspring quality. In evolutionary theory the hypothesis is important in relation to explanations for low seed to ovule ratios and the optimal allocation of resources to male and female reproduction in hermaphroditic plants

The SEA hypothesis is based on the following premises: i) plants abort a substantial part of the embryos, ii) otherwise viable embryos are aborted, iii) the probability of being aborted depends on the genotype of the embryo. In contrast to ample theoretical attention given to the SEA hypothesis (e.g. Kozłowski and Stearns, 1989; Latta, 1995; Burd, 1998), there are only few experimental studies in which all conditions, mentioned above, were considered. The SEA hypothesis received a lot of attention in the 1980s (Stephenson, 1981; Wilson and Burley, 1983; Casper, 1988; Lee, 1988; Andersson, 1990; Andersson, 1993) but, probably due to difficulties with the interpretation of the results of experiments in which the abortion level was manipulated, empirical research on the topic drastically reduced. Most of these problems can now be overcome using molecular techniques. In this paper we want to review the work on this hypothesis and discuss the possibilities and difficulties that are presented by the use of molecular methods to shed new light on the topic.

In crossings, SEA shows up as a deviation from Mendelian segregation for some molecular markers. We will review data on the genetic maps of plants to judge whether there is a potential for SEA to be detected by means of marker segregation analysis. However, SEA is not the only mechanism leading to deviation from Mendelian segregation. We will discuss how we may distinguish SEA from other mechanisms that include e.g. meiotic drive, gametophytic selection, and seedling death.

MECHANISMS OF EMBRYO ABORTION

Two explanations have been proposed for the selective abortion of particular genotypes. 1. Maternal control, 2. Embryo competition.

Since, potentially, SEA may be maternally controlled, it has often been discussed as an aspect of female choice together with pre-fertilisation processes, such as selective inhibition of pollen germination and pollen tube growth (Wilson and Burley, 1983; Marshall and Folsom, 1991). Theoretically, in angiosperms maternal control can be through the endosperm, the tissue that nourishes the embryos, because it contains two copies of the maternal and one copy of the paternal genes, also abortion may be related to an interaction of the maternal and paternal genome. However, Marshall and Folsom (1991) concluded in their review on mate choice in plants that there is little evidence to prove that specific maternal mechanisms produce sorting among compatible donors. The problem with the assumption of maternal control is how the link with offspring quality later in life is brought about, in other words: How can the mother plant “know” which embryos will give the highest fitness contribution? Moreover, it is technically extremely difficult to experimentally test this hypothesis. If changes in the maternal tissue of the seed (nucellus and integuments) precede changes in embryo and endosperm development, this could point to maternal control. If the order of changes is the reverse, this would point to embryo competition (Marshall and Folsom, 1991 and refs. therein). As yet there is very little evidence to decide for either of the two possibilities.

More generally accepted is the idea that SEA is brought about through competition among embryos. Some embryos may be better competitors for resources than others, either because they present a larger sink or they may even release chemical substances, which are most probably indole compounds that inhibit the sucrose uptake of siblings (Mohan Raju et al., 1996; Krishnamurthy et al., 1997; Arathi et al., 1999). In this scenario, maternal “recognition” of the embryos is not necessary. The mother plant can influence offspring quality indirectly by controlling the level of resources and thus setting the selective arena for the embryos. It has been even suggested that endosperm reduces embryo competition since it is more frequently observed in the species with multiovulated ovaries compared to those with uniovulated ones, and it is found more often in the species with multiovulated species that experience less abortion (Uma Shaanker et al., 1996).

However, from an evolutionary ecological perspective the mechanism leading to SEA is not as interesting as the fact whether or not it can increase offspring quality. The increase of offspring quality through SEA would mean that embryo abortion is

potentially adaptive. The correlation between the abortion level and offspring quality is, under the assumption of embryo competition, brought about by genes that control e.g. basic metabolic processes that are important, both during embryo development and during later life, or by genes that have pleiotropic effects. Goldberg et al., (1989) summarise in their review: “More than 90% of the 15,000 diverse mRNAs present in mid maturation stage embryos are represented in both cotyledon stage and fully differentiated, mature embryos. Most of these mRNAs are also present in post-germination cotyledons and in the mature plant leaf.” The fact that most of the genes are expressed both in embryonic stage and later in life, gives ample opportunity for embryo abortion to have an effect on offspring quality later in life.

Most evidence for the fact that abortion depends on the genotype of the embryo comes from studies on inbreeding depression. If selfed embryos have a higher chance of being aborted than outcrossed ones (Montalvo, 1992; Gibbs and Sasaki, 1998), this suggests that recessive deleterious or lethal alleles may influence competitive strength of embryos. Embryonic viability is often assessed in relation to early-acting inbreeding depression. On the basis of information on seed production after selfing and outcrossing, the number of so-called, lethal equivalents is estimated. A lethal equivalent is a lethal gene or a number of deleterious genes that make up for one lethal gene. According to Lynch and Walsh (1998), the number of lethal equivalents per gamete affecting early embryonic survival varies approximately from 1.7 to 5.0 for conifers and from 0.4 to 0.91 for short-lived angiosperms. However, for considering SEA it is essential to make a distinction between lethal and deleterious alleles. If abortion results from recessive lethal alleles, both the level of abortion and the direction of selection are fixed. The embryos, which possess lethal alleles, will die irrespective of the conditions they encounter during development. In such cases it is not likely that the level of inbreeding depression during seed set will be correlated with the level of inbreeding depression later in life. This may explain the absence of such a correlation in the studies of Husband and Schemske (1995) or Koelewijn et al., (1999). On the other hand, if the alleles on which embryo abortions depend are deleterious, embryo abortion may be selective and depend on the conditions the embryo encounters. Remington and O'Malley (2000) studied early acting inbreeding depression in loblolly pine (*Pinus taeda*) using information from a genetic map. They estimated that in this species 19 loci have moderately deleterious or lethal embryonic effects. Moreover, most of the alleles reducing viability are recessive and for 3 loci overdominance was found. There is also another study (Melser et al., 1997) suggesting that embryo abortion may not be a result of action of recessive deleterious alleles. On the basis of comparing seed production after selfing and outcrossing in *E. vulgare* (after sufficient amount of pollen was applied), they found that some individual plants aborted more selfed embryos and others more outcrossed ones. Melser et al., (1997) concluded that in *E. vulgare* the effects of the deleterious alleles are mostly additive.

One can imagine that embryo abortion may be influenced by a number of (mildly) deleterious alleles that each by themselves have only a small effect and are therefore not easily purged from a population.

In the remaining part of the paper we will first present the more traditional phenotypic approach to study SEA and then we will discuss how molecular techniques can be used to study the same research questions.

PHENOTYPIC APPROACH

The level of abortion

Flowering plants commonly produce more ovules than seeds. In many angiosperm species, ovules may not develop into seeds due to pollen limitation (Wolfe, 1983; Zimmerman and Pyke, 1988; and see Burd, 1994 for a review) or because they are involved in self-incompatibility mechanisms (Waser and Price, 1991; Seavey and Carter, 1996). However, even after successful fertilisation, a considerable proportion of the ovules fails to produce seed in many species. Wiens (1984) estimated that seed-ovule ratio equals, on average, about 85% for annuals and 50% for perennials. Wiens's data are based on developing fruits. If ovules in undeveloped fruits are also included, the seed ovule ratios may be even lower. Dissections of ovaries showed that a large fraction of embryos is aborted, for example in *Prunus cerasus* (Bradbury, 1929), *Oxalis magnifica* (Guth and Weller, 1986) and *Epilobium angustifolium* (Wiens et al., 1987). Some species show extremely high abortion rates. In *Dedeckera eurekaensis* the seed-ovule ratio equals 2.5%, although about 90% of the ovaries initiate growth, indicating that fertilisation took place (Wiens et al., 1989). In *Asclepias speciosa* approximately only 3.8% of the ovaries develop into mature fruits, although 82.4% of them were fertilised (Bookman, 1984).

Gymnosperms also abort many seeds. In *Pinus sylvestris* on average 30% seeds are aborted (Karkkainen et al., 1999). The level of embryo abortion is, however, higher due to polyembryony. The most common form is simple polyembryony with independent fertilisations of more than one archegonium within the same ovule of which usually only one develops into a seed (Sorensen, 1982; Willson and Burley, 1983; Haig, 1992 and references there in).

In some cases, it has been argued that embryos are not viable because of high genetic load, as in the *D. eurekaensis* example (Wiens et al., 1989). However, as we will discuss later, even viable embryos are often aborted at a very high rate.

Are potentially viable embryos aborted and does abortion increase offspring quality?

In some species with linearly arranged ovules, developing embryos in the basal end of the ovary are more likely to abort. Nakamura (1988) described a successful *in vitro* culture of embryos from the basal end in *Phaseolus vulgaris*. In *Dalberia sisso*, Ganeshaiah and Uma Shaanker (1988) cut off two distal seeds and implanted the remaining pod in agar to complete maturation of the rest of the seeds. This treatment resulted in an abortion rate in the basal end of the pod as low as in the distal end of intact, control pods. Both Nakamura (1988) and Ganeshaiah and Uma Shaanker (1988) did not relate abortion to offspring quality.

To our knowledge, only four papers present evidence that abortion can increase offspring quality. These experiments compared offspring quality after natural abortion and after random thinning of the ovaries. In contrast to the first, the latter is not selective. Species of the family of the Boraginaceae always produce four ovules in each flower arranged in equal positions, in a square. Although pollen is not limiting seed production, on average there are fewer than four seeds per flower found in many species of the Boraginaceae (e.g. *Cynoglossum officinale* – Jong and Klinkhamer, 1989; *Echium vulgare* – Klinkhamer et al., 1994 and *Cryptantha flava* (Casper, 1988). In *Cr. flava* (Casper, 1988) and in *Cy. officinale* (Melser et al., 2001) the random destruction of three ovules in a flower resulted in doubling of the chance of maturation for the remaining ovule, compared to a control treatment with all ovules intact. This shows that in the control treatment a large fraction of the aborted embryos was potentially viable. In *Cr. flava* seeds from the control group with natural abortion showed higher emergence and survival during two years of growth (Casper, 1988). Melser and Klinkhamer (2001) found that natural abortion resulted in higher offspring survival in *Cy. officinale*. In *Lotus corniculatus* offspring produced after natural embryo abortion showed better germination, produced more leaves, and later in life more inflorescences, flowers and matured more seeds compared to the treatment after random destruction of ovules (Stephenson and Winsor, 1986). In a study on patterns of seed abortion in *P. coccineus*, Rocha and Stephenson (1991) found that ovules at the basal end of the ovary are more likely to abort, due to the fact that they lag behind in development because they were pollinated later, which may result in reduced nutrient availability. Destroying the ovules on the stylar end increased the probability of seed maturation on the basal end. The progeny that resulted from this treatment was significantly less successful compared to the control treatment with regard to germination time, vegetative growth, flowering time and number of flowers.

Is abortion dependent on embryo's genotype?

Stephenson (1981) and Lee (1988) show in their reviews that in many plant species the chance for an embryo of being aborted depends on factors like time of initiation, position within ovary, resource availability and pollen source. Even if the level of embryo abortion is high and viable embryos are aborted, abortions do not necessarily depend on the genotype of the embryo and may not lead to selection. Both single pollen donor and mixed pollen donor experiments have been used to study the relationship between abortion rate and genotype.

The easiest way to detect selection is when each flower of a plant receives pollen from a single pollen donor only. One can then compare the siring success of different potential fathers directly, by counting the seeds in the flowers, without the use of genetic markers. With this approach, it is unlikely that pollen tube competition influences differences among fathers. The disadvantage of single pollen donor experiments is that competition among the embryos within a flower cannot be detected.

Bertin (1982) studied the self-incompatible trumpet creeper (*Campsis radicans*) and applied pollen of different fathers and found that the pollen donors that were favoured by particular recipients were usually those whose pollinations resulted in fruit with many and large seeds. Although prezygotic mechanisms were not all properly

excluded, the author conclude that fruit abortion seems to have been more important in donor selectivity than prezygotic phenomena.

Most single pollen donor experiments aim at comparing abortion after self- and outcross pollination, or comparing outcross pollination with close and distant donors. Such comparisons are interesting, especially because Husband and Schemske (1996) showed that embryo development is one of the most important life stages in which inbreeding depression can act.

For the self-compatible *Aquilegia caerulea* Montalvo (1992) found that the abortion rates for selfing were, on average, 38% higher compared to abortion rates for outcrossing, while there were no significant differences in fertilisation rate for both pollination types. For *E. vulgare*, Melser et al., (1997) found that in some individuals self-pollen was relatively more successful compared to outcrossed pollen while in others the outcrossed pollen was more successful. Pollen donors did not differ in pollen viability, pollen germination and pollen tube growth. Therefore, Melser et al., (1997) concluded that differences in siring success of different pollen donors were most likely caused by selection among embryos.

Gibbs and Sasaki (1998) found for *Dalbergia miscolobium* in the field that 30.0% of crossed flowers and 3.6% of selfed flowers developed mature fruits. This difference was mainly caused by abortion of selfed embryos because, in the ovules dissected 4-6 days after pollination, embryos were found in similar frequency and condition for both treatments.

Marshall and Whittaker (1989) studied effects of identity of a pollen donor on offspring quality in *Raphanus sativus*. They found significant paternal effect on the number of leaves and weight of offspring after eight weeks of growing in a greenhouse. The effects of pollen donor were more pronounced if maternal plants were grown in water stress conditions. Their results suggest that the processes that sort among potential fathers during pollination, fertilisation and seed filling may improve offspring quality.

Multiple donor experiments, where a mixture of pollen from different genotypes is applied to a single flower, combined with paternity analysis, can also provide information about SEA. The advantage of multiple donor experiments is that selection among pollen donors within flowers can be detected. The disadvantage is that, if it is not possible to analyse aborted embryos for their paternity, an appropriate method has to be found to separate the effects of pollen tube competition from SEA. Marshall and Ellstrand (1988) carried out a multiple donor experiment on *Raphanus sativus* under stress conditions. Early water stress can affect both fertilisation and early seed abortion. In contrast, late water stress can only influence seed abortion. The contribution to the progeny of the three pollen donors differed from the control in the late stress treatment but not after early stress. Apparently, only late abortions provide the opportunity to select in this case.

Attributing the abortion rate to the origin of pollen in some gymnosperms is even easier since they have poorly developed prezygotic selection mechanisms (Willson and Burley, 1983). For example, Karkkainen et al., (1999) determined the abortion rate in *Pinus sylvestris* as a proportion of empty seeds, because seed coat formation in this species is an effect of pollination. They found that frequency of abortion increases with

the proportion of self pollen applied to the flowers. The proportion of empty seeds ranges from 23% after outcrossing to 76% after selfing.

Limitations of phenotypic approach

Missing information about the selection among genetically different offspring sired by the same father. Pollination experiments can show that selective abortion exists only if siring success of different fathers is compared, either after single donor pollinations or after mixed donor pollinations combined with paternity analysis. Moreover, it is necessary to eliminate that prezygotic mechanisms may play a role. The big disadvantage of this approach is that a part of post-fertilisation selection, which may occur among genetically different offspring of the same father, can not be observed. Only molecular techniques can provide data on that.

Crushing ovules does not always reduce abortion levels. One can explain Casper's (1988) and Melser and Klinkhamer's (2001) results by assuming that the resources not used by the destroyed ovules are allocated to the remaining ovules within the same flower, thereby increasing the chance for maturation. If the experimental treatment is applied to only a part of the flowers, it is possible that resources that would be used by crushed ovules are divided among all ovules of the plant and not only among those that remained in the hand-thinned flowers. In such a case, the difference among the treatments would be small and could go undetected. Perhaps this may at least partly explain the negative results found in two studies on *Anchusa officinalis* (Andersson, 1990) and *Achillea ptarmica* (Andersson, 1993). The difficulties in the interpretation of the results from the experiments discussed above can be avoided if a single treatment is applied to a whole plant and the same genotypes are used in different treatments (Melser et al., 2001).

Decreased offspring quality after random crushing of ovules may be an artefact. In experiments based on ovule destruction, inferior offspring not necessarily results from genetic differences but may be caused by subtle effects of the mechanical damage itself. Casper (1988) cautions: "Prematurely removing some reproductive structures might upset initial source-sink relationships and thus plant-resource levels, adversely affecting seed quality. In addition, forcing a flower to distribute resources to an ovule that it normally would not mature might itself result in an inferior seed." Moreover, developmental irregularities of the flower can influence the competitive strength of the embryo.

An experiment, as described above, is therefore not sufficient to prove that selective embryo occurs. The best way to show that SEA can increase offspring quality in ovule destruction experiments is to collect genetic evidence as well. We will therefore discuss in the remaining of the paper how molecular data can be used to overcome the problems caused by the traditional approach to the SEA hypothesis.

GENOTYPIC APPROACH

If embryo abortion is selective, certain alleles will be under- or over-represented in the offspring, compared to Mendelian segregation. The upswing in molecular methods in the last decade has led to an easy access of abundant molecular markers in almost every organism (e.g. AFLP). Such molecular markers might be a powerful tool to detect and assess the adaptive value of SEA. Using molecular markers avoids the limitation of pollination experiments because selection among offspring of a single pollen donor can be detected. Even if the plant is self-pollinated, selection among embryos may be observed in the loci for which the parent plant was heterozygous. So far, selection among the offspring of single father has been largely ignored. This may have caused an under-estimation of the level of SEA.

The second advantage of using molecular markers to test the SEA hypothesis is that the presence or absence of alleles that are under- or over-represented in the offspring, compared to Mendelian segregation, can be related to offspring performance in later life. This would be a much better way of assessing the selective advantage of embryo abortion compared to traditional methods, because no manipulations of flowers or plants (e.g. destroying of ovules) are needed and because selection can be directly linked to the genotype of the offspring.

Selection among embryos can be presented on the level of DNA as a deficiency or excess of certain genotypes among the offspring that successfully went through seed maturation, compared to expected Mendelian segregation. We reviewed genetic maps of plants in order to determine the potential for SEA. If the percentage of molecular markers showing non-Mendelian segregation found in genetic maps of plants is as low as expected due to chance alone, we have to conclude that SEA is not an important process. This argument, however, cannot be reversed. If many markers show non-Mendelian segregation, that could be due to SEA but other selective mechanisms cannot be excluded. For instance, meiotic drive and gametophytic selection can lead to non-Mendelian segregation as well (Appendix). The difficulty in distinguishing the cause of non-Mendelian segregation is a disadvantage of this method. An appropriate experimental design should be used to study segregation in plants with different treatments leading to differences in the level of abortion, as will be discussed later.

Is non-Mendelian segregation common in plants?

Data about non-Mendelian segregation in plants can be found in genetic maps. In almost all genetic maps of plants we reviewed, authors refer to a statistically significant departure from Mendelian segregation as *distorted segregation*, although they usually do not present any evidence for the presence of segregation distorter genes *sensu* Lyttle (1991). Lyttle defines segregation distorters as genetic elements that exhibit meiotic drive. That is why, when we consider a statistically significant departure from Mendelian segregation, we will use the more neutral term: non-Mendelian segregation.

It is common practice to test by means of a chi square test at a 5% significance level, whether or not segregation of a certain marker deviates from the expected ratio. If all markers are inherited independently, 5% of all markers should show non-Mendelian segregation, if no selection occurs. However, it is extremely difficult to determine the expected fraction of markers showing non-Mendelian segregation under the null

hypothesis, that no selection occurs. Firstly, non-Mendelian segregation can be over- or under-estimated when judged from the number of loci with a significant non-Mendelian segregation because, in a distorted region of the genetic map, the density of mapped molecular markers may differ from the average. Secondly, an unknown percentage of DNA markers is located in non-functional regions (e.g. not- or loosely linked to functional regions). For such markers, only non-Mendelian segregation due to chance is expected. Nevertheless, tables 1 and 2 (Appendix) provide useful information because, averaged over all species, the first problem should disappear as we have no reason to assume that the density of molecular markers is higher or lower in the region where selection occurs. The second problem can only lead to an under-estimation of selection. Unfortunately, we do not know the quantitative importance of this.

We searched for genetic maps based on intraspecific crosses of cultivated and wild species. Wild species were defined in the broadest sense possible. The basic criterion we used was that artificial selection and inbreeding were minimal. We expected that in genetic maps of cultivated species non-Mendelian segregation is found more often because mapping populations are often derived from crosses between different inbred lines varieties or come from distinct geographical areas (e.g. Loarce et al., 1996; Jenczewski et al., 1997; Liu et al., 1997; Qi et al., 1998). It may happen that genes from one inbred line/variety do not function properly when combined with genes from another inbred line/variety.

Results of the literature survey are presented in tables 1 and 2 (Appendix). The percentage of markers showing non-Mendelian segregation differs significantly from 5% for the 59 analysed species ($t = 9.143$; $df = 58$; $p < 0.001$). It ranges from 0.1 - 40.82% (average: 14.6) for cultivars (Tab.1) and from 0 - 41.0% (average: 11.5) for wild species (Tab.2).

The difference in the average percentage of markers showing non-Mendelian segregation between cultivated and wild species is not significant ($F = 1.099$; $df=1,57$; $p = 0.299$).

Distinguishing between biological phenomena and technical problems.

Sometimes it is argued that sampling error or irreproducibility of the techniques can be responsible for a high percentage of molecular markers showing non-Mendelian segregation. Here we will consider the importance of those problems.

Inconsistent PCR amplification can cause irreproducibility of the method and hence a detection of apparently higher non-Mendelian segregation. RAPD is known as a technique that not always gives fully reproducible results (Jones et al. 1997). We therefore compared the level of non-Mendelian segregation detected in genetic maps using three techniques: AFLP, RAPD, and RFLP. None of the techniques gave significantly higher level of non-Mendelian segregation (paired samples test results for: RAPD vs. RFLP: $df=10$, $p=0.433$; RFLP vs. AFLP: $df=4$, $p=0.222$; RAPD vs. AFLP: $df=4$, $p=0.386$), although PAGE gels used in AFLP give much higher resolution than agarose gels used commonly in RAPD's. Note, however, that the tests are based on a small number of comparisons. The constant warning (e.g. Jones et al. 1997) that RAPD's are not fully reproducible may have caused a severe selection against markers giving non-Mendelian segregation before use in mapping. Many authors using RAPD markers for the construction of a genetic map, only include markers which are efficiently amplified and exhibit unambiguous polymorphism. Jenczewski et al. (1997)

write: "when such precautions are taken, RAPD does not induce higher levels of distortion than restriction fragment length polymorphism (RFLP)". Discarding markers before use in mapping, although to smaller extent may have happened in these of the other techniques as well. Tables 1 and 2 may therefore be an underestimate the level of non-Mendelian segregation.

Other sources of artefacts can be homoplasy, which is the amplification of two fragments of the same length from non-allelic regions, low resolution of agarose-gels, and co-migrating and overlapping polymorphic fragments. However, we expect these explanations to have only a minor influence on the level of non-Mendelian segregation. Rieseberg (1996) tested the homology of 220 RAPD co-migrating fragments in three closely related species of sunflowers and found that 91% of fragments are homologous. This means that artefacts like homoplasy and wrong scoring due to low resolution of agarose gels may be responsible only for 9% co-migrating fragments. However, this number would be much lower if individuals from the same mapping population derived from intraspecific cross were compared.

Moreover, artefacts mentioned above cannot explain why non-Mendelian segregation often occurs in clustered loci. We screened the reviewed genetic maps for the distribution of markers showing non-Mendelian segregation. On the basis of the data from 30 maps (these maps are indicated in tab. 1 and tab 2) we found that 56% of 633 loci segregating in non-Mendelian fashion formed clusters of two or more markers.

A strong argument for the fact that the non-Mendelian segregation is not found due to the chance or sampling error is the repeatability of finding skewed markers in the same species in many crosses, with a different set of parents, in the same chromosomal regions. Xu et al., (1997) mapped chromosomes of rice using many types of crosses: inter subspecific crosses, doubled haploid, and recombinant inbred lines. They detected a non-Mendelian segregation, by means of RFLP in all types of crosses, ranging from 17% for one of the intersubspecific crosses till 70% for one of the doubled haploid populations. 227 distorted markers were clustered in 17 chromosomal regions, and nine of these regions were associated with segregation distortion in more that one population. Repeatability of non-Mendelian segregation in the same region of genetic maps derived from different crosses of the same species was also reported by Price and Tomos (1997).

How to distinguish between SEA and other biological explanations for non-Mendelian segregation?

There is a long list of biological explanation for non-Mendelian segregation in plants, it includes, apart from SEA, meiotic drive, gametophytic selection, selective germination and seedling death, B chromosome, cytoplasmic inheritance, endophytes and chromosomal rearrangements (Appendix). Therefore, it is very difficult to separate between SEA and other causes of non-Mendelian segregation. Below we will present three ways allowing for such a discrimination.

Genotyping embryos.

Direct evidence for SEA may be presented by molecular marker segregation analysis of aborting embryos in comparison to mature seeds. Rigney (1995) performed a successful paternity analysis for aborting embryos by means of the MDH allozyme marker. Rigney (1995) removed embryos that were being aborted from a plant and analysed their paternity in *Erythronium grandiflorum*. Selfed embryos were more likely to abort than outcrossed ones. Moreover, the progeny fertilised by nearby donors are aborted more often than those sired by distant donors.

The difficulty with isolation of embryos and the very small amount of material may limit the feasibility of this method. PCR based techniques, like microsatellites, can be a better alternative to allozyme analysis since they require much smaller amount of plant material. Hufford et al., (2000) have shown that aborting embryos of *Platypodium elegans* can be successfully genotyped by means of microsatellites. Reusch (2000) used also microsatellites to genotype developing embryos in *Zostera marina*. However, isolation of embryos at the stage when they are large enough for analysis makes it impossible to investigate effects of very early stages of abortion.

Analyzing segregation patterns.

When one can not analyze the aborting embryos for their paternity, it is rather difficult to judge what was the cause of observed non-Mendelian segregation that is already detected in a map. An attempt to separate different causes has been made by Pham et al., (1990) who determined whether selection before or after fertilisation took place on the basis of segregation analysis of isozyme loci in a F_2 generation in several crosses of rice. They used successive χ^2 tests for 18 loci in which non-Mendelian segregation was found. Firstly, the equiprobability of alleles (p,q) was tested. Secondly, a χ^2 test was made to test if the distribution of genotype frequencies fits to $p^2:2pq:q^2$ (based on the observed allele frequencies p and q) (see Fig.1) Since, for most of the tested skewed loci, the frequency of alleles was not equal and genotype frequencies fitted to $p^2:2pq:q^2$ distribution, Pham et al., (1990) concluded that prefertilisation (gametophytic) selection was responsible for non-Mendelian segregation. However, for other loci, evidence for post-fertilisation selection was found. Guiderdoni (1991) reported similar results for the crosses between different varieties of rice.

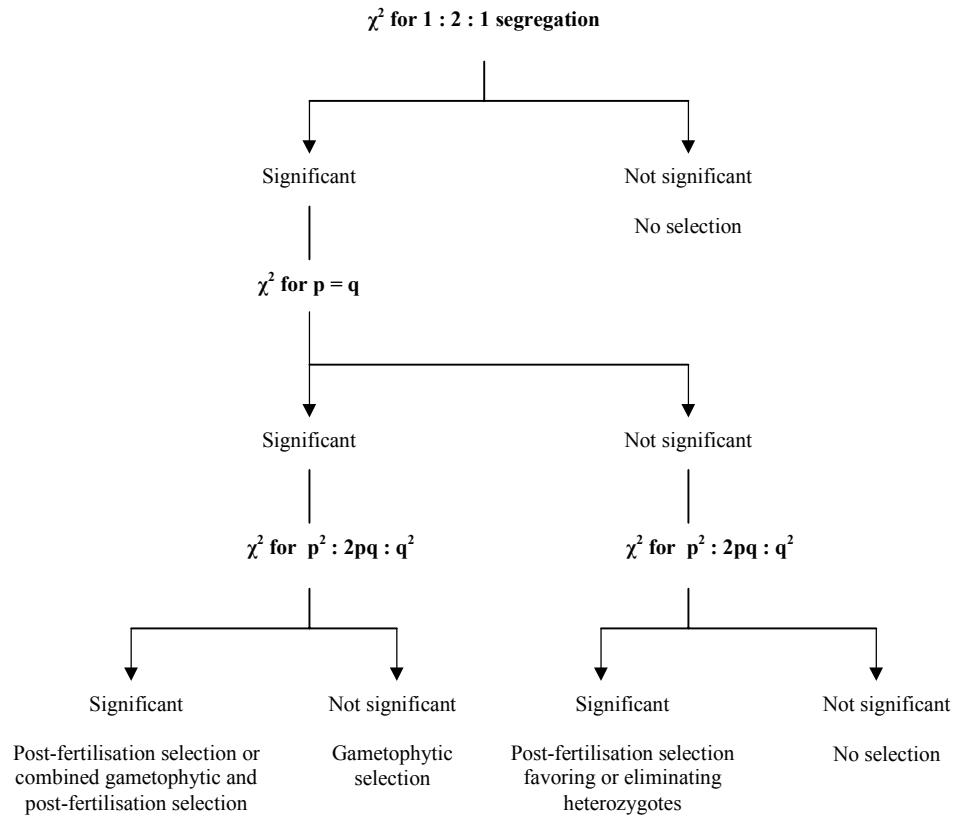


Fig. 1

Key for determining whether gametophytic or post-fertilisation selection takes place in a segregating F_2 population analysed with co-dominant markers (based on Pham, 1990)

We performed such an analysis for the reviewed genetic maps that reported segregation data for the F2 generation for co-dominant markers. We found segregation data for such markers in 6 genetic maps only (Mukai et al., 1995; Baudracco-Arnas and Pitrat, 1996; Katzir et al., 1996; Chen et al., 1998; Korzun et al., 1998; Vanhala – unpublished data). Chi square analysis of 56 loci revealed that, in 31 loci, post-fertilisation selection affecting heterozygotes took place, while in 7 loci, zygotic or a combination of selection before and after fertilisation occurred. In 15 loci gametophytic selection was detected. In 3 loci the stage of selection could not be determined by means of subsequent χ^2 tests. This analysis shows that post-fertilisation selection occurs in the majority of cases (68 %) involved in non-Mendelian segregation.

Another method to track down the cause of non-Mendelian segregation is the analysis of segregation of molecular markers in reciprocal crosses. Korzun et al., (1998) performed such crosses in rye (*Secale cereale*) and found, in one cross, 7 loci showing non-Mendelian segregation, while in the other cross such a skewed segregation was found for 9 loci. Only 2 of those loci were common for both crosses and they are potential loci in which post-fertilisation selection could take place. Asymmetry of segregation data in reciprocal crosses could be caused by post-fertilisation selection due to an interaction between nuclear and cytoplasmic genes which is different, depending on which plant is used as a female in a cross, or gametophytic selection affecting either male or female function of one of the parents. The distinction between pre- and post-fertilisation selection in the latter case could be made if reciprocal backcrosses to both parents are performed. Faris et al., (1998) compared non-Mendelian segregation in 4 such crosses in *Aegilops tauschii*. They attributed nearly the whole observed non-Mendelian segregation on chromosome 5D to gametophytic selection affecting male function, however, they could not exclude nuclear-cytoplasmic interaction in one region on that chromosome.

Possible experimental design to test SEA hypothesis.

Attributing non-Mendelian segregation, in crosses used to make genetic maps, to one of the explanations given in the appendix can be done in some cases if an experiment is carefully planned or additional research is conducted. Some of the explanations (other than SEA) for non-Mendelian segregation can be ruled out by additional studies such as: chromosome counting and observation of pollen germination (Appendix). The best way to separate the effect of SEA from other explanations listed in appendix would be to find an experimental treatment with which the level of abortion is manipulated without influencing the other processes. Nutrient stress would be a good candidate: it is known to influence abortion levels while there are no reports that it influences e.g. meiotic drive. If the deviation from Mendelian segregation for certain molecular markers is positively correlated with the level of embryo abortion this would imply that, indeed, embryo abortion is selective. Using the same genotype (clone) in all treatments could further strengthen the argument because meiotic drive, B chromosomes, cytoplasmic inheritance, chromosomal rearrangements can be excluded since the same nuclear genes (chromosomes) are in the same cytoplasmic environments in all nutrient treatments. If clones are grown in the same environment, they could possibly also contain the same endophytes.

An alternative experiment could be to compare the segregation of molecular markers in the offspring coming from two treatments performed on self-pollinated clonal replicates of one genotype of a plant. The first treatment would have the ovules randomly crushed, while the control treatment would not be manipulated. If selective abortion is playing a role, then it is expected that more loci in the offspring of control plants show non-Mendelian segregation.

The advantages of these two experimental designs are that:

1. non-Mendelian segregation can be attributed to SEA,
2. it can be established if SEA leads to higher offspring quality, and linked to the genotype of the offspring,
3. at least for the control treatment, SEA can be studied in unmanipulated plants,
4. non-Mendelian segregation can also be studied among the offspring of the single father.
5. it is possible to find markers for SEA that can be used on other genotypes.
6. major loci controlling SEA can be detected.

CONCLUSIONS

The traditional experimental phenotypic approach to test the SEA hypothesis has the disadvantage that the treatment itself can also be a source of artefacts. That is why the evidence that SEA is increasing offspring quality is still weak. The molecular genotypic approach links SEA to the presence and absence of particular alleles, which is why offspring quality can be related to specific alleles and, therefore, manipulations like crushing ovules are not necessary.

The level of non-Mendelian segregation found in the published genetic maps suggests that there is an ample opportunity to detect SEA. An appropriate experimental design, which we propose in this paper, would not only detect SEA and provide the possibility to relate this to offspring quality, but it would also provide more information about the genetic mechanisms controlling this process.

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APPENDIX

Table 1 Percentage non-Mendelian segregation found in genetic maps of cultivated species.^{\$}

Species	Segregating population		No. of polymorphic markers	% of markers showing nMS at 0.05	No. of loci showing nMS for each type of marker						References		
					RAPD		RFLP		AFLP			other #	
	nature	no.			total	% nMS	total	% nMS	total	% nMS		total	% nMS
<i>Allium cepa</i> *	F3	58	128	14.84 ^b	14	0.00	112	16.96			2	0.00	King et al. 1998
	F2	90	82	29			82	29					Cloutier et al. 1997
	F2	200-253	62	20.97 ^a	61	21.31 ^a					1	0.00	Sondur et al. 1996
	F2	112	5	20.00							5	20.00	Katzir et al. 1996
<i>Cucumis melo</i>	BC	66	228	14.47	6	0.00			221	14.93	1	0.00	Wang et al. 1997
	F2	218	110	12.73	68	16.18	36	2.78			6	33.33	Baudracco-Arnas and Pitrat 1996
<i>Festuca pratensis</i> *	F2	56	70	37.14 ^a			70	37.14 ^a					Chen et al. 1998
	F2.F3	96	138	8.70			138	8.70					Shappley et al. 1998
<i>Gossypium hirsutum</i> *	F2	65	85	2.35							85	1.18	Wang et al. 1998
	F9 RIL	103	568	8.45					568	6.45			Qi et al. 1998
<i>Hordeum vulgare</i>	F8 RIL	167	113	4.42 ^b	32	9.37 ^b	78	2.56 ^b			3	0.00 ^b	Teulat et al. 1998
<i>Hordeum vulgare</i> *	F2	134 or 40	306	7.52 ^a			22	9.09 ^a	284	7.39 ^a			Richter et al. 1998
<i>Malus domestica</i> *	F1	96	10	0.10							10	0.10	Gianfranceschi et al. 1998
<i>Malus pumila</i> *	F1	152	290	11.38 ^a	133	7.52 ^a	124	14.52 ^a	4	0.00 ^a	29	10.34	Maliepaard et al. 1998
<i>Medicago tornata</i>	F2	80	33	39.39	32	40.62					1	0.00	Jenczewski et al. 1997
<i>Medicago truncatula</i>	F2	122	49	40.82	48	39.58					1	100.0 ⁰	Jenczewski et al. 1997
<i>Oryza sativa</i>	F5 BIL	98	245	4.9 ^{a,b}			245	4.9 ^{a,b}					Lin et al. 1998
<i>Oryza sativa</i>	BC	122	118	12.50			57	c	61	c			Tan et al. 1998
<i>Oryza sativa</i> *	3way cross	230	191	8.38 ^a			191	8.38 ^a					Liu et al. 1997
<i>Oryza sativa</i> *	F2	178	71	12.68 ^a			71	12.68 ^a					Price and Tomos 1997

<i>Phaseolus vulgaris</i>	F8 RIL	72-75	599	17.53	489 ^a	c	100 ^a	c			10 ^a	c	Freyre et al. 1998
<i>Pisum sativum</i>	F2 RIL	139	259	8.11	248	8.06	9	11.11			2	0.00	Laucou et al. 1998
<i>Prunus persica</i> *	F2	63	270	1.85	92	1.09	50	8.00	115	0.00	13	0.00	Dirlewanger et al. 1998
<i>Secale cereale</i> *	F2	258	91	10.99			88	9.09			3	66.67	Korzun et al. 1998
<i>Secale cereale</i> *	F2	54	104	20.19	12	0.00 ^a	77	19.48 ^a					Loarce et al. 1996
<i>Setaria italica</i> *	F2	138	160	21.25 ^a			160	21.25 ^a					Wang et al. 1998
<i>Sorghum bicolor</i> *	F5 RIL	110	155	5.81 ^{a,b}			155	5.81 ^{a,b}					Dufour et al. 1997
<i>Sorghum bicolor</i> *	F5 RIL	91	129	24.03 ^{a,b}			129	24.48 ^{a,b}					Dufour et al. 1997
<i>Theobroma cacao</i> *	BC	131	138	4.35 ^a	104	3.85 ^a	32	6.25 ^a			2	0.00 ^a	Crouzillat et al. 1996
<i>Triticum turgidum</i> *	F7 RIL	65	259	18.53			244	c			15	c	Blanco et al. 1998
<i>Vigna unguiculata</i> *	F8 RIL	94	181	14.36 ^a	133	11.28	19	15.79	25	24.00	4	0.00	Menendez et al. 1997
<i>Zea mays</i>	F2 ^d	112	201	14.43	140	17.14	161	3.11					Beaumont et al. 1996
<i>Zea mays</i> *	F3:4	80	106	8.49 ^a			106	8.49 ^a					Tuberosa et al. 1998

^aWe have searched for the genetic map of cultivated species with the keywords: "genetic map" or "linkage map" and "plant" in the journal "Theoretical and Applied Genetics" from volume 93 (year 1996) till volume 97 (year 1998). We used WINSPIRS 2.0 to search in the Current Contents database. The search resulted in 222 records. Data from 33 papers out of 222 papers could be included in the table. A paper was included if the number of loci with significant non-Mendelian segregation for the genetic map could be calculated. Partial genetic maps and maps based on doubled haploids or intraspecific cross were not included. Many doubled haploid lines are derived from the pollen of one parent. Analysing these lines yields the segregation directly without the necessity of crossing. After pollen germination and regeneration of haploid plants, a chromosome doubling occurs spontaneously or it is induced chemically (by colchicine). The plants grow and then the material is sampled for DNA analysis. During the germination of pollen and while the plants are growing in *in vitro* culture, selective mortality may occur. This mortality might explain the relatively high levels of non-Mendelian segregation found in the doubled haploid offspring (Xu et al. 1997). Since the offspring did not develop from embryos, the genetic maps based on double haploid population were not reviewed in this study. We did not include maps derived from interspecific and intergeneric crosses because, in such wide crosses, chromosome pairing and other phenomena - that are not related to selective embryo abortion - may play roles.

nMS -non-Mendelian segregation, BC -backcross, RIL-recombinant inbred lines, BIL-backcross inbred lines;
^a % of markers showing nMS in the map only (the number of loci that showed nMS and that are not linked in the genetic map could not be retrieved from the article),
^b only markers showing nMS given for the probability level $P < 0.01$ were presented,
^c the type of markers that showed nMS could not be identified in a paper,
^d two mapping populations combined together, #- this category of markers may have included following markers: isoenzymes, minisatellites, microsatellites, IGS, SCAR, CAPS, PCR-markers, rDNA, STS, morphological and biochemical markers.

* indication of a genetic map that could be used for analysis of distribution of loci with non-Mendelian segregation along linkage groups. This analysis is described in a chapter: "distinguishing between biological phenomena and technical problems"

Table 2 Percentage non-Mendelian segregation found in genetic maps of wild species. ^{§§}

Species	Segregating population		No. of polymorphic markers	% of markers showing nMS at 0.05	No. of loci showing nMS for each type of marker								References
	nature	no.			RAPD		RFLP		AFLP		other #		
					total	% nMS	total	% nMS	total	% nMS	total	% nMS	
<i>Acacia magnum</i>	F1	108	153	3.92			153	3.92					Butcher et al. 2000
<i>Acacia magnum</i>	F1	123	169	5.33			169	5.33					Butcher et al. 2000
<i>Aegilops tauschii</i> *	F2	56	546	24.18			447	27.96 ^a	53	11.32 ^a	14	c	Boyko et al. 1999
<i>Arabidopsis thaliana</i>	BC	93	47	10.64 ^a		c	2	c					Kuittinen et al. 1997
<i>Cryptomeria japonica</i> *	F2	73	164	23.78	33	21.21	128	24.22	463	32.61	3	33.33	Mukai et al. 1995
<i>Hordeum chilense</i>	F2	100	463	32.61									Vaz Patto et al. – 2001
<i>Hordeum vulgare</i> ssp. spontaneum	F2	233	321	41.00					308	41.56	13	23.08	Vanhala-unpublished data
<i>Lotus japonicus</i>	F2	50	15	0.00 ^a	12	0.00					3	0.00	Jaing and Gressloff 1997
<i>Picea abies</i>	F1 - M.	17	14	0.00	14	0.00							Skov 1998
<i>Picea abies</i>	F1 - M.	17	11	0.00	11	0.00							Skov 1998
<i>Picea abies</i>	F1 - M.	80	145	3.45	145	3.45							Skov and Wellendorf 1998
<i>Picea abies</i> *	F1 - M.	72	447	5.60					366	6.01	81	3.70	Paglia et al. 1998
<i>Pinus edulis</i>	F1 - M.	40	542	6.09					542	6.09			Travis et al. 1998
<i>Pinus pinaster</i>	F1 - M.	124	473	0.00 ^b	437	0.00					35	0.00	Plomion et al. 1995
<i>Pinus pinaster</i>	F2 - M.	or 34 or 126	120	0.00 ^b	120	0.00 ^b							Plomion et al. 1996
<i>Pinus pinaster</i> *	F2 - M	200	378	6.35	127	5.51			239	7.11	61	0.00	Costa et al. 2000
<i>Pinus radiata</i>	F1 - M.	198	202	38.12	198	37.88					4	50.00	Kuang et al. 1998
<i>Pinus strobus</i> *	F1 - M.	72	103	2.91	97	3.09					6	0.00	Echt and Nelson

<i>Pinus sylvestris</i>	M. F1	29	226	8.40														1997
<i>Populus deltoides</i>	BC	93	523	6.50														Lerceteau and Szmidt 1999
<i>Pseudotsuga menziesii</i> *	F2	48	217	6.45	27													Wu et al. 2000
<i>Pseudotsuga menziesii</i> *	F1 -	80	247	18.22	247													Jermstad et al. 1998
<i>Pseudotsuga menziesii</i> *	M.	80	261	10.34														Krutovskii et al. 1998
<i>Pseudotsuga menziesii</i> *	F1 -	80	261	10.34														Krutovskii et al. 1998
<i>Quercus robur</i>	M.	94	307	19.87 ^a	271													Barreneche et al. 1998
<i>Senecio jacobaea</i> *	F1	140	77	15.58														Vrieling – unpublished
<i>Zizania palustris</i>	F2	172	126	10.32														Kennard et al. 1999

Abbreviations as in tab.1, M. – megagametophytes

^{ss}We searched for genetic maps of wild species by means of Winspirs 2.0 in the Current Contents database until August 2000. We used the same keywords as in the case of search for genetic maps of cultivated species, but in all available journals. The search resulted in 1275 records. We scanned the abstracts of all articles to find genetic maps of intraspecific crosses of wild plant species. The criteria of including the data from a genetic map into this review were the same as for cultivated species.

BIOLOGICAL EXPLANATIONS FOR NON-MENDELIAN SEGREGATION IN GENETIC MAPS OF PLANTS

Meiotic drive. Lyttle (1991) defines meiotic drive as “mechanics of meiotic division that cause one member of a pair of heterozygous alleles or heteromorphic chromosomes to be transmitted to progeny in excess of the expected Mendelian proportion of 50%”. A number of meiotic drive systems are described in details for animals. However, little is known about meiotic drive in plants. In most flowering plants, megasporogenesis may lead to meiotic drive because of an obvious asymmetry of the meiotic division: only one of the four haploid cells develops into a functional egg and this cell may contain preferentially transmitted alleles or chromosomes. An example of such a process is the preferential transmission of chromosomal knobs (large clusters of repetitive DNA) on chromosome 10 into viable megaspores in maize (Buckler et al., 1999). Another example of meiotic drive, although of interspecific origin, is the preferential transmission of alien chromosomes. Such chromosomes, common in Triticeae, are called “cuckoo” chromosomes (Miller, 1983). Finch et al., (1984) described the effects of one chromosome coming from *Aegilops sharonensis* in wheat (*Triticum aestivum*) plants. Such monosomic plants have abnormal female and male meiosis, only meiospores containing the alien chromosome develop into normal gametophytes. Only such a cytological analysis combined with mapping would allow for attributing non-Mendelian segregation found in the map to meiotic drive.

Gametophytic selection includes all phenomena that cause differential success of pollen from different donors or pollen from the same donor but bearing different alleles. Gametophytic selection may occur, for example, during pollen germination and pollen tube growth. Germination of pollen *in vitro* is a standard method used to assess both its viability and pollen tube growth. However, only in very few cases such tests are combined with data on segregation of molecular markers (Lin et al., 1992; Sari-Gorla et al., 1992). Often, gametophytic selection is assumed to occur on the basis of allele frequencies in the offspring for the loci in which non-Mendelian segregation was found. Under-representation of one of the alleles is then attributed to gametophytic selection in one of the parents (see e.g. Wagner et al., 1992). Such studies neglect the fact that post-fertilisation selection affecting homozygotes influences also allele frequency.

Selective germination and seedling death. Kuang et al., (1998) linked non-Mendelian segregation to seedling death in *Pinus radiata*. A comparison of the segregation of RAPD markers was made for megagametophytes, for surviving seedlings and those that died within the first month after germination in order to find markers for which segregation was significantly skewed in opposite directions in both groups. A null allele of one locus was over-represented in dead seedlings while it was strongly under-represented in the seedlings that were still alive. The authors suggested that an allele closely linked to this null allele is responsible for the seedlings' death. Moreover, a segregation analysis at the same locus for unsown seeds showed that the null allele was over-represented in this stage. Kuang et al., (1998) gave two possible explanations: selection favouring this allele prior to germination or a sampling error. If the allele responsible for seedling death is indeed favoured during embryo maturation, this would present a case opposite to what is predicted by the SEA hypothesis.

The elimination of selective seed germination and seedling death as the explanation for non-Mendelian segregation found in the map can be done if seed that did not germinate and dead seedlings are included into the segregation analysis.

B chromosomes are supernumerary chromosomes that are not essential for growth and reproduction of organisms. They have been described in more than 1000 species of plants (see Jones and Rees, 1982 for a review). B chromosomes can be distinguished from normal (A) chromosomes because they are usually smaller and consist of large amounts of heterochromatin. Their number may be variable even within the same individuals due to nondisjunction in the anaphase of the mitosis. B chromosomes are inherited in a non-Mendelian fashion which, according to Jones (1991), can be caused by their nondisjunction in female or/and male meiosis, nondisjunction in mitosis during development of the male gametophyte, or preferential fertilisation by B-containing sperm. B chromosomes usually consist of repetitive DNA and some of such repeats were found to code for ribosomal RNA (Camacho et al., 2000 and references therein). Theoretically, markers of B chromosomes may appear in genetic maps as single, unlinked markers or separate linkage groups. A way to avoid this possibility is careful selection of parents without B chromosomes (by chromosome counting), for the mapping population.

Cytoplasmic inheritance via plastids and mitochondria can show up as non-Mendelian segregation in molecular markers in the case of biparental inheritance of organelles. In angiosperms, inheritance of plastids is predominantly maternal and in gymnosperms – paternal. However, cases of biparental inheritance of those organelles were also described. Examples are alfalfa (*Medicago sativa*), evening primrose (*Oenothera*) and *Pelargonium* cultivars (Mogensen, 1996). Ten out of 68 angiosperm species and 3 out of 11 gymnosperms listed by Reboud and Zeyl (1994) are classified as having biparental inheritance of plastids. A similar picture rises from a review of Smith (1988). Nineteen out of 60 angiosperms showed at least occasional biparental inheritance. Less examples (only four species) are available for biparental inheritance of mitochondria for two reasons. Firstly, this phenomenon did not receive much attention (Reboud and Zeyl, 1994). Secondly, it may occur less often. Species with biparental inheritance of plastids may have strictly maternal transmission of mitochondria (Mogensen, 1996).

Endophytes. A diversity of organisms, like bacteria and fungi, are known to live inside and among plant tissues (Carroll, 1988; Clay, 1988; Hallmann et al., 1997). The DNA from endophytes may be extracted together with plant DNA and eventually give the same effect as contamination. Cytoplasmic inheritance and endophytes can potentially be observed as distorted unlinked markers. However, molecular markers for organelle DNA will never be linked to markers for nuclear genes and if there are two polymorphic markers for organelle DNA, they will be 100% linked to each other because of a lack of recombination. Molecular markers for eucaryotic endophyte DNA may appear in a map (resulting in more groups than chromosomes). However, they will never be linked to the markers that are known to be developed for plants e.g. morphological markers.

Chromosome rearrangements, such as translocation and duplication, are often suggested causes of non-Mendelian segregation found in genetic maps (e.g. Vaillancourt and Slinkard, 1992). However, genetic mapping alone is not sufficient to link non-Mendelian segregation directly to translocation. Belay and Merker (1998) analysed inheritance of the translocation on chromosome 6B in tetraploid wheat (*Triticum turgidum*) by cytogenetic study. They observed C-banding patterns in the F₂ generation derived from two crosses, both with one parent homozygous for a translocation and one parent homozygous for the lack of translocation. In both crosses, non-Mendelian segregation was observed. Homozygotes containing 2 chromosomes without translocation were over-represented.

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SUMMARY

In my thesis, I studied a number processes that have an influence on a genetic constitution of offspring in plants. These processes are (1) mating (pollination), (2) post-pollination selection and (3) selective embryo abortion.

Mating in plants

Plants have little influence on their mate choice before pollination. Since they are unable to move, they can not search for the suitable pollen donor. Instead they depend on pollinators and abiotic factors in transfer of their pollen. The pollinators often transfer pollen among neighboring plants. In populations with a genetic structure, such pollination may lead to crosses among related individuals (biparental inbreeding) and as a result plants may experience inbreeding depression even in seeds that are not selfed. Therefore, a presence of the genetic structure can have an important influence on plant reproduction.

I tested for a genetic structure in two biennial plants *Echium vulgare* and *Cynoglossum officinale* in order to estimate the amount of biparental inbreeding. Both species are pollinated by bumblebees known for their tendency to visit neighboring plants. I mapped and sampled flowering plants from natural populations and analyzed the genetic structure using seven polymorphic microsatellite loci per species. The analysis showed that the genetic structure among the flowering of both species can not intensify inbreeding. The estimated amount of biparental inbreeding does not exceed 2 % for *E. vulgare* and *C. officinale*. The average kinship coefficients per distance class were significantly higher than zero for both species only in the first distance interval, suggesting a genetic structure at a very small scale, probably due to leptocurtosis of gene dispersal curves. The genetic structure of both species appeared to be very weak compared to data published for 17 herbaceous species with similar pollen and seed dispersal.

Post-pollination selection

Because opportunities to choose mates prior to pollination are limited in plants, post-pollination mate choice is essential for sexual selection in plants. Genetically diverse pollen grains landing on a stigma may differ in ability to adhere to the surface of the stigma, to germinate on its surface and to form a pollen tube that can reach the ovule. Many studies have shown also that pollen from different donors may differ with respect to the speed of pollen tube growth and fast growing pollen has higher fertilization success when applied to the stigma in a mixture of pollen from different donors. The term *pollen competition* is frequently used to describe such observations.

Pollen competition is believed to be a mechanism of so called cryptic self-incompatibility (CSI). If self-pollen grows slower than outcross pollen, it will have an equal fertilization success when applied in single donor treatment but a lower success if the two pollen types are applied together to the stigma. The concept of CSI is appealing to many researchers, although it is still unclear whether or not it is a common phenomenon. I studied CSI in *Echium vulgare*, which shows low selfing rates in the field. I chose this species, because its selfing rates are much lower than the theoretical

predictions based on the plant size and pollen dynamics, suggesting that post pollination selection against selfing takes place. I used twenty genotypes, combined in 10 pairs 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 over all 20 genotypes were found. However, for 2 genotypes we found significant CSI. I 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, I found no evidence that CSI in *E. vulgare* is due to pre-zygotic mechanisms.

Selective embryo abortion

Embryo abortion provides also an opportunity to alter the genetic constitution of the offspring if embryos can be selectively aborted depending on their genotype. Selective embryo abortion (SEA) will be adaptive if embryos of genotypes that would perform worse later in life are preferentially aborted. Then SEA would lead to the investment of resources in the offspring with the highest potential fitness only. Many studies have shown that otherwise viable embryos are aborted. However, only few studies, - all on the level of the phenotype, indeed have shown a correlation between the level of abortion and offspring quality and these studies have been challenged for their experimental design.

I searched for another way of studying of SEA – on the level of genotype. Non-random abortion at the level of molecular markers can be observed as a deviation from Mendelian segregation: over- or under-representation of markers in the offspring. If embryo abortion is commonly found in plants, and if it is selective, many genetic maps of plants should detect non-Mendelian segregation of molecular markers. I reviewed the literature on the genetic maps of plants and found that the level of non-Mendelian segregation found in these genetic maps is high. On average, 11.5 % of the tested markers in the genetic maps of wild species and 14.6 % - in the cultivated ones, show a departure from Mendelian segregation. Based on the six genetic maps, providing sufficient data, I calculated that in 68% of loci segregating in non-Mendelian fashion post-fertilisation selection is involved. This shows that there is a potential for detecting selective embryo abortion using a genetic map.

SAMENVATTING

In mijn proefschrift bestudeer ik een aantal processen die de genetische constitutie van de nakomelingen van een plant beïnvloeden. Deze processen zijn: (1) bestuiving, (2) selectie na bestuiving en (3) selectieve embryo-abortus.

Bestuiving bij planten

Omdat planten zich niet kunnen voortbewegen kunnen ze ook niet op zoek naar de meest geschikte partner. Zij hebben dus weinig invloed op de aanvoer van stuifmeel door andere planten. Daarvoor zijn ze afhankelijk van bestuivers of wind- of waterstromen. Bestuivers verspreiden het pollen vaak tussen naburige planten. In populaties met een sterke genetische structuur kunnen zulke bestuivingen leiden tot kruisingen tussen genetisch verwante individuen (biparental inbreeding). Als resultaat daarvan kunnen planten last hebben van inteeltdepressie, zonder dat de zaden door zelfbestuiving zijn ontstaan. De genetische structuur van de populatie kan daarom een belangrijke invloed hebben op het reproductieve succes van de planten.

Ik heb de genetische structuur bestudeerd van twee tweejarige plantensoorten: *Echium vulgare* (slangekruid) en *Cynoglossum officinale* (veldhondstong). Op basis daarvan heb ik een schatting gemaakt van de hoeveelheid biparental inbreeding. Beide soorten worden bestoven door hommels. Van hommels is bekend dat ze heel vaak naburige planten achter elkaar bezoeken. Ik heb de planten in kaart gebracht en verzameld in natuurlijke populaties en de genetische structuur geanalyseerd met behulp van zeven polymorfe microsatellietloci voor iedere soort. De analyse liet zien dat de genetische structuur van de bloeiende planten zo zwak was dat dit niet leidde tot biparental inbreeding. De geschatte hoeveelheid biparental inbreeding bedroeg ten hoogste 2% voor beide soorten. De gemiddelde verwantschapscoëfficiënt voor de verschillende afstandsklassen was alleen significant groter dan nul voor de kleinste afstandsklasse (tot 1,48 m voor *E. vulgare* en tot 6,49 m voor *C. officinale*). Dit laat zien dat alleen op heel kleine schaal een genetische structuur aanwezig was. Dit laatste is waarschijnlijk het gevolg van een leptokurtische vorm van de genverspreidingscurve. In vergelijking met literatuurdata van 17 andere kruidachtige soorten, met vergelijkbare vormen van pollen- en zaadverspreiding, was de genetische structuur van de door mij onderzochte soorten zwak.

Selectie na bestuiving

Omdat de mogelijkheden voor partnerkeuze voor de bestuiving dus zeer gering zijn, spelen bij planten processen na de bestuiving een essentiële rol bij seksuele selectie. Genetische verschillende pollenkorrels die op een stamper landen kunnen verschillen in het vermogen om zich te hechten aan het oppervlak van de stamper, om te kiemen op het oppervlak van de stamper of om een pollenbuis te vormen die de ovula kan bereiken. Veel studies hebben laten zien dat pollenkorrels van verschillende planten niet allemaal even snel groeiende pollenbuizen maken. Ook hebben zij laten zien dat snelgroeiende pollenbuizen een grotere kans hebben om de ovula te bevruchten wanneer een mix van pollen van verschillende vaders op de stamper aanwezig is. In verband hiermee wordt veelal de term pollenconcurrentie (*pollen competition*) gebruikt.

Pollenconcurrentie wordt geacht medeverantwoordelijk te zijn voor zgn. cryptische zelfincompatibiliteit (CSI). Als pollen van dezelfde plant (eigen pollen) langzamer groeit dan pollen van ander genotype (vreemd pollen), kan het nog steeds hetzelfde bestuivingssucces hebben als vreemd pollen indien per bloem maar één type pollen aanwezig is. Echter wanneer beide type pollen tegelijkertijd op een stigma aanwezig zijn, dan verwachten we dat het vreemde pollen de race om de bevruchting van ovula wint en dus succesvoller is. Het idee van CSI spreekt veel onderzoekers aan, hoewel het nog steeds onduidelijk is hoe vaak het voor komt. Ik bestudeerde CSI bij *Echium vulgare*. Deze soort heeft een lage zelfbestuivingsgraad in natuurlijke populaties. Ik koos deze soort omdat de zelfbestuivingsgraad veel lager bleek te zijn dan wat op grond van theoretische modellen over pollen dynamica voorspeld werd. Dit laatste suggereert dat na de bestuiving selectie tegen eigen pollen kan optreden. Ik gebruikte 20 genotypen die gecombineerd werden in 10 paren. Drie bestuivingstypen werden toegepast: zelfbestuiving, kruisbestuiving (wederzijds binnen ieder paar) en bestuiving met een mix van pollen van beide planten. Vervolgens werd het genotype van 10 zaden van het gemengde bestuivingstype per plant bepaald met behulp van microsatelieten. Gemiddeld over alle 20 planten werd er geen aanwijzing voor selectie tegen zelfbestuiving gevonden. Echter, voor twee moederplanten vonden we statistisch significante aanwijzingen voor CSI. Ik bepaalde de maternale en paternale effecten op pollenbuisgroei en de maternale effecten op pollenkieming. Gemiddeld over alle 20 planten waren hierin geen verschillen tussen zelf en kruisbestuiving. Bovendien bleken de pollenkieming en de pollenbuis groei niet verschillend voor de planten met of zonder CSI. Ik vond dus geen aanwijzingen dat CSI het gevolg is van selectie in de periode tussen bestuiving en bevruchting.

Selectieve embryo abortus (SEA)

Het aborteren van embryo's geeft een mogelijkheid om de genetische samenstelling van het nakomelingschap te beïnvloeden, als de selectie genotype-afhankelijk is. SEA kan adaptief zijn als vooral die embryo's geaborteerd worden die later in het leven weinig kans op succes zouden hebben. SEA leidt er dan toe dat er meer geïnvesteerd kan worden in nakomelingen met een grote kans op succes later in het leven. Veel studies hebben laten zien dat potentieel levensvatbare embryo's geaborteerd worden. Weinig studies hebben echter laten zien dat er een verband is tussen de hoeveelheid aborti en de kwaliteit van de wel geproduceerde zaden. De meeste van deze studies zijn alleen gebaseerd op het fenotype van de nakomelingen en zijn aan kritiek onderhevig vanwege de gebruikte onderzoeksmethoden. Ik heb gezocht naar een betere experimentele benadering gebaseerd op het genotype. Selectieve abortus manifesteert zich als niet-Mendelse overerving op het niveau van moleculaire merkers. Sommige merkers zijn dan onder- of over vertegenwoordigd in het nakomelingschap. Ik heb een literatuurstudie gedaan naar het voorkomen van niet-Mendelse overerving in genetische kaarten. Die studie laat zien dat niet-Mendelse overerving frequent voorkomt. Gemiddeld laat 11.5 % van de geteste merkers bij wilde soorten en 14.6% bij gekweekte soorten een significante afwijking van Mendelse overerving zien. Zes van de onderzochte kaarten boden bovendien de mogelijkheid om te onderzoeken of deze

afwijking het gevolg was van selectie na de bevruchting. Dat bleek in 68% van de merkers die niet-Mendels overerfden het geval. Dit hoge percentage laat zien dat het potentieel heel goed mogelijk is om selectieve abortus aan te tonen met behulp van genetische kaarten.

STRESZCZENIE

W niniejszej pracy badałam wiele procesów mających wpływ na genotyp potomstwa u roślin. Te procesy to: (1) kojarzenia (zapylenie) (2) dobór działający po zapyleniu oraz (3) selektywne aborcje zarodków.

Kojarzenia u roślin

Rośliny mają niewielki wpływ na wybór partnera przed zapyleniem. Ponieważ nie mają zdolności poruszania się, nie mogą szukać odpowiedniego dawcy pyłku. Zamiast tego, są zależne od zapylających owadów lub czynników abiotycznych transportujących pyłek. Owady zapylające często przenoszą pyłek w obrębie sąsiadujących z sobą roślin. W populacjach posiadających strukturę genetyczną, takie zapylenia mogą prowadzić do krzyżowania osobników blisko spokrewnionych (obu rodzicielski chów wsobny, *biparental inbreeding*) w wyniku czego rośliny mogą doświadczać depresji wsobnej, nawet jeśli siewki pochodzą z zapylenia krzyżowego. Tak więc, obecność struktury genetycznej może mieć istotny wpływ na reprodukcję roślin.

W mojej pracy badałam strukturę genetyczną dwóch roślin dwuletnich *Echium vulgare* i *Cynoglossum officinale* w celu oszacowania stopnia obu rodzicielskiego chowu wsobnego. Oba gatunki są zapylane przez trzmiele, znane z tendencji do odwiedzania sąsiadujących ze sobą roślin. Zmapowałam i pobrałam próbki kwitnących roślin z populacji naturalnych, oraz zanalizowałam strukturę genetyczną używając siedmiu polimorficznych loci mikrosatelitarnych dla każdego gatunku. Analiza pokazała, że struktura genetyczna wśród kwitnących osobników obu gatunków nie może wzmacniać oburodzicielskiego chowu wsobnego.

Oszacowany poziom inbredu oburodzicielskiego nie przekracza 2% u *E. vulgare* i *C. officinale*. Średnie współczynniki pokrewieństwa na klasę odległości dla obu gatunków, były istotnie wyższe od zera wyłącznie dla pierwszego przedziału odległości, co sugeruje strukturę genetyczną w bardzo małej skali, prawdopodobnie ze względu na rozkład leptokurtyczny krzywych dyspersji genów. Struktura genetyczna obu gatunków wydaje się być bardzo słaba, w porównaniu z danymi dla 17 gatunków innych roślin zielnych, posiadających podobny sposób przenoszenia pyłku i rozsiewania nasion.

Dobór działający po zapyleniu

Ponieważ u roślin możliwości wyboru partnera przed zapyleniem są ograniczone, dobór działający po zapyleniu ma kluczowe znaczenie dla doboru płciowego. Genetycznie różne ziarna pyłku osiadające na znamieniu mogą różnić się co do zdolności przylegania do znamienia, kiełkowania na jego powierzchni, oraz co do formowania łagiewki, która sięga do woreczka zalążkowego. W wielu pracach pokazano, że pyłki pochodzące od różnych dawców, mogą różnić się co do szybkości kiełkowania łagiewki, a szybko kiełkujący pyłek ma większe prawdopodobieństwo zapłodnienia. Termin konkurencja pyłków (*pollen competition*) jest często używany aby opisać podobne zjawisko.

Konkurencja pyłków jest uważana za mechanizm tzw. kryptycznej samo niezgodności (*cryptic self-incompatibility*, CSI). Własny pyłek może kiełkować wolniej niż pyłek pochodzący z innej rośliny. Wolniejsze kiełkowanie nie ma znaczenia gdy pyłek od jednego dawcy sam trafia na znamię. Natomiast gdy własny pyłek i szybciej kiełkujący pyłek od innego dawcy zostaną jednocześnie nałożone na znamię, dochodzi do konkurencji o zapłodnienie zalążków i w efekcie większość nasion będzie efektem zapylenia krzyżowego. Będzie to miało takie same efekty jak proces genetycznej samo niezgodności, ale będzie się ujawniać tylko w warunkach pozwalających na konkurencję pyłków. Do koncepcji CSI u roślin odwołuje się wielu badaczy, mimo że wciąż nie jest do końca wyjaśnione na ile powszechne jest to zjawisko wśród roślin.

Badałam CSI u *Echium vulgare*, który to gatunek w warunkach naturalnych odznacza się niskim stopniem samozapylenia. Wybrałam ten gatunek, ponieważ poziom samozapylenia w populacjach naturalnych jest o wiele niższy niż wynikałoby to z przewidywań teoretycznych opartych na rozmiarach rośliny i dynamice pyłków, sugerując, że dobór działający po zapyleniu ma tutaj miejsce, działając przeciwko samozapyleniu. Użyłam 20 genotypów, połączonych w 10 par, w trzech zabiegach: samozapylenie, zapylenie krzyżowe (wzajemna krzyżówka w obrębie każdej pary) oraz zapylenie z użyciem mieszaniny pyłków pochodzących od obu roślin. Próbkę 10 siewek pochodzących z zabiegu z mieszaniną pyłków została zgenotypowana dla każdej z dwudziestu roślin eksperymentalnych, używając loci mikrosatelitarnych. Nie stwierdzono efektu działającego przeciwko samozapyleniu w analizie uwzględniającej średnie dla wszystkich 20 genotypów. Natomiast w szczegółowej analizie testami nieparametrycznymi, tylko dla dwóch genotypów wykryto istotne CSI. Oszacowałam efekty matczyne i ojcowskie na kiełkowanie pyłku, jednakże nie było istotnej różnicy pomiędzy kiełkowaniem pyłku i wzrostem pomiędzy pyłkiem pochodzącym z samozapylenia oraz zapylenia krzyżowego, dla średniej z 20 genotypów. Wzrost łagiewki i kiełkowanie u dwóch genotypów u których stwierdzono CSI, nie różniły się od roślin, u których nie stwierdzono CSI. Tak więc, nie znalazłam przesłanek, że CSI u *Echium vulgare* jest spowodowane przez mechanizmy prezygotyczne.

Selektywna aborcja zarodków

Aborcja zarodków zapewnia również możliwość wpływu na genotyp potomstwa, jeśli zarodki mogą być selektywnie usuwane w zależności od ich genotypu. Selektywna aborcja zarodków może być przystosowawcza, jeśli zarodki o genotypach, które mają potencjalnie niższe dostosowanie, będą częściej resorbowane. Wówczas selektywna aborcja zarodków będzie prowadzić do zwiększenia inwestycji zasobów tylko w potomstwo o 'wyższej jakości'. W wielu pracach pokazano, że nawet żywotne zarodki są resorbowane. Jednakże tylko kilka prac – wszystkie na poziomie fenotypowym – pokazało rzeczywiście korelację między poziomem aborcji a jakością potomstwa. Niestety wyniki tych prac są kwestionowane z powodu planu eksperymentalnego i możliwości że opisane efekty są artefaktami pochodzącymi z użytej metody.

Szukałam innej metody badania selektywnych aborcji – na poziomie genotypu. Nielosowe aborcje na poziomie markerów molekularnych będą obserwowane jako odchylenia od segregacji mendlowskiej: zbyt częste, lub zbyt rzadkie występowanie markerów (allelów) w potomstwie. Jeśli aborcje zarodków są powszechne u roślin i jeśli

są selektywne, wówczas wiele map genetycznych powinno pokazać nielosową segregację markerów molekularnych. W literaturze na temat map genetycznych u roślin znalazłam, że poziom segregacji niemendłowskiej jest wysoki. Średnio 11,5 % badanych markerów w mapach genetycznych gatunków dzikich i 14,6% u gatunków uprawnych wykazuje odchylenia od segregacji mendlowskiej. W oparciu o sześć map genetycznych, zapewniających dostateczną ilość danych, wyliczyłam, że w 68% loci segregujących w sposób niemendłowski zaangażowany jest dobór postzygotyczny. To pokazuje, że istnieje potencjał dla wykrycia selektywnych aborcji zarodków w oparciu o mapy genetyczne.

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This thesis benefited from the direct or indirect contribution of many people, who I would all like to thank here.

Everything started when Nico de Boer recommended me as a suitable candidate for this PhD position (Nico, thank you for believing in me!) In the beginning, I have had more to do with hydrobiology than with plant ecology, so I had to learn a lot about plants. Tom de Jong, Chantal Melser and Marielle Rademaker shared their experiences with *Cynoglossum* and *Echium*, and Karin van der Veen gave me lots of practical pieces of advice on how to grow plants and use climate rooms. Experiments usually involved hundreds of plants and would not have been possible without the help of technicians of the plant ecology group, especially, Henk Nell, Hans de Heiden and Joep Bovenlander. Further, Jeanette Biemans and William Kerssens did a great job working with *Echium* – being their supervisor was really valuable experience to me. My PhD study also had another important component: molecular analysis, which, at first, was a complete 'abracadabra' to me. Marcel Eurlings and Rene Glas helped me find my way in the lab and answered countless questions on a daily basis (I miss you guys! In the lab I work now I am supposed to have answers, not questions!).

I have lots of nice memories of the plant ecology group. I learned a lot from the journal clubs/work discussions and enjoyed all the social gatherings. 'AIO dinners' with my fellow PhD students, Chantal, Nico, Gera, Mirka, Sonja, Grit, Milena and Martina, were really 'gezellig'. I would like to thank my colleagues here who were very supportive during all the disasters that happened in my project: when my review kept coming back like a boomerang, when the AFLP didn't work for *Cynoglossum*, and when the tetraploidy of *Echium* complicated the analysis. Thanks for listening!

The 9 months I spent at Newcastle on Marie-Curie fellowship was an enormous injection of enthusiasm for me. These months were really active and productive. I especially liked the atmosphere of positive thinking at MC training site. I enjoyed coffee break discussions, badminton games and other gatherings with my Newcastle-colleagues: Łosia, Laurence, Ingo, Marie and Roddy, Nur, Ranja and Kirstin. And about my other activities in Newcastle, well... I will only say I'm glad I didn't end up with a permanent hand injury :)

During all these years, many people made my life outside biology exciting and fun, too. I fondly remember the long evenings over delicious dinners with Nóra, Marta and Natalia, and the many chats with Marcin, Mark, Péter and Thijs, and in Newcastle, dancing with Kostja and Eda. Getting together with Ann, Ansgar, Arjan, Bart, Emilie, Eric, Freek, Martin and Ward in Maneer Jansen to talk about 'everything and nothing' was not only first class entertainment, but also improved my Dutch a lot.

Now let me write few words for my mother. Kochana mamó, dziękuję za popieranie moich decyzji, za cierpliwość w czekaniu kiedy wreszcie przyjadę do domu i za to, że zawsze się o mnie martwisz.

There were three people closest to me in the final, most stressful stage of my project: Anikó Lipták, Sonja Esch and my sister, Anna. If I could have three paranymphs, one on my right, one on the left and one behind, I would choose all of them. They all were wonderful friends to me in the moments when everything was going wrong. Sonja also took care of many arrangements related to my promotion and the printing of my thesis. She did all of that during the most difficult last few months of her own PhD project. (Sonja, I wish I could help you as much as you helped me.)

And a final 'thank you' to Martin Britijn, who made a lot of effort to prepare the graphical design of this thesis. I can't see the final result while typing this text but I am sure it will look nice.

Curriculum vitae

I was born on 22nd August 1974 Rzeszów (south-east Poland). I started my studies in biology at the Jagiellonian University in Kraków in October 1993. During my study I developed an interest in evolutionary ecology and attended many seminars and workshops in this field. I chose ciliates as model organisms to study prey-predator interactions and inducible defences in my research projects under the supervision of dr Janusz Fyda from the Department of Hydrobiology. In 1997, I went to the Netherlands with a 6-month fellowship supported by the European TEMPUS program to study inducible defences in plants at the section of Plant Ecology of Leiden University. I carried out an experiment testing the herbivory induced withdrawal of resources in *Senecio jacobaea* supervised by Nico de Boer and Eddy van der Meijden. Coming back to Kraków, I continued my experiments on ciliates and wrote an MSc thesis under the supervision of Krzysztof Wiąckowski, entitled "The influence of the presence of food and predator on the activity of ciliates". I defended this thesis with a very good result and received the title Master of Science in June 1998.

In October 1998 I went back to the Netherlands to start a PhD study at the section of Plant Ecology at Leiden University. The project was about selective embryo abortion in plants originally, but I broadened it later to include other processes of post-pollination selection and the study of a population genetic structure. I worked with plants (pollination experiments, field studies) and molecular markers like microsatellites and AFLP. I learned to develop microsatellites from Kirsten Wolff during my 9-month Marie-Curie Fellowship at the University of Newcastle (UK) in 2003. During my whole PhD period I gave six oral and two poster presentations at international congresses.

Between February and September 2004, I worked as a molecular analyst at the section of Plant Ecology in Leiden. I helped to introduce a new technique, RAF (randomly amplified DNA fingerprints), in studies on plant and animal species. I performed a RAF analysis on the parasitoid wasp *Mesochorus fascialis* in order to study the relation between its genetic diversity and habitat fragmentation. This project forms part of the PhD project of Sonja Esch.

From 15th September 2004, I work as a postdoctoral researcher in the Institute of Botany at the Polish Academy of Sciences in Kraków. My work in Poland is part of the European project 'IntraBioDiv', which aims at comparing the genetic variation at three different levels (intraspecific, interspecific and habitat) in order to find efficient conservation strategies. This project also has a phylogeographic aspect as the Carpathian and Alpine populations will be compared.

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