Breeding System Evolution and Pollination Success in the Wind-Pollinated Herb *Plantago maritima*

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Dissertation presented at Uppsala University to be publicly examined in Föreläsningssalen, Avdelningen för växtekologi, Uppsala, Saturday, March 19, 2005 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

In this thesis, I examined variation in sex expression and mating patterns in the sexually polymorphic, wind-pollinated herb Plantago maritima. With a combination of field studies, greenhouse experiments, and genetic analyses, I (a) examined factors influencing sex ratio variation in gynodioecious plants (in which hermaphrodites and females coexist), (b) discovered variation in breeding system, (c) investigated density-dependence of seed production, and (d) documented genetic variation within and among populations close to the northern range margin in Europe.

In a survey of 104 P. maritima populations, I documented considerable variation in sex ratio (range 0-70% females, median 6.3% females). As predicted, females were more frequently missing from small than from large populations, and the variance in sex ratio increased with decreasing population size. Among twelve populations sampled for seed production, the frequency of females was positively related to relative fecundity of females and negatively related to population size. The results suggest that the local sex ratio is influenced both by the relative fecundity of females and hermaphrodites, and by stochastic processes in small populations.

A comparative field study showed that plant fecundity decreased with increasing distance to nearest pollen donor both within and among populations in an archipelago in southern Sweden, where self-incompatibility was confirmed in controlled crosses. In contrast, plant fecundity was overall higher and was not density-dependent in the Skeppsvik archipelago in northern Sweden, where controlled crosses showed that plants are self-compatible. The results were consistent with the prediction that evolution of self-fertility should reduce density-dependence of pollination success.

I quantified the genetic structure within and among populations from eastern Sweden and western Finland based on variation at four polymorphic microsatellite loci. The genetic diversity was low in northern Sweden, which may be the result of a history of small population sizes and periods of frequent self-fertilization.

Keywords: Breeding system, mating system, gynodioecy, male sterility, reproductive output, self-compatibility, self-incompatibility, wind-pollination, founder event, genetic structure

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ISSN 1651-6214
ISBN 91-554-6153-0
urn:nbn:se:uu:diva-4790 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4790)
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


II. Nilsson, E., Wolff, K., Ågren, J. Mating system evolution in the wind-pollinated herb Plantago maritima: loss of self-incompatibility close to the northern range margin in Europe. (manuscript)

III. Nilsson, E., Coronel, V., Ågren, J. Population density and seed output in self-compatible and self-incompatible populations of the wind-pollinated herb Plantago maritima. (manuscript)

IV. Nilsson, E. Genetic structure in populations of the perennial plant Plantago maritima across a shift in mating system. (manuscript)

V. Nilsson, E., Wolff, K. Five polymorphic microsatellite markers for Plantago maritima. Molecular Ecology Notes (accepted)

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Fyra män i brudkammaren och ensamma kvinnor komplicerar
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Introduction

Most flowering plants are sessile organisms, destined to live wherever the seeds germinated. Being sessile is a major drawback when it comes to sex. While most free moving organisms seek out their mate prior to mating, plants require a means of transferring male gametes (pollen) between mates. Many plants accomplish this by attracting insects and manipulating their behaviour so that they will transport pollen between plants of the same species (e.g., Darwin, 1877a; Nilsson, 1998; Pellmyr, 2003). However, this plant–insect relationship is not entirely flawless. First, potential insect pollinators are not present in all plant habitats (Proctor et al., 1996), and are not entirely reliable pollen vectors in the habitats where they do occur (Kalisz et al., 2004). Second, animal pollen vectors can carry diseases capable of destroying the reproductive function of stricken plants (Carlsson and Elmqvist, 1992; Marr, 1997). Third, cues that attract pollinators might also attract plant herbivores (Mutikainen and Delph, 1996; Ehrlén et al. 2002). To avoid these problems, some plants rely on wind or water to transport their pollen between mates (Whitehead, 1983; Goodwillie, 1999), others are highly self-fertilizing (Jain, 1976; Hoffmann et al., 2003), and some have given up sex more or less entirely (van Dijk, 2003). However, even though asexual reproduction (Berg & Redbo-Torstensson, 1998) or self-fertilization (Kalisz et al., 2004) could provide a means of securing reproduction, most plants are adapted to increase the proportion of pollen reaching other plants, and to receive pollen from other plants (Barrett, 2002).

About 70 percent of all flowering plant species are co-sexual with only hermaphrodite flowers, i.e. flowers containing both male (stamen) and female (pistil) sexual function (Yampolsky and Yampolsky, 1922). Because of the large number of pollen grains produced by single individuals, matings can potentially occur with many individuals, including the plant, and even the flower, where the pollen originates (Barrett and Harder, 1996; Barrett, 2002). Self-fertilization, however, is commonly costly for the plant as it may generate less fit offspring compared to cross-pollinated progeny (e.g., Ågren and Schenske, 1993; Husband and Schenske, 1996; Koelwijn, 2004). In fact, the separation of male and female function, whether within the same flower, within individuals, or among individuals, is a strategy believed to maximize the number of cross-fertilized progeny (Barrett, 2002). Moreover, some plants have a self-incompatibility system whereby self-pollen is recognized and rejected (Franklin-Tong and Franklin, 2003). Thus, reducing self-
fertilization, and maximizing cross-fertilization, reduces the risk of inbreeding depression. Furthermore, this creates high gene flow within populations, which enables outcrossing populations to maintain a high genetic diversity. This could increase the possibility for an adaptive response to changes in the biotic or abiotic environment (Falconer and Mackay, 1996; Fowler and Whitlock, 1999). Despite these benefits of outcrossing, the transition from self-incompatibility to self-fertility is a common evolutionary shift in plant mating system (Roalson and McCubbin, 2003). This makes flowering plants a suitable study system of the evolutionary significance and maintenance of sex, a highly controversial and unresolved issue in evolutionary biology.

The evolution of plant breeding systems

The earliest fossil evidence of angiosperms (flowering plants) has been dated to early Cretaceous, 125 – 115 million years before present (Friis et al., 2001). The flowers of early angiosperms were most likely hermaphroditic (Friis et al., 1999) and pollinated by insects (Friis et al., 1999; Pellmyr, 1992; Ren, 1998), but their flowers contained no nectar (Crepet et al., 1991). Instead pollen served as food for the pollinators and leftover pollen was transported between plants as the insect pollinator foraged among the flowers.

Because of the negative effects associated with self-fertilization, the evolution of self-incompatibility was a major breakthrough in the history of angiosperm plants. It has been suggested that self-incompatibility evolved early in angiosperm history (Zavada, 1984), and indeed the occurrence of a gametophytic incompatibility system among several groups of eudicots (e.g., Solanaceae, Rosaceae, Scrophulariaceae and Plantaginaceae) suggests an ancient origin of self-incompatibility (Igic and Kohn, 2001). Self-incompatibility opens up for more promiscuous pollination strategies, such as wind pollination. A high number of pollen is commonly produced by wind-pollinated species (Ackerman, 2000). The high probability of self-fertilization in such species could be prevented by the rejection of self-pollen with a self-incompatibility system or a temporal or spatial separation of sex function (Culley et al., 2002).

Gender dimorphism, i.e. when not all members of a population are hermaphrodite (Richards, 1997), is an interesting aspect of plant breeding systems. The sexual function of plants can be expressed as male and female on separate individuals (i.e. dioecy), as female and hermaphrodite individuals (gynodioecy) or as the unusual male and hermaphrodite individuals (androdioecy). Furthermore, the proportion of male and female flowers can vary within individuals. Approximately 19 percent of known species exhibit some kind of gender dimorphism (Yamplosky and Yampolsky, 1922); this in-
cludes a wide variety of gender strategies that involve various combinations of female, male and hermaphroditic flowers at the plant or population level (Barrett, 2002). The invasion of female plants into originally hermaphroditic populations is a common evolutionary pathway to more complex dimorphisms (Charlesworth and Charlesworth, 1978).

An important process in the evolution of plants is polyploidization, i.e. the multiplication of entire chromosomal complements (Ramsey and Schemske, 2002; Popp, 2004). Between 47 and 70 percent of flowering plants have been estimated to be descendants of polyploid ancestors (Masterson, 1994). Polyploidy creates a reproductive barrier between the plant with raised ploidy level and its progenitors, but can also have implications for breeding system evolution. First, it is thought to be common that self-incompatibility is lost following a polyploidization event (e.g., Entani et al., 1999; Miller and Vernable, 2000, but see Brunet and Liston, 2001; Mable, 2004). Second, polyploidy has been proposed to promote the evolution of gender dimorphism (Pannell et al., 2004). However, self-fertility, and gender dimorphism, can evolve without a change in ploidy level (Wallander, 2001; Dorken et al., 2002; Kondo et al., 2002; Tsukamoto et al., 2003; Busch, 2004; Thorogood et al., 2004).

**Wind pollination**

Wind pollination has evolved in numerous lineages of angiosperm plants, and it is found in about 18 percent of angiosperm families today (Ackerman, 2000). It is both ecologically and economically important; many the dominant plants of the boreal forest, savanna and temperate grasslands are pollinated by wind (Kellogg, 2001; Shantz, 1954). However, biologists have largely overlooked wind-pollination, possibly because of the aesthetic enticing of showy, animal pollinated plants (Barrett, 2002).

The evolution of wind pollination could be favoured if animal pollinators are absent (Weller et al., 1990; Cox, 1991), spatially or temporally unreliable (Goodwillie, 1999), or if there is competition among plant species for pollinators. Wind-pollination may allow the maintenance of high levels of cross-pollination in pollinator poor environment (e.g., Dow and Ashley, 1996). Consistent with this view, wind pollination is a common pollination system in habitats where potential insect pollinators are sparse, such as grasslands, savanna, and salt-marshes (Proctor et al., 1996).

Pollen from wind-pollinated plants has been found to travel tremendous distances. Tormo-Molina et al. (2001) recorded substantial airborne Plantago pollen concentrations several kilometres from the nearest population, and van de Knaap (1987) recorded pollen deposition of e.g. Plantago lanceolata and P. maritima in peat on the artic islands Spitsbergen and Jan
Mayen, more than 500 km from nearest pollen source. This has led to the common assumption that pollen limitation of seed production is unlikely in such plants (e.g., Faegri and van der Pijl, 1966). Yet empirical estimates of successful pollen transfer are much shorter (e.g., Bos et al., 1986). Whitehead (1983) suggested that wind pollination is most efficient in large and dense populations, and the limited empirical evidence at hand supports this hypothesis. For example, pollen limitation has been established in one population of *Plantago maritima* (Dinnéetz, 1997) and in areas where the clonal grass *Spartina alterniflora* occurs at low density (Davis et al., 2004).

Thus, wind-pollinated plants might be independent of animal pollinators, but could still be pollen limited if interplant distances becomes large. This could happen during colonization, and could be an important selective agent in the evolution of mating systems in wind-pollinated plants. If self-incompatibility is lost, imprecise pollen transfer in wind-pollinated plants could generate high selfing rates. The highly bimodal distribution of outcrossing rates, with a deficit of mixed mating, could be a response to selection for either of two major mating strategies in wind-pollinated species (Vogler and Kalisz, 2001).

The cost of sex

Despite the common occurrence of sexual reproduction, evolutionary biology has so far failed to thoroughly explain its maintenance (e.g., Agrawal, 2001). Sexual reproduction occurs in a wide range of organisms, from the sharing of genetic material in bacteria, to the complex lifecycle of higher organisms with haploid-diploid generations and recombination during meiosis.

Among flowering plants, the evolution of self-fertile taxa from self-incompatible progenitors (Schoen et al., 1996; Sharma et al., 1992; Stebbins, 1970), asexual strains from sexual ancestors (van Dijk, 2003), and the evolution of dioecy in self-fertile groups (Miller and Vernable, 2000), suggests that the advantage, or cost, of sexual reproduction is spatially and temporally variable.

Traditionally the cost of sex has been assessed using arguments about the reproductive contribution to future generations (i.e. fitness). First, there is a two-fold transmission advantage of the selfer. While an outcrossing individual only transmits half of its genes to its offspring, a selfer transmits all of its genes (Maynard Smith, 1978). Second, an obligate cross-fertilized plant faces the risk of not receiving enough pollen, independent of the resource status of the plant (Ashman et al., 2004). Pollen limitation is a common phenomenon in plant populations (Burd, 1994) and is most frequent in self-incompatible species (Larson and Barrett, 2000), suggesting that a shift in
mating system, from self-incompatibility to self-compatibility, can be partly driven by pollen limitation (Ashman et al., 2004).

The rarity of organisms showing an ancient asexual origin (Judson and Normark, 1996) suggests that sexual reproduction is advantageous for most organisms. It is commonly believed that recombination allows for new combination of alleles to be presented in different individuals, and that these individuals will vary in fitness because of new beneficial allele combinations or the accumulation of deleterious mutations (Archetti, 2003). However, it has proven difficult to provide an experimental set up where these hypotheses can be tested. But because of the lower rate of recombination in self-fertilizing populations (Hagenblad and Nordborg, 2002), selfing populations of otherwise outcrossing plant species could provide the appropriate framework for approaching these problems.

The evolution of self-fertility

Self-incompatibility is a genetic mechanism in flowering plants that prevents self-fertilizations by rejecting pollen from genetically related individuals (Vieira and Charlesworth, 2002). It is a common mechanism, described in 91 out of 275 families of angiosperms (Haring et al., 1990). Despite the general success of self-incompatibility, the shift to self-fertility is common in the history of flowering plants (Stebbins, 1957, 1970; Schoen et al., 1997; Talavera et al., 2001). Self-incompatibility is considered most likely to be lost in situations where pollen limitation is significant, such as during colonization, in isolated populations or at the range margin of a species’ distribution (Baker 1955; 1967; Jarne and Charlesworth, 1992; Pannell, 1997; Pannell and Barrett, 1998). The reproductive assurance provided by self-fertilization is considered the ecological driver promoting self-fertility genes to spread in a population (e.g., Cheptou, 2004). The strength of this selection is determined by the magnitude of inbreeding depression and pollen limitation (Lloyd 1979; Lande and Schemske, 1985; Stebbins, 1957). A shift in life history strategy towards a more short-lived habit both lowers the cost of present reproduction on future reproduction, and could reduce the expression of late-acting inbreeding depression (Charlesworth, 1989; Morgan et al., 1997). Furthermore, while inbreeding depression commonly is treated as a constant, recent empirical evidence suggests that biotic conditions, such as the frequency and density of outcrossed conspecific plants, is of importance for the expression of inbreeding depression (e.g., Koelewijn, 1998; Cheptou et al., 2001; Cheptou and Schoen, 2003; Koelewijn, 2004). Self-fertility might be more likely to evolve under conditions where intraspecific competition is relaxed, pollen limitation severe, such as during the range expansion of a species. The inbreeding depression may be reduced in such situations,
and could be lowered further by several generations of low population sizes (Barrett, 1988).

Once self-fertility has evolved, consequent purging of deleterious mutations can reduce the inbreeding depression among self-fertilizing lines (Crnokrak and Barrett, 2002; Lande and Schemske, 1985, see Byers and Waller, 1999 for review). Populations can become genetically depleted after periods of high selfing rates. This led Stebbins (1957) among others (e.g., Cheptou, 2004) to suggest that selfing could be an evolutionary dead end. However, as of yet, there is no thorough empirical evidence supporting this notion (Takebayashi and Morrell, 2001). Nevertheless, selfing reduces the genetic diversity within populations, and reduces the effective frequency of recombination throughout the genome (Charlesworth, 2003; Hagenblad and Nordborg, 2002; Nordborg, 2000). The evolutionary consequences of these processes are yet to be thoroughly examined.

Re-evolution of self-incompatibility once self-fertility is obtained is unlikely because the re-activation of functional self-incompatibility requires more independent mutations than when it was lost (Charlesworth and Charlesworth, 1998; Takebayashi and Morell, 2001; Nasrallah et al., 2004). Self-incompatibility can be lost due to one or very few mutational events, but re-activation of self-incompatibility is much more complex (e.g., Nasrallah et al., 2004). This is because once stabilising selection stops acting on the genes regulating self-incompatibility, secondary mutations can accumulate in these genes.

Gynodioecy

Gynodioecy i.e. the co-occurrence of females and hermaphrodites in the same population (Darwin, 1877a), is a common gender dimorphism in flowering plants. It occurs in about seven percent of angiosperm species (Yampolsky and Yampolsky, 1922). A major question in the attempts to explain this breeding system has been whether the genetic factors maintaining this gender dimorphism are long lived, or if they are short lived and re-evolve frequently, similar to a host-pathogen system (Charlesworth, 2002). The female (i.e. male sterile) phenotype is commonly governed by cytoplasmically inherited genes, whereas specific nuclear genes can restore male fertility (Couvet et al., 1986; Kaul, 1988; Frank, 1989; Charlesworth and Laporte, 1998). Females are normally in the minority in gynodioecious species, but the proportion of females varies greatly due to the complex dynamics of the nuclear-cytoplasmatic sex determination system (e.g., Gouyon et al., 1991; Couvet et al., 1998; Bailey et al., 2003; Jacobs and Wade, 2003; Ronce and Olivieri, 2004). The theoretical models at hand for explaining the co-existence of females and hermaphrodites consider both sexual differences in
female fertility and the pattern of female inheritance (e.g., Lewis, 1941; Delanny et al., 1981; Gouyon et al., 1991; Bailey et al., 2003; Jacobs and Wade, 2003).

Stochastic processes and founder events can increase the overall frequency of females in gynodioecious populations. First, a lack of restorer genes in young populations may create a purely cytoplasmic inheritance of females. In such a situation, the proportion of females should increase until pollen limitation prevents any further increase in frequency (McCauley et al., 2000). Second, a mismatch between cytoplasm and restorer genes may allow for cytoplasmic inheritance of sex until the invasion of matching restorer genes, and a consequent reduction in the proportion of females (Couvet et al., 1986; Couvet et al., 1998; Ronce and Olivieri, 2004). However, stochastic processes and founder events could also increase the probability that females are missing altogether from a local population. Thus the evolution of nuclear-cytoplasmic gynodioecy can resemble an epidemic disease, where new male sterilizing mutants constantly sweep through populations followed by their corresponding restorer genes (Ingvarsson and Taylor, 2002).

Theoretical models predict that the maintenance of females requires a female fecundity advantage over hermaphrodites, and this has been found in most gynodioecious species (reviewed in Gouyon and Couvet, 1987). This female fecundity advantage can have two components: the reallocation of resources due to the lack of male function (Darwin, 1877a) and the higher outcrossing rate in females (Charlesworth and Charlesworth, 1978). The higher fecundity of females in self-incompatible species, such as *Plantago lanceolata* (van Damme 1984; van Damme and van Delden 1984), suggests that reallocation could be important for the maintenance of females. The outcrossing advantage of females, as compared to hermaphrodites where self-fertilized offspring could suffer from inbreeding depression, has been emphasized in several models of nuclear-cytoplasmic gynodioecy (e.g., Charlesworth and Charlesworth, 1978; Sakai et al., 1997). A higher female outcrossing rate, and a higher female germination rate, has been observed in *Schiedea salicaria* (Weller and Sakai, 2004). In contrast, inbreeding depression among hermaphrodites cannot on its own explain the female fecundity advantage in *Thymus vulgaris* (Thompson and Tarayre, 2000).

**Breeding system variation in *Plantago***

A vast diversity of sexual strategies is displayed in flowering plants, and considerable amount of variation can be found within the genus *Plantago* (Fig. 1). The genus is, however, less diverse in terms of in pollination strategies. All species are either mostly wind pollinated, or produce cleistogamous
(closed) flowers (Primack, 1978; Sharma et al., 1992). The genus belongs to
the family Plantaginaceae (Hoggard et al., 2003; Rønsted et al., 2002), the
phylogeny of which was recently widened to include several taxa from the
former Scrophulariaceae, such as *Antirrhinum, Digitalis* and *Veronica*
(Olmstead et al., 2001; Albach et al., 2005). Interestingly, these newly in-
cluded taxa exhibit characters highly adapted to insect pollination (e.g.,
Parachnowitsch and Elle, 2004), indicating that the genus *Plantago* ori-
ginates from insect pollinated progenitors. Furthermore, even though appar-
ently adapted to wind pollination, insect pollination has been reported in
*Plantago*. For example, *P. media* is frequently visited by potential pollina-
tors (Proctor et al., 1996), and Stelleman (1978) found syrphid fly visits to
account for some seed set in *P. lanceolata*. *Plantago* has been estimated to
be approximately 6 million years old, which suggests a rapid recent radiation
and successful worldwide colonisation (Rønsted et al., 2002).

Gynodioecy is a common sexual system in the genus *Plantago* and occurs
in at least seven of 36 European species (van Damme, 1985). Interestingly,
unlike gynodioecious species in many other families, gynodioecy co-occurs
with self-incompatibility in many species of *Plantago* (Fig. 1).

![Figure 1. The incidence of self-incompatibility and gynodioecy in some European *Plantago* species (van Damme 1985) and their phylogenetic interrelationship (Rønsted et al. 2002).](image-url)
Aims of this thesis

The general objective of this thesis was to explore the evolution and maintenance of gynodioecy and self-incompatibility in natural populations of *Plantago maritima*. I approached this problem by combining estimates of seed production from natural populations with controlled crosses and analysis of genetic variation inferred from molecular markers.

More specifically, I address the following questions:

- Is the proportion of females in gynodioecious populations higher in small populations? (I)
- Can differences in population size and relative female fitness of hermaphrodites and females together explain variation in the proportion of females among populations? (I)
- Are there self-compatible populations of *Plantago maritima* close to the northern range margin, and if so, how does self-compatibility affect the outcrossing rate? (II, V)
- Are self-fertile populations polyploid?
- Is seed output density-dependent in self-incompatible populations, and density independent in self-compatible populations of *Plantago maritima*? (III)
- Is the within-population genetic diversity lower, and the among population diversity higher, in self-compatible than in self-incompatible populations? (IV, V)
Material and Methods

Study species

*Plantago maritima* L. (Plantaginaceae) in the wide sense has a broad distribution in the northern hemisphere (Hultén and Fries, 1986). However the taxonomy is somewhat confused (Ross, 1970). The North American variants are commonly placed in the subspecies *californica* (Hultén and Fries, 1986) or *juncoides* (Ross, 1970). The subspecies *juncoides* is said to inhabit Greenland, Iceland and even the arctic coast of Norway and western Russia (Hultén and Fries, 1986), although the taxonomic status of the Norwegian and Russian plants is unclear (Tutin et al., 1976). Gregor (1939) found North American variants to be self-compatible and hermaphroditic, unlike the self-incompatible and gynodioecious European plants. Several morphologically different varieties occur in Europe, but their systematic status is not investigated (Tutin et al., 1976; Pignatti, 1982). Both subspecies *serpentina*, which grows in mountains areas, and *P. alpina* have been given species status (Tutin et al., 1976). The common chromosome number for all varieties is $2n = 12$ (Tutin et al., 1976), but Gregor (1939) reported tetraploid ($2n = 24$) as well as diploid ($2n = 12$) populations of *P. alpina*. Even though there is a great deal of taxonomical confusion within this species and between its close relatives, the plants used in this study belong to the nominal subspecies *maritima* as described by Linnaeus (1755).

*Plantago maritima* was chosen as the study species of this thesis mainly because of its gynodioecious breeding system (Ross, 1970). However, the species has other convenient features for studies of reproductive biology. It is salt tolerant (Staal et al., 1991) and is consequently common in marine habitats throughout Europe (Tutin et al., 1976). In Sweden, the species is common along the coastline, and inhabits many of the islands in the archipelagos of eastern Sweden and southwestern Finland, but becomes less frequent in the northern part of the country (Hultén, 1971). Individual rosettes are easily distinguished and vegetative propagation is rare (Dinnétz and Jerling, 1997). Furthermore, the seeds are easy to germinate if they are given plenty of light (Jerling, 1982).
The species is predominantly self-incompatible in Europe (Ross, 1970; Dinnéetz, 1996) but I have observed seed set following self-pollination in pilot studies of plants from northern Sweden and northernmost Norway. Self-incompatibility in *Plantago maritima* is likely to be a single-locus gametophytic system, similar to that of *P. lanceolata* (Ross, 1973).

The flowers of *P. maritima* are arranged in a dense racemose inflorescence (Fig. 2). A single plant can produce from one up to more than a hundred inflorescences during one season (pers. obs.). The hermaphrodite flower possesses a long style with a hairy stigma during female phase, and four stamens with bright yellow anthers during male phase (Fig. 3). The conscious anthers can attract syrphid flies (pers. obs.) Female flowers have shorter filaments and thin brown anthers, and the morphology of female types is highly variable even though some distinct types can be recognized (Dinnéetz, 1988). Typically, the stigma is longer and erect for a longer period in female flowers as compared to hermaphrodite flowers (Dinnéetz, 1996).
Figure 3. A detailed study of (A) a single hermaphrodite and (B) female Plantago maritima flower from the plants in Fig. 2. The hermaphrodite is in male phase, with erect anthers and a withered pistil. The female flower (B) has a large feathery pistil and rudimentary stamens by the base of the pistil.

The species is protogynous, i.e. the pistil matures before the anthers on the stamen are ripe, but the degree of protogyny is highly variable. Within an individual, the temporal separation between female and male function can vary from one to 10 days (Dinnétz, 1997). However, the variation in temporal separation of sexual function in individual flowers has not been investigated. The flowering time is highly variable among regions, with a more extended flowering time in southern plants. I have observed differences in flowering time both in the field and in plants growing in the greenhouse.

Study areas

I used seeds or plants originating from Plantago maritima populations in archipelagos on the east coast of Sweden (Gryt, Öregrund and Skeppsvik) and southwestern Finland (Korpoo; Fig. 4), except for (V) where plant material originates from Longhoughton, United Kingdom. The northern Skeppsvik archipelago is situated 880 km north of the southernmost Gryt archipelago, and is subjected to an isostatic land-uplift of about 8 mm per year. In Skeppsvik, the age of the investigated islands range from 46 to 700 years (Carlsson et al., 1990). In contrast, the land uplift is about 2 mm per year in the southern Gryt archipelago. The islands are much higher in Gryt, and the estimated age of the younger islands does not fall below 1600 years (Nilsson, 1999). The salinity of the seawater declines towards the north, with
brackish waters in the Gryt archipelago (~ 7‰), but virtually fresh water in
the northern Skeppsvik archipelago (~ 4 ‰; Snoeijs, 1999).

![Map of archipelagos in Sweden and Finland](image)

*Figure 4. The location of the Gryt (I, II, III, IV), Korpoo (I, IV), Öregrund (I, II, IV) and Skeppsvik (I, II, III, IV) archipelagos in Sweden and Finland.*

**Field estimates, fruit and seed production (I, II, III)**

For each study population, I estimated the proportion of females by deter-
mining the sex of 200 plants, or all plants if the population consisted of
fewer than 200 flowering individuals. Plants with partially reduced male
function were pooled with the hermaphrodite plants when the proportion of
females was calculated. Population size was estimated as number of flower-
ing plants. In this thesis, a population was defined as all plants on an island,
or a group of plants separated by more than 50 meters to the next plants.

Fruit and seed set were estimated in 30 plants per sex morph (I) or in 20 -
30 hermaphrodites (III) for each population. Plants were scored for number
of inflorescences and distance to nearest pollen donor. Three inflorescences,
or all inflorescences if fewer than three, were collected and stored at 4 °C
until further analysis. I estimated seed production by counting the number of
flowers and fruits on each plant, counted seeds from ten ripe fruits weighed
the seeds.
Plant material and controlled crosses (II, IV)

I germinated seeds under artificial light to obtain adult reproducing plants, for the self-compatibility experiment (II), and fresh plant material for DNA extraction (II, IV). The germination frequencies were overall high and most seeds germinated within two weeks. I cultivated plants from one population from the Gryt archipelago, and one population from the Skeppsvik archipelago, in pots filled with a commercial soil mixture (P-jord, Weibulls, Hammenhög, Sweden). On each plant, one inflorescence was pollinated with self-pollen only, and one inflorescence received additional cross pollen from another plant of the same population. To avoid pollen contamination, each inflorescence was isolated in a transparent cellophane bag prior to, and during, treatment. The bag was removed once flowering stopped.

Molecular markers (V)

I developed five polymorphic microsatellite markers, following a modified protocol of Edwards et al. (1996), for Plantago maritima (V). This provided a valuable tool for assessing the variation in the mating system between populations (II) and the population genetic structure (IV). Microsatellite loci, i.e. fragments of DNA that vary in the number a simple DNA sequence is repeated, are often highly polymorphic and relatively easy to survey (Slatkin, 1995). As markers, they are considered neutral and follow Mendelian inheritance (Jarne and Lagoda, 1996). In fact, microsatellites studies proved to be essential for the articles (II) and (VI) in this thesis. Several allozyme marker systems showed no variation in a population from the northern Skeppsvik archipelago. However, the allozyme systems 6PGD, IDH, PGI, PGM and GOT displayed polymorphisms in plants originating from the southern Gryt archipelago (E. Nilsson and P. J. van Dijk, unpublished results).

Cytological investigation

I used two methods to assess the chromosome number in plant material from Sweden, Spain, Portugal, USA (California) and Norway.

The ploidy level of the plants was first estimated by measuring the nuclear DNA amount with a UV flow cytometer following the protocol described in Tas and van Dijk (1999). The nuclear DNA amount was corrected against Taraxacum plants of known ploidy level. Flow cytometry estimates are highly correlated to chromosome counts (Tas and van Dijk, 1999).
Second, chromosome numbers in cell extracts from root tips were counted manually. For chromosome counts, seeds from 10 different populations were germinated on moist filter paper. Two seedlings from each motherplant were pre-treated with 10 – 20 ml of 0.002 M 8-hydroxyquinoline for 4 – 5 hours at 4 – 5 °C, and then fixed in vials half full with 1:3 acetic acid and ethanol (70%) for 24 hours. The seedlings were then hydrolysed in test tubes with 1M HCl for 8 – 10 minutes in a water bath of 60 °C. Subsequently, the seedlings were cleaned with distilled water and dried on clean paper tissue. When dry, seedlings were transferred to a vial and stained in lacto-aceto-orcein for 4 – 5 hours at 20 °C in darkness. The root tip was transferred to an objective glass where a drop of acid was added and the non-stained part of the root removed. An analysing glass was placed on the tissue and the root was squashed by gently pressing the glass with a toothpick. Finally, the numbers of chromosomes in cells undergoing the appropriate stage of mitosis were counted under a microscope.
Results and discussion

Proportion of females (I)

As expected under nuclear-cytoplasmic inheritance of gynodioecy, the proportion of females in populations of Plantago maritima was highly variable (I), as would be expected under the nuclear-cytoplasmic inheritance of gynodioecy. Females were more frequently missing from small populations, and the variance in sex ratio decreased with increasing population size. There was also a decrease in female frequency with increasing population size. This is consistent with the idea that a mismatch between cytoplasmic male-sterility genes and restorers may be particularly likely in small and young populations (Belhassen et al., 1991; Manicacci et al., 1996; Couvet et al., 1998; Ronce and Olivieri, 2004). The proportion of females was highest in the northernmost Skeppsvik archipelago and lowest in the southernmost Gryt archipelago (I).

The proportion of females increased with the relative female fecundity, and decreased with increasing population size (Fig. 5). It thus seems plausible that both selection and stochastic processes govern sex ratios in Plantago maritima.

Surprisingly, ecological processes, such as pollen limitation, have received relatively little attention in both the theoretical modelling and in empirical investigations of gynodioecious species (But see Ashman, 2002, 2003; Asikainen and Mutikainen, 2003). Furthermore, the general female fecundity advantage reported in the literature is commonly estimated from greenhouse grown plants. It cannot be assumed that females would gain a similar advantage in natural populations, where pollen limitation (e.g., Ashman, 2004) and sex-biased herbivory (Mutikainen and Delph, 1996; Ågren et al., 1999; Ashman, 2003) could be present. Moreover, characters of reproductive success, rather than estimates of total reproductive success, have commonly been used as evidence for a female fecundity advantage, and there may be trade-offs among components of reproductive success.
Figure 5. The relationship between proportion of females, relative female fecundity and population size ($F_{2.9} = 16.8, P < 0.001, R^2 = 0.79$) among 12 populations of *Plantago maritima* as described by added variable plot. The plots depict (A) the relationship between proportion of females and relative female fecundity when variation in population size is accounted for, and (B) the relationship between proportion of females and population size when female frequency is accounted for.

Self-fertility (II)

Controlled crosses and genetic analysis of progeny arrays demonstrated that the Skeppsvik population of *P. maritima* was self-compatible and mixed-mating, while the south Swedish population was self-incompatible and predominantly outcrossing (II). Gregor (1939) has previously described North American populations as self-compatible, but this is the first evidence of self-fertile *P. maritima* from Europe. Gametophytic self-incompatibility is likely to be ancestral in eudicots (Igic and Kohn, 2001), and it has been shown that there is a substantial sequence homology between the Rosaceae, Solanaceae, Scrophulariaceae and Plantaginaceae (Vieira and Charlesworth, 2002). The self-incompatibility system in *P. maritima* is most probably gametophytic as in *P. lanceolata* (Ross, 1973). Thus, it is likely that similar processes are responsible for the breakdown of self-incompatibility in *Plantago maritima*.

The breakdown of gametophytic self-incompatibility has been thoroughly investigated in the genera *Lycopersicon* (Kondo et al., 2003) and *Petunia* (Tsukamoto et al., 2003) of the Solanaceae In both *Lycopersicon* and *Petu-
nia, an enzyme in the style recognizes self-pollen and rejects it. In the self-compatible *Petunia axillaris* ssp. *axillaris* from Uruguay, a modifier locus suppresses the expression of this enzyme, and thus allows self-fertilization (Tsukamoto et al., 2003). The expression of the self-incompatibility enzyme is also suppressed in self-compatible *Lycopersicon*, but in this species the gene transcribing the enzyme has been altered (Kondo et al., 2003).

**Cytology**

With one exception, the cytological studies did not indicate any deviation from the basic chromosome number $2n = 12$. Spanish plants from the island of Mallorca appeared to have $2n = 20$ from the flow cytometry; no visual chromosome count was performed with this material. As this would be a deviation from a simple duplication, further investigations are required with visual confirmation of this somewhat odd chromosome number (cf. Dart et al., 2004). The chromosome number of $2n = 12$ was visually confirmed for plant material from northern Norway, Sweden and Finland. Consequently, it is most probable that the plant material used in this thesis is diploid ($2n = 12$).

**Density dependent seed output (III)**

The self-incompatible populations of *Plantago maritima* in the Gryt archipelago displayed a dramatic decrease in seed output with distance to nearest pollen donor (III; Fig. 6). The rapid decline in seed output with distance in the Gryt populations suggests that the pollen dispersal distance is short.

In contrast, seed output did not decrease with increasing distance to nearest pollen donor in the Skeppsvik archipelago, suggesting that self-fertilization provides reproductive assurance in the absence of outcrossed pollen. This suggests that hermaphrodites in the self-compatible populations may have an advantage over females during colonisation of empty habitats, as previously suggested in the theoretical models of Pannell (1997). However, the high frequency of females among populations in the Skeppsvik archipelago suggests that females have some advantage over hermaphrodites, possibly by being fully outcrossed.
Figure 6. The relationship between fruits per flower (arcsine square-root transformed) and mean distance to nearest pollen donor in Plantago maritima populations from the Gryt (N = 21) and the Skeppsvik (N = 20) archipelagos.

Genetic diversity (IV)

The genetic diversity was high in populations of Plantago maritima in southern Sweden (IV), but decreased towards the north. The northern Skeppsvik archipelago had the lowest observed heterozygosity and no private alleles. The low level of genetic diversity among and within the northern populations suggest that they have had a history of small population sizes, where periods of extensive selfing could have further reduced genetic diversity.

However, only one population from the Skeppsvik archipelago have a significant deviation from Hardy-Weinberg equilibrium due to an excess of homozygotes. This suggests that most individuals are the results of outcrossings in these populations, despite their ability to self-fertilize.
General discussion

I have demonstrated the occurrence of self-fertility in north Swedish populations of the otherwise predominantly outcrossing *Plantago maritima*. The self-fertilizing ability of these populations apparently facilitates density-independent seed production. In contrast, seed production in populations from the south Swedish Gryt archipelago showed a markedly positive density dependence. Furthermore, southern populations had a higher genetic diversity than the northern populations. The colonizing ability of self-fertile plants (Pannell and Barrett, 1998) could have accelerated the northward post-glacial expansion, but at the same time contributed to the loss of genetic diversity by the joint action of founding events and self-fertilization (Ingvarsson, 2002).

Interestingly, the proportion of females in populations tends to increase towards the north and is highest in the northern Skeppsvik archipelago. The importance of self-fertility, and inbreeding depression among selfed progeny, for the maintenance of females in gynodioecious populations has been under debate (e.g., Charlesworth and Charlesworth, 1978; Gouyon et al., 1991). The joint occurrence of both self-incompatible and self-compatible gynodioecious populations of *Plantago maritima* makes it an interesting species for the evolution of this breeding system. The higher incidence of females among populations in the northern Skeppsvik archipelago suggests that the female reproductive success of females relative hermaphrodites may be higher in self-compatible than in self-incompatible populations. At the same time, the occurrence of females in the southernmost Gryt archipelago demonstrates that self-fertility in hermaphrodites is by no means imperative for the evolution of gynodioecy.

Theory predicts that mixed-mating, i.e. both self- and cross-fertilisations, can be adaptive if it provides reproductive assurance (Lloyd, 1992; but see Cheptou, 2004). However, inbreeding depression among selfed progeny can not be severe, and self-fertilizations should not hinder outcrossing for mixed-mating to provide reproductive assurance (Lloyd, 1992). Further studies are required to assess the amount of inbreeding depression experienced by selfed progeny in natural populations.

The evolution of breeding systems is a process that alters the very fundament of evolution by changing the way by which gene combinations are presented for natural selection (Bell, 2005). Once self-fertility is achieved,
high selfing rates can evolve and with that the frequency of recombination is lowered (Hagenblad and Nordborg, 2002). Consequently, a lower number of allele variants will be presented to natural selection. The high number of allele variants produced by recombination is commonly believed to be the advantage of sexual reproduction (Archetti, 2003). However, effectively high outcrossing rates can be maintained even in self-fertile populations. First, in the case of Plantago maritima, a temporal separation between female and male function (i.e. dichogamy) would decrease the probability of selfing. Second, with severe inbreeding depression among selfed progeny, most adult plants will be the results of outcrossing even though a substantial part of the seed output stems from self-pollinations (cf. Herlihy and Eckert, 2002). Moreover, it is likely that hermaphrodites experience a high cost of reproduction if selfed seeds do not contribute to fitness (Ashman, 1994). Females, on the other hand, would avoid such a cost.
Conclusions

In this thesis, I have documented considerable variation in the breeding system of *Plantago maritima*. The sex ratio variation can be attributed to both stochastic processes and selection, because females are more abundant in populations where they set more seeds than hermaphrodites. The relative difference in seed production between females and hermaphrodites could be influenced by the breeding system of a local population. I documented the loss of self-incompatibility in a population from northern Sweden, and confirmed a functional self-incompatibility system in southern Sweden.

A comparative field study was performed to evaluate the importance of this shift in breeding system in this wind-pollinated plant. Plant fecundity decreased with increasing distance to nearest pollen donor both within and among populations in the south Swedish Gryt archipelago where self-incompatibility have been confirmed. My results suggest, in accordance with the limited empirical evidence at hand, that effective pollen dispersal distance is low in wind-pollinated herbs. In contrast, plant fecundity was overall higher and was not density-dependent in the Skeppsvik archipelago in northern Sweden where controlled crosses showed that plants are self-compatible. These results are consistent with the prediction that evolution of self-fertility should reduce the density-dependence of pollination success, and has not previously been indicated in a wind-pollinated plant. I found low genetic diversity within populations from northern Sweden, which may be the result of a history of small population sizes and periods of frequent self-fertilization.
Acknowledgements

Fyra män i brudkammaren och ensamma kvinnor komplicerar gulkämparnas kärlekliv


En brudkammare fylld av pollen


En ensam kvinna i brudkammaren

terna sätter med sig själva drabbas av inavelsdepression och bildar mindre livskraftiga planter kan de konkurreras ut av andra gulkämpar, däribland honor. Kanske klarar sig honorna bättre genom att de alltid sätter frön med hjälp av pollen från andra gulkämpar?

Jag har visat att honor är vanligare på öar där de sätter mer från än hermafroditerna, och ovanligare där honorna sätter färre frön. Det verkar med andra ord som att honorna har en fördel under vissa omständigheter, men en nackdel i andra. Ännu så länge kan jag inte sätta fingret på vilka omständigheter det är som gynnar honorna. Men i södra Sverige är honor inte lika vanliga. Här kan hermafroditerna inte befrukta sig själva utan är också de beroende av pollen från andra individer.

Skärgården – Sveriges Galapagos?

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Acta Universitatis Upsaliensis

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