Evolutionary dynamics of mating systems in populations of North American *Arabidopsis lyrata*

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Abstract

Plants can vary in their mating systems from completely inbreeding to completely outcrossing, with intermediate forms referred to as mixed mating systems. *Arabidopsis lyrata* is a strongly outcrossing perennial due to a sporophytic self incompatibility (SI) system. The species occurs in temperate regions of the Northern hemisphere where in Europe its SI system is fully working but around the Great Lakes of North America some populations of *A. lyrata* show a breakdown in SI. Consequently these North American populations are inbreeding or have a mixed mating system next to outcrossing populations with a working SI system. In this thesis I used North American *A. lyrata* to investigate the evolutionary consequences involving variation in mating systems.

First of all I was interested in the time that populations had been isolated from each other in the past that could explain differences in mating systems. In order to determine whether populations experienced a breakdown of SI independently or whether this originated from a single event I used chloroplast DNA (cpDNA) markers to reveal deep phylogeny and microsatellite markers to determine recent population genetic patterns. The results showed a loss of SI in populations from all three detected cpDNA haplotypes. Microsatellite data showed that predominantly inbreeding populations sharing one of these haplotypes showed high levels of homozygosity and that in all three haplotype lineages self-compatible individuals always had reduced heterozygosity compared to self-incompatible individuals. The data further showed that there had likely been at least two independent postglacial colonization routes to the north of the great lakes. This was consistent with phylogeographic studies of other organisms with limited dispersal such as reptiles and amphibians.

The next question was the role of inbreeding depression in the loss of SI. Inbreeding depression is defined as the decline of fitness after an inbreeding event. Inbreeding causes an increase in homozygosity that exposes recessive deleterious mutations, which would normally be sheltered in a heterozygous state, and causes a fitness decline. Individuals experiencing a loss of SI will have higher inbreeding levels and can result in inbreeding depression, which is thought to maintain the SI system. To gain more insight into the role of inbreeding depression in the shift from self-incompatibility to self-compatibility, I conducted an experiment in which I created outcrossed and selfed offspring from self-compatible and self-incompatible mothers from populations with different outcrossing histories. I monitored the offspring for early- and late acting fitness traits like germination rate, growth and time to flowering. I found inbreeding depression in

only one late acting fitness trait, the increase in leaves 5 weeks after germination, to be significantly higher for self-incompatible than self-compatible individuals. I also conducted a regression analysis where relative fitness (the ratio of the fitness trait values of selfed and outcrossed offspring) per mother was regressed against population heterozygosity and found a significantly negative regression. This result suggested that individuals from a population with a relatively high heterozygosity suffered more from inbreeding depression than individuals from populations with a relatively low heterozygosity. This indicated that the history of outcrossing of a population, or purging, played an important role in the shift from outcrossing to inbreeding.

The detection of inbreeding depression could not be evident by only looking at life history traits under greenhouse conditions. But stressful environmental conditions like a pathogen infection could magnify inbreeding depression. I would expect that predominantly outcrossing populations would have a higher heterozygosity than predominantly inbreeding populations and therefore be able to show a higher fitness when exposed to a pathogen. To test this hypothesis I used four outcrossing and four inbreeding populations, which I infected with the crucifer pathogen *Albugo candida* and measured relative growth rates (RGR) and monitored resistance rates. The results showed that there were three infection phenotypes: resistant (no signs of infection), partially resistant (only the initially infected parts showed symptoms) and susceptible (symptoms present on the whole plant). The inbreeding populations showed a bimodal distribution of resistance as two populations showed a high rate of resistance and two showed a low rate of resistance. The outcrossing populations showed a much more uniform distribution of resistant individuals with a higher rate of partially infected individuals across populations than inbreeding populations. Resistant and partially resistant individuals did not differ significantly in their RGR from each other but both had a significantly lower RGR than the untreated control group and a significantly higher RGR than the susceptible individuals. This suggested a cost of resistance that was lower than a cost of being susceptible in the presence of a pathogen. There was no effect of mating system on RGR, which was primarily caused by the fact that two inbreeding populations contained a high amount of resistant individuals and an outcrossing population that showed a very low amount of partially resistant and resistant individuals. The difference in resistance to *A. candida* in *A. lyrata* differed much more between inbreeding than between outcrossing populations. This suggested that alleles responsible for resistance were concentrated in homozygous form in inbreeding populations and both homozygous and heterozygous form in outcrossing populations. This would mean that mating system plays a role in susceptibility, as

resistance genes would be concentrated in certain individuals in inbreeding populations as opposed to a more modal distribution in outcrossing populations.

A shift in mating system often has an effect on floral traits, as there is a lack of necessity to attract pollinators. I wanted to test whether these changes were apparent in *A. lyrata* by comparing pollinator attractants and sexual floral traits between strongly outcrossing and strongly inbreeding populations. I hypothesized that individuals depending on pollinators for outcrossing would show a higher emission of volatiles and floral traits that had evolved to optimize pollen transmission to conspecifics. Autonomously selfing individuals would be independent of pollinators so should show a reduced volatile emission pattern, a floral trait composition that evolved to transmit pollen to their own stigma, and a reduction in floral display compared to outcrossers. My results showed a somewhat contradicting pattern as self-compatible individuals showed higher volatile emission than self-incompatible individuals but self-incompatible individuals showed larger petal size than self-compatible individuals. Pistil height and stamen length were strongly correlated but petal size seemed to co-vary relatively independent from pistil and stamen length. I found no effect of mating system on the evolvement of floral traits to optimize pollen to the stigma and contradicting patterns for pollinator attractant traits. Due to low sample sizes this study turned out to be a pilot study for further research so the results in this study were not conclusive at this stage.

Finally I conclude that SI has been lost independently several times and the low observed genetic load in the North American populations compared to the European populations could be responsible for that. There have probably been two independent colonization routes to the North of the Great Lakes following the last glaciation in which a Northern distributed cpDNA haplotype lineage seems to have a lower frequency of SC individuals than a southern cpDNA haplotype lineage. Inbreeding populations showed a bimodal distribution of infection phenotypes among individuals compared to outcrossing populations that showed a more evenly distributed of infection phenotypes among individuals which is thought to be caused by higher heterozygosity in outcrossing populations. Traits involved with pollinator attraction show a contradicting pattern with high volatile emissions for self-compatible individuals, which could be due to the dependence on pollinators for self-fertilization in self-compatible plants.

Candidate's declaration

I declare that the work recorded in this thesis is entirely my own, except where otherwise stated, and that it is also of my own composition. Much of the material included in this thesis has been produced in co-authorship with others, and my personal contribution to each chapter is as follows:

Chapter 2. *Published as:* Hoebe, P.N., Stift, M., Tedder, A., Mable, B.K. 2009 'Multiple losses of self-incompatibility in North American *Arabidopsis lyrata*?: Phylogeographic context and population genetic consequences' *Molecular Ecology* **18**: 4924-4939. Data collection facilitated by PNH, MS, AT, and BKM. Analysis conducted and manuscript drafted by PNH. Final draft enhanced by PNH, MS, and BKM.

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I further declare that no part of this work has been submitted as part of any other degree.

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1 General introduction

1.1 Introduction

1.1.1 Mating system variation

In plants there are many different ways to produce offspring but can be divided in two main categories: sexual and asexual reproduction (Kirkpatrick & Jenkins 1989; Maynard Smith 1978). In sexual reproduction male and female gametes fuse, resulting in offspring that are genetically distinct from the mother (except for self-fertilized offspring) as opposed to asexual reproduction, which has an identical genetic makeup as the mother (Eckert 2001). In terms of sexual reproduction there are two extremes, completely outcrossing and complete self-fertilization, with mixed mating systems in between (Jarne & Charlesworth 1993; Lloyd & Schoen 1992; Schoen & Brown 1991). Self-fertilizing plants use their pollen to either fertilize the stigma of the flower to which the anthers belong (self-pollination) with closed (cleistogamy) or open flowers (autogamy) or the stigma of another flower on the same plant (geitonogamy). Self-fertilizers are either depended on pollinators (facilitated) or not (autonomous). Outcrossing plants can have flowers that are hermaphroditic, hermaphroditic and female (gynomonoecious), hermaphroditic and male (andromonoecious), or separated into two genders (monoecious and dioecious), as well as various combinations of these sexual breeding systems (Barrett & Harder 1996a). Outcrossing plants with hermaphrodite flowers often have developed a self-incompatibility (SI) system to prevent self-fertilization, which can be heteromorphic or homomorphic (Hiscock & McInnis 2003). Heteromorphic SI systems prevent selfing through temporal or spatial barriers between male and female traits. In homomorphic SI systems there is a genetically determined physiological barrier where the pollen carries specific proteins that can be recognized by the stigma if they carry the same specificity, in which case the pollen gets rejected (Hiscock & McInnis 2004).

Self-incompatibility, rather than self-compatibility, is thought to be an ancestral state according to the distribution of self-incompatibility states of several species mapped on an angiosperm phylogeny (Allen & Hiscock 2008; Igic *et al.* 2004; Takebayashi & Morrell 2001). Self-incompatibility systems in different angiosperm families are thought to have independent origins where gametophytic systems probably evolved earlier than sporophytic systems (Allan & Hiscock, 2008). Mating system at the population level is described as a certain proportion of inbreeding and outcrossing individuals present in a population, where inbreeding individuals persist or arise by the breakdown of self-

incompatibility in outcrossing individuals. Opposed to earlier theoretical studies which outcomes described mating systems as fully outcrossing or inbreeding (Lande & Schemske 1985), it is now believed that mixed mating systems can be stable throughout time. For example, a study (Vogler & Kalisz 2001) showed that 49% of all the animal pollinated plant species in this study had an outcrossing rate between 20% and 80%. However, there is very little known about the exact mechanisms behind the origin of populations although it can be linked with for example mate assurance by selfing in an unpredictable pollinator presence (Kalisz & Vogler 2003). The persistence in time of mixed mating systems is still unclear as there are studies that suggest that it is an intermediate stage to complete inbreeding (Lande & Schemske 1985) where others find prove for it to be stable in time (Goodwillie *et al.* 2005).

1.1.2 Evolution of plant mating systems

There are several evolutionary scenarios that could lead up to either a selfing or outcrossing strategy (Barrett 2003). Theoretical work on mating systems suggested an endpoint in either outcrossing or inbreeding within a population where not only consequences but also causes and mechanisms were researched. Genetic research looked at variation at genome level and a possible relation with inbreeding depression and predicted a similar scenario (Charlesworth & Wright 2001). Contrary to these studies, not only female but also male function should be considered when fertility will play a role in an outcrossing or inbreeding strategy (Barrett 2003). The evolution towards separated floral sexual organs did not only prevent self fertilization, also avoiding pollen waste has driven this development. It is important to consider different evolutionary forces that shaped mating systems in plants to understand the mating strategy present in a population or individual (Barrett & Harder 1996a). Growth habitat and clonal architecture leads to evolutionary transitions betweens sexual systems (Barrett & Shore 2008). Considering mating systems as only outcrossing or selfing would be an oversimplification as intermediate outcrossing rates are very common (Vogler & Kalisz 2001). This could be explained by environment depended pollination and inbreeding depression (Pannell & Barrett 1998). A problem is obviously the maintenance of inbreeding depression in outcrossing individuals and the floral cost for selfers. Many studies do find a high amount of inbreeding depression (Carr & Dudash 1997) so it is not clear how mechanisms overcome this in mixed mating systems . Mixed mating systems could occur through delayed selfing in which selfing only occurs when an individual was unsuccessful in

outcrossing and it SI system breaks down after a certain amount of time (Vogler & Kalisz 2001; Vogler & Stephenson 2001). Another possibility is an open pollination system in which individuals are in principal self fertilizing but outcross pollen lands on their stigma by wind pollination or visiting insects. Another mechanism, which maintains both strategies, occurs when pollinators visit flowers on the same plant and will transfer outcross pollen to the first flower but after visiting other flowers on the same plant the outcross pollen on the pollinator will dilute and self pollen will increase and further deposited on the plant its stigmas (Barrett 2003). This will result in fruits with seeds that are a result of outcross and self pollinations. In the tristylous species *Eichhornia paniculata*, which has a heteromorphic SI system and outcrossing rates varying from zero to one, modifier genes controlling stamen length play a role in maintaining the mixed mating systems in combination with demographic factors that influence pollinator presence and determine the selection on either outcrossing and selfing strategies. (Barrett *et al.* 1989).

1.1.3 *Inbreeding depression*

Hermaphroditic plants are capable of both outcrossing and self-fertilizing (Takebayashi & Morrell 2001). Self-fertilizers are thought to be more successful than outcrossers in terms of gene transmission to the next generation as they fertilize their own ovules and ovules of outcrossers, whereas outcrossers fertilize only ovules of other outcrossing individuals (Barrett & Harder 1996a; Barton & Charlesworth 1998). Mutations responsible for selfing can therefore increase their transmission by 50% if they appear in an outcrossing population as their pollen not only fertilizes other ovules but also their own (Fisher 1941).
Inbreeding depression is the fitness difference between outcrossed and inbred progeny or in formula: Lnz_O-Lnz_I=BF, in which the difference of the natural logarithm of fitness component z of outbred (Lnz_O) and inbred progeny (Lnz_I) equals the inbreeding coefficient (F) times the reduction in log fitness dealing with inbreeding (B) (Charlesworth & Charlesworth 1999). Inbreeding depression would even out the advantage of high gene transmission in self-fertilizing individuals.

If inbreeding depression outweighs the advantage of increased gene transmission to the next generation (Herlihy & Eckert 2002), then it is expected that outcrossing is favoured over inbreeding. Self-incompatibility (SI), which prevents selfing and promotes outcrossing, is thought to be maintained with inbreeding depression as a driving force as

there will be a disadvantage compared to other selfing individuals in terms of gene transmission (Charlesworth & Charlesworth 1979). There are heteromorphic and homomorphic SI systems, where the first one has temporal (dichogamy) or spatial (herkogamy) barriers and the latter one has genetically determined physiological barriers between male and female reproductive parts of a hermaphroditic plant (deNettancourt 1997). Within homomorphic SI systems there are gametophytic (GSI) and sporophytic SI systems (SSI) (Bateman 1952). In the GSI system the haploid pollen genotype determines the pollen its specificity whereas in SSI the paternal genotype determines the pollen its specificity (Hiscock & McInnis 2003). In a sporophytic homomorphic SI system, the male gene (*S*-locus *C*ysteine *R*ich, *SCR*) encodes ligands that are deposited on the paternal pollen coat by the anther tapetum cells, while the female gene (*S*-locus *R*eceptor *K*inase, *SRK*) encodes proteins deposited on the maternal stigmatic surface (Charlesworth *et al.* 2005). Pollen that has the same specificity as the maternal proteins on the stigma will be rejected (Hiscock & McInnis 2004). This recognition mechanism involves that *SRK* genes have to evolve in concert with the associated *SCR* genes (Nasrallah & Nasrallah 1993). The *SRK* and *SCR* genes are tightly linked, with recombination being suppressed in this area to maintain the paired specificity (Awadalla & Charlesworth 1999).

The *S*-locus is under frequency dependent selection, resulting in many different specificities (*S*-alleles) and can lead to a high accumulation of mutational load, which is thought to complicate the transition from outcrossing to inbreeding (Glemin *et al.* 2001). Sporophytic SI systems could be operational at very small numbers of *S*-alleles (Brennan *et al.* 2005). Nevertheless, if the number of *S*-alleles drop below a certain threshold, because the population goes through a bottleneck for example (Foxe *et al.* 2009), individuals will suffer from limited mating opportunity. Under such a scenario, only selfing individuals, despite inbreeding depression, would be able to contribute offspring to the next generation (Guo *et al.* 2009). Selfing individuals would also face a decline in heterozygosity, as inbreeding results in higher homozygosity in a population (Wright 1977). This decline could result in lower fitness and two, non mutually exclusive, hypotheses have been proposed to explain this fitness decline: 1) Overdominance, where the fitness advantage of heterozygotes over homozygotes is caused by their heterozygous state irrespective of the underlying mechanism (Shull 1908). 2) Partial dominance, which is a form of overdominance, where inbreeding depression is caused by recessive deleterious mutations that are sheltered in a heterozygous state but become exposed in a homozygous state (Carr & Dudash 2003; Davenport 1908). According to the partial dominance hypothesis, deleterious mutations that would be exposed after an inbreeding

event could be removed from a population through selection (Bijlsma *et al.* 1999). This is commonly referred to as purging but its role in maintaining mixed mating systems is still unclear. Although most inbreeding depression seems to be caused by deleterious recessive alleles (Charlesworth & Charlesworth 1999), the role of over- and partial dominance to explain inbreeding depression is still unresolved.

Inbreeding depression could take place in early (*i.e.* seed abortion, germination success) and late (*i.e.* growth, number of flowers) life stages of the plant where early acting inbreeding depression is thought to be caused mainly by recessive lethal mutations and late acting inbreeding depression by weakly deleterious mutations that are harder to purge (Husband & Schemske 1996). Purging of deleterious load could happen in a lineage specific manner, with variation in inbreeding depression between maternal lineages, or at the population or species level (Byers & Waller 1999; Schultz & Willis 1995) but this is still under debate. Finally, there are also studies that show early acting inbreeding depression that is very difficult to purge (Koelewijn *et al.* 1999) and studies that show more severe inbreeding depression taking place in later life stages (Glaettli & Goudet 2006). These contradicting results makes the role of the timing of inbreeding depression still unresolved.

A sheltered deleterious load revealed by inbreeding could expose lethal mutations such as chlorophyll deficiency, which would be fatal in an early stage of an individuals' development (Karkkainen *et al.* 1999). Mildly deleterious mutations such as a low growth rate or seed set might only be negative in competition with conspecifics so isolated selfing individuals could purge their deleterious load over generations by selection on their exposed mutational load (Barrett & Charlesworth 1991). Although purging could overcome the negative consequences of inbreeding in the short term, consequently there will be a loss of genetic variation. This could result in a possible lower adaptability to novel environments and a decline of the effective population size, increasing the role of genetic drift, which results eventually in a further loss of genetic variation (Wang *et al.* 1999). Inevitably, purging complicates the detection of inbreeding depression when looking at life history traits in greenhouse conditions only.

1.1.4 *Self-Incompatibility systems*

Mechanisms that prevent self fertilization are generally referred to as self incompatibility systems. There are different ways that these SI systems can act like for example by spatial or temporal separation of the male and female parts of the flower, which is generally referred to as heteromorphic self incompatibility. In Daffodils (*Narcissus*) (Barrett & Harder 2005) there is a heteromorphic spatial self incompatibility system present where the female parts and the male parts are spatially separated but variation in length between the anthers and the pistil between individuals promotes outcrossing. Many plants have a genetically controlled self-incompatibility system, in which self-fertilization is avoided by a physiological response (Takayama & Isogai 2005). There are different types of molecular mechanisms underlying homomorphic SI between different taxa (McClure *et al.* 2000). Early work on the discovery of components involved in the gametophtyic SI system was on *Nicotinia alata* where proteins on the stigmatic surface were related to recognition of received pollen (Anderson *et al.* 1986). These S-proteins showed ribonuclease activity and were further referred to as S-RNases (Kawata *et al.* 1988). The S-RNases showed a dual role as recognition molecules as well as pollen tube growth inhibition. The alleles underlying the different S-RNases show a high amount of diversity with normally only 50% similarity between alleles (Ioerger *et al.* 1990). Generally the S-locus determines the compatibility between to specificities. Only in *Papaver* (Franklintong *et al.* 1995) is the product of the S-locus sufficient to initiate a SI reaction, where in Brassica and the S-RNase systems (*Nicotinia, Petunia* and *Solanum*) there is a whole range of modifiers involved. Besides the recognition of self pollen by stigma there is a whole cascade of modifiers underlying the SI reaction preventing self pollen to germinate. These modifiers can be divided in three groups; group 1 consists of modifiers directly affecting the expression of genes determining specificity, group 2 consists of factors interacting either genetically or biochemically with determinants and are involved with pollen recognition but not with pollination, group 3 consists of genes that are involved with pollen recognition but also with other pollen-pistil interactions. In sporophytic self-incompatibility systems in Brassicaceae, the male gene (*S*-locus Cysteine Rich, *SCR*) encodes specific ligands that are deposited onto the surface of the pollen grain by the anther tapetum cells, and the female gene (*S*-locus receptor kinase, *SRK*) encodes proteins deposited on the stigmatic surface. Pollen that carries ligands with the same specificity as proteins on the stigmatic surface are recognised and rejected by the stigma. The *SRK* and *SCR* genes are physically tightly linked at the *S*-locus and little or no recombination occurs between them (Charlesworth *et al.* 2000; Hatakeyama *et al.* 2001), which is necessary to maintain their paired specificity

(Awadalla & Charlesworth 1999; Hiscock & McInnis 2004). The *S*-locus is under balancing selection, which maintains many different specificities (commonly referred to as *S*-alleles) and can lead to the build up of a mutational load (Charlesworth 1988; Karkkainen *et al.* 1999; Uyenoyama 1988). This load is thought to make transitions from outcrossing to selfing difficult. The mechanism by which the SI reaction is induced is mostly unknown although some pathways with their modifiers are recognized.

1.1.5 *Causes and mechanisms of SI breakdown and the evolution of SC*

In terms of mechanistic possibilities for the breakdown of SI there are several possibilities: a) weak expression certain S-alleles, expression of modifier genes, composition pollen load (self pollen vs. mix pollen), environmental conditions like temperature, and internal stylar conditions like flower age (Levin 1996). These variations in SI expression are usually referred to as partial self incompatibility or leaky SI (Vogler *et al.* 1999). In a study on delayed self fertilization (Stephenson *et al.* 2000) old flowers show a mixture of outcross and self pollen where young flowers showed just outcross pollen. There were three selfing phenotypes distinguished: strong S-allele expression, weak S-allele expression, and a breakdown of the SI system. They found a heritable S-allele expression suggesting that SI is influenced by natural selection. Inbreeding depression was less for weakly than for strongly expressed S-alleles. The plasticity seems to combine a strategy that combines advantages of outcrossing and self fertilizing in a mixed mating system. A study on *Senecio squalidus* (Hiscock 2000) found reduced SI or partial SC (PSC), where SI is affected by modifiers unlinked to the S locus and a underlying gametophytic element influencing the SSI system which gives flexibility to the SI system resulting in PSC in the female part of the flower as the male component is unaffected. Partial SC could be an intermediate state between fully SI and SC allowing partial self fertilization when mating partners are limited for example.

Another possibility of breakdown of SI is hybridisation where modifier genes or recognition loci (epigenetic mechanisms) get affected and causes a breakdown in SI. (Nasrallah *et al.* 2007). When hybridisation takes place the resulting offspring would have

an advantage being self fertile as there will be no potential mating partners other than siblings. A study looking at hybrids between crucifer species suggested that hybridisation caused epigenetic changes resulting in changes of the S-locus genes expression. This caused a loss of SI in the stigmas of *Arabidopsis thaliana-lyrata* and *Capsella rubella-grandiflora* hybrids and their homoploid progenies. The abnormal expression of SRK gene transcripts in Arabidopsis and suppression of SCR in Capsella are reversible mechanisms that could produce self fertile hybrids.

1.1.6 ***Population History***

One of the possible conditions that allow a shift from outcrossing to inbreeding or mixed mating systems is a small population size, which can result in limited mating opportunities. For example, individuals with a homomorphic SI system are restricted to mating partners that are not closely related, which becomes problematic in a limited population size. During colonization of new habitats, where low densities of colonizers are expected, self-fertilizing individuals have an advantage over outcrossing individuals, as they are independent of a mating partner (Baker 1955). When new habitats become available, after changing climatical conditions for example, opportunities for populations to colonize these new areas arise. During ice ages, many species inhabiting higher latitudes were pushed back to lower latitudes due to lower temperatures and uninhabitable landscapes at the higher latitudes. During interglacial periods, species would colonize the available habitats in probably small groups of individuals from refugia at lower latitudes (Schmitt *et al.* 2006). Populations inhabiting these refugia would be allopatrically isolated from other refugia and would promote diversification between these populations, which would be still detectable in conserved genetic markers (Hewitt 1996). If a shift in mating system had occurred during postglacial expansion independently from each refugium, this would be evident in slowly evolving genetic markers.

In order to reveal population history, genetic markers with different characteristics can be applied to look at genetic diversity, heterozygosity, outcrossing history, and inter-population relationships like gene flow and maternal history (Avise 2000; Avise *et al.* 1987; Wright 1951). Fast evolving markers like microsatellites can reveal recent states of neutral diversity and heterozygosity and can be applied at both individual and population levels (Bowcock *et al.* 1994). Several microsatellite loci on different chromosomes can be

used to gain a genome wide estimate of neutral diversity (Ellegren 2000; Schlotterer 2000). By comparing these markers between populations it is possible to get an estimate of gene flow and population structure.

To reveal deeper phylogenies in plants, chloroplast DNA regions are generally used, which are slow evolving, non-recombinant, and uniparentally inherited markers (Avise *et al.* 1987; Birky 1995). Reconstructing colonization events using chloroplast DNA markers could elucidate population historical events like population expansion dynamics, gene flow, colonization routes and locality of refugia and hybrid zones (Avise 2000; Hewitt 1993; Hewitt 2004).

In order to reveal mechanisms that promoted shifts in mating system, cpDNA markers could be used to determine deeper phylogeny and microsatellite markers could give an indication of recent population genetic patterns. However, there are not many studies that have looked at mating system variation in a phylogeographic background and the conditions that allowed a shift from outcrossing to inbreeding.

1.1.7 *Pathogen susceptibility in relation to mating system variation*

Novel environments or stressful conditions impose novel evolutionary pressures on populations and can select different variants from a gene pool to survive to the next generation (Bijlsma *et al.* 1999). One such pressure could be a pathogen spreading through a population. A population that experienced a loss of genetic variation through inbreeding might not be able to adapt to these new circumstances and even if it could respond initially, it might not be able to keep up an arms race with an equally evolving pathogen (Parker 1991b).

Plants have general and specialized responses for detecting and acting on pathogens attacking them (Jones & Dangl 2006). The susceptibility of the host depends on the genotype of both the host and the pathogen (Decaestecker *et al.* 2007). In plants, the gene for gene (GFG) model describes the genetic interaction between host resistance and pathogen antigenic loci; each host resistance (R) gene relates specifically to a corresponding avirulence gene in the parasite (Flor 1955; Flor 1971; Thompson & Burdon

1992). Resistance genes of the host recognize corresponding avirulence factors released by the pathogen and trigger programmed cell death in the infected cells (Soanes & Talbot 2008). In this model, the virulence allele is 'universally virulent' as it can infect any host genotype (R and susceptible) whereas the avirulent genotype can only infect susceptible hosts. A particular characteristic of the model is the high fitness cost involving resistance (Laine & Tellier 2008). In the GFG model several genes in both host and parasite are involved in an arms race of adaptation. A non-adapted pathogen can adapt to become virulent in a host until the host becomes resistant, which makes the parasite avirulent. The original GFG model is supposed to be oversimplified as it chose to ignore polygenic resistance. A multigene model, where several resistance loci of the host are in an arms race with specific virulence loci in the pathogen, was proposed to explain intermediate stages of infection (Ellingboe 2001).The multigene model would also be more suitable to describe the occurrence of polymorphisms at several loci under pathogen selective pressure (Holub 2006). The matching alleles model (MAM) is used more in animal-parasite interactions to explain polymorphisms in genotypes related to immune systems (Little *et al.* 2006). The MAM assumes that parasite and the host match up at one specific recognition locus, which means that universal virulence does not exist in this model. It predicts that negative frequency dependent selection plays a role in maintaining polymorphisms in hosts and parasites at recognition loci without the cost of virulence (Laine & Tellier 2008). Within this model, either a stable state can be reached, where the parasite and the host have a long term maintenance of polymorphisms at both host and parasite loci, or an unstable state, where there is continuing fixation of one host and parasite allele resulting in high polymorphisms at these loci in a population (Holub 2001). Relocation of resources for immunity against a pathogen has a certain cost (Rolff & Siva-Jothy 2003) and can have significant effects on fitness traits like fecundity, life span (Yan *et al.* 1997), and reproductive success (Biere & Antonovics 1996). A study where a resistance locus, recognizing *Pseudomonas syringae*, was inserted in *A. thaliana* showed on average a 9% reduction in seed set in the absence of the pathogen compared to the control (Tian *et al.* 2003). The role of the host its mating system on resistance is not clear as not many studies have tested populations with varying outcrossing rates of a species against the rate of resistance either to evaluate inbreeding depression in a stressed environment or to detect whether an increase of homozygosity due to inbreeding affected pathogen resistance. Also, the role of purging in inbreeding populations is not clear as some studies found a higher resistance rate in inbreeding individuals compared to outcrossing individuals in populations with a mixed mating system (Koslow & Clay 2007) where others found that inbreeding individuals were showing more susceptibility to the pathogen (Carr *et al.* 2003).

1.1.8 *Floral consequences of mixed mating systems*

Outcrossing in plants involves transport of pollen from the paternal anthers to the maternal stigma. As plants are sessile they are in need of vectors, *i.e.* insects, to transport their pollen to conspecifics (Gross & Werner 1983). Reproductive parts, like anthers and stigma's, of outcrossing plants evolve in concert to optimize the relocation of pollen of the paternal anthers to the maternal stigma, with insects transferring the pollen on specific parts of their body (Cresswell 1999). Next to this development of reproductive parts, olfactory and visual cues, associated with certain rewards like nectar, have also evolved to attract pollinators (Thomson & Plowright 1980). A study on European *Arabidopsis lyrata* showed that experimental manipulation by hand pollinations decreased the number of flowers produced and decreased flowering time (Sandring *et al.* 2007). Insects usually detect flowers at large distances by ultraviolet patterns expressed on the petals of a plant, whereas volatile compounds, released from the flowers, are attractants at a closer range (Chittka & Raine 2006; Pichersky & Gershenzon 2002). In this context, outcrossing plants benefit from occurrence at high densities as this will attract pollinators from large distances as their mutual display is increased and mating partners are close (Lazaro *et al.* 2009; Sih & Baltus 1987). When plants get isolated, by colonization of new habitats for example, distances between conspecifics increase and compatible mating partners become scarcer. When outcrossing is not possible anymore, plants that can reproduce without a mating partner have a benefit over plants that are solely outcrossing (Pannell & Barrett 1998). Reproductive assurance in the form of inbreeding in to different degrees, most extremely autonomous self-fertilization, might become beneficial in circumstances of few mating partners or low pollinator densities (Kalisz & Vogler 2003). Despite inbreeding depression, offspring of inbred crosses might survive due to low interspecific competition. However, the developmental consequences for floral characters and pollinator attractants have not been tested in combination with each other.

1.1.9 *Case study*

A study on the aquatic flowering plant *Eichhornia paniculata* (Barrett *et al.* 1989) showed possible evolution from an outcrossing system with trimorphic hetermorphic SI system towards a self fertilizing monomorphic system. *E. paniculata* is an obligate outcrosser and

pollinated by co-evolved long tongue pollinators. The species occurs in the North West of Brazil and Jamaica where vast lake areas are its main habitat. There are three genetically determined morphs distinguished: in all morphs one anther is always longer than another but in the L morph, the style is longer than both anthers; in the M morph the style is intermediate to the anthers; and in the S morph the style is shorter than the shortest anther. Populations can have a combination of all three morphs (tristylous), the L and M morph (distylous), or only the M morph (monostylous). The tristylous system is mostly associated with an outcrossing mating system where the monomorphic system with a selfing mating system. The S morph was associated with a lower fertility, which explained its rarity in the tri- and distylous populations. Despite its lower fertility, the S morph was fixed in a number of populations in Brazil and all populations in Jamaica, which was explained by founder effects and evolutionary pressure towards self fertilization as mating assurance in the absence of pollinators.

1.2 Model system

The species used in this study, *Arabidopsis lyrata*, occurs in the Northern hemisphere in temperate regions (Jalas & Suominen 1994). It is closely related to *Arabidopsis thaliana* (Koch *et al.* 2000) and occurs like its sister species in a wide range of temperatures but *A. lyrata* is more distributed in isolated patches (Leinonen *et al.* 2009). It is a diploid perennial that occurs in colder climates in Europe but is a successional species of post-glacial dune landscapes in North America (Spence 1959). The North American subspecies *A. lyrata lyrata* shows reduced genetic variation detected by chloroplast DNA and nuclear ribosomal sequences compared to European populations (Schmickl *et al.* 2008a). It is mostly outcrossing in Europe whereas around the Great Lakes of North America, populations have been found that show highly outcrossing, highly inbreeding or mixed mating systems (Mable & Adam 2007; Mable *et al.* 2005). North American *A. lyrata* has a sporophytic self-incompatibility system which apparently has become defective in self-compatible individuals from inbreeding and mixed mating populations (Mable *et al.* 2005). Based on microsatellite markers and controlled greenhouse pollinations, predominantly selfing populations showed significantly lower outcrossing rates based on progeny arrays, genetic diversity, and observed heterozygosity than outcrossing populations (Mable & Adam 2007; Mable *et al.* 2005). This makes North American *A. lyrata* an excellent model system to test hypotheses concerning the evolution and consequences of mixed mating systems.

1.3 Objectives and Chapter Outlines

1.3.1 *Chapter 2: Population history*

A possible scenario for the difference in outcrossing rates between the populations of *A. lyrata* around the Great Lakes could be a different population history. After the last glacial maximum (~18,000 yrs ago) the ice sheet that covered northern parts of North America withdrew and species from southern refugia colonized the novel northern habitats available. *A. lyrata* has a physiological mechanism that recognizes self pollen by certain proteins in the pollen coat. Pollen will be prevented from growing a pollen tube if one of the self-incompatibility genes is shared with the fertilized plant. Loss of self-incompatibility could be due to bottleneck effects in which a major loss of S-allelic diversity enabled the selfing individuals to maintain themselves whereas the outcrossing ones were unable to find compatible mating partners. The lake habitats are still continuously changing and *A. lyrata* is an early successional species. Self-compatible individuals could have thrived in harsh but open habitats without the necessity of finding mating partners. The populations of *A. lyrata* around the Great Lakes show a geographical distribution that relates to their predominant mating system. The more southern populations are mostly inbreeding and the more Northern populations are mostly outcrossing. In order to determine whether population history was responsible for the loss of SI and if this loss happened multiple times I was interested in looking at the history of the populations around the Great Lakes by means of a slowly evolving uniparental inherited genetic markers. Using a chloroplast DNA marker I set out to determine a relation between post-glacial expansion and the loss of SI. If there had been more than one refugium from where this species colonized the post-glacial landscape this could be reflected in the cpDNA haplotype pattern. The predominantly inbreeding populations could be related to a common refugium, which would indicate a single loss of SI. Similarly, if the predominantly outcrossing populations shared a cpDNA pattern that differed from the inbreeding populations that would indicate that they had a different population history and support a single loss event. The reasons and the timing for the loss of SI could be detected by microsatellite analyses where diversity, private alleles and indications of gene flow between populations would give indications of possible bottlenecks or isolation.

1.3.2 *Chapter 3: Inbreeding depression*

In reproductive strategies in most organisms, outcrossing is favored over inbreeding in terms of fitness costs. Overdominance, the masking of recessive deleterious mutations by dominant non-deleterious ones, and heterozygote advantage, in which heterozygous individuals are supposed to be fitter than homozygotes, are two theories that explain this difference in fitness. This raises the question of how selfing individuals maintain themselves within populations consisting of outcrossing and inbreeding individuals. *Arabidopsis lyrata* is a small plant that occurs on the Northern hemisphere. It is mainly outcrossing due to a genetically determined self-incompatibility system. Around the Great Lakes of North America, populations of *Arabidopsis lyrata* exist which differ in the ratio of individuals capable of setting selfed seed and which differ in their realized outcrossing rates. I was using this system to test whether there are fitness differences between (forced) selfed and outcrossed offspring from parents from predominantly selfing compared to predominantly outcrossing populations and if populations of *A. lyrata* around the Great Lakes have experienced different (recent) histories.If an absence of inbreeding depression in individuals with a defective SI system were detected this could be due to purging of exposed deleterious mutations. Levels of outcrossing history present in each population varied from completely inbreeding to completely outcrossing with inbetween mixed mating system populations. To assess inbreeding depression at an individual level, I compared the fitness differences of outcrossed and inbred progeny of mothers with different selfing phenotypes and originating from populations with different outcrossing histories. In this way I would correct for any maternal effects and would be able to compare different maternal lines across populations. I tested the effect, using a regression analysis, of outcrossing history on relative fitness at the individual level, by using the mothers' selfing phenotype and average observed heterozygosity across loci within individuals, and at the population level, by average observed heterozygosity across individuals within populations.

1.3.3 *Chapter 4: Pathogen susceptibility*

Purging could obscure the detection of inbreeding depression when life history traits are assessed in greenhouse conditions where environmental stress could reveal inbreeding depression. Inbred populations show a higher homozygosity than outcrossing populations,

which could also influence their vulnerability to pathogens. Variation in outcrossing history could explain the capability to adapt to novel conditions like the presence of a pathogen. I infected individuals from populations with different outcrossing histories with the pathogen *Albugo candida*. If outcrossing history played a role in the response to stressful conditions I would expect inbreeding populations to suffer more from infection than the outcrossing populations.

1.3.4 ***Chapter 5: Pollinator attraction***

Outcrossing and facilitated self-fertilizing plants are in need of pollinators to fertilize their ovules where autonomous self-fertilizing plants are independent of pollinators. Flower morphology traits like petals, pistils, and stamens are expected to co-evolve with each other according to the presence of a pollinator and whether or not they are self –fertilizing or outcrossing. In order to detect the consequences of variation of mating systems, flower morphology traits like pistil and stamen lengths were measured. Also pollinator attraction traits like petal lengths and the amount and composition of volatiles were measured. I would expect that outcrossing plants would put more energy in the production of volatiles to attract pollinators. Also the length of the pistil in respect to the stamens is probably different between the two strategies, as outcrossing plants should show more adaptation towards effective pollen transfer to other plant stigmas through pollinators. Finally, I expect petal length, which functions as a pollinator attractor, to be different between the two mating systems. In an ideal world I would have measured both the volatile secretion and the effect it had on visiting insects, which I unfortunately did not manage to perform in full. Because of bad summers and other circumstances I only managed to measure volatile secretion in a small number of individuals but not the effect they might have had on the pollinators in terms of attraction in the field. This chapter is thus more of a pilot study than a complete data chapter but it could be used to motivate a follow up study on the same topic.

1.3.5 ***Overall conclusions***

In the final chapter I will summarize all the main results and conclusions on the evolutionary consequences of mixed mating system in *Arabidopsis lyrata* subsp. *lyrata*. I will combine all main results per chapter and discuss these in an attempt to draw a

complete picture of all the different approaches towards the causes and consequences of mixed mating systems in this species. I will discuss unanswered questions and possible future directions for follow up research. Finally I will discuss broader implications of this research.

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2 Population history in relation to the loss of SI

2.1 Introduction

Hermaphrodites inherently have the potential to self-fertilize. However, to prevent potentially negative consequences of inbreeding, they often have mechanisms in place to prevent it. Many plants have a genetically controlled self-incompatibility system, in which self-fertilization is avoided by a physiological response (Takayama & Isogai 2005). The *S*-locus is under balancing selection, which maintains many different specificities (commonly referred to as *S*-alleles as they underlie different phenotypes contrasting haplotypes) and can lead to the build up of a mutational load (Charlesworth 1988; Karkkainen *et al.* 1999; Uyenoyama 1988). This load is thought to make transitions from outcrossing to selfing difficult.

In natural plant populations there is often a trade-off between outcrossing and self-fertilization, and outcrossing rates can differ even within populations of a single species (Takebayashi & Morrell 2001). Demographic factors and population history can influence which strategy is most advantageous (Maynard Smith 1978), depending on whether reproductive assurance or maintaining genetic diversity are more important. For example, in a changing environment, loss of genetic diversity would reduce adaptive potential (Charlesworth 1976; Van Valen 1977) and outcrossed individuals with greater variation and higher rates of effective recombination would be selectively favoured (Busch *et al.* 2004). On the other hand, in isolated populations with mate limitation, reproductive assurance is more important than adaptive potential, and individuals that can self-fertilize would be selectively favoured (Amos & Balmford 2001; Grindeland 2008; Richards *et al.* 2003).

Arabidopsis lyrata has become a focus for the study of mating system evolution due to its close relatedness to *A. thaliana* and good understanding of its sporophytic self-incompatibility system (Charlesworth *et al.* 2000; Charlesworth *et al.* 2003; Ross-Ibarra *et al.* 2008; Schierup *et al.* 2001). *A. thaliana* is highly selfing annual (Abbott & Gomes 1989) and is thought to have lost its self-incompatibility system independently multiple times and with different genetic bases (Charlesworth & Vekemans 2005; Kusaba *et al.* 2001; Nasrallah *et al.* 2004; Shimizu *et al.* 2008). *A. lyrata,* on the other hand, is considered a predominantly outcrossing perennial, with high inbreeding depression found in enforced selfings in European populations (Karkkainen *et al.* 1999). In contrast to European populations of *A. lyrata* (ssp *petraea*), North-American populations (ssp *lyrata*) have been found that have experienced a breakdown of self-incompatibility, as well as a

shift to inbreeding at a population level (Mable & Adam 2007; Mable *et al.* 2005). Based on microsatellite markers, populations showing a large proportion of individuals capable of setting selfed seed showed significantly lower outcrossing rates, genetic diversity and observed heterozygosity than those where most individuals retained a strong SI system. This led to the hypothesis that the loss of self-incompatibility could be related to postglacial expansion. Glaciation periods have had an enormous impact on current population structure in postglacial landscapes. At least some *A. lyrata* populations inhabiting present-day Europe and eastern North America survived the last glacial maximum (21,000-18,000 BP) in non-glaciated refugia (Hewitt 1996; Hewitt 2004; Soltis *et al.* 2006), with subsequent expansion into new habitats as the ice receded. Bottleneck effects in small founding populations can cause depletion of genetic diversity and increase the influence of genetic drift (Keller & Taylor 2008; Nei *et al.* 1975). In plants with genetic self-incompatibility systems, this could lead to a reduction in the number of *S*-alleles present compared to the central population and, as a consequence, fewer compatible mating partners (Vekemans *et al.* 1998). Additional mate limitation may be caused by low plant densities that attract less pollinators (Levin & Kerster 1969). Therefore, it is predicted that small marginal populations (for example after postglacial colonization) are more likely to evolve self-fertilization than central populations, because reproductive assurance could outweigh the potentially negative effects of inbreeding (Baker 1955; Pannell & Barrett 1998). Selection for self-fertilization as a way of reproductive assurance might therefore be expected during periods of postglacial expansion (Baker 1966; Busch & Schoen 2008; Pannell & Barrett 1998).

In Europe, lack of a north-south gradient in diversity among populations of *A. lyrata* ssp. *petraea* and high within-population diversity throughout Central Europe has been interpreted as evidence that some populations survived north of the ice sheets in the alps (Clauss & Mitchell-Olds 2006). Populations of North American *A. lyrata* ssp *lyrata* have not been studied as extensively in a phylogeographic context, but it has been hypothesized that colonization of glaciated areas was more recent than in Europe (Koch & Matschinger 2007; Wright *et al.* 2003). During the last glacial maximum (21,000-18,000 BP), parts of North America were covered by the Laurentian ice sheet, which covered the Great Lakes area and extended as far south as the 40°N line of latitude (Lewis *et al.* 2008). There is increasing evidence for the importance of southern Appalachian refugia and refugia close to the Laurentian ice sheet for colonization of the Great Lakes region in both plants and animals (Soltis *et al.* 2006). For example, mitochondrial DNA sequences suggested the presence of three distinctive lineages in the common garter snake (*Thamnophis sirtalis*),

which the authors concluded could reflect three separate postglacial invasions to Ontario (Placyk *et al.* 2007). Similarly, a study on spring peepers (*Pseudacris crucifer*) suggested three postglacial invasions into the region north of the Great Lakes (Austin *et al.* 2002). As reptiles and amphibians are bound to specific habitats that allow only certain migration routes (restricted mobility), they could be compared to plants in this context. Phylogeographic studies on jack pine (*Pinus banksiana*) using cpDNA sequences (Godbout *et al.* 2005) and the small herb white trillium (*Trillium grandiflorum*) based on RFLP of chloroplast DNA and allozyme diversity (Griffin & Barrett 2004) both suggested multiple colonization routes to the north of the Great Lakes.

Previous studies on *A. lyrata lyrata* established a spatial pattern of predominantly outcrossing populations in the west and predominantly selfing populations in the east (Mable & Adam 2007; Mable *et al.* 2005; Mable *et al.* 2003). This could be explained by two independent postglacial invasions of this area. To test this hypothesis I assessed chloroplast haplotype variation in relation to mating system and nuclear microsatellite variation. Specifically, I addressed the following questions: 1) Was self-incompatibility in *A. lyrata lyrata* lost once or multiple times and could the loss be related to post glacial expansion after the last glacial maximum? 2) Does the pattern of re-colonization of *A. lyrata lyrata* after the last glacial maximum match postglacial expansion patterns from previous phylogeographic studies on other organisms? 3) What is the effect of mating system transition on microsatellite-based genetic diversity and observed heterozygosity?

2.2 Materials & Methods

2.2.1 Sampling

In 2003, seven sand dune populations of *Arabidopsis lyrata* were sampled in protected park areas on the Ontario side of the Great Lakes of North America: Lake Superior Provincial Park (LSP) on Lake Superior, Pinery Provincial Park (PIN) on Lake Huron, Manitoulin Island (MAN) in Georgian Bay (between Lakes Huron and Superior), Tobermory Singing Sands (TSS) in Bruce Peninsula National Park (BPNP) on Lake Huron, Long Point Provincial (LPT) on Lake Erie, Point Pelee National Park (PTP) on Lake Erie, and Rondeau Provincial Park (RON) on Lake Erie (Figure 2.1). All of these

populations had been studied before (Mable & Adam 2007; Mable *et al.* 2005), but I also included two new populations which were suspected to demonstrate a mixed mating system based on preliminary self-pollination experiments: 1) Old Woman's Bay (OWB), which was located several km from the LSP population and 2) a population growing on limestone pavements (alvars) (TSSA), which was located 1 km from the TSS population. I collected seeds from up to 30 independent focal plants per population. These were used to raise individuals for assessment of variation in strength of SI in relation to cpDNA variation.

In order to assess chloroplast DNA variation across the range that had been previously studied, I analysed DNA of samples from four additional populations (TC: n=6; PUK: n=10; IND: n=6) that had been used in former studies (Mable & Adam 2007; Mable *et al.* 2005). For preliminary assessment of cpDNA haplotype variation to the south and west of Lake Michigan, I extracted DNA from herbarium samples, two from Jo Davies (JDV, collected from a sand area south of Blanding, Illinois, INHS number 40772, year: 1949), and two from Lake (LKE, collected from a sand prairie habitat at Illinois Beach State Park, INHS number 98313, year: 1961). The herbarium samples were generously made available by the Illinois Natural History Survey (INHS). These "extra" populations were not included in the microsatellite-based analyses of population structure or heterozygosity estimates. Although mating system 'status' could theoretically differ from year to year, I did not find any significant deviation between population 'statuses' of different years.

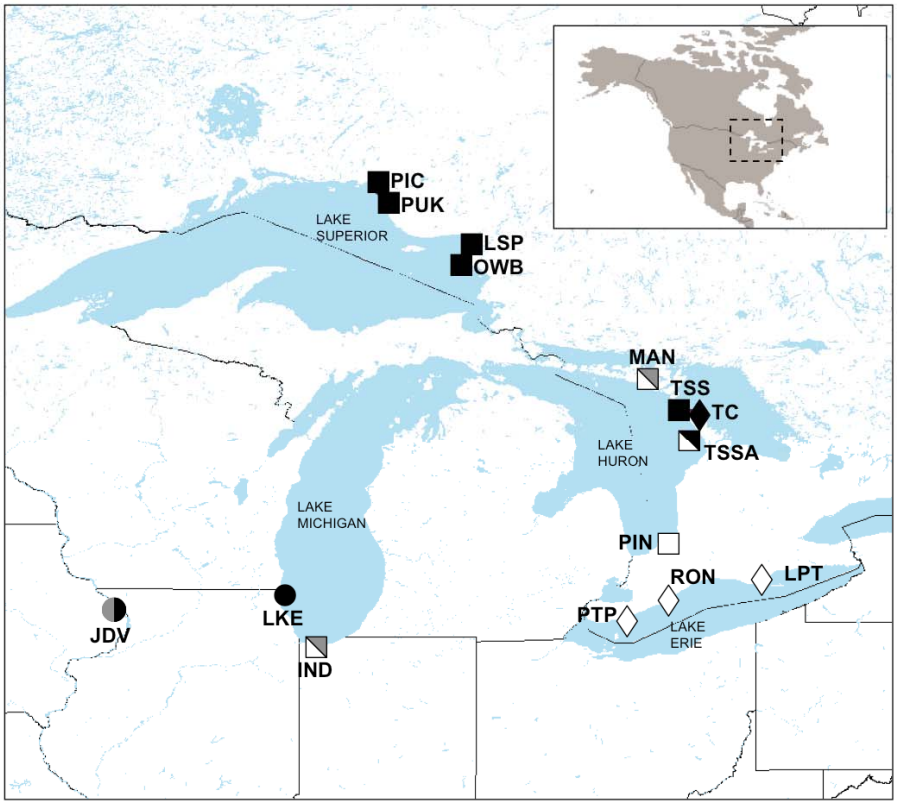


Figure 2.1. Distribution of three different chloroplast (cp) DNA haplotypes (L1, L2, S1) detected in individuals from populations around the Great Lakes area in North America. Population shapes indicate the mating system based on microsatellite outcrossing rates (*Tm*), where squares are predominantly outcrossing populations (*Tm* >0.60), diamonds are predominantly inbreeding populations (*Tm* <0.60) and circles are populations that were not tested for mating system. Population names are indicated by abbreviations of their area of origin (see text). Black shapes indicate the short cpDNA haplotype (S1), white indicates the more predominant long haplotype (L1) and grey indicates the second more rare long haplotype (L2). L1 and L2 differed from each other at two positions by base pair substitutions.

2.2.2 Selfing phenotype determination

Ten seeds from 25 different mothers per population were germinated in Levington S2 + Sand mix (Scotts Professional, Ipswich) in controlled climatic incubators under a regime of 16 hour light (20ºC) and 8 hour dark (16ºC) with 80% relative humidity. Two weeks after germination, plants were moved to the Scottish Crop Research Institute, Invergowrie and further grown in a common greenhouse environment under a 16 hour light (20ºC): 8 hour dark (16ºC) regime. A single plant per maternal seed family was tested for strength of self-incompatibility (SI) by manual self-pollination of six flowers to classify its selfing phenotype. To compare the results with former studies (Mable et al, 2005; Mable & Adam, 2007), I used the same methods to determine selfing phenotype. Fruits were scored as either negative (no seeds), small (short fruits with no more than 3 seeds) or positive (a fruit with more than three seeds). For each plant, selfing phenotype was classified according to the following scheme: 1) self-incompatible (SI) = individuals producing zero or one (out of six) positive selfed fruits; 2) self-compatible (SC) = individuals producing five or six (out of six) positive fruits; and 3) partially self-compatible (PC) = individuals producing two, three or four out of six positive selfed fruits. The proportion of plants belonging to each selfing phenotype was compared to microsatellite-based outcrossing rates previously estimated from progeny arrays for the seven populations also used in Mable & Adam (2007). Six to ten seeds from 25 mothers per population were raised from the OWB and TSSA populations to establish outcrossing rates using microsatellite markers on progeny arrays (described below).

2.2.3 DNA sequencing

From each plant used in the study DNA was extracted from 100 mg of silica-dried tissue using the FastDNA kit, using the manufacturer's instructions (QBiogene 101, MP Biomedicals), but with an additional 10 sec pulverization of dried leaf tissue in the FASTPREP instrument prior to adding buffers. The noncoding cpDNA region *trn*L(UAA)3'exon-TrnF(GAA) was amplified by PCR with primers E 5'-GGTTCAAGTCCCTCTATCCC-3' and F 5'-ATTTGAACTGGTGACACGAG-3' from Taberlet *et al* (Taberlet *et al.* 1991). DNA was amplified in a 20 µl PCR by adding 15 mM PCR buffer (10x), 25 mM MgCl, 1 mM dNTP, 10 µM forward primer (*trn*L), 10 µM reverse primer (*trn*F), 5 U/µl TAQ polymerase, and 1 µl DNA. The PCR temperature

cycles were: 3 minutes at 94˚C followed by 35 cycles of 40 seconds at 94˚C, 1 min at 56˚C, and 1 min at 72˚C, followed by a final extension of 3 min at 72˚C and a hold at 4˚C. A negative control was included for all PCR runs. The PCR products were visualized on 2% TBE agarose gels. Bands to be sequenced were excised and subsequently purified using QiaQuick gel extraction kits (Qiagen Inc.). Purified PCR products were directly sequenced on an ABI 3730 sequencer (by The Sequencing Service, University of Dundee). Sequences were aligned, visually checked for false base assignment using Sequencher 4.7 (Gene Codes) and sorted into haplotypes. A BLAST search on GenBank was performed to confirm amplification of the *trn*L(UAA)3'exon-TrnF(GAA) region by comparing the sequences to other submitted *A. lyrata* sequences from the same cpDNA region.

2.2.4 Microsatellite genotyping

Nine microsatellite loci previously used by Mable & Adam (2007) were screened for variation: ADH-1, AthZFPG, ATTS0392, F20D22, ICE12, ICE9 (Clauss *et al* 2002), LYR104, LYR133, LYR417 (obtained from V. Castric and X. Vekemans, personal communication). Products were amplified by multiplex PCR, using the default reagent concentrations recommended by the kit instruction manual (QIAGEN Inc, exact primer concentrations can be requested from the authors). Thermocycling was performed on PTC-200 (MJ research) machines using the following programme: initial denaturation at 95°C for 15 min followed by 34 cycles of 94° for 30 s, 55°C for 90 s (ramp to 72°C at 0.7°C/s) and a final 72°C extension for 10 min. Multiplex products (1:160 dilutions) were genotyped using an ABI 3730 sequencer (by The Sequencing Service, University of Dundee). Genotypes were analysed using GENEMAPPER 4.0 (Applied Biosystems) and corrected manually. Multi-locus genotypes were obtained for two purposes: 1) to estimate genetic diversity and population structure based on population samples (25 individuals per population), for which I used individuals tested for strength of self-incompatibility; and 2) for the two populations not used in previous studies (OWB and TSSA), to determine realized outcrossing rates based on progeny arrays, using 6-10 siblings of the individuals tested for strength of self-incompatibility (as described in Mable & Adam 2007).

2.2.5 Outcrossing rates

I calculated multi-locus outcrossing rates (T_m) for populations TSSA and OWB using MLTR version 2.3 (Ritland 2002), which implements the mixed-mating model described by Ritland & Jain (Ritland & Jain 1981). Using a maximum likelihood approach, this program calculates single (T_s) and multi-locus (T_m) outcrossing rates and predicted allele frequencies in pollen (p) and ovules (o). Standard errors were calculated by bootstrapping across progeny arrays using 1,000 replicates. Values of the starting parameters were: 0.1 increasing in steps of 0.1 to a maximum of 0.9 for t; unconstrained pollen and ovule gene frequencies; all the other parameters were set to default values. Results with the lowest standard error and highest likelihoods are reported.

2.2.6 Population genetic analyses

The program microsatellite analyser (MSA) (Dieringer & Schlotterer 2003) was used to compute population allele frequencies at individual microsatellite loci, as well as observed and expected heterozygosity (H_o and H_e). The average number of alleles per locus (N_a) and the number of private alleles (N_p) were calculated from the allele frequency data. I used a one-way ANOVA with a Tukey-Kramer post hoc test, as implemented in JMP (version 5 SAS business), to test for differences in H_o between selfing phenotypes (SI, PC, SC) and a 2-tailed t-test (implemented in R for OS Mac statistical computing) to test for differences in N_a, and N_p between mating systems (predominantly inbreeding or outcrossing). The program CREATE (Coombs *et al.* 2008) was used to create input files for HP-RARE, ARLEQUIN, and STRUCTURE. HP-Rare (Kalinowski 2005) was used to calculate allelic richness and private allelic richness to estimate genetic variation using rarefaction to correct for sample size (Kalinowski 2004). Since the microsatellites that I used have imperfect repeats, which makes them inappropriate for models of stepwise mutation (Muller *et al.* 2008; Slatkin 1995), I performed AMOVA based on pairwise *Fst* (Slatkin 1995) as implemented in ARLEQUIN 3.11 (Excoffier *et al.* 2005) to evaluate variation with respect to different scenarios of population groupings: 1) geographic location (lakefront of origin), 2) population mating system classification (predominantly outcrossing or predominantly inbreeding based on T_m), and 3) cpDNA haplotypes. Also, partial Mantel tests as implemented in ARLEQUIN 3.11 were performed to test for isolation by distance; i.e., to test for a correlation between geographic (km) and genetic distances (1/(1-*Fst*)).

Finally, I used a Bayesian clustering algorithm implemented in STRUCTURE 2.1 (Pritchard et al 2000) to analyze population and individual clustering; i.e., to infer the number of unique clusters (*K*) explained by the data. This method uses a multi-locus genotype to probabilistically assign individuals to one or more (if populations are admixed) clusters. I ran 10 simulations per prior *K* (*K*=1 to *K*=10); a burn-in period of 1,000; number of MCMC replicates after burn-in = 100,000; and used the admixture model with default settings. I plotted *K* vs. the likelihoods obtained over all simulations and inferred *K* as recommended by the STRUCTURE manual. I also tried an alternative method, that calculates the first order derivative of the distribution of *–Ln* probability (*P*) of the data (*d*) (*–LnP*(*d*) (*L*'(*K*))), the second order derivative (*L*''(*K*)), and the second order derivative divided by the standard deviation (*dK* (*L*''(*K*)/*stdev*)) to detect the optimal number of clusters (Evanno *et al.* 2005).

2.2.7 Individual effects of selfing phenotypes

I calculated individual observed heterozygosities manually by computing the fraction of heterozygous loci over all loci per individual based on MSA output (Dieringer & Schlotterer 2003). In a one-way ANOVA with selfing phenotype and population as fixed factors, I tested the effect of selfing phenotype (SI, PC, SC) on individual heterozygosity, both overall and for the two predominant cpDNA haplotypes (L1 and S1) separately.

2.3 Results

2.3.1 Selfing phenotypes and outcrossing rates

Based on self-pollinations, most populations were mixed in the sense that both self-incompatible (SI) or partially self-compatible (PC) and self-compatible (SC) plants were found (Table 2.1). It is unclear whether PC individuals represent individuals with a functional self-incompatibility system that occasionally fails, or individuals that are self-compatible with pollen that has a suboptimal fertility. I found two populations that contained no self-compatible individuals (PIN, TSS) and two populations that contained no self-incompatible individuals (PTP, RON); all other populations contained a certain amount of individuals in all three selfing phenotype classes. The outcrossing rates based on

multi-locus microsatellite-based progeny arrays for TSSA (T_m= 0.414±0.09; T_s = 0.342±0.069) and OWB (T_m = 0.644±0.087; T_s= 0.461±0.081) were intermediate to the outcrossing rates for the other populations (Mable and Adam 2007; Table 2.1). The relative proportions of the selfing phenotypes within a population corresponded to the population outcrossing rates relatively well (i.e., populations with a relatively large proportion of SI individuals had high outcrossing rates, populations with relatively large proportions of SC individuals had lower outcrossing rates). I classified populations based on outcrossing rates. For simplicity, I assigned OWB (T_m > 0.6) to the outcrossing group, and TSSA (T_m < 0.6) to the inbreeding group in further analyses. Average outcrossing rates of outcrossing and inbreeding populations were 0.84 and 0.24, respectively.

Table 2.1. Sampled populations (ordered by increasing outcrossing rate (T_m)), geographic coordinates, selfing phenotypes, multi-locus outcrossing rate (T_m), chloroplast DNA (cpDNA) electrophoretic screening of fragment lengths, and haplotype assignment based on cpDNA sequencing. Values in bold were taken from Mable & Adam (2007).

Population	Coordinates		Selfing phenotype[1]					cpDNA electrophoretic screening		cpDNA sequencing	
	Latitude	Longitude	Number of plants tested	Proportion of plants SI	PC	SC	T_m[2]	Number of plants screened	Fragment length[3] (bp)	Number of plants sequenced	Haplotype assignment[4]
PTP	41˚55'	-82˚30'	23	0	0	1	**0.02**	15	741	3	L1
TC	45˚14'	-81˚30'	**32**	**0.34**	**0.38**	**0.28**	**0.19**	12	515	6	S1
RON	42˚15'	-81˚50'	21	0	0.1	0.9	**0.25**	15	741	4	L1
LPT	42˚34'	-80˚23'	23	0.17	0.17	0.65	**0.29**	18	741	3	L1
TSSA	45˚11'	-81˚34'	12	0.42	0.42	0.16	0.41	17	515 (13), 741 (4)	13	S1 (9) , L1 (4)
OWB	47˚47'	-84˚53'	18	0.61	0.17	0.22	0.64	22	515	19	S1
TSS	45˚11'	-81˚35'	11	0.73	0.27	0	**0.84**	15	515	3	S1
MAN	45˚39'	-82˚15'	28	0.79	0.14	0.07	**0.88**	26	741	24	L1 (1) , L2 (23)
LSP	47˚34'	-84˚58'	16	0.81	0	0.19	**0.88**	20	515	16	S1
IND	42˚37'	-87˚12'	**72**	**0.76**	**0.17**	**0.07**	**0.95**	12	741	6	L1 (3) , L2 (3)
PIN	43˚16'	-81˚49'	25	0.84	0.16	0	**0.96**	10	741	3	L1
PUK	48˚23'	-86˚11'	**16**	**0.81**	**0.13**	**0.06**	**0.98**	18	515	8	S1
PIC	48˚60'	-86˚30'	Insufficient flowering to test				-	3	515	3	S1

[1] SI: self-incompatible (0 or 1 positive self fruits); PC: partially self –compatible (2, 3 or 4 positive self fruits); SC: self-compatible (5 or 6 positive self fruits).

[2] Outcrossing rates based on microsatellites using MLTR (Ritland & Jain (1981)).

[3] For populations with more than one cpDNA length variation, values in parentheses indicate the number of individuals with each length variation.

[4] L1: long fragment (741 bp); L2: long fragment (741 bp) differing from L1 at 2 bp positions (substitutions) in the sequence; S1: short fragment (515 bp) identical to L2 but for a 226 bp insert. For populations with more than one cpDNA haplotype, values in parentheses indicate the number of individuals with each haplotype.

2.3.2 cpDNA variation and correspondence with selfing phenotype

Amplification of the cpDNA *trn*F(GAA) region yielded a short (515 base pairs) and a long fragment (741 bases pairs). Three populations (LSP, OWB, and TSS) were fixed for the short fragment, and five populations (PIN, MAN, LPT, RON, and PTP) were fixed for the long fragment. One population (TSSA) included individuals with both short and long fragments (Table 2.1). For the additional populations screened, PUK (n=10) and TC (n=6) were fixed for the short fragment, while IND (n=6) was fixed for the long fragment. The two herbarium specimens from Lake (LKE) had the short fragment, while the JDV population had one sample with the long fragment, and one with the short.

Sequencing of the fragments indicated that there was no variation in the first region of the chloroplast *trn*L(UAA)-*trn*F(GAA) spacer (base pair position 1-180, following (Ansell *et al.* 2007; Koch *et al.* 2005)). Using variation across the entire cpDNA *trn*F(GAA) region sequenced, which includes a region with pseudogene copies of the *trn* gene (Ansell *et al.* 2007; Koch *et al.* 2005), I identified three distinctive cpDNA haplotypes. The first corresponded to the short fragment (515 bp) and will be referred to as haplotype S1. It was mainly found in the most northerly distributed populations (LSP, OWB, PIC, PUK, TSS, TC as well as in 9 out of the 13 individuals from TSSA), in both herbarium samples from LKE, and in one of the herbarium samples from JDV (Figure 2.1). The second and third haplotypes corresponded to the long fragment (741 bp) and will be referred to as haplotypes L1 and L2. They differed from each other in the pseudogene region at positions 252 (base pair substitution: T (L1) to G (L2)) and 425 (base pair substitution: A (L1) to G (L2)) (from the start of my alignment). Haplotype L2 was identical to the short haplotype (S1) except for a 226 bp deletion in the latter, and was found in the MAN population (in 23 out of 24 individuals), the IND population (in 3 out of 6 individuals) and in one of the two herbarium samples from JDV. Haplotype L1 was found in the more southerly distributed populations (PTP, RON, LPT, PIN, IND), as well as some of the northern populations (1 out of 24 individuals from MAN, 4 out of 13 individuals from TSSA; Figure 2.1).
The loss of self-incompatibility, i.e., the presence of SC individuals, was associated with all three haplotypes. Within the L1 haplotype, populations existed where the majority of individuals was SC, whereas in the S1 lineage SC individuals were present in several populations, but always at relatively low frequency (Table 2.1). The L2 haplotype was only found in two populations (IND and MAN) and was only associated with one SC individual in the MAN population.

2.3.3 Microsatellite variation and population structure

Our data showed clustering of homozygous null alleles (> three individuals where alleles did not amplify for one locus within a population) at three loci for five populations. Locus AtHZ often failed to amplify in populations OWB and LSP, in which reactions failed for 15 out of 20 and 13 out of 18 individuals, respectively. Locus LYR417 often failed in populations PTP, RON, and LPT (8 out of 18, 9 out of 16, 14 out of 27, respectively). Locus ICE9 often failed in LPT (10 out of 27). Loci with null alleles were excluded from subsequent analyses. Measures of allelic diversity are summarised in Table 2.2 and were calculated only for the primary populations used in this study (i.e. excluding IND, PIC, PUK, TC that were screened for cpDNA variation). The rarefacted allelic richness was significantly higher for predominantly outcrossing populations (1.82) compared to predominantly inbreeding populations (1.36) (two-tailed t-test, t=3.28, p=0.002). Populations LSP and OWB (classified as outcrossing) showed a relatively low rarefacted allelic richness, whereas TSSA (classified as inbreeding) had a relatively high allelic richness (Table 2). In all populations observed heterozygosity (H_o) was lower than expected (H_e) based on Hardy-Weinberg equilibrium (Table 2.2).

There was significant pairwise genetic distance (*Fst*) between all populations (Table 2.3). With cpDNA haplotype (S1, L1, L2) as the grouping variable, a locus by locus AMOVA based on *Fst* showed that 12% of the variation was contained among groups, 53% among populations within groups, and 34% within populations. With lake of origin (Lake Huron, Lake Erie, Lake Superior) as the grouping variable, 15% of the variation was contained among groups, 76% among populations within groups, and 9% within populations. With mating system (outcrossing or inbreeding, based on T_m) as the grouping variable, 13% of the variation was contained among groups, 51% among populations within groups, and 36% within populations. Mantel partial correlation between geographic and genetic distances (based on *Fst*/1-*Fst*) showed no significant isolation by distance (r^2 = 0.059, p=0.154). This was also true after splitting populations into two groups based on cpDNA haplotype (S1 versus L1/L2), mating system (outcrossing versus inbreeding based on T_m), or predominant selfing phenotype (SI, PC, SC).

Table **2.2 Sampled populations (ordered by increasing T_m (see Table 2.1)), total number of private alleles (N_p), total number of alleles (N_a), rarefacted (private (RN_p)) allelic richness (RN_a) to correct for sample size, observed (H_o) and expected (H_e) heterozygosity, with averages indicated for predominantly outcrossing and inbreeding populations.**

Population	N_p	N_a	RN_p[3]	RN_a[4]	H_o	H_e
PTP	1	10	0.03	1.2	0.03	0.08
LPT	1	11	0.14	1.3	0.02	0.10
RON	0	8	0	1.1	0.04	0.06
TSSA	1	12	0.05	1.6	0.11	0.15
OWB	1	11	0.05	1.5	0.18	0.25
PIN	1	11	0.03	1.7	0.23	0.26
MAN	5	13	0.17	1.6	0.23	0.31
TSS	1	16	0.10	2.0	0.24	0.37
LSP	2	10	0.11	1.3	0.08	0.11
Inbreeding[1]	3	10	0.06	1.3	0.05	0.10
Outcrossing[2]	10	12	0.09	1.6	0.19	0.26

[1]Populations with an outcrossing rate $Tm < 0.60$: RON, PTP, LPT, TSSA (see Table 2.1)

[2]Populations with an outcrossing rate $Tm \geq 0.60$: LSP, OWB, PIN, MAN, TSS (see Table 2.1)

[3,4]Rarefacted (private)[3] allelic[4] richness: average number of (private) allele(s) per locus corrected for sample size (using the method by Kalinowski (2004)).

Table 2.3. **Comparison between geographic and genetic distances, with pairwise geographic distances (km) above the diagonal and pairwise *Fst* values below the diagonal (* indicates significant differences).**

	LSP	RON	PIN	TSSA	TSS	LPT	MAN	OWB	PTP
LSP	-	640	538	371	371	662	297	25	658
RON	0.86*	-	112	327	327	125	380	660	66
PIN	0.67*	0.51*	-	215	215	140	268	557	160
TSSA	0.82*	0.81*	0.61*	-	0.8	306	75	385	372
TSS	0.42*	0.72*	0.58*	0.26*	-	306	74	384	372
LPT	0.83*	0.84*	0.63*	0.82*	0.78*	-	374	679	190
MAN	0.45*	0.66*	0.54*	0.30*	0.21*	0.74*	-	311	417
OWB	0.72*	0.39*	0.42*	0.66*	0.57*	0.68*	0.42*	-	680
PTP	0.76*	0.15*	0.47*	0.70*	0.68*	0.79*	0.61*	0.39*	-

(* Indicates significant differences ($p < 0.05$) based on 1023 permutations)

2.3.4 STRUCTURE analyses

I followed the procedures outlined in the manual of STRUCTURE and chose K=6 (Ln$P(d)$ = -897) as the most parsimonious and meaningful number of clusters. At K=6, the likelihood starts to reach a plateau (Figure 2.2) and although the simulations for K=9 (Ln$P(d)$ = -844) had a better average likelihood than those for K=6, the improvement in *Ln* likelihood is minimal and the variance of the likelihoods across simulations increased. An alternative (potentially less arbitrary) method described in Evanno et al (2005) did not reveal clear maxima at the first or second order derivative, or at the second order derivative divided by the standard deviation (data not shown), and hence provided no alternative means of identifying K for my data. At K=6, the first cluster was formed by RON and PTP, the second by OWB and LSP, the third by TSSA and TSS, with MAN, PIN and LPT each forming an independent cluster (Figure 2.3). Neither the cpDNA haplotypes (S1 and L1/L2), nor the selfing phenotypes and the mating system were associated with any of the clusters (data not shown).

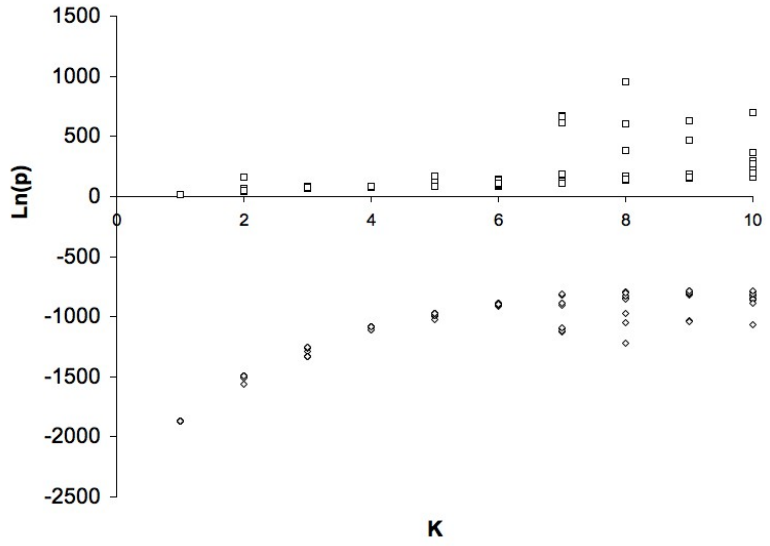


Figure **2.2** **Distribution of the *–Ln* probability (*LnP*) of the microsatellite data d (*LnP*(*d*)) (diamonds) given a *K* (number of populations) and the variance (*Var*[*LnP*(*d*)]) (squares) using STRUCTURE. Ten iterations per *K* and the variance are displayed.**

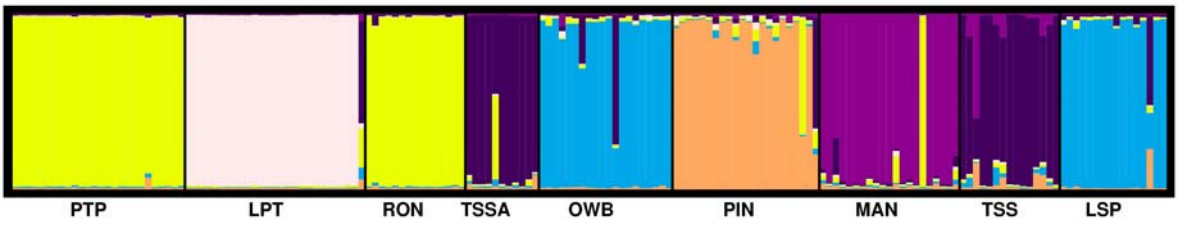


Figure **2.3**. **STRUCTURE plot with K=6. Names on the x-axis indicate different sampled populations ordered by outcrossing rate (T_m). Matching colours indicate individuals assigned to the same cluster.**

2.3.5 Individual microsatellite heterozygosity and selfing phenotype

Overall, there was a significant effect of selfing phenotype (SI, PC, SC) on individual heterozygosity (one way ANOVA, df = 2, F = 11, $p < 0.0001$). Self-compatible (SC) individuals had a significantly lower individual heterozygosity (average $H_o = 0.06$) than SI (average $H_o = 0.19$) or PC (average $H_o = 0.20$) individuals, which were not significantly different from one another (Figure 2.4a). For individuals with the S1 haplotype there was no significant effect of selfing phenotype (Figure 2.4b, one way ANOVA, df = 2, F = 21, p = 0.98). In contrast, for individuals with the L1 haplotype selfing phenotype did have a significant effect on individual heterozygosity (Figure 2.4c, one way ANOVA, df = 2, F = 0.02, $p < 0.0001$). SC individuals had a significantly lower individual heterozygosity (average $H_o = 0.05$) compared to SI (average $H_o = 0.27$) and PC individuals (average $H_o = 0.18$) (Tukey-Kramer post-hoc test, $p<0.05$). I excluded individuals with the L2 haplotype, as the sample size for this group was too small to compare to the other two haplotypes (S1 and L1).

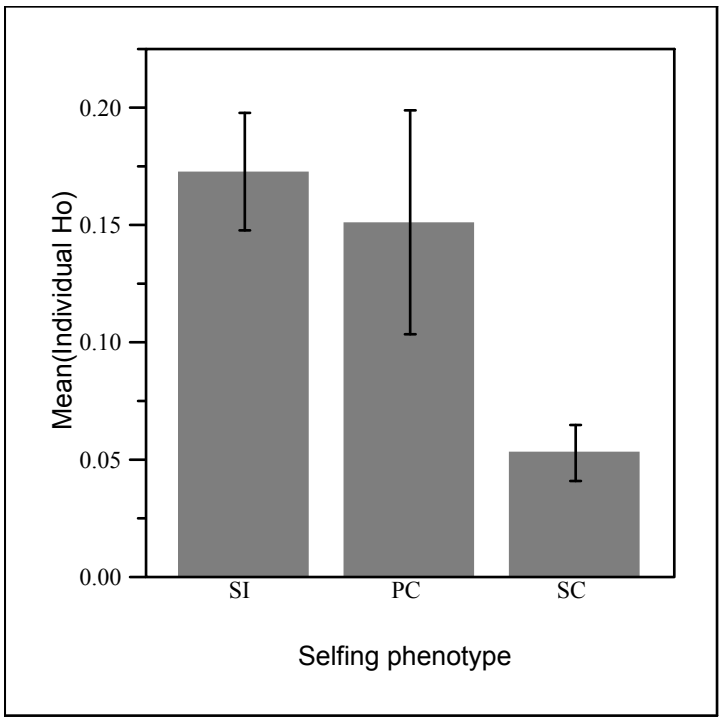
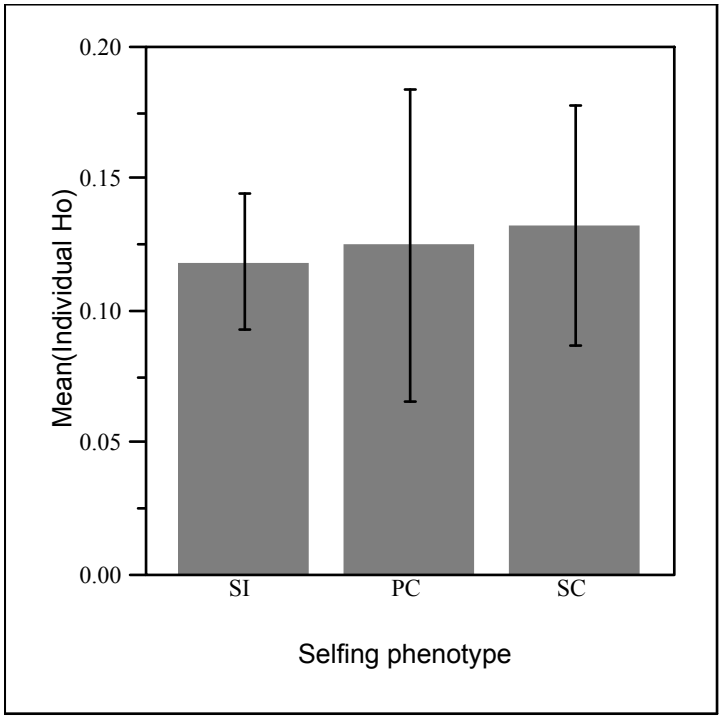
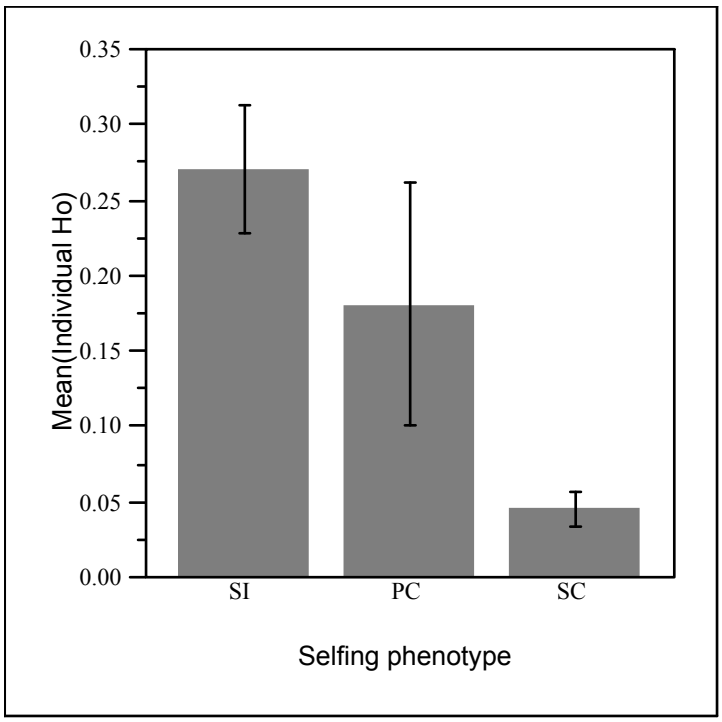


Figure 2.4. The effect of selfing phenotype on individual heterozygosity. One-way ANOVA ($p < 0.0001$) and difference between groups tested by a Tukey-Kramer post-hoc test in which the mean heterozygosity of self-compatible (SC) individuals was significantly lower than of self-incompatible (SI) and partially self-compatible (PC) individuals. Error bars indicate ±standard error of the mean.

(b,c) **The effect of selfing phenotype on individual microsatellite-based heterozygosity calculated separately for the two main chloroplast haplotype lineages found in this study:**



(b) **No significant difference (one-way ANOVA, p = 0.98) in heterozygosity between selfing phenotypes was found for individuals with cpDNA lineage S1, which did not include entire populations that had shifted to an inbreeding mating system.**



(c) **Significantly reduced heterozygosity (one-way ANOVA, p < 0.0001) was found in SC compared to SI and PC individuals with cpDNA haplotype L1 (Tukey-Kramer post-hoc test).**

2.4 Discussion

In this study I report chloroplast haplotype sequence variation, selfing phenotypes, microsatellite-based outcrossing rates and microsatellite variation for North American *Arabidopsis lyrata*, a predominantly self-incompatible species in which self-compatible individuals had been reported previously (Mable & Adam 2007; Mable *et al.* 2005). Based on comparisons of genetic data with differences in selfing phenotypes and mating systems, I discuss the phylogeographic history of these populations and compare potential postglacial colonization scenarios with other phylogeographic studies in the Great Lakes region. I also assess the consequences of the loss of self-incompatibility at the individual and population genetic levels.

2.4.1 Multiple losses of self-incompatibility

I found self-compatible individuals in all except two populations sampled, but only five populations (LPT, PTP, RON, TSSA, TC) showed a substantial shift to inbreeding based on realized outcrossing rates (Table 2.1; Figure 2.1). In general, the proportion of self-compatible individuals in a population based on self-pollinations was consistent with population-based outcrossing rates (Table 2.1). Exceptions to this were TSSA and TC, which each showed a higher proportion of partially compatible than self-compatible individuals and included a relatively large proportion of self-incompatible individuals but showed outcrossing rates less than 0.5. It was unclear if PC individuals had a partially working self-incompatibility system, whether the environment influenced the self-incompatibility system, or that they were SC individuals that received suboptimal pollen. Individual heterozygosity based on microsatellite data grouped them with SI individuals (Figure 4a). This could indicate that PC individuals are an early transition phase from the state of being SI to becoming SC or that the environment could have induced the partial breakdown of the self-incompatibility system but rules out the possibility that they were SC individuals pollinated by suboptimal pollen. The transition from SI to SC could be because of mutations in the SRK/SCR genes but also because of modifiers playing a role but I will only consider the effective selfing phenotype without considering mechanisms responsible for this loss. Intriguingly, while a loss of self-incompatibility occurred in individuals with all three chloroplast haplotypes identified, a shift to inbreeding at the

population level predominantly occurred in only one of these lineages. In this lineage, characterized by a long (741bp) PCR length variant of the *trn*L-F intergenic spacer (haplotype L1), three (RON, PTP, LPT) out of seven populations showed a high proportion (>0.6) of self-compatible (SC) plants (Table 2.1) and showed multi-locus outcrossing rates well below 0.5. Altogether, this suggests that in those populations the loss of self-incompatibility has led to a complete transition to selfing. This is corroborated by the observation that some individuals in predominantly selfing populations are autonomous selfers (i.e., they do not need pollinators; (Mable & Adam 2007). It is noteworthy that I also found outcrossing populations in the L1 lineage (PIN, IND). These populations had few or no self-compatible individuals and high outcrossing rates (Tables 2.1 & 2.2; Mable & Adam 2007). The other long cpDNA type (haplotype L2) was only found in two populations that also included individuals with haplotype L1 namely MAN and IND (Table 2.1). One individual with haplotype L2 was SC, whereas all other individuals were either SI or PC. The fact that the only other SC individual in the MAN population had the L1 haplotype may be explained by migration of SC-L1 individuals to this population. Subsequent gene-flow may then have transferred SC into an L2 background. Alternatively, self-incompatibility has been lost independently in the L2 lineage, but my data cannot distinguish between these scenarios. In IND I did not have selfing phenotype information of the sequenced individuals.

In contrast, the loss of self-incompatibility has not led to a complete transition to selfing in most of the populations within the chloroplast lineage characterized by a short (515 bp) PCR length variant of the *trn*L-F intergenic spacer (haplotype S1). The exception is TC, which had an outcrossing rate of 0.19 and mixed proportions of self-incompatible, partially compatible and self-compatible individuals. However, only 6 individuals were screened for cpDNA haplotypes and the selfing phenotype was not determined for these individuals. The largest proportions of self-compatible individuals were observed in the OWB and LSP populations, but the majority of plants were self-incompatible. Outcrossing rates for OWB were intermediate between selfing and outcrossing (Table 2.1), whereas in LSP outcrossing rates were very high (Table 2.1). This could mean that the loss of self-incompatibility in these populations is too recent to have led to a complete shift to a mating system with increased inbreeding, or that there is a stable mixed mating system with intermediate outcrossing rates. Theory predicts that there are only two ultimate endpoints for populations with a mixed mating system (Lande & Schemske 1985). Either the transmission advantage of selfers would outweigh the negative effects of inbreeding, so that purging of recessive deleterious load would result in a totally selfing population

(Cheptou 2004; Dornier *et al.* 2008), or the higher expected fitness for outcrossing individuals would outweigh the transmission advantage of selfers (Lande & Schemske 1985). Contradicting this theory, mixed mating in natural populations has sometimes been found to be a stable state (Goodwillie *et al.* 2005). Intermediate outcrossing rates are actually predicted to be common in plants with biotic pollen dispersal (Vogler & Kalisz 2001). The presence of multiple *Arabidopsis lyrata* populations with different proportions of self-compatible individuals and variable established outcrossing rates provides a good system to empirically test theoretical predictions regarding mating system evolution.

Alternatively, the loss of self-incompatibility may have occurred once, and subsequently spread throughout the continent. As self-compatible plants in different populations do not share their maternally inherited chloroplast haplotype, this would imply such spread to have been mediated through pollen-flow. Population TSSA is interesting in this context., because it is mixed in terms of chloroplast haplotype, and selfing phenotype. It had intermediate outcrossing rates (Table 2.1). Self-compatibility was only present in individuals with the L1 haplotype, but not in all. Patterns of structuring of microsatellite variation did not suggest that the TSSA population consists of two clusters, which shows that substantial gene flow must occur between the haplotypes at least within this population. The STRUCTURE analysis further shows that there is only very limited admixture between populations. With the exception of the RON and PTP population, that probably have a recent common ancestry (Mable & Adam 2007), pairwise genetic distances were very large ($Fst > 0.35$, Table 2.3) in comparison to those observed between European populations (Clauss & Mitchell-Olds 2006; Gaudeul *et al.* 2007). Despite this strong differentiation, partial Mantel tests were not significant, indicating no isolation by distance. This pattern remained if tested within the two cpDNA lineages separately, in agreement with previous work (Mable & Adam 2007).

More evidence for a scenario of multiple losses of self-compatibility is provided by Analysis of Molecular Variance (AMOVA), in which population (i.e., geographic location) explained most variation, rather than selfing phenotype or mating system. Under a scenario of a single origin of the loss of self-incompatibility, all self-compatible lineages would be expected to carry similar alleles due to a shared origin, and hence should share variance components. Note however, that microsatellite variation was not explained by chloroplast haplotype either, which indicates that these microsatellites markers may be too quickly evolving to allow conclusions regarding the origin of self-compatibility, but are more useful at the population genetic level. A STRUCTURE analysis divided the individuals

into six clusters, according to geographic origin rather than selfing phenotype or mating system (i.e., inbreeding or outbreeding). Populations in close vicinity tended to cluster together and hardly any admixture was detected between clusters (Figure 2.3). Fully self-compatible individuals occur in almost all of these clusters (except for PIN), which provides more support for multiple losses of self-incompatibility, since under the scenario of a single origin either substantial admixture between clusters would be expected (migration of self-compatible plants between clusters), or all self-compatible plants would be expected to form a single cluster. The latter was found in *Capsella rubella*, where self-compatibility has likely evolved only once (Foxe et al 2009).

2.4.2 Two independent colonization routes after the last glacial maximum

The first part of the chloroplast *trn*L(UAA)-*trn*F(GAA) intergenic spacer (Taberlet et al 1991) that I sequenced (base pair position 1-180) is followed by a tandemly repeated *trn*F pseudogene in *A. lyrata* and some other Brassicaceae (Ansell *et al.* 2007; Koch *et al.* 2005; Schmickl *et al.* 2008b). Since pseudogene copy evolution is not well understood and violates general assumptions about sequence evolution, I cannot infer the relation between the three chloroplast haplotypes. Nevertheless, the haplotypes are useful to reconstruct the phylogeographic colonization history of *A. lyrata* in the Great Lakes region. The geographic distributions of the haplotypes indicate the existence of at least two independent colonization routes following the last glaciation (21,000-18,000 BP), during which the Wisconsin ice sheet covered the whole Great Lakes region until just south of present day Lake Michigan (Lewis *et al.* 2008). After the retreat of the ice, the S1 chloroplast haplotype may have expanded north along the western side of Lake Michigan to colonize the area north of Lake Superior, while lineage L1 (and possibly L2) expanded northward more to the east to colonize the area north of Lake Erie. A similar scenario of postglacial expansion was reconstructed for garter snakes, for which mitochondrial sequences also suggested independent colonization routes on the west and east side of Lake Michigan (Placyk *et al.* 2007). Holman (Holman 1995) suggested a primary postglacial colonization route by amphibians and reptiles from present-day Indiana and Ohio into Michigan, followed by later expansions from the north and the eastern Appalachian mountain range. The S1 and L1 chloroplast lineages co-occur in the triangle between Lakes Erie, Huron and Ontario, which had previously been suggested as a secondary contact zone in *A. lyrata* (Mable and Adam 2007). Other studies also suggested

southern Ontario as secondary contact zone for several species of amphibians (Austin *et al.* 2002; Holman 1995; Smith & Green 2004; Zamudio & Savage 2003).

2.4.3 Population and individual specific consequences of the loss of self-incompatibility

The transition from self-incompatibility to self-compatibility should have a major effect on genetic variation, as selfing is expected to reduce heterozygosity by 50% in each generation (Hartl & Clark 1989). However, the loss of self-incompatibility *per se* does not mean that plants become obligate selfers, and the established outcrossing rates may well depend on population-specific selection pressures. Besides comparing population level effects, I set out to test for effects of loss of self-incompatibility at the individual level. At the population level, I found reduced observed heterozygosity and genetic diversity in the three predominantly inbreeding populations (LPT, PTP, RON) compared to the more outcrossing populations, and the highest number of rarefacted private allelic richness was found among predominantly outcrossing populations (Table 2.2). Loss of genetic variation with selfing has been found previously both within *A. lyrata* (Mable & Adam 2007) and in interspecific comparisons between *A. thaliana* and European (self-incompatible and obligately outcrossing) *A. lyrata*. The latter was shown to have higher heterozygosity based on microsatellite markers (Clauss & Mitchell-Olds 2006), threefold higher nucleotide diversity (Ross-Ibarra *et al.* 2008), and higher effective rates of recombination (Wright *et al.* 2006). In my study, SC individuals had an overall reduced observed heterozygosity compared to both SI and PC individuals, which had similar levels of heterozygosity to one another (Figure 2.4a). This indicates that PC individuals, though capable of setting selfed seeds, either remain predominantly outbreeding or have experience recent weakening of the self-incompatibility system. I also found that within the L1 chloroplast lineage SC individuals had a significantly lower individual heterozygosity compared to PC and SI individuals (Figure 2.4c), whereas within the S1 haplotype this pattern was not apparent (Figure 2.4b). This striking difference could reflect a difference in the time of the loss of self-incompatibility in the two lineages.

2.5 Conclusions

Self-incompatibility in *Arabidopsis lyrata* occurring in the Great Lakes region of Eastern North America has been lost independently at least three times, as self-compatible phenotypes occurred in three distinct chloroplast lineages. The exact phylogeographic history of these lineages remains elusive, but fits general patterns of recolonization along a western and eastern route. The loss of self-incompatibility has led to a complete transition to selfing predominantly in one of the lineages. In the other lineages, despite relatively high frequencies of self-compatible individuals, outcrossing rates were generally high and no reduction in heterozygosity or allelic diversity was found, although some populations showed intermediate outcrossing rates and one showed a shift towards inbreeding. Whether this is a stable state driven by a constant trade-off between the advantages of selfing and those of outcrossing, or represents a snapshot of populations on the way to becoming completely selfing may be addressed by following these populations over time. Further extensive sampling of more southern and eastern populations, including potential refugial areas should provide a better understanding of postglacial colonization routes and their impact on mating system evolution. Examination of the consequences of shifts in mating system within a species, such as described here, provide great potential for elucidating the genetic and ecological consequences of reproductive strategies.

3 Inbreeding depression in relation to the loss of SI

3.1 Introduction

The presence of both male and female reproductive components within an individual allows many plants species to outcross as well as self-pollinate (Takebayashi & Morrell 2001). Mating systems in natural populations can vary from entirely outcrossing to complete inbreeding, depending on factors such as ecology, population history, natural enemies, size of the population, density of individuals within the population, and pollinator environment (Amos & Balmford 2001; Richards *et al.* 2003; Steets *et al.* 2007). Self-fertilizing individuals could be offspring from self-fertilizing parents or appear as offspring from outcrossing parents if a mechanism against self-pollination became defective (Pannell & Barrett 1998). An increased autogamous selfing rate was found to be adaptive in an unpredictable pollinator environment in a study on the normally outcrossing annual *Collinsia verna,* suggesting mixed mating as an expected result in these populations (Kalisz & Vogler 2003).

Self-fertilizing individuals pass their complete genome to the next generation and can reproduce independently of a mating partner. However, this would also mean that variation in the offspring and admixture of beneficial mutations in the population is decreased, which could be disadvantageous in the case of changing environmental conditions. Outcrossing individuals, on the other hand, have a dilution of their genetic material in the next generation (Fisher 1941) and are in need of a mating partner but have high variation in their offspring and the potential for effective gene flow within the population that enables them to respond to changing environmental conditions (Van Valen 1977).

Selfing individuals do undergo crossing-over during meiosis but with little effective recombination of chromosome fragments (Morrell *et al.* 2006). Due to inbreeding, homozygosity increases, which means that recombination between heterozygous loci decreases and levels of linkage disequilibrium increase (Glemin *et al.* 2006). In addition, effective population size (*Ne*) in a selfing population is half that of an outcrossing population, making genetic drift play a more significant role. This means that natural selection would have a smaller effect on eliminating both advantageous and disadvantageous mutations, which could result in fixation of mildly deleterious mutations (Glemin 2003). Most deleterious mutations are present in a recessive form (Peters *et al.* 2003) and can thus be maintained in a heterozygous state masked by a dominant allele in outbred offspring (Carr & Dudash 2003; Davenport 1908). Alleles carrying these deleterious mutations contribute to the mutational load (Carr & Dudash 2003), which is

exposed with the increased homozygosity due to inbreeding and is expected to have negative fitness consequences under a model of partial dominance (Charlesworth & Charlesworth 1999). Alternatively, the loss of mean heterozygosity across the genome itself could cause a reduction in fitness due to overdominance (Shull 1908; Wright 1977). Because of these negative effects, self-fertilizing individuals are expected to have lower fitness due to inbreeding compared to outcrossing individuals, which is commonly referred to as inbreeding depression. There have been other studies that looked at variation in inbreeding depression in plants but these focused on variation in inbreeding depression between populations with different mating systems (Busch 2005) instead of individual plants or worked with a species that did not have a sporophytic self incompatibility system (Willis 1993).

The scale of inbreeding depression is thought to vary among families within populations (Kelly 2005) and to have a different magnitude throughout various life stages (Charlesworth & Charlesworth 1987). The timing of inbreeding depression is expected to differ between species with distinct histories of outcrossing (Lande & Schemske 1985). A comparative study found that selfing species experienced most inbreeding depression late in their life cycle, whereas outcrossing species experienced inbreeding depression in both late and early life stages (Husband & Schemske 1996). Their results supported the hypothesis that early acting inbreeding depression was due to recessive lethal mutations, which can be effectively removed by soft selection, and late acting inbreeding depression due to weakly deleterious mutations, which is much harder to remove because of the need for strong selection (Charlesworth 1993).

The history of outcrossing is expected to influence the intensity of inbreeding depression (Goodwillie *et al.* 2005), which means that individuals with a long history of outcrossing would suffer more from inbreeding depression when they self fertilize than individuals with a selfing past (Puurtinen *et al.* 2007; Ross-Gillespie *et al.* 2007). This is because selection on individuals exposed to multiple generations of inbreeding could remove the deleterious load from the population, which is referred to as purging (Fox *et al.* 2008; Lande & Schemske 1985). However, the concept of purging remains somewhat controversial because selection might not constantly reduce this load, and purging could be ineffective in small populations (Byers & Waller 1999). Outcrossing events after a long period of inbreeding could result in an increase in fitness due to heterosis (Ebert *et al.* 2002; Keller & Waller 2002), so that a small amount of outcrossing might be sufficient to allow long-term survival of predominantly selfing populations without complete purging.

In addition, it has also been proposed that individual variation in fitness contributes more to inbreeding depression than outcrossing history, so that purging would play a minor role in the observed variation of inbreeding depression in populations (Schultz & Willis 1995). Most studies have focussed on differences of inbreeding depression between populations with different mating systems. The idea that variation in individual fitness within populations is playing a role in differences between mating systems, remains yet to be tested.

Regardless of population history, if inbred progeny do show reduced fitness relative to outcrossed progeny, which is often observed in natural and artificial populations (Barrett & Harder 1996b; Busch 2005; Charlesworth & Charlesworth 1999), mechanisms to prevent self-fertilization would be favoured by selection. Most studies have tested inbreeding depression in heteromorphic self-incompatibility systems (Willis 1993) and the ones that used homomorphic SI systems were either gametophytic (Porcher & Lande 2005) or had not yet identified the *S*-locus of the species (Busch 2005).

The mate assurance provided by self fertilization allows individuals to persist at low population densities where outcrossing individuals might not be able to maintain their presence due to mate limitation (Charlesworth 2006; Cheptou 2004; Harder *et al.* 2008; Mackiewicz *et al.* 2006) which is thought to provide selfing individuals higher potential to colonize new habitats (Baker 1955). In new environments isolated from competing, potentially fitter, outcrossing relatives, selfing individuals would be able to undergo a period of purging of deleterious mutations (Moeller & Geber 2005; Pannell & Barrett 1998). Nevertheless, not many studies have looked at geographic variation and the history of selfing in relation to purging by comparing individuals with different outcrossing histories.

3.1.1 Model system

Arabidopsis lyrata populations around the Great Lakes in North America have been found where the SI system has broken down while being maintained in others. This has resulted in populations where all individuals are self-compatible, self-incompatible, or have a mixture of both (Mable *et al.* 2005; Mable *et al.* 2003). Based on microsatellite markers and controlled greenhouse pollinations, predominantly selfing populations showed significantly lower outcrossing rates based on progeny arrays, genetic diversity, and

observed heterozygosity than outcrossing populations (Mable & Adam 2007; Mable *et al.* 2005; Mable *et al.* 2003). However, the fitness consequences of the shift in mating system were not examined.

In order to evaluate inbreeding depression in relation to history of inbreeding in natural populations of *A. lyrata*, I conducted an experiment in which I compared the relative fitness of progeny from outcrosses and (forced) self crosses from self-incompatible and self-compatible individuals originating from populations with different outcrossing rates. I examined both early acting fitness traits (seed set, germination rates, and germination time) and late acting fitness traits (relative growth rates and flowering time). The questions I asked were: 1) Do offspring resulting from self fertilizations suffer from reduced fitness compared to those from outcrosses if self-incompatibility state of the mother is considered? 2) Does outcrossing history of maternal plants affect the magnitude of inbreeding depression in their progeny? 3) Is there a difference between the magnitude of early and late acting inbreeding depression in relation to maternal outcrossing history?

3.2 Materials & methods

3.2.1 Sampling

Seeds were collected from the field in 2003 from seven populations, from 30 independent focal plants per population, on the Ontario side of the Great Lakes of North America (Mable & Adam 2007; Mable *et al.* 2005): Pinery Provincial Park (PIN) on Lake Huron, Manitoulin Island (MAN) on Georgian Bay, Tobermory Singing Sands (TSS) at Bruce Peninsula National Park (BPNP) on Lake Huron, Point Pelee National Park (PTP) on Lake Erie, and Rondeau Provincial Park (RON) on Lake Erie. I also included two new populations that were collected at the same time but were not included in previous studies: Old Woman's Bay (OWB) on Lake Superior and Tobermory Singing Sands, Alvar site (TSSA) adjacent to TSS at Bruce Peninsula National Park (BPNP) on Lake Huron. These populations were included because they show a mixed mating system and are located within several kilometres of outcrossing populations used previously (Figure 3.1). All populations were located on sand dunes, except for TSSA, which was located on limestone pavement (alvar).

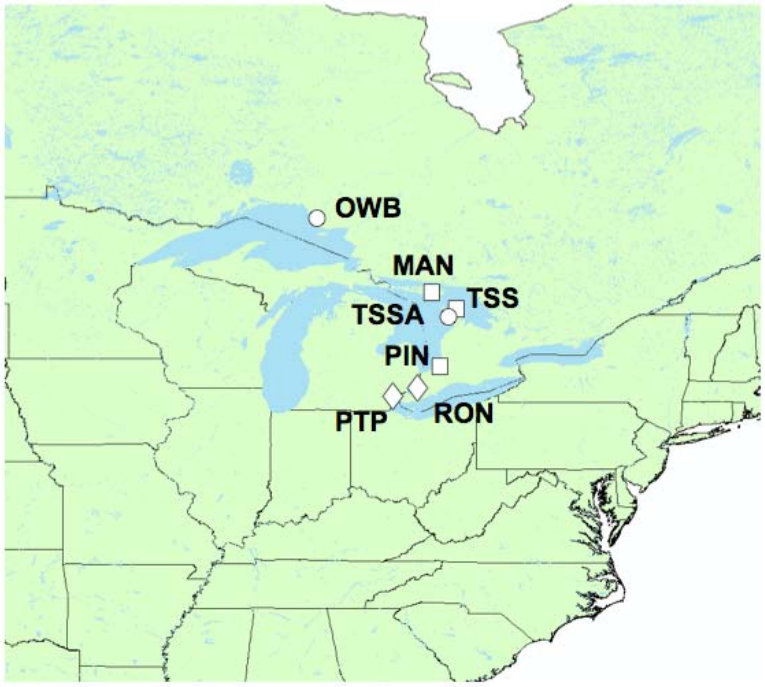


Figure 3.1. Geographical distribution of the populations used in this study around the Great Lakes of North America. Population shapes indicate mating system based on selfing phenotypes (*SP*), where squares are predominantly SI populations (SI individuals >0.75), circles are intermediate populations (0.25< SI individuals <0.75), and diamonds are predominantly SC populations (SI individuals <0.25).

3.2.2 Establishment of maternal selfing phenotype

Ten seeds per mother from 25 independent mothers per population were germinated in Levington S2 + Sand mix (Scotts Professional, Ipswich) in controlled climatic incubators (16 hour light at 20ºC: 8 hour dark at 16ºC, with 80% relative humidity). Two weeks after germination plants were moved and grown under the same temperature and light regime in greenhouses at the Scottish Crop Research Institute, Invergowrie. A single plant per maternal seed family was tested for strength of self-incompatibility (SI), which I will refer to as selfing phenotype (SP), by manual self-pollination of six flowers per plant where three pairs of flowers were rubbed against each other. Based on a former study (Mable & Adam 2007; Mable *et al.* 2005) three classes of SP were distinguished: self incompatible (SI), partially self compatible (PC), and self compatible (SC), based on the relative proportion of full-sized fruits produced on selfing. All fruits were measured with digital callipers and the length of fruits measured to classify them as positive (fruits longer than 9

mm with more than 3 seeds), “small” (fruits less than 9 mm with no more than 3 seeds) and negative (fruits with no seeds). As in previous studies, small fruits were classified as leakiness rather than a breakdown of the SI system and so classification of individual plants was as follows: SI (more than 75% small or negative fruits on selfing); PC (between 25% and 75% small or negative fruits); and SC (fewer than 25% small or negative fruits).

3.2.3 Generation of selfed and outcrossed progeny for fitness trials

For each maternal plant, selfed and outcrossed progeny were generated by controlled crossing experiments, in order to evaluate relative differences in fitness between the two types of progeny. For self-compatible plants, sufficient seeds were produced from selfed fruits to generate progeny for testing. However, for strongly self-incompatible (SI) plants it was necessary to develop a method to bypass the SI system because it was not possible to obtain full length fruits from selfings. This was achieved by enforced selfings of SI plants in a CO_2-rich environment. Although there are also other methods available (e.g. salt treatment of the stigma (Brennan *et al.* 2005)) Six flowers of an individual plant were selfed and the plant placed in a container where 5% CO_2 rich air was led through for up to six hours. After this, the plants were placed back in the green house and fruits were collected when mature (1-2 months, depending on time of year).

Outcrossed progeny for both SI and SC plants were generated by performing crosses within populations, using up to two different fathers per plant (in case of incompatible combinations due to sharing of alleles at the *S*-locus) and three flowers per father to obtain sufficient progeny for testing. Maternal flowers were emasculated by removing the anthers as soon as the flower opened. Stigmas were then pollinated by rubbing anthers from the pollen donor across the surface, using at least 3 different anthers. Fruits were scored as positive or negative based on the number of seeds present in the fruit and positive fruits were collected, seeds counted and lengths measured using digital callipers.

3.2.4 DNA extractions

Leaves were collected and DNA was extracted from 100 mg of silica-dried tissue using the FastDNA kit (QBiogene 101, MP Biomedicals) for each individual used in the experiment, including both parents and offspring raised for fitness experiments. Dried leaf tissue of the parents was pulverized before adding 800 µl CLS-VF and 200 µl of PPS. The solution was homogenized using the FASTPREP instrument for 30 sec at speed of 4.0. Samples were incubated at 55˚C for 30 min, centrifuged at 14,000 g for 10 min. and 600 µl of supernatant was transferred to clean 1.5-ml microcentrifuge tube. Six hundred µl of Binding Matrix was added, mixed by inverting, and incubated at room temperature for 5 min. The solution was centrifuged at 14,000 g for 10 sec and the supernatant discarded. The pellet was re-suspended in 500 µl of SEWS-M and centrifuged at 14,000 g for 60 sec. The supernatant was discarded by pouring off and the tubes centrifuged for 10 sec, after which the remaining supernatant was discarded using a pipette tip. DNA was eluted by resuspending the Binding Matrix in 100 µl of ddH_20 and incubating at 55˚C for 5 minutes, followed by centrifuging at 14,000 g for 60 sec. The supernatant was transferred to a labelled 1.5-ml microcentrifuge tube and briefly centrifuged to pellet out any remaining Binding Matrix. Dried leaf tissue of the offspring was sent to Norwich (DNA extraction service, John Innes Centre) for extraction in 96-well plates.

3.2.5 Microsatellite Analyses

Eight microsatellite loci that had been used previously (Mable & Adam 2007) were screened: ADH-1, AthZFPG, ATTS0392, F20D22, ICE12, LYR104, LYR133, LYR417. The forward primer of each pair was labelled with the ABI fluorescent dyes NED (yellow), HEX (green) or 6-FAM (blue). Products were amplified by multiplex polymerase chain reaction (PCR), using the default reagent concentrations recommended by the kit instruction manual (QIAGEN Inc). Thermocycling was performed on PTC-200 (MJ research) machines using the following programme: initial denaturation at 95°C for 15 min followed by 34 cycles of 94° for 30 s, 55°C for 90 s (ramp to 72°C at 0.7°C/s) and a final 72°C extension for 10 min. Multiplex products (1:160 dilutions) were genotyped using an ABI3730 sequencer (by The Sequencing Service, University of Dundee). Genotypes were read, corrected by eye and analysed using GENEMAPPER 4.0 (Applied Biosystems). Multilocus genotypes were obtained for three purposes: 1) to calculate observed

heterozygosity levels of individuals; 2) to determine realized outcrossing rates for each population; and 3) to confirm that controlled selfings and crosses were actually pollinated by the intended donor plant, by comparing progeny genotypes with those from their mothers and pollen donors.

The program microsatellite analyser (MSA) (Dieringer & Schlotterer 2003) was used to compute population level and individual (maternal) observed heterozygosity (H_o) to determine whether outcrossing history could be predicted based on this parameter and to examine the relationship between H_o and relative fitness. Individual heterozygosity was also used to test whether there was a significant difference between selfing phenotypes (SI, PC, SC) using a one-way ANOVA with Tukey-Kramer post-hoc test. Population-level heterozygosity was regressed against multi-locus outcrossing rates (T_m) using a regression analysis in order to determine whether heterozygosity could be used as an indicator of population history.

In order to classify the mating system of the populations from which individuals were sampled, multi-locus outcrossing rates (*Tm*) were estimated using progeny arrays. Estimates for populations used previously were taken from Mable and Adam (2007) but those for populations TSSA and OWB were established here using 10 progeny per mother from at least 6 mothers per population. Outcrossing rates were calculated using MLTR version 2.3 (Ritland 2002), which implements a mixed-mating model described in Ritland and Jain (Ritland & Jain 1981). Using a maximum likelihood approach. this program calculates single (T_s) and multi locus (T_m) outcrossing rates and estimates allele frequencies in pollen (*p*) and ovules (*o*). Standard errors were calculated by bootstrapping across progeny arrays using 10,000 replicates. Values of the starting parameters were set as follows: 0.1 increasing in steps of 0.1 to a maximum of 0.9 for *t*; unconstrained pollen and ovule gene frequencies; all the other parameters were set to default values. Results with the lowest standard error and highest likelihoods are reported.

3.2.6 Validation of pollination treatments for self-compatible mothers

Although I emasculated receiving flowers prior to cross-pollination, in self-compatible plants there is always a risk of contamination with self-pollen and production of selfed progeny. For this reason, I tested the paternity of all outcrossed progeny from self-compatible plants using the following microsatellite loci: ADH-1, AthZFPG, ATTS0392, F20D22, ICE12, LYR104, LYR133, LYR417. In the self-pollination treatments, the risk of

contamination with cross-pollen is obviously much less due to the close proximity of compatible self-pollen. Therefore, I only tested the paternity of one individual from each family.

3.2.7 Validation of pollination treatments for self-incompatible mothers

Due to the inherent rejection of self-pollen by self-incompatible plants, in the cross-pollination treatment of self-incompatible plants, the risk of contamination with self-pollen is low. Accordingly, I only tested the paternity of one individual from each family in this treatment. The risk of contamination with cross-pollen in the self-pollination and enforced self-pollination treatment is higher, because compatible cross-pollen is likely to outcompete self-pollen. Therefore, I tested the paternity of all the progeny in these treatments. I have not used bags to prevent contamination as flowers were too small and turned out to be unpractical with several crosses per plant.

3.2.8 Parameters used to assess relative fitness

3.2.8.1 Seed Abortion

For each fruit containing seeds from the controlled selfings and outcrosses that had been positively validated with microsatellite analysis, early-acting inbreeding depression was first evaluated by measuring silique lengths and seed numbers in relation to type of cross. Next, the proportion of aborted seeds within a silique was estimated by generating a standardised curve for the number of seeds in individual siliques regressed against silique length for each population. A specific formula for the relationship between the length of the silique and the number of seeds was calculated for each population based on the length of and the number of seeds in fruits resulting from both outcrosses and selfings. The rationale was that the difference between the observed number of seeds and that expected based on the standardized regression for each population would reflect the number of aborted seeds in each silique.

3.2.8.2 Life History Trait Measurements

Relative fitness of selfed and outcrossed progeny was determined by comparing the following life history traits: 1) germination rates and timings; 2) number of leaves after 5 weeks and a proxy for leaf area; 3) bolting and flowering time.

1) Seeds were germinated from mothers that had at least one fruit with four seeds per cross type (outcrossed or selfed). I germinated up to a maximum of 16 seeds per fruit, to allow enough space in the incubators, under the same conditions as the parental generation. A seed was considered to be germinated if the emerged seedling showed two leaves and considered to be not germinated if it had not germinated after 6 weeks. Germination rates were calculated by dividing the number of germinated seeds by the number of sowed seeds.

Two weeks after germination seedlings were transplanted from the germination tray to individual conetainers and chlorophyll deficient seedlings were scored. To prevent biased germination results through positioning of seeds in the germination trays I randomly positioned progeny from particular mothers in the germination trays, and divided seeds from a given fruit between two incubators (due to space limitations). I decided to keep different crosses (outcross and selfings) per mother together in order to determine relative fitness per mother. Determination of the fitness components was blind, which meant that only after entering the fitness component data in a computer file it became apparent which identity or cross type a certain individual had.

2) A proxy for leaf area at two weeks after germination was determined by multiplying the maximum length and diameter of each of the three largest leaves, adding these products up and taking the square root. These measurements were repeated five weeks after germination when the number of leaves as an alternative growth rate was also recorded. Growth rate by leaf surface increase was defined as the natural log of the ratio of the square root of the products after 2 and 5 weeks.

3) Five weeks after germination the plants were moved from the incubator to a greenhouse where there was no humidity and temperature control. Following this, the plants were monitored every day for date of bolting and date of flowering, and once they flowered leaf material was collected for cross validation using microsatellites (see above).

3.2.9 Computation of Relative Fitness Measures

3.2.9.1 Relative fitness of offspring produced by enforced selfings compared to natural selfings

Determination of the relative fitness of selfed vs. outcrossed progeny from the same mothers was complicated by the necessity to use CO_2 treatment to produce selfed progeny from self-incompatible mothers whereas selfed progeny could be produced without this treatment for mothers capable of self-fertilisation. While ideally only CO_2 treatments would have been used for both types of comparison, CO_2 treatment was extremely time consuming (i.e. around six hours) and together with the fact that only five plants at a time could be treated and a limited time frame in which all plants flowered, only a subset of SC mothers were exposed to this treatment to assess whether the CO_2 treatment itself affect progeny fitness. In order to determine whether naturally selfed progeny from self-compatible individuals could be compared fairly with those from enforced selfings of self-incompatible individuals, for each life history parameter the relative fitness of offspring produced by enforced selfings and those produced by natural selfings was compared for all of the self-compatible mothers where both treatments were conducted. To test whether there was a difference between natural selfing and the CO_2 treatment on fitness, I tested for a significant effect of type of selfing on fitness traits like germination time, number of leaves after five weeks, increase in leaf surface in three weeks, time to bolting, and time to flowering, using a t-test as implemented in the program JMP (version 5.0, SAS business). For the not normally distributed data (germination success) I used a logistic regression option that transformed the data into a normal distribution.

3.2.9.2 Effect of interaction of selfing phenotype and treatment on fitness traits

I tested for a possible effect of selfing phenotype (SI and SC), treatment (cross and selfing), and their interaction on fitness traits using a one-way ANOVA as implemented in

the program JMP (version 5.0, SAS business) for traits of which the residuals were normally distributed (days to germination, number of leaves after five weeks, proxy for increase of leaf surface area, time to bolting, and time to flowering). For data of traits for which the residuals were not normally distributed I used a logistic regression option that transformed the data into a normal distribution. Selfing phenotype and treatment were treated as fixed effects.

3.2.9.3 Relative fitness of selfed vs. outcrossed progeny in relation to maternal outcrossing history

Outcrossing history of mothers was assessed in four ways: 1) selfing phenotype of the mother (SP) which was transformed in a continuous variable by using the number (zero to six) of positive selfed fruits; 2) outcrossing rate of the population from which the mother was sampled (Tm); 3) observed heterozygosity of the mother (H_o mother); and 4) observed heterozygosity of the maternal population (H_o pop). The purpose of this was to evaluate whether individual variation in history of outcrossing (SP and H_o mother) or population history (Tm or H_o pop) were more important in determining the strength of inbreeding depression.

The difference in fitness (∂W) between outcrossed (W_O) and selfed progeny (W_s) was defined as relative fitness and calculated as $\partial W = W_O / W_s$ (if $W_s \leq W_o$ for a given trait) or $\partial W = W_s / W_O$ (if $W_s > W_o$ for a given trait) (Agren & Schemske 1993). I tested whether relative fitness was significantly regressed against outcrossing history using a linear regression fit as implemented in the program JMP (version 5.0, SAS business).

3.2.9.4 Comparison of early and late-acting inbreeding depression in relation to outcrossing history

In order to compare early acting (seed abortion, germination rate and time) and late acting (number of leaves after 5 weeks, proxy for leaf surface increase, flowering time) inbreeding depression in relation to outcrossing history, I combined different fitness traits by multiplying the relative fitness measures (∂W) per trait (Willis 1999) for each developmental stage. These combined values were than compared whether they significantly regressed against three different measures (SP, H_o mother, H_o pop) of outcrossing history using a linear regression fit as implemented in JMP (version 5.0, SAS business).

3.3 Results

3.3.1 Establishment of Parental Outcrossing History

Self pollinations showed populations with high proportions of individuals in the SC class: RON (0.9), PTP (1.0); high proportions of individuals in the SI class: PIN (0.84), TSS (0.73), MAN (0.79); and a mixture of individuals in the SI, PC, and SC classes: TSSA (0.42; 0.42; 0.2) and OWB (0.61; 0.17; 0.22) (Table 3.1). This generally reflected population level outcrossing rates (Table 3.1), with TSSA and OWB showing more intermediate values (T_m= 0.41 ± 0.09; T_m= 0.64 ± 0.09) than the populations where most individuals were clearly SI or clearly SC. Based on these results, the outcrossing status of the populations was interpreted as follows: PIN, TSS and MAN were classified as outcrossing; RON and PTP were classified as inbreeding; and TSSA and OWB were classified as intermediate.

Population-level heterozygosity showed a significant positive correlation with population outcrossing rates (p = 0.046, r^2 = 0.45), with the strongly outcrossing populations showing the highest H_o, strongly inbreeding populations the lowest and intermediate populations showing a H_o falling in between outcrossing and inbreeding populations (Table 3.1). Because these two measures (H_o population and T_m) were positively correlated, results are shown only for population heterozygosity as a measure of outcrossing history.

Table 3.1. **Sampled populations (ordered by T_m) showing proportion of tested plants classified as self incompatible, partially self compatible, and self compatible; number of mothers per population, number of mothers with both pollination treatments (outcross, selfing; mothers complete), number of mothers with positively validated pollination treatments (mothers validated), number of mothers with sufficient germination success (mothers germinated); geographic location based on latitude and longitude; observed heterozygosity (H_o) based on microsatellites, multilocus outcrossing rates estimated from progeny arrays (T_m, values in bold were taken from Mable & Adam (Mable & Adam 2007)), and amount of variation explained by the regression model (R^2) of the relationship between length of fruit and number of seeds and its significance (P).**

Population	SI[1]	PC[1]	SC[1]	Number mothers	Mothers complete	Mothers validated	Mothers germinated	Latitude	Longitude	H_o	T_m[2]	R^2	P
PTP	0	0	1	23	11	3	3	41˚55’	-82˚30’	0.01	**0.02**	0.56	<.0001
RON	0	0.1	0.9	21	6	3	3	42˚15’	-81˚50’	0.06	**0.29**	0.74	<.0001
TSSA	0.42	0.42	0.2	12	8	8	7	45˚11’	-81˚34’	0.17	0.41	0.11	0.002
OWB	0.61	0.17	0.22	18	6	6	5	47˚47’	-84˚53’	0.29	0.64	0.91	<.0001
PIN	0.84	0.16	0	25	8	8	7	43˚16’	-81˚49’	0.31	**0.84**	0.74	<.0001
TSS	0.73	0.27	0	11	4	3	3	45˚11’	-81˚35’	0.42	**0.88**	0.59	0.0002
MAN	0.79	0.14	0.07	28	9	8	6	45˚39’	-82˚15’	0.30	**0.88**	0.6	<.0001

[1]SI<0.25 positive self fruits, PC 0.25<positive self fruits<0.75, SC>0.75 positive self fruits

[2]T_m estimated from progeny arrays using a method described by Ritland & Jain (1981)

In terms of individual H_o, mothers classified as SC showed a significantly lower average (H_o = 0.059) than those classified as SI (H_o = 0.19) or PC (H_o = 0.20) ($p < 0.0001$, $F = 11.1$), but there was no significant difference between individuals in the SI or PC classes (Figure 3.2). For this reason, PC and SI individuals were grouped together for the purposes of simplicity and because they basically showed the same magnitude for outcrossing history (based on individual heterozygosity). SP and individual H_o did not show a linear positive relationship, which meant that SP likely did not reflect the history of outcrossing in the mothers, but only their current ability to produce selfed offspring.

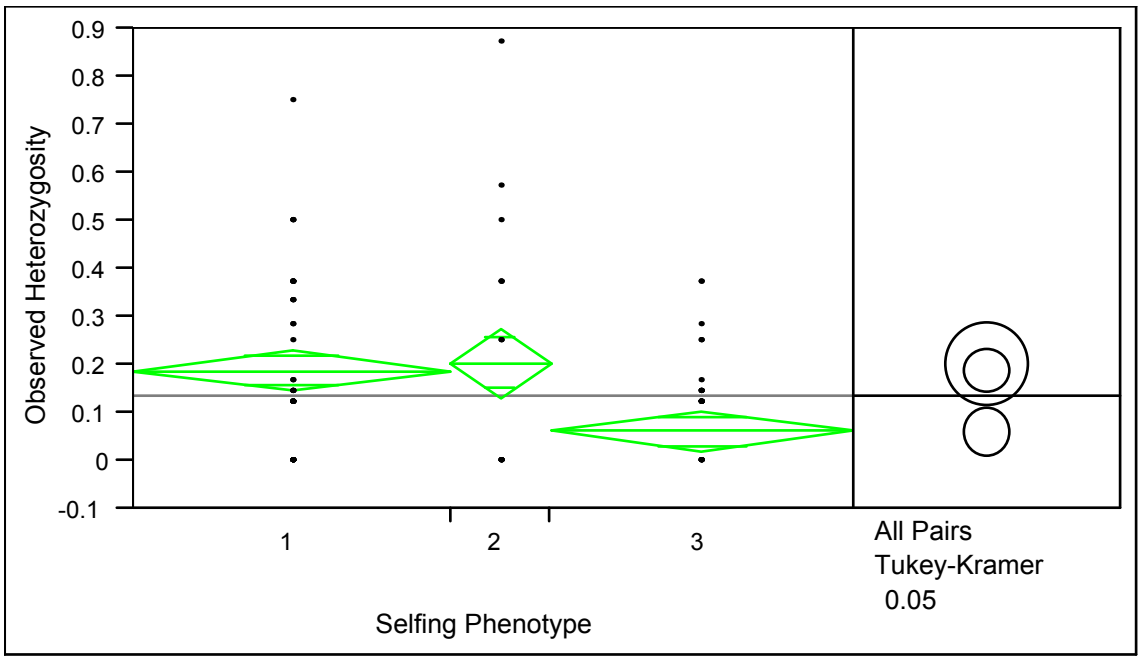


Figure **3.2. Observed heterozygosity (H_o) by selfing phenotype (1=SI, 2=PC, 3=SC. Widths of diamonds indicate sample size; height indicates variance; horizontal midline indicates the overall mean; top and bottom lines indicate 95% confidence intervals. One-way ANOVA ($p<0.0001$) and difference between groups was tested by a Tukey-Kramer post-hoc test in which the mean H_o of SI and PC individuals were significantly different from SC individuals.**

3.3.2 Generation of Selfed and Outcrossed Progeny

From the 138 mothers I grew initially up, I used 39 mothers (19 SI, 11 PC, 9 SC; Table 3.1) that had at least one fruit per pollination treatment (cross and selfing). The main reason that mothers ended up with less than one fruit per pollination treatment was because of the low success rate of the enforced selfings in the SI mothers and the fact that

temperature conditions in the green house created a small window of opportunity for simultaneous flowering of all mothers within a population to do all the necessary crosses. Overall, of the crosses performed in SI individuals 15 mothers completely failed where the remaining 50 mothers showed varying success of fruits per cross; of the enforced selfings (CO_2 treatment) in SI mothers, 10 mothers failed whereas 31 mothers showed varying success of fruits per cross; 32 out of 67 SI mothers produced naturally self-fertilized fruits after self pollination. Paternity analysis for the pollination treatments (described below) further reduced sample sizes (Table 3.1). There was variation in germination rate for all mothers used in the experiment but from all sowed seeds (1485) a subset germinated (1191), which mildly reduced the sample size for maternal level (Table 3.1) but especially affected the sample size per mother. Additionally I also included 13 SC mothers with outcrossings and selfings but without enforced selfings but did not use these after testing whether selfings and enforced selfings differed significantly from each other (Table 3.1).

3.3.3 Validation of crosses

3.3.3.1 Validation of crosses in SC plants

Paternity analyses confirmed that in five mothers all progeny resulting from the cross-pollination treatment was indeed outcrossed, however, six mothers showed offspring that was due to inadvertent self-fertilisation. In 4 of these cases, all offspring was selfed, and the mother was excluded from further analysis. In the 2 remaining cases, only truly outcrossed progeny was included in the analyses. Within populations with predominantly SC plants I found very little variation at microsatellite loci, which complicated paternity analyses for these individuals and I was not able to determine paternity for 10 mothers. These were excluded from the analyses.

Of the selfings performed using SC individuals I validated a subset of offspring of 21 mothers of which 18 showed to be pollinated by the intended pollen donor where I extended the validation to the rest of the progeny, whereas 3 mothers showed offspring that was varying in validation results, as some offspring of the same mother was crossed and other was selfed, and I only included progeny that was truly the result of self-pollination.

3.3.3.2 Validation of crosses in SI plants

Of the enforced selfings using SI mothers, microsatellite-based paternity analyses confirmed that 14 mothers showed all progeny to be pollinated by the intended pollen donor whereas 16 mothers showed offspring that was varying in validation results, as some progeny was the result of cross-pollination, and I only included offspring that was truly self-pollinated.

Of a subset of the crosses performed for SI individuals, microsatellite-based paternity analyses confirmed that 28 mothers showed all validated progeny to be pollinated by the intended pollen donor and I extended the validation to the rest of the progeny, whereas 2 mothers showed offspring that was varying in validation results where I only included progeny that was the result of cross-pollination.

After validation of all the crosses done there were 22 mothers (8 SC, 6 PC, and 8 SI mothers) that had at least one surviving progeny that was verified for each pollination treatment (cross, selfing). Only progeny from these mothers were considered in the inbreeding depression analyses.

3.3.4 Estimation of Seed Abortion

The relationships between fruit length and number of seeds for different populations showed an r^2 (amount of variation explained by the model) above 0.5 for all populations but TSSA and a p-value that was significant (<0.002) for all populations (Table 3.1, Figure 3.3, Appendix) which indicated that inferring such a relationship from silique length and the number of seeds per population could be a reliable method to predict the number of seeds expected for a given silique length.

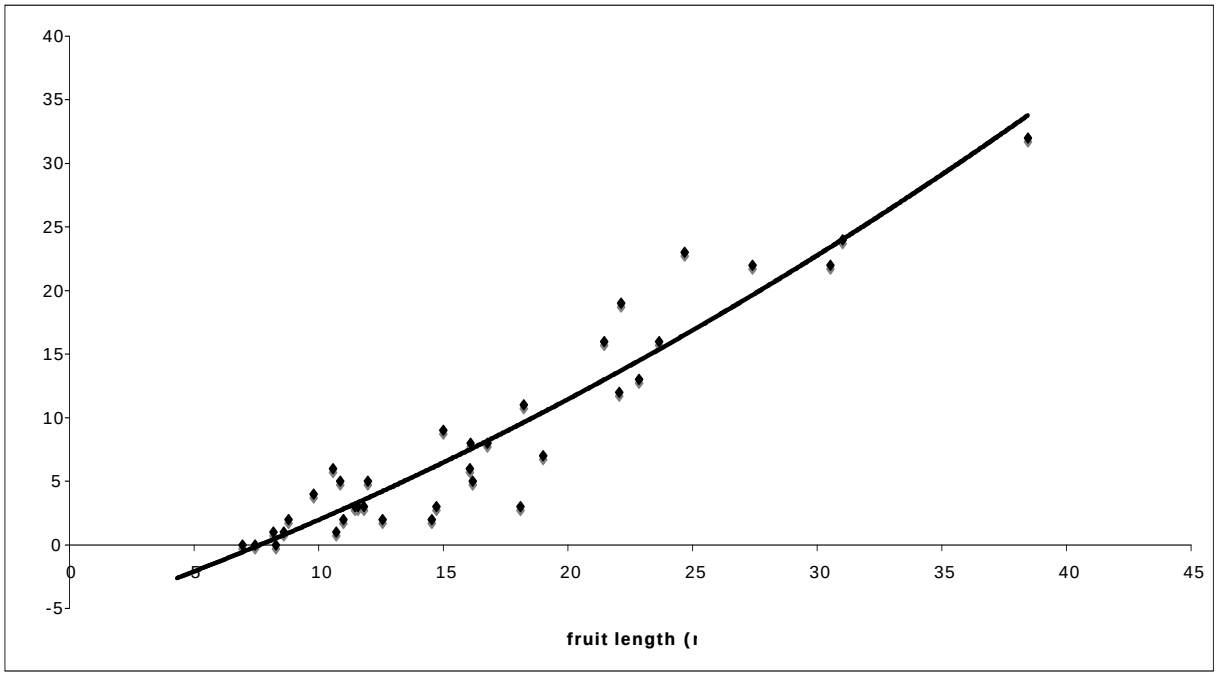


Figure 3.3.. Relationship between fruit length and number of seeds for population OWB explained by $y = 0.0092x^2 + 0.67x - 5.68$ with $R^2 = 0.91$ and $p < 0.0001$. The formula describes the relationship between fruit length and number of seeds based on a population sample and R^2 indicates the amount of variation explained by the model.

3.3.5 Inbreeding depression in life history traits

3.3.5.1 Relative fitness of offspring produced by enforced selfings compared to natural selfings

Comparison of selfed vs. enforced selfed offspring for self-compatible mothers showed no significant difference for germination score ($p = 0.09$), days to germination ($p = 0.42$), number of leaves after five weeks ($p = 0.82$), increase in leaf surface area in three weeks ($p = 0.90$), time to bolting ($p = 0.77$), or time to flowering ($p = 0.90$) (Table 3.2). According to these results, selfings from self-compatible individuals appear to be comparable to enforced selfings (Table 3.2). To increase sample sizes, I thus used all SI and PC mothers that had progeny from crosses and enforced selfings and all SC mothers that had progeny from crosses and natural selfings. This provided me with a final sample size of 34 mothers (20 SC, 6 PC, and 8 SI mothers).

Table **3.2 Comparison between enforced (CO2) and natural selfings (Self) measured in different fitness traits from self-compatible mothers. Sample size is indicated for all traits. Statistical test shows type of test with Bonferroni corrected p-value, mean squares (MS), and the F-ratio.**

Fitness trait	*Selfing*	*Mean*	Statistical test	P-value	MS	d.f.	F
Seed abortion	*Self*	*3*	*T-test*	*0.33*	13	1	0.49
	CO2	*1.7*					
Germination score	*Self*	*0.89*	*T-test*	*0.30*	0.12	1	0.5
	CO2	*0.86*					
Germination Time	*Self*	*14*	*T-test*	*0.43*	*83*	*1*	*1.7*
	CO2	*11*					
Number of leaves	*Self*	*8.5*	*T-test*	*0.44*	*1.4*	*1*	*0.30*
	CO2	*8.1*					
Increase surface	*Self*	*0.93*	*T-test*	*0.73*	*0.04*	*1*	*0.05*
	CO2	*0.91*					
Days to bolting	*Self*	*68*	*T-test*	*0.31*	*138*	*1*	*0.5*
	CO2	*73*					
Days to flowering	*Self*	*83*	*T-test*	*0.07*	*489*	*1*	*1.7*
	CO2	*90*					

3.3.5.2 Effect of interaction of selfing phenotype and treatment on fitness traits considering the maternal selfing phenotype

There was a significant effect of treatment on germination time where crosses (10) showed a shorter germination time than selfings (12). The interaction between selfing phenotype and treatment showed a significant effect for the number of leaves five weeks after germination where outcrosses in SI individuals (8.4) and selfings in SC (8.4) individuals had significantly more leaves than enforced selfings of SI individuals (7.2) (ANOVA $p < 0.0001$, Tukey-Kramer post hoc test, Table 3.3). There was a significant effect of selfing phenotype on the proxy for surface increase where SI individuals (0.85) had a lower increase of surface than SC individuals (0.93) ($p = 0.02$). There was a significant effect of selfing phenotype on days to bolting where SI individuals showed a longer (72) time bolt than SC individuals (69).

Table **3.3 Effect of selfing phenotype (SP, determined by self pollination of 6 flowers where an individual could be: self-incompatible (0 or 1 positive self fruits); partially self –compatible (2, 3 or 4 positive self fruits); self-compatible (5 or 6 positive self fruits)), treatment (outcross or selfing)), and their interaction on different fitness traits (seed abortion, germination score, germination time, number of leaves, increase (leaf) surface, days to bolting, days to flowering). Treatment and selfing phenotype are fixed effects. Bonferroni correction was performed for each significant p value.**

	Seed abortion				Germination score				Germination time			
Source of variation	d.f.	MS	F	P-value	d.f.	MS	F	P-value	d.f.	MS	F	P-value
Treatment	1	8.9	0.22	0.64	1	0.01	0.04	0.85	1	187	9.6	0.002*
SP	1	174	4.2	0.28	1	0.24	2.2	0.14	1	44	2.2	0.13
SP*treatment	1	56	1.4	0.24	1	0.32	2.9	0.09	1	34	1.8	0.18
Error	319	41			653				568	19		

	Number of leaves				Increase surface				Days to bolting				Days to flowering			
Source of variation	d.f.	MS	F	P-value	d.f.	MS	F	P-value	d.f.	MS	F	P-value	d.f.	MS	F	P-value
Treatment	1	14	4.7	0.21	1	0.01	0.12	0.73	1	20	0.14	0.7	1	76	0.45	0.5
SP	1	0.19	0.06	0.8	1	0.51	8.8	0.02*	1	808	5.7	0.02*	1	152	0.9	0.34
SP*treatment	1	89	29	<0.0001*	1	0.04	0.7	0.4	1	7	0.05	0.82	1	132	0.79	0.38
Error	374	3.1			374	0.06			374	141			374			

***** Indicates significant p-value (<0.05; Bonferroni corrected)

3.3.5.3 Relative fitness of selfed and outcrossed progeny in relation to outcrossing history

Most relative fitness traits did not show a significant relationship with outcrossing history, measured in terms of the selfing phenotype of the mother, the H_o of the mother and the H_o of the population (Table 3.4). The exceptions were that there was a significantly positive relationship between 1) the number of leaves at 5 weeks and H_o at the population level ($p = 0.02$, $r^2 = 0.45$; figure 3.4a); 2) the number of leaves at 5 weeks and the selfing phenotype (from mostly selfed fruits to mostly outcrossed fruits) ($p = 0.04$); 3) the number of days to flowering and H_o at the population level ($p = 0.04$; Figure 3.4b) (Table 3.4). However, after a Bonferonni correction only the number of leaves after 5 weeks was still significant ($p=0.02$).

Table 3.4 **Regression analyses of outcrossed and selfed progeny in relation to outcrossing history measured in three different ways: population heterozygosity (H_o pop), individual heterozygosity (H_o individual), and selfing phenotype (SP). P-values (after Bonferroni correction) indicate probability of the regression and the value of R^2 indicates the amount of variation explained by the regression.**

Fitness trait	OC history	Statistical test	P-value	R^2
Seed abortion	Ho pop	Regression analysis	0.13	0.07
	Ho mother		0.97	0.1
	SP		0.84	0.1
Germination success	Ho pop	Regression analysis	0.5	0.03
	Ho mother		0.84	0.002
	SP		0.3	0.05
Germination Time	Ho pop	Regression analysis	0.66	0.01
	Ho mother		0.3	0.06
	SP		0.47	0.03
Number of leaves	Ho pop	Regression analysis	0.02*	0.45
	Ho mother		0.09	0.15
	SP		0.05	0.19
Increase surface	Ho pop	Regression analysis	0.5	0.02
	Ho mother		0.4	0.04
	SP		0.26	0.07
Days to bolting	Ho pop	Regression analysis	0.16	0.1
	Ho mother		0.16	0.1
	SP		0.4	0.04
Days to flowering	Ho pop	Regression analysis	0.2	0.2
	Ho mother		0.1	0.13
	SP		0.1	0.12

***** Indicates significant p-value (<0.05; Bonferroni corrected)

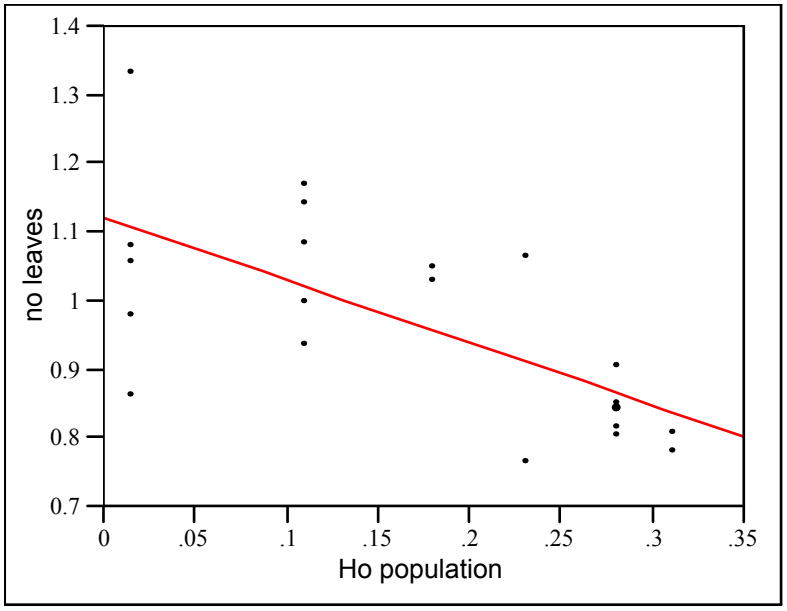


Figure 3.4 a Regression of the number of leaves after 5 weeks by the observed heterozygosity of the population.

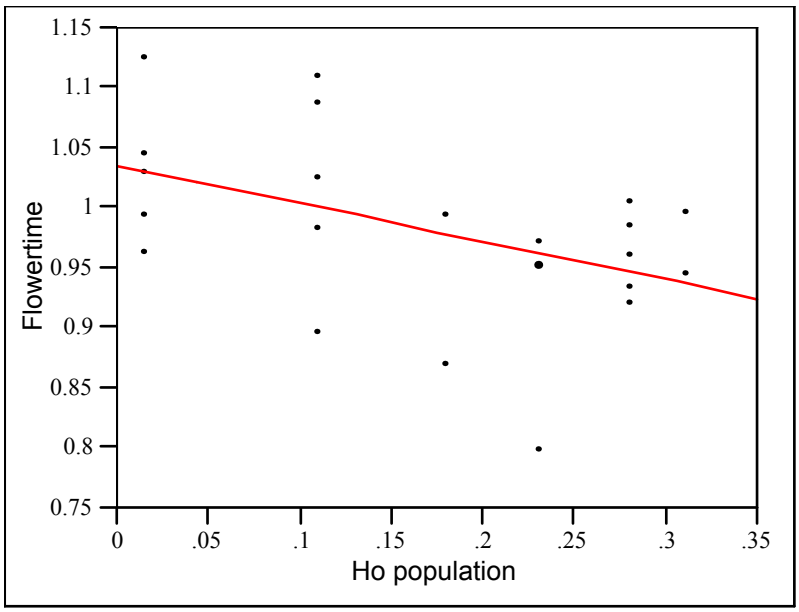


Figure 3.4 b **Regression of the time to flower after germination by the observed heterozygosity of the population.**

3.3.5.4 Comparison of early and late-acting inbreeding depression in relation to outcrossing history

The cumulative measure of early inbreeding depression (seed abortion, germination success, days to germination) did not show a significant relationship with any of the outcrossing history measures (H_o pop, H_o mother, SP, Table 3.5). The combined measure for late acting inbreeding depression showed a significant effect for all measures of outcrossing history (H_o population and mother, Table 3.5) except selfing phenotype after a Bonferroni correction.

Table **3.5 Regression analyses of combined parameters for early and late acting inbreeding depression (IBD) in relation to outcrossing history measured in three different ways: population heterozygosity (Ho pop), individual heterozygosity (Ho individual), and selfing phenotype (SP). P-values (after Bonferroni correction) indicate probability of the regression and the value of R^2 indicates the amount of variation explained by the regression.**

Type of IBD	OC history	Statistical test	P-value	R^2
Early acting IBD	Ho pop	Regression analysis	0.79	0.004
	Ho mother		0.18	0.09
	SP		0.59	0.02
Late acting IBD	Ho pop	Regression analysis	0.04*	0.33
	Ho mother		0.2	0.21
	SP		0.2	0.22

***** Indicates significant p-value (<0.05; Bonferroni corrected)

3.4 Discussion

3.4.1 Validation of selfings and crosses

Using microsatellite based paternity analyses to confirm the intended pollen donors resulted in a dataset with reliable pollination treatments although the dataset became much smaller than intended. Validation of the crosses with microsatellites revealed difficulties of achieving outcrossing in (autogamous) SC plants and enforced self-pollinations of SI individuals in an enriched CO_2 environment. For the highly SC plants non-self pollen, which can be advantageous in SC plants (Rigney *et al.* 1993), did not outcompete outcross pollen in almost half of the outcrosses attempted. This could be due to timing of pollen deposition if self fertilization takes place before the flower opens, or higher compatibility of self- than outcross pollen, which could be due to outcrossing depression (Lankinen & Skogsmyr 2002). However, my approach was conservative, as I excluded highly self-fertilizing individuals (especially population PTP) that showed very little variation at microsatellite loci that made it not possible to determine paternity for a varying number of offspring of 10 SC mothers. Contamination with non-self pollen in 8 of the 27 validated forced selfings of SI individuals could be explained by insects that made their way into the green house, or accidental transfer of pollen to the focal plant when they were moved into the CO_2 container with 3 other plants. The occurrence of accidental selfing could be explained by the fact that SI and PC individuals where pooled in the SI selfing phenotype class which increased the possibility of self-pollination for the self-incompatible phenotype.

These results call for a need to validate paternity using variable genetic markers of crosses in studies with organism that are highly self fertilizing or enforced selfings in self-incompatible species. They also highlight the problem with strongly outcrossing species due to a sporophytic SI system that is difficult to overcome. By using a method that breaks down the SI reaction allowing self-pollen to fertilize the ovule, success of overcoming the SI reaction and seed abortion due to strong inbreeding depression are hard to disentangle. Most other studies on inbreeding depression (Willis 1993) have used species that show a heteromorphic SI system, which is easier to manipulate. Other studies that looked at inbreeding depression in species with a homomorphic SI system (Busch 2005), used bud pollinations which could give complications as a study on three mustard species (Cabin *et al.* 1996) pointed out that the fertilization of immature ovules could lead to misleading results as the treatment influenced fitness, especially when comparing seeds per fruit.

An issue with comparisons of outcrossing rates and heterozygosities between populations with mixed mating systems is the difference in variability of genetic markers. Groups of inbreeding individuals will have very little variation compared to outcrossing individuals for similar loci. When a limited number of loci is used or if loci were developed for outcrossing populations, it can be very hard to detect the presence of any variation in highly inbreeding individuals (Grueber *et al.* 2008b) and skew estimations of heterozygosity and outcrossing rates, resulting in a much lower estimate of actual variation present. Studies on small insular bird populations also found difficulties in comparing genetic diversity with mainland populations as the island populations showed no variation with a multilocus microsatellite approach (Grueber *et al.* 2008a; Miller *et al.* 2003). Increasing the number of microsatellite loci or using different markers like SNP's or minisatellites could be necessary to obtain sufficient polymorphisms to compare populations with a high and a low amount of genetic variation (Grueber *et al.* 2008b).

3.4.2 Inbreeding depression in selfing and outcrossing A. lyrata

3.4.2.1 Are there differences in fitness of selfed vs. outcrossed progeny considering the maternal selfing phenotype?

I found no significant difference between fitness traits of selfed and outcrossed progeny considering the maternal selfing phenotype for any trait except the number of leaves five weeks after germination (Table 3.3). I found significant effects of pollination treatment and selfing phenotype for germination and bolting time, but the actual biological differences were minor (2 days). Selfing phenotype showed a small significant effect on the proxy for leaf surface increase, which was higher for SC than for SI mothers, which is probably due to a maternal effect where SC mothers invest more energy in the increase of leaf surface than SI mothers. One of the possibilities of the low amount of inbreeding depression detected would be due to not enough fitness traits used. Compared to other similar studies (Busch 2006; Karkkainen *et al.* 1999; Brennan *et al.* 2005) where they did find inbreeding depression I have used a similar or higher amount (3 compared to 1 (Busch 2006), 2 in Brennan *et al.* 2005; 4 in Karkkainen *et al.* 1999) of early acting fitness traits. For the late acting fitness traits I have used two measures of growth and two measures of reproductive traits which is less than other studies (8 in Busch 2006, 7 in Brennan *et al.* 2005; 5 in Karkkainen *et al.* 1999) but expected more inbreeding depression in early life stages conform the study on European *A. lyrata* (Karkkainen *et al.* 1999). Varying rates of outcrossing between mothers could have different fitness implications for offspring

resulting from either a crossing or selfing event. In a study on inbreeding depression on *Leavenworthia alabamica* (Busch 2005) it was found that SC individuals from outcrossing populations suffered more from inbreeding depression than those from inbreeding population, indicating that outcrossing history plays a role in determining the magnitude of inbreeding depression. A review on several studies on purging among plant species and between and within populations, showed that 20 out of 52 studies reviewed, showed evidence for purging which showed to be an inconsistent force within populations (Byers & Waller 1999). Thus, comparing relative fitness without considering history of outcrossing could obscure overall effects of inbreeding.

A theoretical study showed that if the genetic load is not too high and due to the result of lethals it will be purged quickly, but if it is due to detrimentals of small effect, the genetic load becomes fixed and overall fitness will be reduced (Hedrick 1994).

3.4.2.2 Is there a difference in relative fitness of selfed vs. outcrossed progeny in relation to maternal outcrossing history?

When I assessed how relative fitness of offspring resulting from outcrosses and selfings varied according to their mother's outcrossing history, I found significant relationships for number of leaves five weeks after germination. For this fitness trait I found that individuals with a relatively high population heterozygosity suffered from more inbreeding depression than individuals from populations with relatively low population heterozygosity. Given the strong correlation between population heterozygosity and population outcrossing rates, this suggests that recessive deleterious mutations may be purged from highly inbreeding populations, whereas inbreeding depression may be more pronounced in outcrossing populations. This also suggests that population history of outcrossing could be more important for purging of inbreeding depression than maternal effects due to the mother's current outcrossing status (selfing phenotype) or recent history of outcrossing (maternal H_o). In contrary, a theoretical study found that individual variation in inbreeding depression played a far bigger role in variation of inbreeding depression than population history regardless of selfing rate (Schultz & Willis 1995). Also, an empirical study on inbreeding depression in *Mimulus guttatus* found significant differences in inbreeding depression between families where between population inbreeding depression showed very little differences (Carr & Dudash 1997). These studies all support the partial dominance hypothesis for inbreeding depression where certain deleterious alleles are responsible for inbreeding depression and revealed with increasing homozygosity. The opposing, though

not mutually exclusive, theory is the overdominance hypothesis, which explains inbreeding depression by the fact that heterozygote individuals are fitter than homozygotes. Our study would support the partial dominance theory as I find evidence of purging in populations with relatively shorter outcrossing history, but the outcrossing history predictor (H_o population, H_o mother, selfing phenotype mother) indicated that the history of outcrossing is better explained on a population than a family level.

Mixed mating systems can exist as a stable state according to many different studies (Johnston *et al.* 2009; Vogler & Kalisz 2001), although original theories predicted that selection should favour the extremes of complete inbreeding or complete outcrossing (Lande & Schemske 1985). My research showed that populations with intermediate outcrossing rates had values of observed heterozygosity that did not differ significantly from highly outcrossing populations. Also, PC individuals did not differ significantly from SI individuals for observed heterozygosity whereas SC individuals did. If partial selfing does not have significant implications for heterozygosity levels then this could mean that some outcrossing in combination with some inbreeding is sufficient for maintaining levels of heterozygosity that are comparable to outcrossing individuals. This would be concordant with Wright's island theory (Wright 1931) and subsequent studies (Tallmon *et al.* 2004; Wang 2004) where they show that one migrant per generation would be enough gene flow to maintain sufficient genetic diversity and avoid inbreeding depression in isolated populations.

A previous study evaluating inbreeding depression in European *A. lyrata* (Karkkainen *et al.* 1999) compared selfed and outcrossed offspring across different families from a single population. They found significant differences in fitness traits measured as the number of chlorophyll deficient offspring, germination time, and number of leaves after 33 days. Although I did not look at exactly the same fitness traits as that study, I found no significant differences between the germination time or the number of leaves between outcrossed and selfed progeny but if I took outcrossing history into account I did find a significant difference between selfed and outcrossed progeny from mothers from populations with a relatively longer outcrossing history. In the European *A. lyrata* study the percentage of families that produced chlorophyll deficient seedlings was 70%. I only found this type of response in 3 out of 50 families resulting from selfings of self-incompatible mothers where I found none in self-compatible mothers. Chlorophyll deficiency is a result of exposure of deleterious, often recessive, alleles (Willis 1992) and suggests that the mutational load for this trait in the European subspecies was higher than

in the North-American subspecies of *A. lyrata*. If North American populations of *A. lyrata* would have a lower deleterious load this could explain the higher incidence for mixed mating in the populations present around the Great Lakes compared to European populations that are strongly outcrossing. In my former chapter (Chapter 2) it was evident that there were multiple independent losses of SI between different cpDNA varieties, which could be explained by a lower fitness cost of inbreeding due to a reduced deleterious load in North American populations around the Great Lakes.

3.4.2.3 Is there a difference between the magnitude of early and late acting inbreeding depression in relation to outcrossing history?

There was a striking difference between early- and late acting inbreeding depression. There was no significant effect of early acting inbreeding depression, as measured by seed abortion, germination success, and germination time. Late acting inbreeding depression showed a significant effect for outcrossing history determined on population level (H_o population). The number of leaves and time to flowering appeared to be the main determinants for late-acting inbreeding depression. Other studies have also found that the magnitude of inbreeding depression sometimes differs between life history stages. A study on inbreeding depression between outcrossed and selfed progeny in three populations of *Silene vulgaris* (Glaettli & Goudet 2006) showed variation in the intensity of inbreeding depression between the number of aborted seeds, germination rate, and probability of flowering, where the latter two had a much higher magnitude than the former. This would be in concordance with my study as I also found a higher rate of inbreeding depression in later life stages. A study on inbreeding depression in Scots pine between maternal lines on the other hand, showed very high inbreeding depression in an early life stage as they found that seed set in open pollinated fruits was five times as high as for self pollinated fruits (Koelewijn *et al.* 1999). They suggested that purging was not very effective and strong inbreeding depression maintained outcrossing individuals. A study on *Mimulus guttatus* (Willis 1999), on the other hand, showed effective purging of deleterious load in an experiment where inbred and outcrossed lines were created from wild samples. The fitness trait that showed the most significant amount of inbreeding depression in the outcrossed population was germination rate. In my study I did not find a significant difference in early acting inbreeding depression traits like seed abortion or germination success. I did find evidence of purging for the number of leaves after five weeks as selfings of SC individuals showed no significant decrease in the number of leaves compared to crosses, whereas selfings in SI individuals did show a significant reduction of leaf number compared to SI

crosses. It is thought that late acting inbreeding depression is more difficult to purge than early acting inbreeding depression, which would maintain inbreeding depression in later life stages after a purging event (Husband & Schemske 1996). This is not consistent with my data as I found inbreeding depression to be significantly regressed against population level heterozygosity in late acting inbreeding depression like the number of leaves after 5 weeks.

3.5 Conclusion

In this study I found that difficulties with producing outcrosses using self compatible plants and enforced selfings using outcrossing plants could potentially interfere with the reliability of interpretation of results based on relative fitness of progeny. This strongly highlights a need for paternity analyses using molecular markers when doing these type of crosses. Inbreeding depression was revealed in (enforced) selfings in individuals with relatively high population level heterozygosity compared to selfings of individuals with relatively low population level heterozygosity. This was most apparent for late acting inbreeding depression, measured in terms of growth patterns in this study. Purging of deleterious load seems to have taken place in individuals that have a low outcrossing rate and do not seem to suffer from inbreeding depression. Population level heterozygosity rather than individual heterozygosity and selfing phenotype shows to be the best predictor for estimating the rate of outcrossing in relation to fitness consequences and the effect of purging.

Following up on this study it would be interesting to see how the magnitude of inbreeding depression changes over several generations of inbreeding for individuals with a high outcrossing rate. One way of getting a more precise idea about very early inbreeding depression is to compare the number of fertilized ovules to the number of germinated seeds, which would give stronger insights into post zygotic mortality than the method used here to infer the number of aborted seeds.

The role of inbreeding depression in maintaining an outcrossing mating system was confirmed here as strongly outcrossing populations revealed late life inbreeding depression. On the other hand I also showed that populations that show a high rate of inbreeding have apparently overcome the fitness cost of inbreeding depression that allows them to adopt an autogamous self-fertilization strategy.

4 Pathogen susceptibility in relation to the loss of SI

4.1 Introduction

Organisms have to co-evolve with their (a)biotic environment in order to maintain their fitness in a competitive background. The Red Queen hypothesis (Van Valen 1977) is based on the idea that in an ever-changing environment, individuals should vary to compete with others for resources and to stay adapted to the environment. Sexual reproduction and recombination allow individuals to produce new variation in each generation (Decaestecker *et al.* 2007). The lack of effective recombination within an asexual genome, on the other hand, allows an accumulation of deleterious mutations and leads to a progressive decrease in fitness and loss of genetic variability through generations (Muller's ratchet: (Muller 1932)). In host-parasite interactions there will be a rapid change of environment (arms race between host and parasite) between generations, with the expectation that sexually reproducing hosts will have a better chance of "keeping up" than those reproducing asexually (Carius *et al.* 2001).

Muller's ratchet and The Red Queen hypothesis are also applicable to variation in sexual mating systems in plants, where inbreeding can extend to complete self fertilization in hermaphrodites, which reduces effective population size (N_e) and effective recombination rates, increasing the extent of linkage disequilibrium and diminishing polymorphism across the genome compared to outcrossing relatives (Glemin *et al.* 2006). This loss of heterozygosity and polymorphism can lead to a reduction in fitness of selfed versus outcrossed progeny (inbreeding depression) (Lande & Schemske 1985).

The mechanisms by which inbreeding depression is exposed are still under debate. There are two widely described hypotheses to explain inbreeding depression. 1) overdominance and 2) the partial dominance hypothesis. Despite the fact that inbreeding is expected to have negative impacts on fitness, there are many studies where there is a lack of evidence for such a reduction (Milot *et al.* 2007; Swindell & Bouzat 2006). The absence of this fitness decline is usually attributed to selection during a period of inbreeding, when deleterious mutations are thought to be purged from the population (Bijlsma *et al.* 1999).

Inbreeding depression can be hard to measure by looking at life history traits alone (Schultz & Willis 1995). A study on *Sabatia angularis* in three different environments (greenhouse, garden, field) showed that inbreeding depression was detected in all environments but that environmental stress played a significant role as the highest amount of inbreeding depression was detected in the field, intermediate in the garden and least in

the greenhouse (Dudash 1990). A stressful, novel environment to an organism could reveal or magnify inbreeding depression, as well as the purged deleterious load (Bijlsma *et al.* 1999; Ross-Gillespie *et al.* 2007). Experimental infection by a pathogen, for example, could reveal both variation in resistance and magnification of inbreeding depression (Ross-Gillespie *et al.* 2007). For example, in house mice (*Mus musculus*), first cousin inbreeding showed an infection rate twice as high as the control group (Ilmonen *et al.* 2008). In contrast, no inbreeding depression was observed between outcrossing and inbreeding individuals of a freshwater snail (*Lymnaea*), after exposure to natural occurring trematode parasites in a field experiment (Puurtinen *et al.* 2004; Trouve *et al.* 2003). But none of these studies have actually looked at exposure to a novel pathogen in combination with a good knowledge of their inbreeding history. To reveal the inbreeding depression present in a population or species it is necessary expose the system to a novel stressful environment where possible purging of deleterious mutations will also be revealed.

Many plants have mixed mating systems with different levels of inbreeding and are also hosts to a wide range of pathogens. Mating system could alter the range of pathogens to which a particular host species could be susceptible. In a study on 182 outcrossing plant species, a positive relationship was found between the number of fungal pathogen species that can infect a plant host and increasing outcrossing rate of the host (Busch *et al.* 2004). In relation to pathogen susceptibility this would mean that inbreeding individuals would be more likely to be susceptible and suffer from infection (Carr *et al.* 2003). On the other hand, inbreeding could also be beneficial as an experimental infection study on *Impatiens capensis*, an annual with a mixed mating system, and sympatric and allopatric varieties of a foliar rust pathogen showed that outcrossing caused the breaking up of resistant genotypes whereas inbreeding maintained the resistant genotype (Koslow & Clay 2007). But this study did not test their observations against a genetical background to reveal the actual heterozygosity rates of the individuals used in their study. Outcrossing in plants involves the need for pollinators, which can also serve as vectors for pathogen transmission. Exposure to pathogens could be reduced as pathogen vectors are diminished due to a lower selection for pollinator attraction in autogamous self fertilizing plants (Alexander & Antonovics 1988; Bucheli & Shykoff 1999; Collin *et al.* 2002). This would mean that a pathogen would be less likely to spread through an inbreeding than an outcrossing population (DeAngelis *et al.* 2008). A lot of studies have considered the effect of mating system on pathogen susceptibility but many of these were theoretical studies and field studies that used host sympatric pathogens or did not test their findings with molecular markers to reveal actual outcrossing rates and histories. In order to elucidate the effects of

outcrossing rate on fitness in relation to pathogen exposure, individuals with different outcrossing histories should be exposed to a pathogen.

4.2 Model system

The pathogen *Albugo candida* is a pathogen occurring on wide range of crucifers and includes strains that are often highly specialized on certain Brassicaceae species (Hiura 1930; Petrie 1988). Although there are also varieties that are generalists and infecting many different Brassicaceae species (Thines *et al.* 2009). Different specialized, phylogenetically distinct, strains of *A. candida* occur on species like *A. thaliana* and *Capsella bursa-pastoris,* (Voglmayr & Riethmuller 2006) and certain host genes play a role in immunity such as the *RPP13*-gene in *A. thaliana* that controls resistance for powdery mildew (*Peronospora parasitica*) (Rose *et al.* 2004). These resistance genes commonly exhibit a polymorphic pattern that reflects resistance and susceptibility to distinct pathogen varieties and are believed to be targets for natural selection, for example for the *RPP13*-gene it was found that the amino acid diversity on this locus was on average six times greater than the average amino acid gene comparisons in several accessions of wild populations of *A. thaliana* (Gos & Wright 2008; Rose *et al.* 2004). Of several *A. thaliana* host accessions from Europe, Africa, Asia, and North America, only 15 % were able to recognize two isolates (*A. thaliana* host strains: Acem1 and Acks1) of *A. candida,* with three host genes involved, which was in sharp contrast to recognition by *A. thaliana* of another pathogen, powdery mildew (*P. parasitica*), where there were no incompatible accessions and genes for recognition were numerous (Holub *et al.* 1995). As the compatibility of pathogen-host interactions seems to be initiated by mutual historical interactions, it is not clear what role the genetic variation of the host plays in terms of susceptibility.

Several studies have suggested that the interaction between pathogen and host play an important role in shaping the genetic structure of plant populations (Burdon *et al.* 2006; Holub 2007). Many studies (Alignan *et al.* 2006; Wang *et al.* 2009) have looked at specific host-pathogen interactions in a laboratory environment but there is little knowledge of how much variation for both hosts and parasites is present in natural populations and how environmental dynamics shape the polymorphisms observed there (Holub 2008).

In this study I examined whether the outcrossing history of populations of *A. lyrata* affect their ability to respond to infection with *A. candida*. Specifically, I was testing the hypothesis that populations of *A. lyrata* that are strongly outcrossing, will be more resistant to *A. candida* than those with a history of self-fertilization. This is because I would predict that there would be more variation in terms of resistance genes present in outcrossing than in inbreeding populations.

4.3 Materials & methods

4.3.1 Sampling

Seeds were collected from the field in 2007 from sand dune populations in protected park areas, from 30 independent focal plants per population, around the Great Lakes of North America (Mable & Adam 2007; Mable *et al.* 2005). Four were collected from Ontario: Pinery Provincial Park (PIN) on Lake Huron, Long Point Provincial (LPT) on Lake Erie, Point Pelee National Park (PTP) on Lake Erie (collected in 2003), and Rondeau Provincial Park (RON) on Lake Erie; one from Michigan: Indiana Dunes National Lakeshore, West Beach (IND) on Lake Michigan; two from Ohio: Headland Dunes State Nature reserve (HDC) on Lake Erie and Kitty Todd Nature Preserve (KTT) in Toledo; and one from a rock substrate in New York State: Iona Marsh (IOM), near New York City (Figure 4.1, Table 4.1).

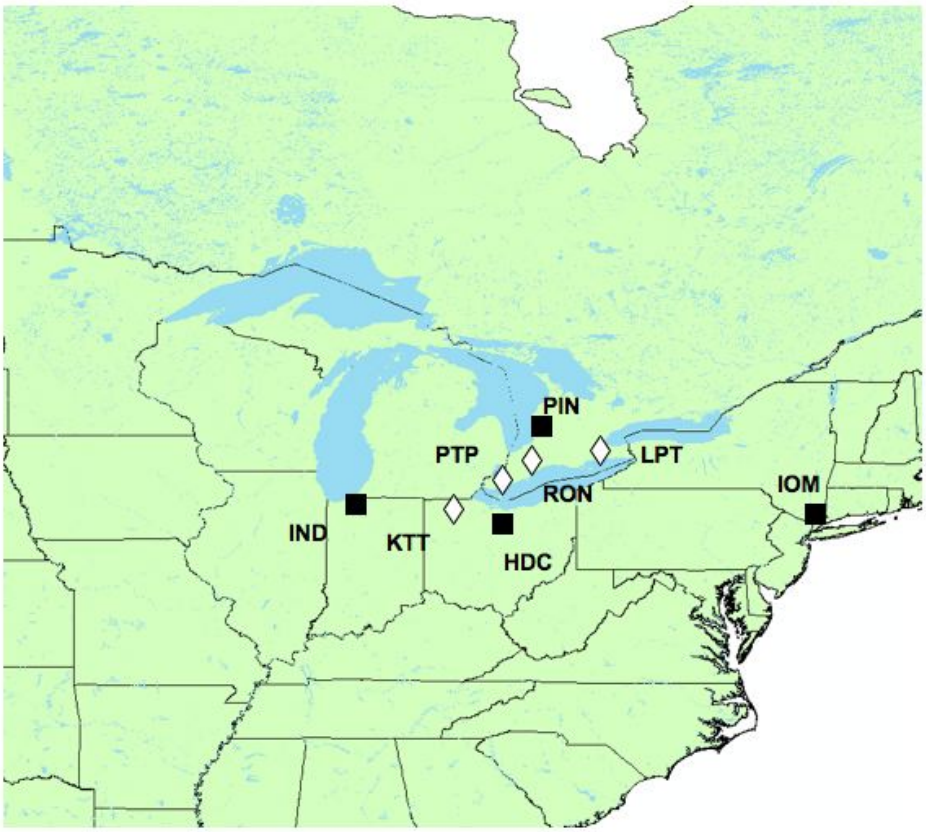


Figure 4.1. **Geographic location of populations used in this study around the Great Lakes of North America. Population shapes indicate mating system based on microsatellite outcrossing rates (*Tm*). Black squares are predominantly outcrossing populations (*Tm* >0.50) and white diamonds are predominantly inbreeding populations (*Tm* <0.50 individuals).**

Table **4.1 Overview of population infection rates: population; mating system based on outcrossing rates; geographical location indicated by latitude and longitude (in degrees and minutes); total number of individuals that showed symptoms of infection on the whole plant (susceptible), only on the inoculated leaves (partially resistant), or no symptoms; the proportion of individuals in the population that show no symptoms of infection (resistance fraction).**

Population	Mating system	Latitude	Longitude	Susceptible individuals	Partially resistant individuals	Resistant individuals	Resistance fraction
IOM	Outcrossing	41° 10’	73° 34’	2	13	4	0.21
IND	Outcrossing	42° 37’	87° 12’	7	10	3	0.15
HDC	Outcrossing	41° 45’	81° 17’	8	9	3	0.15
PIN	Outcrossing	43° 16’	81° 49’	9	3	2	0.14
	Average			7	9	3	**0.16**
KTT	Inbreeding	41 37’	83° 47’	8	4	8	0.40
PTP	Inbreeding	41° 55’	82° 30’	10	2	8	0.40
LPT	Inbreeding	42° 34’	80° 23’	12	3	1	0.06
RON	Inbreeding	42° 15’	81° 50’	15	5	0	0.00
				11	4	2	**0.22**
All	**Average**			71	49	29	**0.19**

4.3.2 Population classification

In order to classify populations as predominantly outcrossing or predominantly inbreeding, ten seeds from 25 mothers per population were raised to seedlings to establish outcrossing rates using microsatellite markers on progeny arrays. Seeds were grown up in Levington S2 + Sand mix (Scotts Professional, Ipswich) in controlled climatic incubators (16 hour light (20ºC): 8 hour dark (16ºC) regime with 80% relative humidity). Leaves were either dried in silica gel or placed fresh into tubes and sent to the DNA extraction service (John Innes Centre, Norwich UK) for DNA extraction. Eight microsatellite loci: ADH-1, AthZFPG, ATTS0392, F20D22, ICE12, LYR104, LYR133, LYR417) were genotyped (Mable & Adam 2007). The forward primer of each pair was labelled with the ABI fluorescent dyes NED (yellow), HEX (green) or 6-FAM (blue) (Table 2.1). Products were amplified by multiplex polymerase chain reaction (PCR), using the default reagent concentrations recommended by the kit instruction manual (QIAGEN Inc). Thermocycling was performed on PTC-200 (MJ research) machines using the following programme: initial denaturation at 95°C for 15 min followed by 34 cycles of 94° for 30 s, 55°C for 90 s (ramp to 72°C at 0.7°C/s) and a final 72°C extension for 10 min. Multiplex products (1:160 dilutions) were genotyped using an ABI 3730 sequencer (by The Sequencing Service, University of Dundee). Genotypes were read, corrected by eye and analysed using GENEMAPPER 4.0 (Applied Biosystems).

Multilocus genotypes were used to establish outcrossing rates (*Tm*) for populations HDC, RON and KTT, using MLTR version 2.3 (Ritland 2002), which implements the mixed-mating model described in Ritland & Jain (Ritland & Jain 1981) as described in chapter 2. Outcrossing rates for the other populations used in the experiment were obtained from a former study (IND, PIN (Mable *et al.* 2005); PTP, LPT (Mable & Adam 2007)). Too few seeds were available from IOM so its outcrossing rate was obtained from Yvonne Willi (personal observation), who originally collected the seeds. Populations with $Tm \geq 0.5$ were classified as outcrossing (OC) and those with $Tm < 0.5$ were classified as inbreeding (IB).

4.3.3 Pathogen Experiment: Seed germination and inoculation

To diminish the loss of seedlings in an early stage, an excess of four seeds per mother were germinated in Levington S2 + Sand mix (Scotts Professional, Ipswich) in controlled climatic incubators (16 hour light (20ºC): 8 hour dark (16ºC) regime with 80% relative humidity). Eventually I used two seeds per mother, which I divided in an inoculated and a control group, which were transplanted to a new soil tray. Per population I used 20 independent mothers. I divided the populations over four trays so that each tray would include a row of five individuals from each population, resulting in a total of 40 individuals per tray. Considering the control and the treated groups there were eventually 8 trays.

The inoculum was prepared by dipping four leaves of *Capsella bursa-pastoris* infected by ACEM2 in distilled water of 10°C. The concentration of spores was determined by haemocytometer counts (Brite-Line haemocytometer) using a Leitz microscope (model SM-LUX) and diluted to 5 x 10^4 spores/ml.

Seedlings were infected with this inoculum by pipetting 3µl onto each leaf. This was done at a developmental stage where other species (*A. thaliana, C. bursa-pastoris*) have been found to be most susceptible to their respective strains of *Albugo* (Holub *et al.* 1995), one to nine days after germination. The control populations were treated in the same way but with distilled water. If for some reason transplanted individuals died before they were treated with inoculum, I replaced them with one of their siblings in a second stage infection treatment after one week, which was also done for late germinating individuals (sample sizes: PTP (1), LPT (3), KTT (2), PIN (2), IOM (1), HDC (1)). Silique samples from IOM, PIN, and LPT had a high proportion of immature seeds resulting in negative germinations, which explains a lower sample size (Table 4.1). Both infected and control seedlings were kept in closed germination trays in the same incubator. Conditions in the incubator were 16 hour days, 18°C for the first five days and after that 20°C day and 16°C night, with no controlled humidity. The germination trays were rotated every two days by moving them one position up or down in the incubator.

4.3.4 Fitness in relation to infection

Host plants showed symptoms of infection by sporulation in the form of white dusty spots on the leaves (Holub *et al.* 1995). These infection symptoms were scored at two levels: susceptible, where the symptoms were present on the whole plant (Figure 4.2c), and partially resistant, where the symptoms were restricted only to the inoculated leaves (Figure 4.2b). Plants that showed no symptoms were classified as being resistant (Figure

4.2a). These three phenotypic outcomes after infection are further referred to as infection phenotype. Negative inoculations in apparently resistant individuals could not be distinguished from completely immune individuals but former experiments with the same inoculation method for compatible host parasite combinations showed no negative inoculations (E. Holub, pers. comm.). Seedlings were monitored for pathogen symptoms as present or absent every two days for five weeks.

One and four weeks after inoculation treatment, for each plant, the numbers of leaves were counted and the maximum length and width of the three largest leaves were measured to determine growth rates. Relative growth rate (RGR) was determined by the natural log (*Ln*) of the ratio of the leaf surface after four weeks (A_4) and one week (A_1). To correct for maternal effect I divided the RGR of each individual infected plant (RGR inf) by its sibling control RGR (RGR ctrl) resulting in a corrected RGR (RGR inf/ctrl).

After four weeks the lids were removed from the germination trays to accommodate plant growth. Both the control and inoculated plants were further monitored for infection for another week.

To estimate variation in the degree of susceptibility, the area of infection on the entire plant was estimated by the average coverage of the pathogen symptoms on all the leaves after four weeks and the number of infected leaves relative to the total number of leaves was determined after five weeks.

a) Control/Resistant **b) Partially Resistant** **c) Susceptible**

Figure **4.2 Pictures of individuals from different groups of susceptibility to *Albugo*.** a. **resistant individual showing no symptoms;** b. **partially resistant individual showing only symptoms on the inoculated leaves;** c. **susceptible individual, showing symptoms on the whole plant.**

4.3.5 Outcrossing history in relation to pathogen response

Leaves were collected from each individual that was experimentally infected and that survived until five weeks and frozen until DNA extraction. Microsatellite genotypes were obtained from these individuals using the same markers as for the progeny arrays in order to compare levels of heterozygosity with infection status. The program microsatellite analyser (MSA) (Dieringer & Schlotterer 2003) was used to compute average observed heterozygosity (H_o) of individuals across microsatellite loci

Correspondence analyses between mating system and infection phenotype was used to test whether there was a difference in assignment of individuals with inbreeding or outcrossing mating systems (based on *Tm* values) to susceptible, partially resistant, or resistant infection phenotypes using a likelihood ratio chi-square test, as implemented in JMP (version 5 SAS business). I also used a likelihood ratio chi-square test to test for a difference in the amount of individuals with susceptible, partially resistant, or resistant infection phenotypes between populations within mating systems.

I used an ANOVA as implemented in JMP (version 5 SAS business) to test for an effect of infection treatment (infected vs. control) of all infection phenotypes on RGR, and restricted to the resistant (including partially resistant individuals) infection phenotype, to test the cost of resistance per population, on RGR; the effect of mating system (with nested population effect), infection phenotype, and the interaction between mating system and infection phenotype on corrected RGR and H_o; the effect of mating system on surface of leaf area covered by pathogen symptoms and days to infection. I tested for a significant positive regression of corrected RGR on H_o to see whether there was a positive heterozygosity-fitness relationship present, using a regression analyses as implemented in JMP (version 5 SAS business).

4.4 Results

4.4.1 Mating system classification

Outcrossing rates based on multilocus microsatellite-based progeny arrays generally corresponded strongly with the population heterozygosity levels (see chapter 3). Although population HDC showed an intermediate outcrossing rate (0.78) compared to the other populations, I assigned it to the outcrossing group of populations, as my threshold was 0.50 because this reflected the divide in both low and high outcrossing rates and observed heterozygosity.

4.4.2 Infection rates

After inoculation it took six days before the first plants showed symptoms of infection by sporulation. In no case did control plants show any symptoms of infection before or after removal of the lids from the germination trays. Overall, 19% of all inoculated plants showed resistance to pathogen infection, as indicated by an absence of external signs of infection. Plants from predominantly outcrossing populations (PIN, HDC, IOM, IND) together had an average resistance rate of 16% (SD = 0.82) whereas those from predominantly inbreeding populations (PTP, RON, LPT, KTT) together had an average resistance rate of 22% (SD = 4.3). Among the outcrossing populations, resistance rates were relatively uniform (21%, 15%, 15%, and 14%: Table 4.1, Figure 4.3a) whereas the inbreeding populations were more dichotomous in their responses (40%, 40%, 6%, and

0%: Table 4.1, Figure 4.3a). A likelihood ratio chi-square test showed a significantly higher number of susceptible individuals (P=0.002, χ^2=12) for predominantly inbreeding compared to outcrossing populations and a significantly higher number of partially resistant individuals in outcrossing populations than in inbreeding populations (P=0.01, χ^2=8.9) (Figure 4.3b). Within the inbreeding populations, PTP and KTT had significantly more resistant individuals than RON and LPT (p = 0.0001, χ^2 = 27).

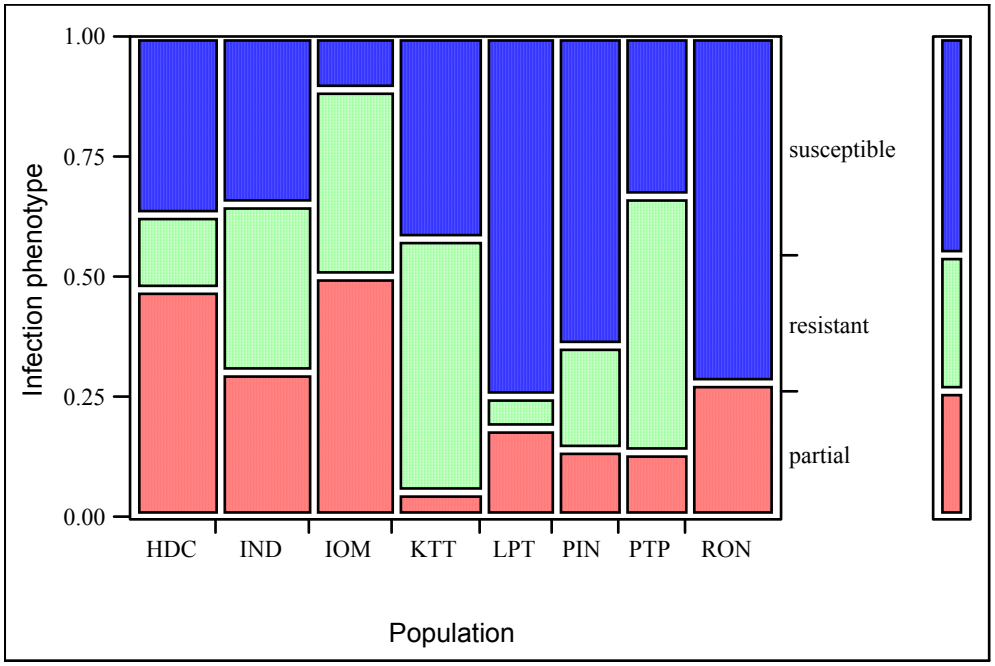


Figure 4.3a **Relative proportion of the three infection states of treated individuals (resistant, partially resistant, and susceptible) of four predominantly outcrossing (HDC, IND, IOM, PIN) and four predominantly inbreeding populations (KTT, LPT, PTP, RON). A Likelihood Ratio Chi Square test showed that within the predominantly inbreeding populations LPT and RON had significantly less resistant individuals than PTP and KTT (p = 0.0001, χ^2 = 27).**

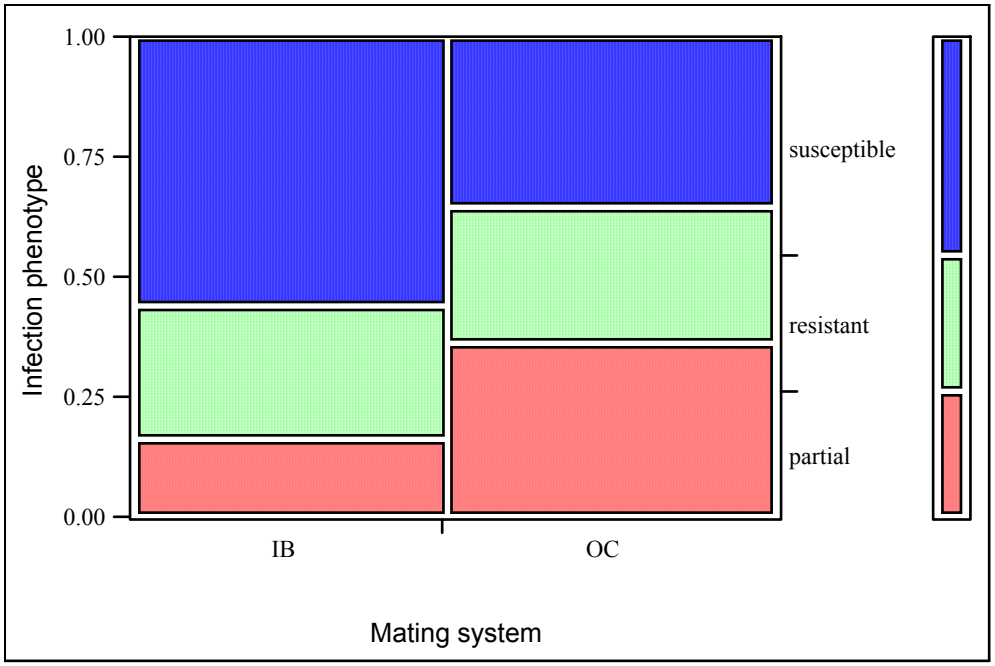


Figure **4.3** b **Relative proportion of the three infection states of treated individuals (resistant, partially resistant, and susceptible) in relation to mating system (IB = predominantly inbreeding; OC = predominantly outcrossing). A contingency analysis with a Likelihood Ratio Chi Square test showed a significantly higher proportion of susceptible individuals in the inbreeding populations compared to the outcrossing populations (p = 0.002, χ^2=12).**

4.4.3 Effect of mating system and infection phenotype on growth response

There was a significant effect of treatment (infected vs. control) on RGR, with control plants consistently showing higher rates than their infected siblings ($p<0.0001$, Table 4.2, Figure 4.4). There was a significant effect of treatment on RGR for the resistant and partially resistant infection phenotypes where populations IOM and PIN untreated control individuals showed higher rates than their treated siblings ($p<0.0001$, Figure 4.4).

There was no effect of mating system on the corrected RGR but there was a significant effect (Table 4.3, $p = 0.02$) when the population factor was nested in mating system with outcrossing population HDC showing a significantly higher corrected RGR (0.69) than outcrossing population PIN (0.21). There was a significant effect of infection status on growth rate ($p<0.0001$), with susceptible plants (mean RGR = 0.33) showing a significantly reduced growth rate relative to partially resistant (mean RGR = 0.92) and resistant individuals (mean RGR = 1.0) but no significant differences between partially and fully resistant individuals (Figure 4.5). There were no significant effects of mating system or the interaction of mating system and infection phenotype on corrected growth rate.

There was no significant effect of mating system, infection phenotype or their interaction on relative leaf area that showed pathogen infection symptoms or days to first symptom.

Table **4.2 Analyses of variance for corrected relative growth rate (RGR inf/ctrl) and the observed heterozygosity (H_o). Mating system and infection phenotypes are fixed effects and population is a random effect that is nested in mating system.**

Source of variation	Analysis	*RGR inf/ctrl*				H_o			
	ANOVA	*d.f.*	*SS*	*F*	*p-value*	*d.f.*	*SS*	*F*	*p-value*
Mating system		1	0.19	1.4	0.24	1	1.5	3.7	<0.0001
Population (mating system)		6	2.2	2.6	0.02	6	1.1	15	<0.0001
Infection phenotype		2	5.8	28.6	<0.0001	2	0.16	3.7	0.03
Infection phenotype * mating system		2	0.14	0.68	0.51	2	0.17	4.2	0.02

4.4.4 Effect of mating system and infection phenotype on heterozygosity

There was a significant effect of mating system and population nested in mating system on observed heterozygosity ($p < 0.0001$, Table 4.3). Outcrossing populations had a significantly higher mean H_o (0.31) than inbreeding (0.039) populations (Tukey-Kramer post-hoc test).

There was a significant effect of infection phenotype on H_o ($p = 0.03$) where susceptible individuals showed an observed heterozygosity (mean $H_o = 0.11$) that was lower than both partially resistant (mean $H_o = 0.24$) and resistant individuals (mean $H_o = 0.23$) but only significantly different compared to partially resistant individuals.

There was a significant effect of the interaction of mating system and infection phenotype on H_o ($p=0.02$), with resistant and partially resistant individuals from outcrossing populations showing a significantly higher H_o than all infection phenotypes of inbreeding populations. The resistant individuals from outcrossing populations also showed a significantly higher H_o compared to susceptible individuals from outcrossing populations. The outcrossing susceptible individuals showed a significantly higher H_o compared to inbreeding susceptible and resistant individuals ($p= 0.02$; Table 4.3). There was no significant relationship between the individual observed heterozygosity and corrected RGR ($p = 0.09$, $R^2=0.033$) based on a regression analysis.

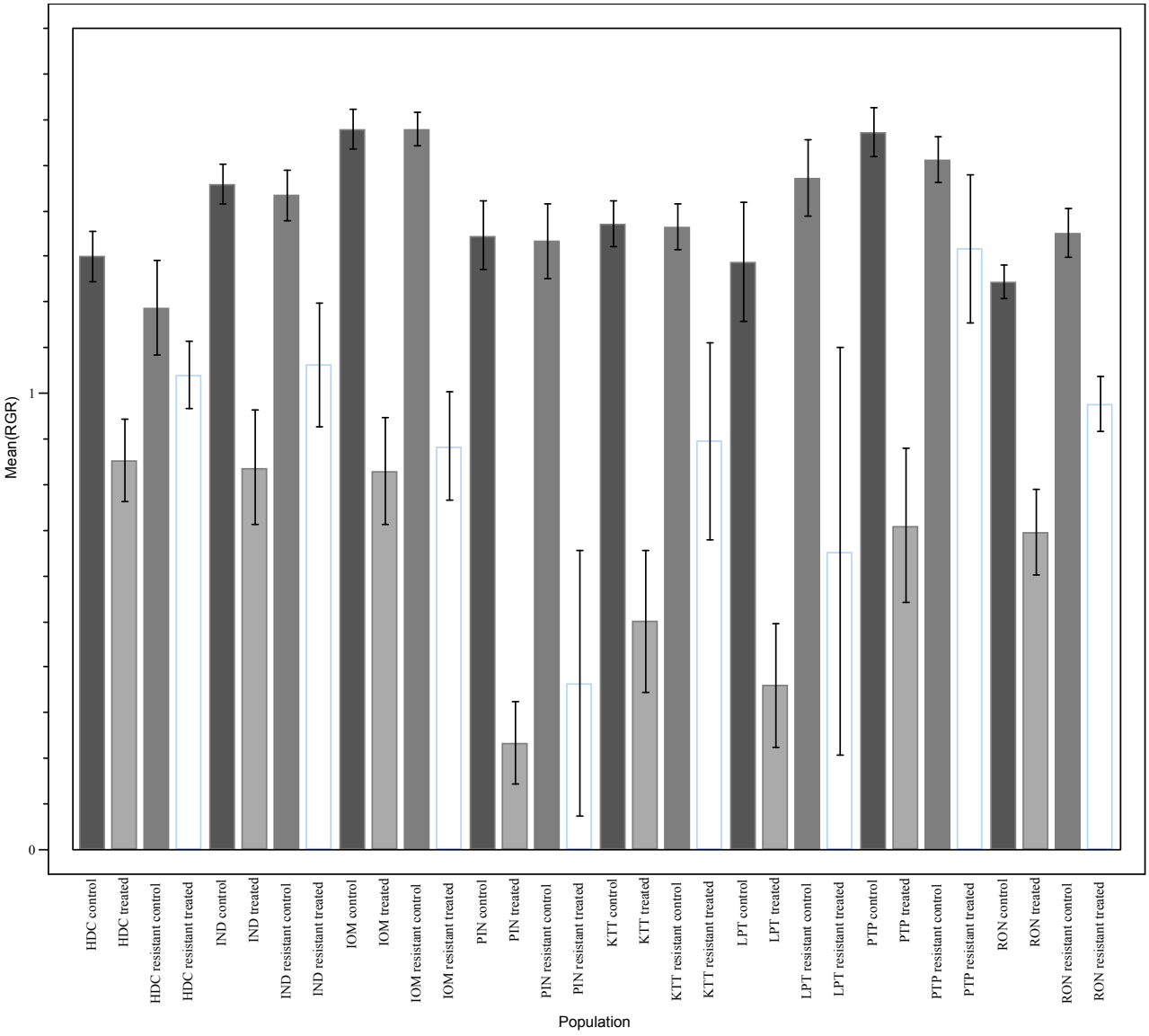


Figure **4.4 Bar plot of the Relative Growth Rate (RGR) with standard error bars for each population, where the dark grey bars indicate RGR of the untreated control group, the light grey bars indicate RGR of the treated group, the intermediate grey bars indicate RGR for the resistant and partially resistant untreated control individuals, and the white bars indicate RGR for the resistant and partial resistant treated individuals. The untreated control group had a significantly higher RGR compared to the treated group for each population ($p<0.0001$). For the resistant and partial resistant individuals, the untreated control group had a significantly higher RGR compared to the treated group for populations IOM and PIN ($p<0.0001$).**

Table **4.3 Overview of growth rate, heterozygosity and mating system determination: population, mean RGR of infected (inf) and control (ctrl) populations and values corrected for population effect (inf/ctrl), observed heterozygosity (H_o), and outcrossing rate (T_m).**

Population	RGR inf	RGR ctrl	RGR inf/ctrl	H_o	T_m
IND	0.84	1.45	0.60	0.47	0.95[1]
IOM	0.83	1.58	0.51	0.33	0.94[3]
PIN	0.23	1.38	0.21	0.27	0.84[1]
HDC	0.85	1.30	0.69	0.11	0.78
KTT	0.50	1.36	0.38	0.05	0.31
RON	0.70	1.27	0.55	0.06	0.29[1]
LPT	0.36	1.31	0.34	0.04	0.25[2]
PTP	0.71	1.56	0.43	0.02	0.03[2]

[1] from (Mable et al., 2005)

[2] from (Mable & Adam, 2007)

[3] from Yvonne Willi (personal observation)

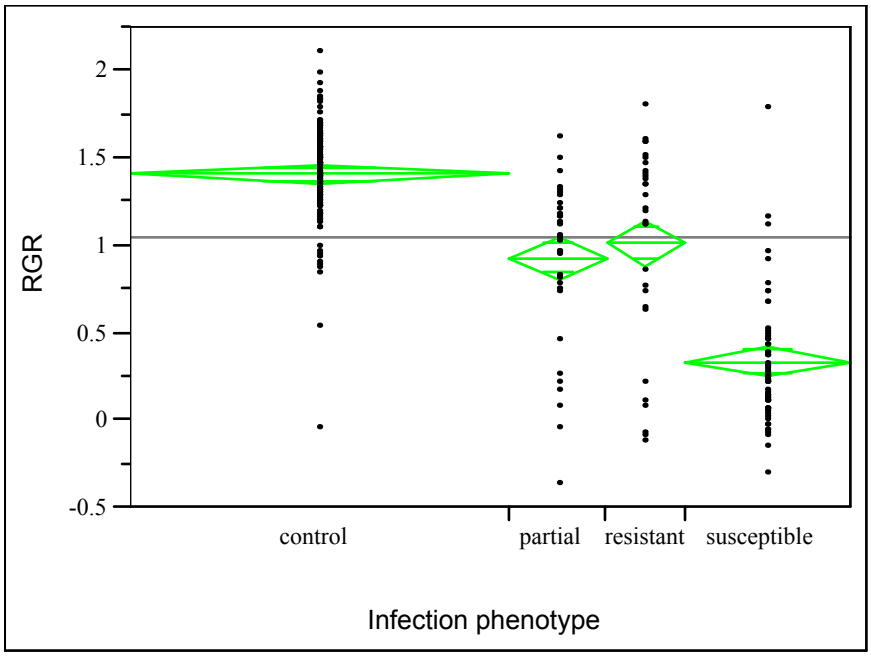


Figure **4.5** Effect of infection grade on growth rates. **The control group are uninfected individuals, the partial group showed only symptoms on the initially infected leaves, the resistant group showed no symptoms after infection, and the susceptible group showed symptoms across the whole plant. The relative growth rate (RGR) was calculated using the natural log of the increase in leaf surface over three weeks. Width of diamonds indicates sample size, height indicates variance, central line in the diamond indicates the mean, the top and bottom lines indicate the 95% confidence interval, with individual values indicated by black squares. There was no significant difference in RGR between the partial and susceptible groups but the control group had a significantly higher RGR than all infected plants and the susceptible group had a significantly lower RGR than the partial and resistant groups ($p < 0.0001$).**

4.5 Discussion

4.5.1 Relationship between mating system and fitness

Overall, there was no significant effect of mating system on fitness (RGR) after exposure to *Albugo candida*. The generalization of mating system assigning might be responsible for this as I classified a population to a particular mating system (outcrossing or inbreeding) based on the population outcrossing rates. Ideally, mating system would be tested by self-pollinations of the individuals used in the experiment but most plants did not survive to flowering to allow direct measurement of the strength of self-incompatibility. Relative growth rate (RGR) did not show a significantly positive regression against observed heterozygosity (H_o) and the actual outcrossing rates (T_m). There were significantly more susceptible individuals in the inbreeding group than in the outcrossing group, which could have implications for fitness traits later in life like further growth or time of flowering (Alexander & Antonovics 1995) but this remains to be tested in follow up experiments.

The overdominance hypothesis posits a fitness superiority of heterozygous over homozygous individuals, which was not supported by my data that showed a non-significant regression between observed heterozygosity measured by neutral microsatellite markers and fitness traits (RGR). Growth rate (measured by a proxy for leaf surface increase and increase of number of leaves) after infection was the major fitness trait I have used in this study, other fitness traits could show a different effect, although traits like area of infection and days to first symptoms did not show an effect. A study on juvenile survival in roe deer did not find a positive correlation between genome-wide heterozygosity and fitness but found a relationship between fitness and inbreeding coefficient, suggesting a locus specific rather than a genome-wide effect of heterozygosity (Da Silva *et al.* 2009). Another study on ectoparasites in salmon found a nonlinear relationship between H_o and fitness, with a higher infection rate in individuals with an intermediate level of heterozygosity compared to individuals with a low or high H_o (Blanchet *et al.* 2009). In my study this could mean that there were specific loci responsible for fitness traits or that there was an unknown relationship present between fitness traits and H_o (Hansson & Westerberg 2002). A possibility for the absence of a relation ship between H_o and fitness could be that fitness is in this case is determined by the presence or absence of a resistant genotype. In a study on the effect of bacterial

infections on inbred populations of *Drosophila* a study found wide variation in disease resistance in replicate populations indicating specific resistance alleles playing a role rather than general inbreeding effects (Spielman *et al.* 2004). In another study on powdery mildew resistance in European *A. lyrata* it was found that certain alleles of a resistance gene were associated with susceptibility and others with resistance to this pathogen (Jorgensen & Emerson 2009). They further indicated that local selective sweeps could favour different alleles in different populations contributing to variation at resistance loci.

Selection for resistance against a certain pathogen when there are only homozygous states of alleles present in a population could result in a bimodal distribution ('all or nothing') in terms of susceptibility, as populations fixed for the homozygous state of the resistance allele would be immune and those without it would be susceptible. This could explain why some inbreeding populations (PTP 40% and KTT 40%) showed significantly more resistance to *Albugo* than other inbreeding populations (LPT 6% and RON 0%), which could indicate that these less susceptible populations had a resistant genotype for almost half of the individuals in the population. The resistant and partially resistant group probably reflected variation in the resistance response from the plant on the pathogen as both infection phenotypes showed a similar magnitude of RGR and H_o. Partially resistant individuals were either able to restrict their infection to inoculated leaves or were able to 'heal' infected parts, whereas resistant individuals did not show any symptoms after five weeks. A study on the susceptibility of *Lolium perenne* to a fungal pathogen showed that within species genetic diversity had a negative correlation with the intensity of the fungal infection symptoms in the plants (Roscher *et al.* 2007) suggesting that higher levels of genetic variation in hosts are not always beneficial with pathogen interactions.

A selective sweep induced by pathogens could also result in low genetic diversity and high homozygosity due to linkage of loci throughout the genome related to selection on resistance genes (Moeller & Tiffin 2008; Stranger & Mitchell-Olds 2005; Tiffin *et al.* 2004). The inbreeding populations had individuals that are homozygous for most microsatellite loci and their resistance was concentrated in certain individuals, which could suggest that these patterns are caused by strong selection, possibly a pathogen, inducing a selective sweep.

In a population with co-dominant heterozygous states next to homozygous states of alleles related to resistance and additive effects of individual resistance alleles could result in intermediate immunity and would predict an infection rate that would be much more evenly distributed across individuals in the population than in a population were alleles

related to resistance would be dominant (Westerdahl *et al.* 2005). A theoretical model showed that if a single dominant allele were sufficient for resistance, selfing could be beneficial and most resistance alleles would be present in homozygotes, whereas in outcrossing individuals they would be more present in heterozygotes but only under certain conditions like the absence of inbreeding depression and a low cost of resistance (Koslow & DeAngelis 2006). This would be in concordance with my observation that in the outcrossing populations resistant individuals had a significantly higher H_o at microsatellite loci than the susceptible individuals, whereas in the inbreeding populations there was no overall difference in H_o between the different infection phenotypes.

Another possibility could be that some inbreeding populations are more geographically isolated than others and have purged their mutational load (PTP and KTT) from the population related to pathogen susceptibility because they show a high proportion of individuals with a resistant infection phenotype. Other populations could still be on their way to be completely fixed for resistance genotypes, as they would be less geographically isolated and experience small amounts of gene flow with neighbouring populations (RON and LPT). This would mean that they would import novel genetic material from other populations, which could possibly be a 'top up' of maladapted genetic variation. Other studies have also found a lower susceptibility for inbreeding compared to outcrossing individuals, suggesting that outcrossing could 'break up' the successful genotypes responsible for immunity (Koslow & Clay 2007). A study on a selfing annual legume found several fixed genes which were highly associated with certain resistance loci against a specialist pathogen (Parker 1991a). A follow up study showed a decline in population size due to disease mortality, suggesting that the mating system could put restrictions on further adaptation to the pathogen (Parker 1991b).

4.5.2 Cost of resistance

Across populations, the difference in RGR between the control group and the resistant group potentially indicated a cost of resistance. Growth rates were significantly higher for individuals that were exposed to *Albugo sp.* but showed no infection after five weeks (classified as resistant) or had cleared most of the infection (classified as partially resistant) than for exposed individuals that showed widespread signs of infection (classified as susceptible) but significantly lower than control individuals. The growth rates indicated that the cost of resistance for resistant or partially resistant individuals was not as high as the cost of being totally susceptible (Figure 4.5). In my study, resistant or partially resistant

individuals had a significantly lower growth rate compared to the control group probably as a result of relocation of resources from growth towards defence to the pathogen (Mauricio 1998). Interestingly, there was variation in the cost of resistance between populations where populations IOM and PIN showed a high cost of resistance as the RGR for treated resistant or partially resistant individuals was significantly different from their untreated control siblings.

A study on *A. thaliana* showed that a gene, which was involved in resistance against powdery mildew and also present in *A. lyrata*, showed either fitness benefits or costs depending on whether it was expressed in the presence or absence of the pathogen (Orgil *et al.* 2007). A field study on *A. thaliana* showed that isogenic lines differing in the presence or absence of a resistance locus *(RPM1)* showed a big cost (9% lower seed production) in the presence of this resistance gene (Tian *et al.* 2003). From these studies it was concluded that the cost of resistance would only be worthwhile if there were a constant pressure of the presence of a pathogen to maintain these resistance genes. This would also suggest that North American *A. lyrata* has already experienced some exposure to *A. candida* where there would be a possibility of pathogen influx from Brassicaea crop species carrying crucifer specific pathogens with them (Jacobson *et al.* 1998) or that the resistance genes present in North American *A. lyrata* are effective against the Capsella specialized *A. candida* pathogen.

4.5.3 Variation in host susceptibility to a pathogen

There appeared to be variation in the resistance of *A. lyrata* to *A. candida* between populations with different mating systems but overall the population effects tended to be stronger than the effects of mating system. The pathogen (*A. candida*) was isolated from a European population *of C. bursa pastoris* and has not been reported to naturally infect *A. lyrata*. The presence and variation of resistance in North American *A. lyrata* could indicate a general immunity response against pathogens from the host and a general infection ability of the pathogen (Heath 1991) but most oomycetes are host specific and are usually not compatible with other hosts (Holub *et al.* 1995). Although the Albugo variant in this study is a generalist in European populations of Brassicaseae species (Thines *et al.* 2009). Nevertheless, other studies have also shown that host pathogen resistance against a novel pathogen could pre-date the introduction of the pathogen, although a suitable

environment for the pathogen and its life history traits would determine the success of the virulence of the pathogen (Parker & Gilbert 2004).

4.5.4 Future work

I plan to genotype *A. lyrata* individuals for specific resistance genes in comparison with genotyping the *A. candida* isolates used for particular avirulance genes, in order to determine whether there is a gene-for-gene type response or a more generalized response to a novel pathogen. I also plan to perform crosses using siblings of susceptible and infected individuals used in this study in order to evaluate the heritability of responses. A repeat of the experiment with a different strain (i.e. ACEM1, *A. thaliana* host) of *A. candida* also could be used to test the ability of *A. lyrata* to deal with different pathogens and provide more information about variation of immunity in these populations. Also, follow-up infection experiments by transferring offspring of surviving infected plants from each stage to the next could tell me more about the plasticity of populations with different levels of genetic diversity.

4.5.5 Conclusions

The cost of resistance, which was lower than the cost of being susceptible, suggested that there could be an evolutionary pressure selecting for the maintenance of general pathogen resistance. The apparent specialization of *Albugo* seemed not as strict as former studies indicated and unexpected susceptibility and immunity were observed in North American *A. lyrata*. The mating system did not seem to play a significant role in fitness in relation to environmental stress but inbreeding populations contained more susceptible individuals than outcrossing populations. The difference in resistance to pathogens differed much more between inbreeding populations than between outcrossing populations. That difference could suggest that alleles responsible for resistance were present in homozygous form in inbreeding populations and in both homozygous and heterozygous form in outcrossing populations. This would mean that mating system would play a role in susceptibility, as resistance genes would be concentrated in certain individuals in inbreeding populations opposed to a more modal distribution of resistance genes across individuals in outcrossing populations.

5 Consequences of reproductive character evolution in relation to variation in mating system

5.1 Introduction

Hermaphroditic plants have different strategies to reproduce sexually, such as self-pollination or outcrossing with a conspecific. A way for both reproductive strategies to transfer pollen from the male parts to the female parts of the flower can be through an external vector (Darwin 1862). Plants that are capable of self pollination without external factors are considered to be autonomous (Lloyd 1992). Many species of angiosperms have insects as pollinators where in some species combinations of plant and insect morphology have co-evolved showing highly specialized plant pollinator relationships (Nilsson 1988). Pollen production is costly so flower morphology is thought to evolve to allow optimal transfer of pollen to stigmas (Cresswell 1998). There are different mechanisms in plant mating systems to promote an efficient way of transferring pollen from male parts to female parts of a plant (Campbell 1991). The distance between the stigma and the anthers is expected to co-evolve to promote optimal pollen to stigma transport by visiting insects (Conner & Sterling 1995). The comparison between the insect pollination of outcrossing and self-fertilizing plants has not been researched in many studies.

Insect attraction is often realized by floral characters like petal size, petal colour reflectance, and nectar production in combination with volatile secretion. (Barrett & Harder 1996a). With self-fertilization, reproductive organs are expected to be under different types of selection than under an outcrossing scenario (Thompson *et al.* 1998). Self fertilizing individuals are expected to show an adaptation towards optimal transfer of pollen from anthers to the stigma of the same plant (Runions & Geber 2000) When self-fertilizing plants are independent of a pollinator (autonomous) for self-fertilization, this will decrease selection on pollinator attractants like petal size (Foxe *et al.* 2009). But if they are still dependent on a pollinator for self-fertilizing (facilitated) they would still need to attract insects (Schoen & Lloyd 1992). The timing of flowering is important in a population with pollinator dependent individuals as co-flowering conspecifics attract more potential pollinators together than alone (Allee 1931; Stephens *et al.* 1999). However, it is not clear if this density effect has an effect on individual petal size of pollinator dependent outcrossing and inbreeding populations.

The part of the flower that is observed by visiting insects, is often the petals' UV reflectance of the spectrum (Endler 1990). Insects use floral characters and scent to discriminate between preferred species. The volatiles perceived by insects are not necessarily individual components but often a combination of certain compounds (bouquet) that trigger the insects' awareness (Waelti *et al.* 2008). Volatiles are usually by-

products of nectar production produced by glands in the petals (Dudareva *et al.* 2004) and their production is generally costly for plants (Pichersky & Gershenzon 2002) so it is expected that there will be substantial selection pressure on this trait and plants that are independent of pollinators for self-fertilization are expected to show less production of volatiles (Ferrari *et al.* 2006). However, there are not many studies that have looked specifically at volatile components and insect attraction in relation to mating system.

In an outcrossing population dependent on insects for pollination, lack of pollinators could drive evolution towards autonomous selfing (Lloyd 1992). This would result in different adaptations of floral trait components than pollinator dependent individuals (Anderson & Busch 2006; Lloyd & Schoen 1992). But in studies that have looked at these type of adaptations (Anderson & Busch 2006) it was not always clear whether or not the self-fertilizing plants were autonomous or facilitated self-fertilizing individuals.

5.1.1 Model system

Previous studies on North American *A. lyrata* have shown that the main volatile component that is produced is *phenylacetaldehyde* (Peer & Murphy 2003) and the majority of pollinators are syrphid flies and small butterflies (Grundel *et al.* 2000). Although comparisons between European *A. lyrata* and *A. thaliana* volatile emissions have been made (Abel *et al.* 2009), the role of floral volatiles in pollinator attraction in relation to mating system has not been tested within a species.

Different mating systems are thought to have evolved in concordance with their environment. These different strategies would result in different selective pressures on flower morphology. But if environmental conditions would result in local adaptation, this would outweigh the effects of mating system and result in more variation within than between mating systems.

Petal display is thought to be a measure for pollinator attraction as a study found that with increasing petal damage in *Fragaria virginia* by florivory there was an increasing rate of selfing (Penet *et al.* 2009). Another study showed that pollinators visiting the genus Mimulus preferred large flowers (Schemske & Bradshaw 1999) and in *Raphanus sativus* not only pollinator visits increased with larger petal size, but also more pollen was produced in large than in small flowers (Stanton & Preston 1988). Petal length, which in

this study was used as a proxy for pollinator attraction, would be expected to differ between outcrossing and facilitated selfing (pollinator dependent) and autonomous (pollinator independent) selfing individuals. I wanted to test whether petal length was longer in pollinator dependent individuals compared to pollinator independent individuals and/or there was a difference between populations.

A previous study has suggested that the correlation between petal length and either the stamen or the pistil should be stronger in outcrossing and facilitated self-fertilizing individuals than in autonomous self-fertilizing individuals, as this would be favored due to pollinator attraction (Anderson & Busch 2006). I wanted to test whether the correlation between petal and either pistil or stamen length was also stronger in pollinator dependent than in pollinator independent individuals of North American *A. lyrata* and/or there was a difference between populations.

A study on outcrossing wild radish (*Raphanus sativus*) showed that stamen and pistil had a similar height to promote effective pollen transfer by pollinators (Conner & Via 1993; Conner & Sterling 1995). Pistil height and stamen length promoting pollen deposition on the stigma of the same individual, are also expected in pollinator independent individuals (Affre & Thompson 1998). I wanted to test whether the length of the stamen in relation to the pistil differed between outcrossing, facilitated selfing, and autonomous selfing individuals.

In *A. lyrata* there are long and short stamens present that could have different functions in pollen deposition (Harder & Wilson 1998). I wanted to test whether the difference between long and short stamens varied between outcrossing, facilitated selfing, and autonomous selfing individuals and/or there was a difference between populations.

Outcrossing and facilitated self-fertilizing plants would be expected to put more energy into the quantitative and qualitative production of volatiles to attract pollinators than autonomous plants. I wanted to test whether there was a difference between pollinator dependent and independent individuals in terms of volatile compound composition and the amount of volatiles produced and/or there was a difference between populations.

5.2 Methods

5.2.1 Sampling

Seeds were collected in 2003 from four populations in the field, from 20 independent focal plants per population, on the Ontario side of the Great Lakes of North America (Mable & Adam 2007; Mable *et al.* 2005): Pinery Provincial Park (PIN) and Tobermory Singing Sands (TSS) at Bruce Peninsula National Park (BPNP) on Lake Huron; Long Point Provincial Park (LPT) and Rondeau Provincial Park (RON) on Lake Erie (see Figure 2.1 chapter 2). These populations were chosen for comparison because they represented two predominantly outcrossing and two predominantly inbreeding populations.

5.2.2 Germination and Selfing Phenotype Determination

Fifteen seeds per mother from 20 independent focal plants per population were germinated on moist filter paper in petri dishes in a growth cabinet (Sanyo MLR-350: 16 hour light (20ºC): 8 hour dark (15ºC) regime). Seeds that developed green cotyledons and a root were considered to be germinated and five germinated seedlings per mother were transferred to soil and further grown under a 16 hour light (20ºC): 8 hour dark (16ºC) regime in a common greenhouse environment (at the Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam). A single plant per maternal seed family was tested for strength of self-incompatibility by manual self-pollination of six flowers (See chapter 2 for methods). An individual was considered to be autonomously self-compatible (SC) when fruits appeared without manual self-pollination. In order to prevent self-pollination by any other mechanism than autonomous self-pollination, water was added to the soil rather than to the plant stems or leaves and other tactile contamination mechanisms like airflow or insect presence were prevented as much as possible. Previous microsatellite paternity analyses (Chapter 3) showed autonomous selfing even after outcrossing with emasculation after the flower opened.

5.2.3 Morphological character determination

For each mother three flowers were collected on the day that the flower opened (when the stigma and the anthers were revealed) and preserved in 96% ethanol. The timing of measurement of the different morphological traits is crucial, as the pistil will increase in

size as soon as the ovule is fertilized and the fruit starts developing. I measured lengths of four petals, four long stamens, two short stamens, the ovary and the pistil (Figure 5.1 a) per flowering individuals over a period of five weeks. For each petal, I measured the length of the horizontal, visible part (Figure 5.1 b) to the 100^{th} mm with a digital caliper. I measured the stamens, ovary, and pistil from the base of the flower where the stamens and pistil come together (figure 5.1 a). The top of the ovary was where the broad shape narrowed to form the beginning of the style (figure 5.1 a). I calculated one average value per floral trait (petal, pistil, ovary, long- and short stamen) per individual with 12 individuals per population. I used a Principal Component Analysis (implemented in SPSS) to detect the amount of co-variation between the different morphological traits measured and any clustering of populations or autogamous and non-autogamous individuals.

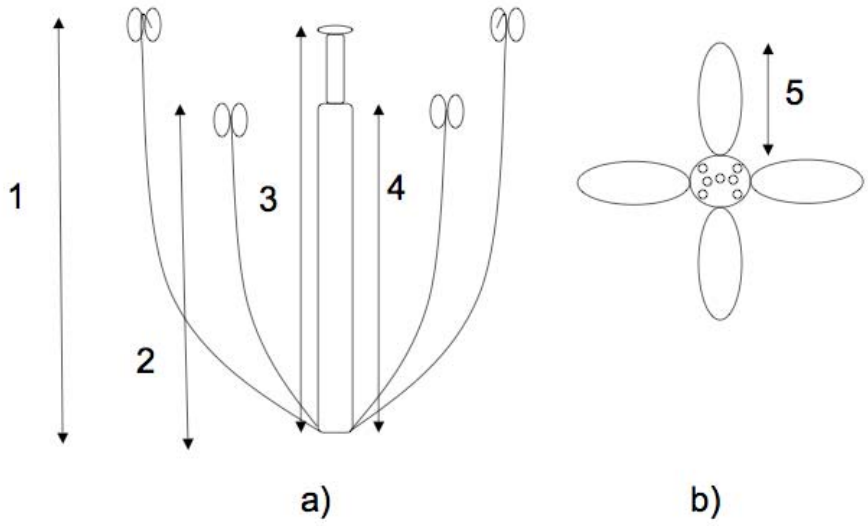


Figure **5.1Schematic representation of the flower morphology of a transverse section** (a) **and a top view** (b) **of an *A. lyrata* flower. Arrows indicate the length of the long stamens** (1)**, the short stamens** (2)**, the pistil** (3)**, the ovary** (4)**, and the petals** (5)**.**

5.2.4 Volatile collection

Headspace measurements of plant volatiles were done for a minimum of 11 plants per population. I had to use siblings of individuals used in the floral measurement experiment in some cases, as not all individuals flowered all the way through the experiment, which also explains dissimilar sample sizes for floral and volatile measurements. These siblings were additionally tested for their selfing phenotype but showed similar results as the individuals used in the floral measurement experiment, therefore these results were not included in the table (Table 5.1). Flowering plants were measured in a closed incubator where filtered air was pumped through for 24 hours. I measured volatiles of non-flowering individuals as a control to confirm that the volatile components measured originated from the floral organs. On the outlet of the incubator volatiles were trapped on a binding matrix (Tenax). Volatiles were eluted with pentane, which serves as a carrier, and injected into a Gas Chromatographer (ATAS 6890N) Mass Spectrometer (LECO Pegasus III) (GCMS). Separation was performed on a silica capillary column (30 m x 0.25 i.d. x 0.25 µm thickness) coated with poly [5% phenyl] methylsiloxane with helium as carrier gas (flow rate 2 ml min^{-1}). Mass spectrometry was performed at an ionization potential of 70eV and scan range of 35 to 350 atomic mass units. Volatile components were identified using reference components in a chromatographic library (LECO Pegasus III software). Detected peaks were only used when they showed twice the signal compared to background noise level.

5.2.5 Volatile analyses

To test for significant differences of volatile compounds and amounts of certain volatile compounds between populations and selfing phenotypes (average of all individuals) I used a non-parametric Kruskal-Wallis test, as the residuals strongly deviated from normality.

5.3 Results

5.3.1 Selfing phenotype determination

Self-pollinations showed populations with high proportions of individuals in the SC class: RON (0.9), LPT (0.65) further referred to as inbreeding populations; and high proportions of individuals in the SI class: PIN (0.84), TSS (0.73); further referred to as outcrossing populations (Table 5.1). As in former chapters I lumped the SI and PC class together into one SI class (chapters 2 and 3). These self- pollination results were consistent with former

results and showed comparable outcomes in terms of inbreeding or outcrossing determination with multilocus outcrossing rates (see Chapters 2, 3, 4). The inbreeding populations showed almost half of their families to be autonomous where the outcrossing populations showed none (Table 5.1).

Table **5.1Populations used in this study ordered by autonomous fruit set ability, self-pollination results, where the proportion of self-incompatible, partially self-compatible, self-incompatible individuals, the number of observed autonomous families, and the number of families tested in a population are indicated for selfing phenotype, flower morphology determination and volatile emission; geographic location is indicated as latitude and longitude expressed in degrees and minutes.**

Population	SI[1]	PC[1]	SC[1]	Number of autonomous	Sample size selfing phenotype and morphology	Sample size volatiles	Latitude	Longitude
TSS	0.6	0.4	0	0	5	15	45˚11’	-81˚35’
PIN	0.8	0.2	0	0	20	12	43˚16’	-81˚49’
LPT	0	0.2	0.8	7	16	15	42˚34’	-80˚23’
RON	0	0.1	0.9	9	12	11	42˚15’	-81˚50’

[1] SI=0 or 1 positive fruits, PC = 2, 3 or 4 positive fruits, SC=5 or 6 positive fruits.

5.3.2 Flower morphology determination

The PCA showed two main principal components (Table 5.2 a, b); PC1 summarized the variance in sexual floral traits (pistil, ovary, long- and short stamens) and PC2 summarized variance in petal length. I observed no clustering among populations or mating system when PC2 was plotted against PC1 (Figure 5.2). There was a significant difference of petal length between selfing phenotypes; SI individuals (mean = 50 mm) had significantly longer petals than SC individuals (47 mm) (df = 1, t-ratio = -3.3, p = 0.001). There was a significant difference of petal length between populations (d.f. = 3, MS = 14680, F-ratio = 6.2, p = 0.0006) where PIN (50 mm) had significantly longer petals than LPT (47 mm) and RON (46 mm) but TSS (48 mm) did not differ from other populations. The autonomous individuals did not show significantly reduced petal size compared to non-autogamous individuals. There was no significant difference of difference in stamen and pistil height between selfing phenotypes but there was a significant difference between populations (t-ratio = 5.7 d.f. = 3 $p < 0.0001$; Figure 5.3); populations TSS (mean = -4.2 mm) and LPT (mean = 0.81 mm) had significantly different pistil and stamen differences than populations PIN (mean = 6.2 mm) and RON (mean = 9.2 mm). There was no significant difference in pistil and stamen difference between autogamous and non-autogamous individuals. There were no significant differences between long and short stamens or ovary and long stamen differences for selfing phenotypes or populations.

Table **5.2**a **Principal component matrix with five principal components (PC) extracted from five morphological characters (petal length, ovary length, pistil length, long anther length, and short anther length) where each PC summarizes variation for a certain trait.**

	PC 1	PC 2	PC 3	PC 4	PC 5
Petal	0.136	0.990	0.027	-0.005	0.001
Ovary	0.916	-0.016	-0.391	0.026	-0.085
Pistil	0.921	-0.026	-0.379	0.016	0.09
Long anther	0.903	-0.063	0.342	-0.254	-0.003
Short anther	0.858	-0.046	0.461	0.224	0.001

Table 5.2 b **Total, percentage and cumulative percentage of total variance (described in eigenvalues which are the variances of the factors) of all five morphological characters explained by each component (factor).**

Component	Initial Eigenvalues		
	Total	Percentage	Cumulative
1	3.26	65.10	65.10
2	0.99	19.75	84.86
3	0.63	12.54	97.40
4	0.12	2.31	99.70
5	0.01	0.30	100.00

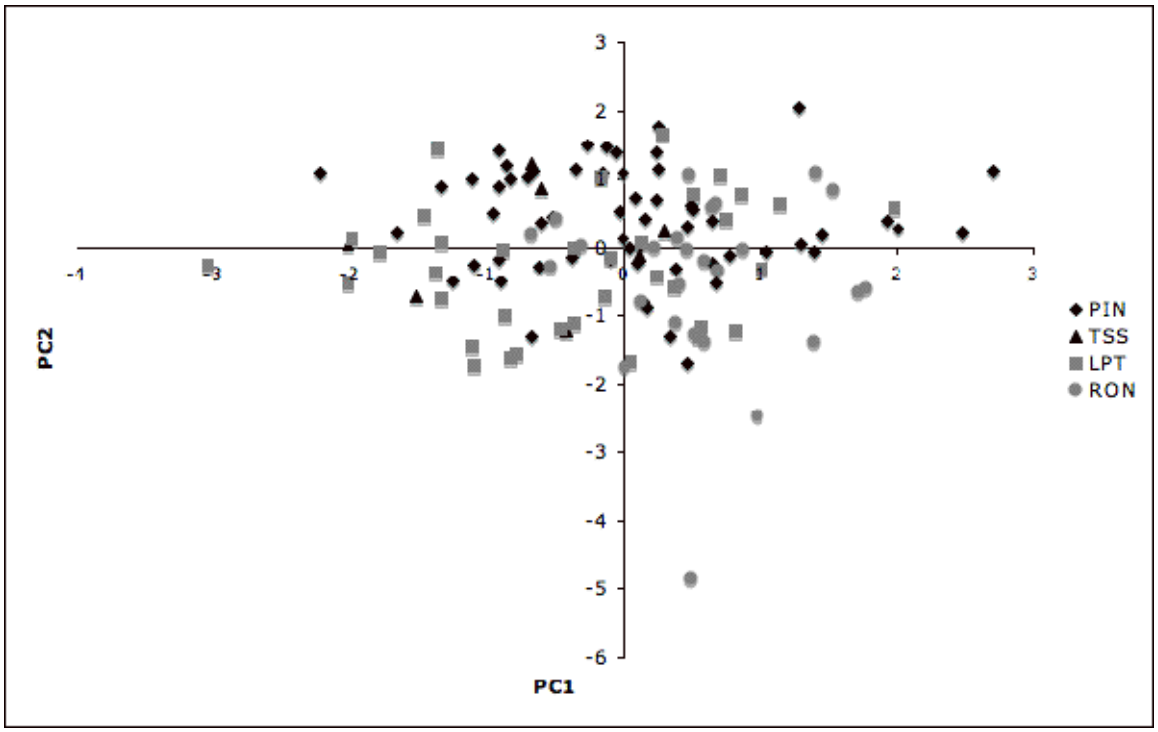


Figure **5.2 PCA plot of predominantly outcrossing populations PIN (black diamonds) and TSS (black triangle), and predominantly inbreeding populations LPT (grey square) and RON (grey circle). Principal Component 1 (PC1) on the x-axis shows the summary of mainly sexual floral traits (pistil, long and short stamen length) and principal component 2 (PC2) on the y-axis shows mainly petal length. There is no particular clustering of population or mating system visible.**

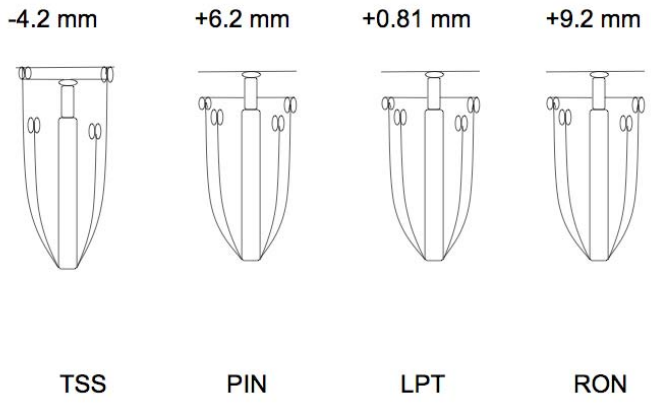


Figure 5.3. Schematic representation of the average flower morphology per population of a transverse section of an A. lyrata flower. Horizontal lines and values above indicate the mean difference in the length of the pistil and the length of the long stamen (mm).

5.3.3 Volatile production

Two dominant volatile components (*p-cymene* and *phenylacetaldehyde*) were identified in all populations. The compound *phenylacetaldehyde* was the dominant volatile component present in the predominantly self-compatible population LPT (detected in 11 out of 15 individuals) but was detected in fewer individuals in the other populations (7 out of 12 in PIN, 3 out of 11 in RON, and 2 out of 15 in TSS). The compound *p-cymene* was detected in all populations (detected in 11 out of 12 individuals in PIN, in 11 out of 15 in TSS, in 8 out of 11 in RON, and in 11 out of 15 in LPT). Population LPT produced significantly more *phenylacetaldehyde* than any other population (χ^2 = 25.5, d.f. = 3, p < 0.0001) (Figure 5.4 a). There was no significant difference for *p-cymene* production between populations.

Across populations, self-compatible individuals produced significantly more *phenylacetaldehyde* then self-incompatible individuals (χ^2 = 11.5, d.f. = 1, p = 0.001) (Table 5.3; Figures 5.4 a and b). There was no significant difference in the production of *p-cymene* between selfing phenotypes. There was no significant difference between autonomous and non-autonomous individuals in the production of *phenylacetaldehyde* or *p-cymene*.

Table 5.3 Mean production of *p-cymene* and *phenylacetaldehyde* per population and mating system in pg/flower/24 hours ± standard error

Population	*p-cymene*	*phenylacetaldehyde*
LPT	0.29 ± 0.1	80 ± 0.7
PIN	0.35 ± 0.01	1.4 ± 0.1
RON	7.8 ± 0.05	0.30 ± 0.02
TSS	33 ± 0.2	0.11 ± 0.01
Mating system		
SI	16 ± 0.1	0.91 ± 0.01
SC	3.7 ± 0.04	35 ± 0.3

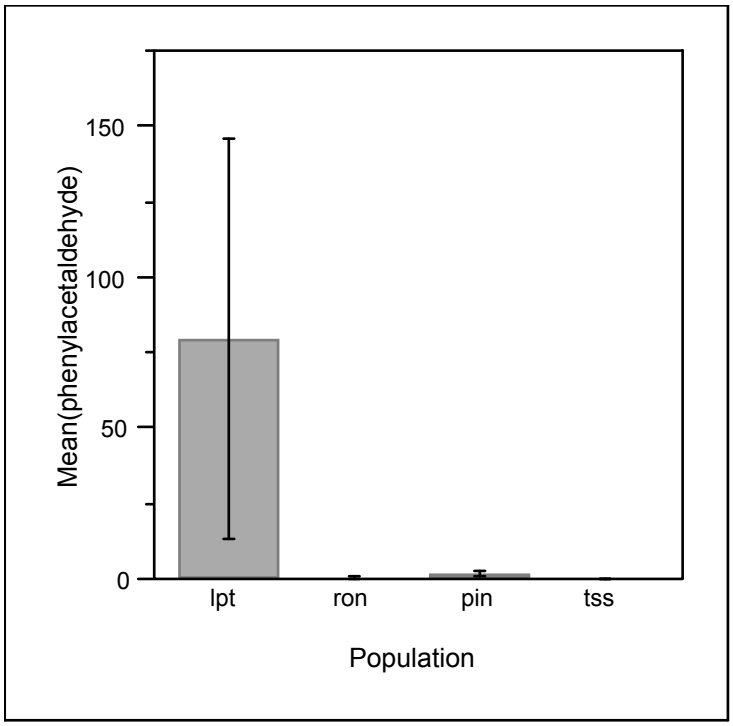


Figure 5.4 a. Bar chart of the mean amount of *phenylacetaldehyde* produced per population (where LPT and RON are inbreeding, and PIN and TSS are outcrossing populations) measured in pg/flower/24hours, with the standard error indicated

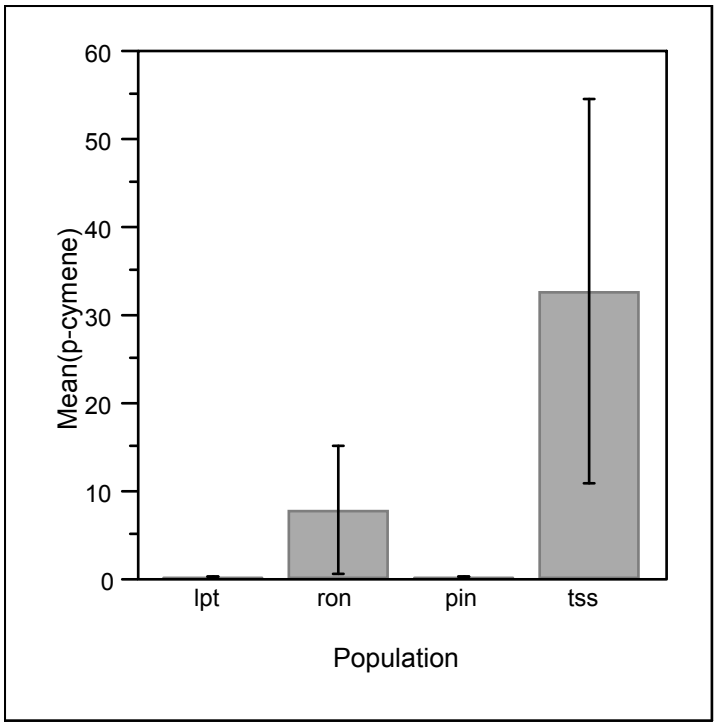


Figure 5.4b **Bar chart of the mean amount of *p-cymene* produced per population (where LPT and RON are inbreeding, and PIN and TSS are outcrossing populations) measured in pg/flower/24hours with the standard error indicated.**

5.4 Discussion

5.4.1 Flower morphology

Petal length was longer in SI than in SC individuals but there was no difference between autonomous and non-autonomous individuals. This would be an expected result to a certain extent, as outcrossing individuals would have to invest in the attraction of pollinators. On the other hand, it would also be expected that facilitated SC individuals would show adaptation towards the attraction of pollinators. Strongly autonomous selfing species such as *Arabidopsis thaliana* also show a strong reduction in petal length compared to outcrossing relatives such as *A. lyrata*. Similarly, within the genus *Capsella* the autogamous selfing species *C. bursa-pastoris* shows a strong reduction in flower size compared to its outcrossing relative *C. grandiflora* (Foxe *et al.* 2009). This could indicate that the facilitated self-fertilizers are autonomous but this could not be detected or they attract pollinator in another way than by petal display.

The difference between pistil length and stamen height showed no significant difference between selfing phenotypes. There was a significant difference between populations in the difference between long stamen length and pistil height. In the case of the outcrossing populations TSS and PIN it is unknown in which spatial position on the pollinator the pollen is picked up and deposited on the stigma of the receiving mother plant. There could be different strategies of stamen pistil positioning that have to do with different pollinators present in different outcrossing populations that would all be successful in translocating pollen from one plant to another. A study on the outcrossing perennial *Polenium brandegei* showed large continuous variation in the positioning of the height of the anthers with respect to the pistil from approach (anthers below pistil) to reverse (anthers above pistil) herkagomy that temporally fluctuated with pollinator abundance (Kulbaba & Worley 2008). Also for *A. lyrata*, different pollinators present at different times and populations could be responsible for a difference in flower morphology (Medan 2003).

For the autonomous selfing individuals in inbreeding populations LPT and RON it is expected that the stamen would be located above the stigma to aid in deposition of the pollen onto the stigma but unexpectedly the anthers were positioned under the stigma. This could mean that insects would still be involved in transmitting the pollen from the anthers to the stigma but it could also mean that the pistil grows through the anthers to cause self-

fertilization (Barrett & Harder 1996a). Autonomous fruit set is a difficult trait to measure as environmental influences (e.g. temperature, humidity) could be responsible for the strength of the self-incompatibility reaction (Pandey 1973). There was a lot of variation observed on maternal level for autonomous fruit set. So if autonomous self-fertilization was falsely observed due to environmental influences this could be an explanation for the lack of observed flower morphology associated with autonomous self-fertilization.

I found evidence showing phenotypic correlation between pistil, ovary, long stamen and short stamen length. A principal component summarizing variance in stamen and pistil length for the inbreeding populations LPT and RON was detected, but there was no particular clustering of these populations compared to the outcrossing populations PIN and TSS (Figure 5.3). A correlation analysis for each floral trait separately also did not show significant differences between autonomous and non-autonomous individuals. In contrast, a study on SI and SC races of *Leavenworthia alabamica* showed that SI races had a stronger correlation between petal and stamen length than SC races (Anderson & Busch 2006). It could be that the shift in mating system from outcrossing to inbreeding is too recent in *A. lyrata* to detect correlations between morphological floral characters (Williams & Conner 2001) or that environmental influences play a role.

Interestingly, the four sexual floral traits, pistil, ovary, long- and short stamen length, were explained by one principal component explaining most of the variance (65%), which indicated the general size of the flower. The second principal component explained most of the variance (20%) associated with petal length for all populations. Petal length thus appeared to co-vary more independently from the sexual floral traits than the sexual floral traits did from each other. When I tested each combination of traits separately for correlation, I found strong correlation between each floral trait with no difference between selfing phenotypes or between populations. This contradicted a study on genetic correlations of floral morphology in *Arabidopsis thaliana* using QTL mapping, where there was a high correlation of pistil, stamen and petal lengths (Juenger *et al.* 2000). On the other hand, a study on the aquatic heterostylous plant *Eichhornia paniculata,* which is exposed to heterogeneous environmental factors during its life stages, showed independent development for individual floral traits dependent on the specific environment, allowing dynamic control of its mating system (Vallejo-Marin & Barrett 2009). A common garden and green house study on *Datura wrightii* showed that environmental factors like water availability and the presence of herbivory influenced petal morphology directly and indirectly outcrossing rate (Elle & Hare 2002). This could mean that in North American *A.*

lyrata, environmental factors influence sexual floral traits and petal size independently and could account for inter-population variation for these traits.

5.4.2 Volatile emission

I found no evidence that individuals that had adopted an autonomous self-fertilizing mating system, produced fewer volatiles than the ones that did not. Another study on the effect of mating system on volatile production in *Cucurbita pepo* subsp. *texana* found that inbreeding reduced both total volatile production and the relative composition, as some compounds showed a reduction where others did not (Ferrari *et al.* 2006). In my study there was a significantly higher amount of *phenylacetaldehyde* produced in SC individuals compared to SI individuals but their role in pollinator attraction was not established. The volatile compound *phenylacetaldehyde* is known to occur in Brassicaceae and has a role in pollinator attraction (Abel *et al.* 2009; Omura *et al.* 1999). It could also be that the combination of certain volatile compounds is more crucial for pollinator attraction than each part separately as a study showed that certain volatile components expressed together with specific other volatile components, would determine the attractiveness of a plant to pollinators (Waelti *et al.* 2008). Additionally, populations RON and LPT still show low levels of outcrossing (see outcrossing rates chapter 2) so if self-compatible individuals from these inbreeding populations were occurring in lower densities, they would be isolated from other co-flowering individuals and would have to invest more resources in attraction of pollinators necessary for self-pollination. However, data from former studies on North American *A. lyrata* showed that there was no difference in density of individual plants between predominantly outcrossing and inbreeding populations (Mable 2008). But these populations could have a strategy of 'bet-hedging' where a small amount of outcrossing, is combined with the reliability of a mating partner by selfing.

6 General Discussion

There are many different strategies in plants to secure genetic material for the next generation. The different reproductive strategies observed in natural populations have evolved due to evolutionary factors working on mating systems of plants. In my study I found that the inbreeding load must have been purged resulting in individuals from inbreeding populations capable of self fertilization without fitness consequences (Growth by number of leaves; sections 3.3.5.2 & 3). Consequently, these populations have lost a severe amount of genetic diversity but there are mixed results about the consequences of this loss.

This thesis reports the results of research on the evolutionary consequences of mixed mating system in *Arabidopsis lyrata* subsp. *lyrata*. The presumable causes and mechanisms proposed in my thesis for a shift in mating system were explained by population history, strength of inbreeding in relation to outcrossing history, pathogen susceptibility, and pollinator densities. It is evident from the population history study that most populations have very low gene flow between them, which could result in local adaptation. In case of inbreeding depression in life history traits, relative fitness showed a negative relationship with the population observed heterozygosity. Population effect explained most of the variation for fitness in the presence of the pathogen *Albugo candida* as outcrossing population PIN showed a level of resistance comparable to inbreeding populations LPT and RON, whereas two other inbreeding populations showed high levels of resistance comparable to the three other outcrossing populations. Presumably local effects of exposure to a pathogen determined this pattern. Variation in volatile production and the position of the anthers with respect to the pistil were explained by a population effect. A study on local adaptation of European *A. lyrata* also showed highly local adaptation for traits like rosette area, survival, and flowering propensity, and were presumably explained by small population sizes in isolated locations (Leinonen *et al.* 2009).

In the next section I will discuss the possible causes, mechanisms, and consequences underlying the causes of a shift in mating system. From my phylogeographic study it became clear that there were three different cpDNA haplotypes of the TrnL-F locus present among the researched populations around the Great Lakes of North America. Almost every population was fixed for a certain haplotype with exceptions in populations TSSA, MAN and IND, where two haplotypes were present. The presence of two haplotypes could be a region of secondary contact as individuals with certain haplotypes were associated with certain selfing phenotypes and showed differences in heterozygosity according their haplotype background.

A loss of SI in populations with a mixed mating system compared to predominantly inbreeding populations seemed to have very different consequences in terms of inbreeding depression, as in populations with a fixed state of self-compatibility (PTP and RON) I found the strongest evidence for purging. These were also the populations that shared a cpDNA haplotype (L1) with LPT and PIN. Populations LPT and PIN, which showed respectively inbreeding and outcrossing mating systems, showed much more inbreeding depression than highly inbreeding populations PTP and RON. I explained this by a difference in outcrossing history estimated from population level observed heterozygosity. Outcrossing history of the mother seemed not to explain purging but population levels did. Populations that shared cpDNA haplotype S1 showed significantly fewer individuals with a loss of SI than populations with haplotype L1, and inbreeding in these individuals showed a severe decline in fitness. There was a difference between self-compatible individuals that had cpDNA haplotype S1 compared to L1 for individual heterozygosity levels, indicating a different history of outcrossing between these haplotypes.

Pathogen susceptibility seemed to be quite severe in certain inbreeding populations (LPT and RON) compared to other strongly inbreeding populations (PTP and KTT). It is interesting that population RON and PTP seem indistinguishable from microsatellite and cpDNA data but showed very different responses to pathogen infection. It might be that PTP experienced a selective sweep due to a pathogen, which RON did not experience. In inbreeding populations there were more susceptible individuals than in outcrossing populations. In outcrossing populations there were more partially resistant individuals than in inbreeding populations and heterozygosity levels in outcrossing populations were significantly higher than in inbreeding populations. This could indicate that there is more than one gene involved in resistance or that more than one allele is involved in resistance with co-dominant states in heterozygotes that show intermediate resistance.

As for the flower morphology there was no particular floral sexual trait explained by mating system but rather a population specific effect. There was no evidence of flower morphology expected for autonomously selfing plants but as the study on inbreeding depression showed that outcrosses in many mothers from the PTP and RON populations turned out to be selfings after microsatellite paternity analysis, there must be a mechanism causing self-fertilization in an early developmental stage in these populations. Mating system was an explanatory factor for pollinator attractants but the values were in the direction of the inbreeding individuals as they showed significantly higher volatile (*phenylacetaldehyde*) emission than outcrossing individuals but for petal size in the direction of the outcrossing individuals as they showed larger petals than the inbreeding

individuals. Although the biological significance of *phenylacetaldehyde* was not tested with behavioural insect attractant experiments, in other studies *phenylacetaldehyde* was found to be an insect attractant. However, local adaptation seemed to play a big role, as the high volatile emission was dominantly present in population LPT. Population LPT could still depend on insects for self-fertilization or outcrossing as the population genetics showed higher outcrossing rates and heterozygosity than RON and PTP, which could be due to a more recent loss of SI in LPT than in RON and PTP.

The high *Fst* values indicate isolation of most populations but the exact time since isolation is hard to determine, as microsatellites evolve too fast and chloroplast markers too slowly to determine this. As these plants are mostly confined to successional landscapes where they meet little competition, it could be that habitat destruction of postglacial dune landscapes could have played a role in their isolation. The exact origin of the populations around the Great Lakes is not clear. The origin of the species is believed to be central Europe, where most of the genetic diversity is found (Koch *et al.* 2005). Present day *A. lyrata* around the Great Lakes could have come from southern refugia, which meant that the species arrived in North America before the last ice age. Alternatively, they could have come from Eurasia after the last ice age following the retreating Laurentide ice sheet but this colonization route is not fully resolved yet (Schmickl *et al.* 2008a). North American *A. lyrata* differ from European populations in their ability to experience a breakdown of SI without the same amount of inbreeding depression (Karkkainen *et al.* 1999), as for example, they do not show the same proportions of chloroplast deficient offspring after (enforced) selfing. This would explain multiple losses of SI, as observed in different cpDNA haplotypes, as fitness costs are not so severe and would ease the transition from SI to SC. Additionally, the observed inbreeding depression in North American *A. lyrata* takes place late in life, which would allow inbred plants to succeed in circumstances with little competition. This could also make the subspecies *A. l. lyrata* more successful colonizers than subspecies *A. l. petreae,* as flexibility in selfing phenotype would give a reproductive assurance at low densities of conspecifics. The European subspecies is mostly confined to undisturbed rocky outcrops in mountainous areas as opposed to successional sand dune landscapes in North America. These ecological differences put different evolutionary pressures on the European and the North American subspecies and could result in a more flexible mating system to cope with heterogeneous environments.

Unanswered questions and future directions

Pathogens

Following up on the pathogen experiments, it would be interesting to see what underlying genetics are responsible for resistance against *Albugo* species. There has already been some studies on the population genetics of European *A. lyrata* and Powdery Mildew (*Erysiphe graminis*) showing different levels of resistance, which could not be explained by geography but rather by local adaptation (selective sweeps) (Jorgensen & Emerson 2009). A gene that is responsible for controlling White rust (*Albugo candida*) resistance in *Arabidopsis thaliana* is WRR4, which encodes a protein that confers resistance against white rust (Borhan *et al.* 2008). Sequencing the loci responsible for *Albugo* resistance in *A.l. lyrata* could show the actual variation present for the R genes and would give a better answer on the implication of mating system variation on pathogen susceptibility.

Inbreeding depression

As a follow up on my inbreeding depression experiments, it would be interesting to test the amount of inbreeding depression in enforced selfings of individuals with a working SI system over several generations. To test whether the inbreeding load was associated with certain *S*-alleles, it would interesting to genotype *S*-alleles and compare them against the amount of inbreeding depression they are associated with. It would be interesting to compare this against European *S*-alleles and possibly shed light on the difference in observed inbreeding depression between these two subspecies. However, as S-alleles are relatively short sequences and have similar homologues throughout the genome, genotyping is difficult which is the reason why I have not been able to genotype some individuals.

Ecological knowledge and field experiments

In addition to all the genetical and experimental studies on North American *A. lyrata*, this would be a good opportunity to increase the ecological knowledge as there is still not much known about the exact ecological differences between populations with different mating

systems, for example. An ecological survey could show differences in for example pH, rainfall, or soil type between populations. Survival of individual plants (annual vs. perennial) could give insight into the longevity of individuals, which could reveal whether mating strategies change to cope with low pollinator densities in certain years. Clonal growth could also be a factor that influences long term survival as an alternative reproduction mechanism in case of low pollinator densities. Transplantation experiments could reveal local adaptation in fitness related traits.

Pollinator attraction

Due to circumstances like bad weather and failing logistics I was not able to perform a more extensive study on the role of pollinator attractants in particular volatile emission. It could be that inbreeding could play a role on the quality or the amount of volatiles produced (Ferrari *et al.* 2006). In order to determine the biological significance of the volatiles produced, it is necessary to test the isolated compounds measured against pollinator attraction in an experimental choice (Y-tube) setup. Additionally, field experiments with individual plants, that are determined for volatile emission, are needed to test pollinator behaviour on the volatile compounds released by the plants.

Herbivory

Next to my experiment in which I infected different populations with the pathogen *A. candida* to look at inbreeding depression in a stressful environment, the impact of herbivory would be an alternative approach. It would be interesting to see how effectively inbred and outcrossed generations respond to herbivores. In combination with that it would be interesting to see whether these different mating systems release different 'warning signals' or trigger different defence mechanisms.

Broader implications

Overall, the observation of purging and the patterns of pathogen susceptibility show that inbreeding is not necessarily 'bad' in North American populations of *A. lyrata*. It seems more of a reproductive strategy, possibly when outcrossing is not an option. There are many studies that have found similar results in terms of purging of the inbreeding load but also many others that found opposing results as inbreeding depression was maintained even after several generations of inbreeding. Generally, plants are more flexible than most other higher organism with their mating system, as they are mostly hermaphrodites. In

studies associated with conservation genetics the consensus is mostly that the loss of diversity, through habitat destruction for example, can only be resolved due to outbreeding with conspecifics from other populations. Associated with this is the 'dead end' hypothesis that describes the loss of variation resulting in the inability to adapt to changing environmental conditions (Takebayashi & Morrell 2001), which remains to be tested in species that do not seem to be affected by severe inbreeding like for example wild Barley (*Hordeum vulgare*) (Verhoeven *et al.* 2004), Soay sheep (*Ovis aries*) (Overall *et al.* 2005), and the Mauritius Kestrel (*Falco punctatus*) (Ewing *et al.* 2008).

7 Appendix

Additional data Chapter 2:

Table app1 Microsatellite primers used for genetic diversity study and progeny arrays indicating the forward and reverse primer sequence, the repeat unit sequence, dye incorporated in the forward primer and allelic size range (from (Mable & Adam 2007)

Primer*	Sequence (5'-3')	Repeat unit	Dye	Size range
ADH-1	ACCACCGGACAGATTATTCG	GGT	NED	300–354
	CCCAGAAGTAAACATCGGTGTG			
ATTS0392	TTTGGAGTTAGACACGGATCTG	AAG	HEX	140–157
	GTTGATCGCAGCTTGATAAGC			
ICE12	CTCATGGCAAAAGAGGGAAA	CT	HEX	222–238
	GCTCTCTCACCTCGAACGTC			
ATHZFPG†	TTGCGTTTCCACATTTGTTT	CT	6-FAM	138–158
	TGGGTCAATTCACATGTAGAGA			
F20D22	CCCAAGTGACGTCTGGTTTC	GTTT	6-FAM	171–195
	AACAAAATGAGTTTCTCTGCATG			
ICE9†	TTCCTTGCTCAAATTGAAGG	CTT	6-FAM	117–120
	TTTCCCCACACAAAATCTCC			
LYR104	CTCCATCATCGATCTCAGCA	TTC	HEX	128–131
	GAGGCGAATGTAGTGGAAGG			
LYR133	GTTGCTGCTGCTGATGGTT	GTA	NED	131–134
	CAAGGAAGGCAGCAAAGAAA			
LYR417†	AATCCCATCTCTTTCCGCTT	CAT	6-FAM	174–190
	GGAAGGAGAACCAACGATCA			

*The first six primers were from Clauss et al (2002); the last three from V. Castric & X. Vekemans. † Not used in progeny arrays

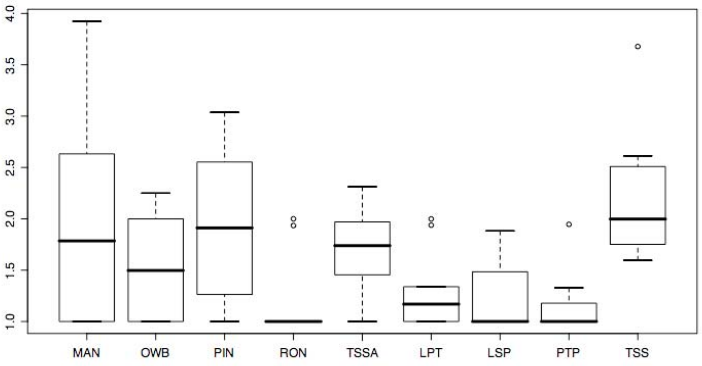


Figure app3a a Box plot of rarefacted mean number of alleles (y-axis) per population across loci. Boxes show 95% interval, whiskers show 5% of the data, horizontal line is the median, and circles represent outliers.

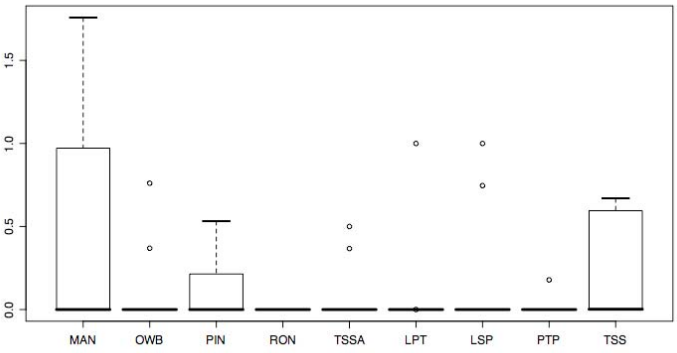


Figure app3b Box plot of rarefacted mean number of private alleles (y-axis) per population across loci. Boxes show 95% interval, whiskers show 5% of the data, horizontal line is the median, and circles represent outliers.

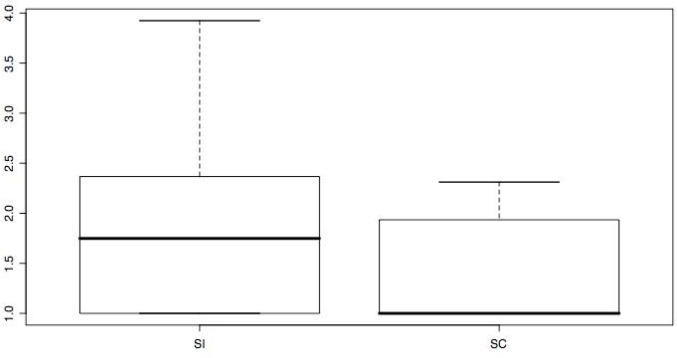


Figure app4a Mean number of alleles (y-axis) per mating type across loci. Boxes show 95% interval, whiskers show 5% of the data, horizontal line is the median, and circles represent outliers.

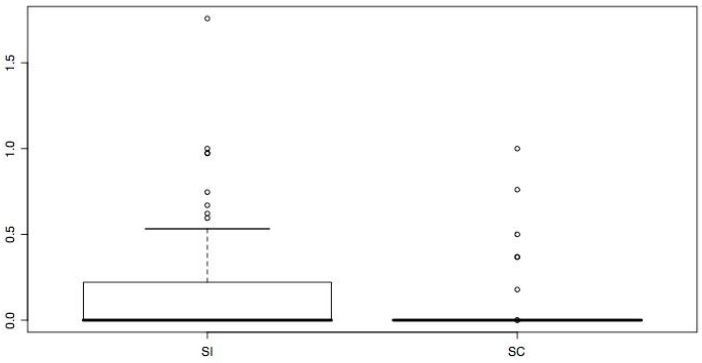


Figure app4b Mean number of private alleles (y-axis) per population mating type across loci

Table app5 AMOVA based on pairwise Fst. The corresponding F-statistics estimated were Fst=0.64, Fsc=0.59, and Fct=0.13. Groups are SI and SC populations.

Source of variation	Sum of squares	Variance components	Percentag variation
Among groups	66.923	0.19807	13.06
Among populations within groups	206.054	0.77382	51.03
Within populations	183.540	0.54463	35.91
Total	456.517	1.51652	

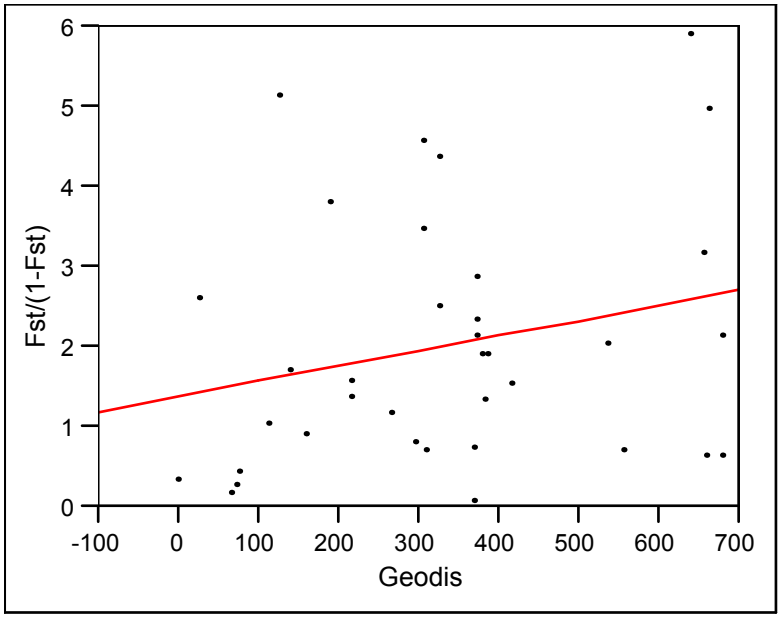


Figure app5 Isolation by Distance plotted with geographical distance in km on the x-axis and genetical distance on the y-axis

Table app7 Estimation of average null allele frequency per population (Est pop) and per locus (Est loc).

Populations	Est pop	Locus	Est loc
MAN	0.18	ADH	0.07
OWB	0.17	AtHz	0.25
PIN	0.07	ATTS	0.01
RON	0.11	F20d22	0.05
TSSA	0.10	ICE12	0.15
LPT	0.24	Lyr104	0.04
LSP	0.27	Lyr133	0.09
PTP	0.14	ICE9	0.32
TSS	0.07	Lyr417	0.36

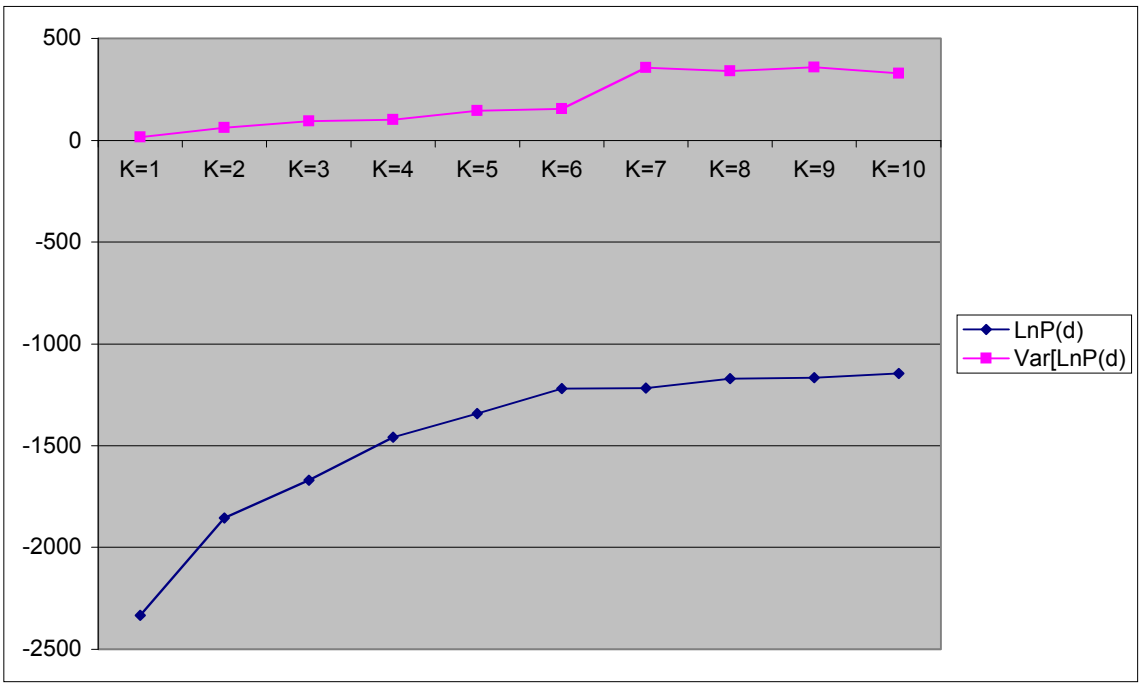


Figure app6 a

Distribution of the –Ln probability of the microsatellite data (LnP(d)) given a K (number of populations) and the variance (Var[LnP(d)]) including missing data values using STRUCTURE.

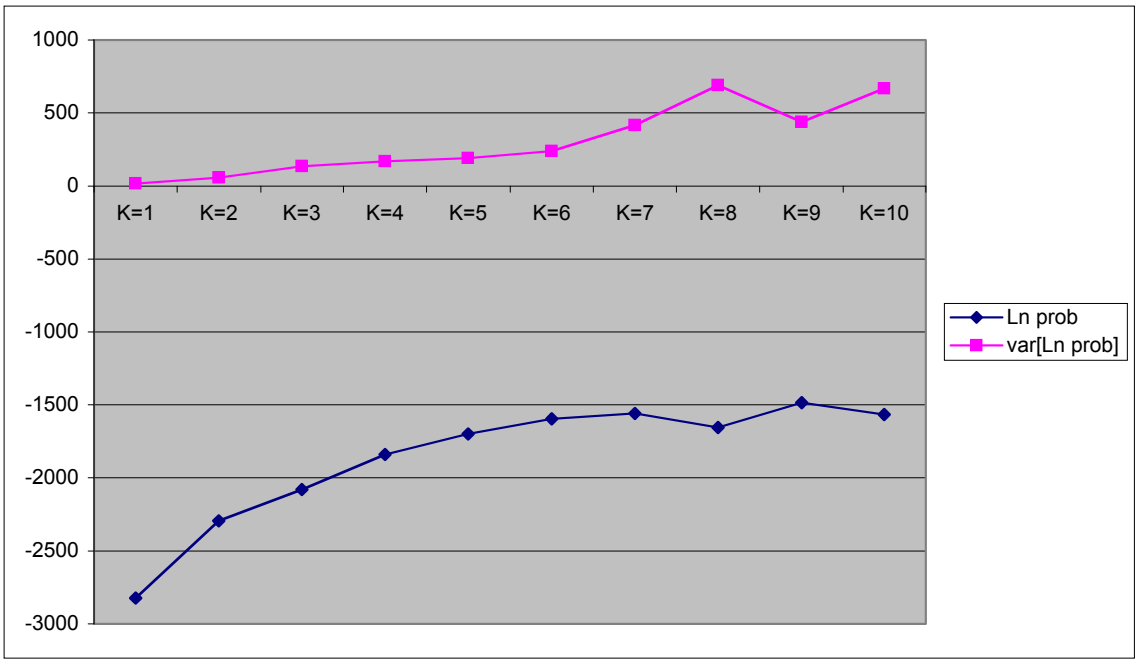


Figure app6 b

Distribution of the –Ln probability of the microsatellite data (LnP(d)) given a K (number of populations) and the variance (Var[LnP(d)]) including missing data values replaced by an assigned allele using STRUCTURE.

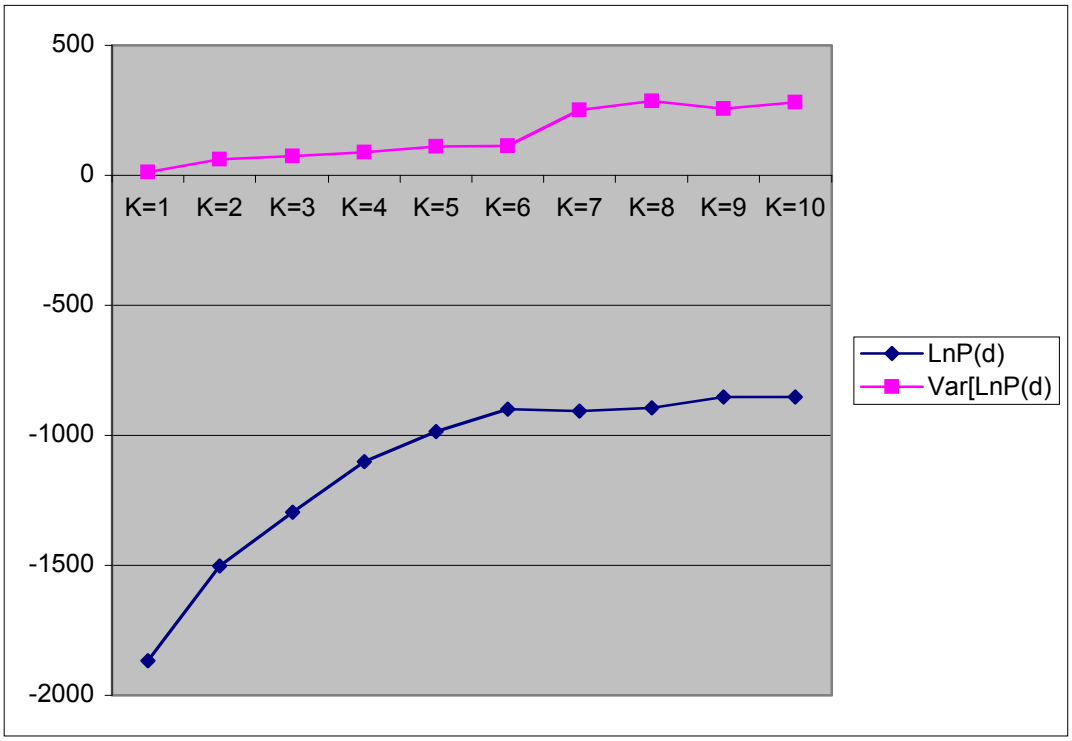


Figure app6 c

Distribution of the –Ln probability of the microsatellite data (LnP(d)) given a K (number of populations) and the variance (Var[LnP(d)]) excluding loci with missing data values using STRUCTURE.

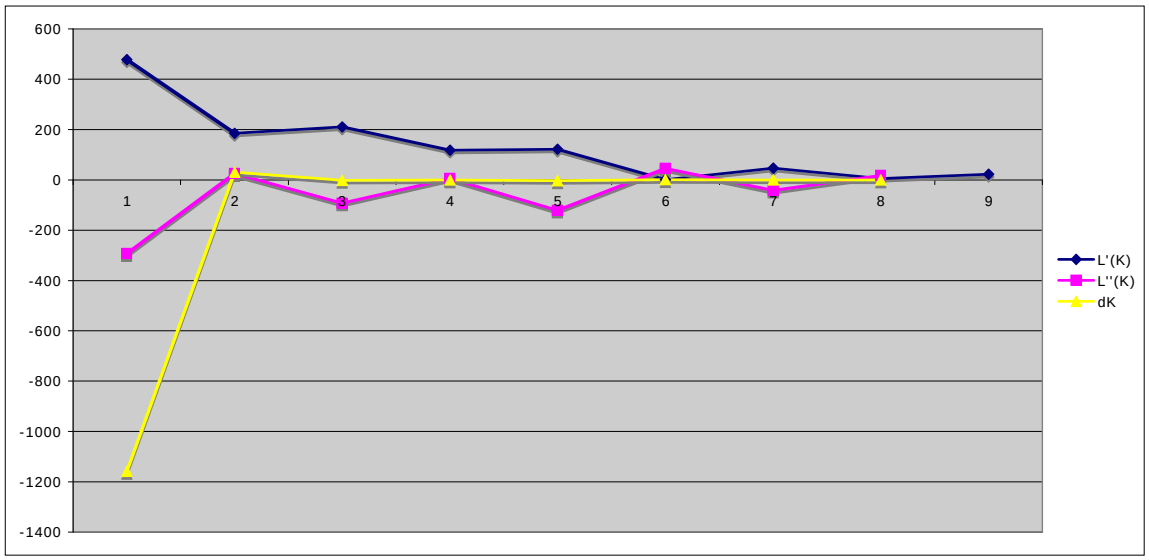


Figure app6d

Distribution of the first L’(K) (circles) and second L’’(K) (squares) order derivative of the distribution of –LnP(d) and the dK (L’’(K)/standard deviation) (triangles) including missing data values using STRUCTURE.

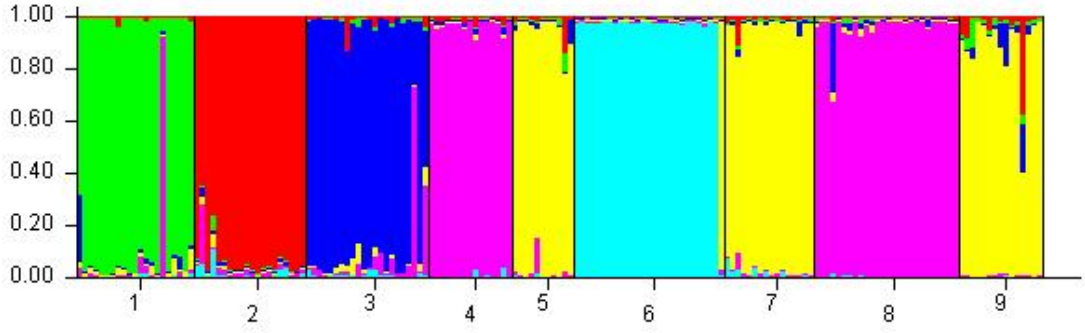


Figure app7a STRUCTURE plot with K=6 and including missing data values. Numbers on the x-axis indicate different populations (1=MAN, 2=OWB, 3=PIN, 4=RON, 5=TSSA, 6=LPT, 7=LSP, 8=PTP, 9=TSS for figures jj until jj g). Same colours indicate individuals assigned to the same cluster.

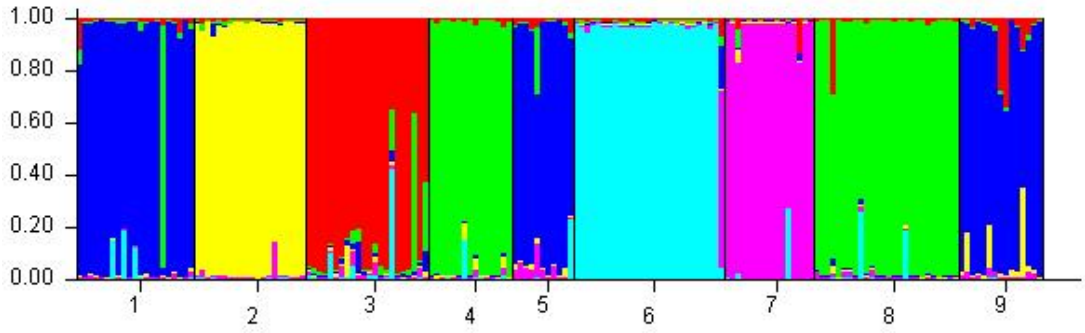


Figure app7b STRUCTURE plot with K=6 with assigned alleles for missing data values.

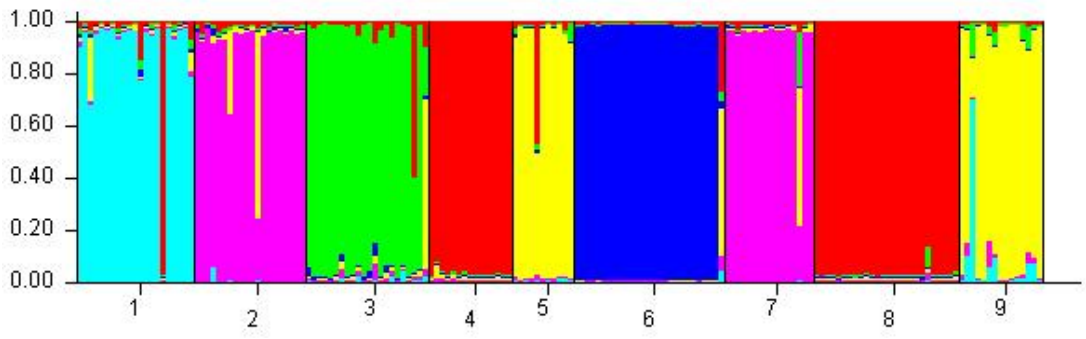


Figure app 7c STRUCTURE plot with K=6 and excluding loci with missing data values.

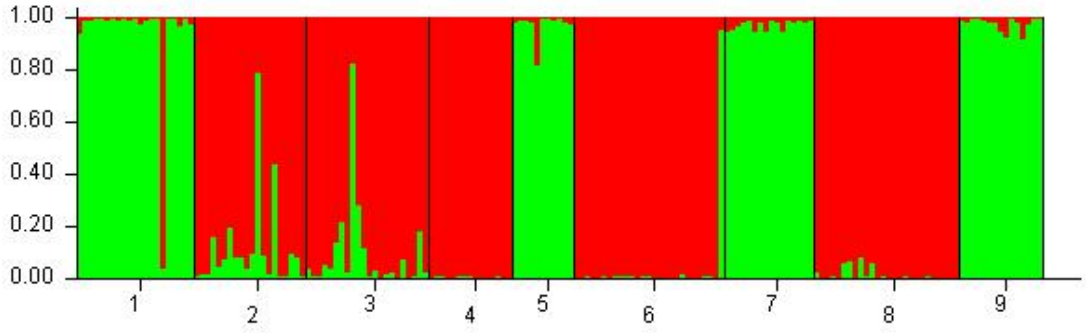


Figure app 7 d STRUCTURE plot with K=2 and including loci with missing data values.

Chapter 3 Correlation fruit lengths and number of seeds per population:

The r^2 (amount of variation explained) and relationships between fruit length and number of seeds for different populations showed: $R^2 = 0.91$, $y = 0.0092x^2 + 0.67x - 5.68$ for OWB (Figure 3.1); $R^2 = 0.59$, $y = 0.11x^2 – 1.48x + 6.26$ for TSS; $R^2 = 0.60$, $y = 0.018x^2 + 0.33x – 1.29$ for MAN; $R^2 = 0.74$, $y = 0.13x^2 – 1.35x + 7.08$ for PIN; $R^2 = 0.56$, $y = 0.12x^{1.70}$ for PTP; $R^2 = 0.11$, $y = 1E\text{-}05x^2 + 0.55x + 3.07$ for TSSA; $R^2 = 0.74$, $y = 0.0011x^2 + 1.49x - 5.89$ for RON.

Inbreeding depression & pathogens

Methods:

In order to evaluate inbreeding depression in relation to history of inbreeding in natural populations, I designed an experiment in which I compare outcrosses and (forced) selfcrosses from self incompatible and self compatible plants. I first germinated ten seeds from one mother and up to 29 mothers per population from nine populations, which had previously been characterised for outcrossing rates. Germination rates and times were recorded for all individuals in order to see whether there were any differences between individuals differing in their mating system. All surviving progeny were grown to flowering and leaves collected for DNA extractions, which will be used to establish outcrossing rates based on microsatellite variation (according to Mable et al. 2005). I kept one plant per maternal line and monitored the extra plants for a natural pathogen infection trial to see whether certain maternal lines or individuals were more affected by certain pathogens.

Previous observations have suggested that populations classified as self compatible (SC) show a high proportion of individuals that can set selfed seed without a pollinator (autogamous fruit set, Mable, personal observation). In order to assess whether this could be used as a reliable indication of the shift in mating system, autogamous fruit set was recorded for all individuals that flowered, A single individual per maternal field plant was used to evaluate the strength of self-incompatibility based on enforced self-pollinations. I tested each parental plant for self compatibility by looking for the ability to set selfed seed, by selfing six flowers per plant, and scoring fruits (negative if there was no seed, small if there were only one or two seeds and full fruit if the silique contained more then three seeds) and monitoring the ability of setting autogamous fruits (self fertilization without the necessity of pollinators). Any selfed fruits produced were collected and measured as a relative fitness measure. The length of the fruit is correlated with the number of seeds it contains. The fitness measure is defined as the ratio of the observed number of seeds and the expected number of seeds. Fruit lengths were measured with a digital calliper in order to confirm classification as negative, small or positive (where negative has no seeds, small a fruit with no more then three seeds and positive a fruit which exceeds three seeds) and as an indication of maternal fertility. For self-compatible plants, seeds from positive fruits can be used to generate selfed lines but for self-incompatible plants it has been necessary to develop a method to bypass the SI system because it is not possible to obtain full length fruits from selfings, using individuals that are strongly self incompatible (SI). This was

achieved by enforced selfings of SI plants in a CO_2 rich environment. Six flowers of an individual plant were selfed and placed in a container where 5% CO_2 rich air was led through for up to six hours. After this the plants were placed back in the green house and fruits were collected after two to three weeks.

For the outcrossings within populations I used up to three different fathers per plant and three flowers per father to detect strong paternal effects. I emasculated the flowers of the mother by removing the anthers as soon as the flower opened. I then pollinated the stigma of the mother plant with the paternal anthers. Resulting fruit was collected and measured. The length of the fruit is correlated with the amount of seeds they could contain and I used this to calculate the ratio of the actual amount of seeds and the expected amount of seeds as a fitness measure.

From these crosses I grew up the seeds (F1) and I am doing the same measurements again. I am also measuring the length and the width of three leaves of each seedling two weeks after germination. I will repeat these measurements five weeks after germination.

I monitored all plants from the parental generation, which were grown up to flower, for natural infection development as they got infected with powdery mildew and white rust.

A novel part of this experiment will be using pathogen response as a measure of relative fitness between outcrossed and inbred lines of progeny. Seedlings will be infected with different pathogens like powdery mildew and white rust and monitored for the effects on mortality and leaf size.

Results

Germination trial

I observed average germination times (Table 1) between populations from the parental generation ranging from 11.6 (MAN) up to 22.2 (TSS) days. The average germination success (Table 1) ranged from 14 % (MAN) up to 78% (TSS).

SI strength

I tested the maternal populations for their strength of self-incompatibility and found that no population was 100% self incompatible (Table 2). Two populations showed a very high frequency of self-compatible individuals (RON: N=19, % SC = 95; PTP:,N=19, % SC = 100) (Table 2).

This indicates that every population is predominantly SI or SC but the ratios of SI and SC plants within a population differ.

Fruit Lengths

There was no difference in average fruit length between SI and SC populations when selfed and outcrossed fruits were pooled (Table 3). Later analyses with more samples will take a closer look at the ratio of observed and expected number of seeds from selfed and outcrossed fruits from SI and SC individuals.

Pathogen Response in Relation to Inbreeding

The inbreeding populations PTP (43%, N=160), TSSA (27%, N=93) and RON (23%, N=86) had the highest rates of infection. The populations OWB (6%, N=134, SC), LSP (10%, N=131, SI) and PIN (11%, N=203, SI) had the lowest infection rates (Table 4). Pathogen infection rate was, however, very variable both within and between populations. The infection rate seemed to affect certain families more severely, which is indicated by the observation that neighbouring plants did not necessarily infect each other but certain maternal lines seemed to be more vulnerable to certain pathogens. This could be an indication that the susceptibility to certain pathogens is genotypically determined. The fact that the most inbred populations have the highest rate of infection could indicate that homozygous individuals are more vulnerable for infection.

Flower morphology

Methods:

Petal lengths of SI, PC and SC plants of the paternal generation were measured. I measured the visible part of each petal and three flowers per plant with a digital caliper to the 100^{th} mm. I used a one-way ANOVA to look for significant differences between SI, PC and SC plants.

Currently I am measuring more petal lengths together with stamen and pistil lengths of the parental generation.

Headspace measurements of plant volatiles were done on four populations (PIN (SI), TSS (SI), LPT (SC) and RON (SC)) with 20 plants per population. Each plant was tested for their mating system by selfing six flowers per plant as described in the former chapter. Plants were kept in a closed incubator where filtered air was led through for 24 hours. On the outlet volatiles were trapped on a binding matrix, which were later on eluded with pentane and injected in a Gas Chromatographer Mass Spectrometer (GCMS). Volatile components were identified using reference components in a chromatographic library.

Results

Petal length

I found no significant difference between one group consisting from SC and PC individuals and the other group consisting from SI individuals (N=64, P=0.16).

There was a significant difference though between predominantly SI and SC populations (N=30, P=0.003).

Volatile production

There seemed to be two dominant volatile components (p-cymene and phenylacetaldehyde) among all populations. I used a non-parametric Kruskal-Wallis Test as the data were not normally distributed. The volatile component p-cymene did not differ significantly between populations (N=96, P=0.9393) or between SI and SC individuals (N=96, P=0.4021). Phenylacetaldehyde differed significantly between populations (N=96, P<0.0001), LPT (which is highly selfing) the highest mean production of phenylacetaldehyde and TSS (which is predominantly outcrossing) had the lowest mean production of phenylacetaldehyde. Also SC individuals produced significantly more phenylacetaldehyde then SI individuals (N=96, P=0.0004) (Table 5).

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