Microbotryum violaceum on *Silene dioica*

- understanding traits that influence plant-pathogen interactions

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ABSTRACT: The dynamics of a plant-pathogen interaction vary both within and among species. Both spatial structuring and specific genetic and life-history characteristics will affect the interaction and the outcome of a potential co-evolution between the two organisms. In this thesis I have studied the interaction between the wild perennial herb *Silene dioica* and its automictic, obligate anther smut *Microbotryum violaceum MvSd*. From the plant perspective, I have examined different aspects of biochemical resistance in *S. dioica* to *M. violaceum MvSd*. From the pathogen perspective, I have focused on the breeding system of *M. violaceum MvSd* and its connection to fitness and distribution of genetic diversity. I have used varying methods; glasshouse trails involving inoculation of plants with the pathogen, classical Mendelian analysis involving controlled crosses between plants, microscopic studies of spores and molecular DNA-analysis.

With the results I demonstrate that resistance to *M. violaceum MvSd* in *S. dioica* can be specific to the attacking pathogen strain and also spatially highly diverse both within and among populations within a metapopulation. Together, these factors are likely to delay the establishment of the disease within host populations and reduce the spread and amount of disease, once it has been established. The results also suggest that the specific resistance expressed against two different *M. violaceum MvSd* strains were determined by separate gene systems and that, in both cases, the resistance was simply inherited. This implies a potential for relatively rapid response to *M. violaceum*-induced selection in *S. dioica* populations variable for resistance.

My results also show that automixis clearly is the predominating breeding system of *M. violaceum MvSd*, similarly to what earlier has been shown for *M. violaceum MvSl*. Furthermore, I found lower levels of neutral genetic diversity in *M. violaceum MvSd* in the northern parts of Sweden, compared to what has been found in populations in more southern Europe. This result is consistent with predictions that populations in the outer regions of a species distribution have lower levels of genetic variation. Moreover, populations were highly differentiated in northern Sweden, which could have been generated by high selfing rates, genetic drift and high population turnover rates, all factors that coincide with life-history and ecology of *M. violaceum MvSd*. However, despite the general low variability in neutral genetic markers, I did find variation in fitness related traits, both within and among populations, as well as differences in infection ability between strains, suggesting there is a potential for co-evolution between *S. dioica* and *M. violaceum MvSd* in the area.

To summarize, this thesis reflect a plant-pathogen system that is highly influenced by constant colonisation-extinction dynamics, which is likely to have influenced both the genetics of resistance in the plant and the breeding system of the pathogen and thus also the interaction between the two organisms.

KEYWORDS: *Silene dioica*, *Microbotryum violaceum*, plant-pathogen interaction, biochemical resistance, breeding system, automixis, race specific resistance, extinction-colonisation dynamics

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Cover: Front: Flowers of male *Silene dioica*
Back: Flowers of *S. dioica* filled with spores of *Microbotryum violaceum*

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LIST OF PAPERS

This thesis is a summary and discussion of the following papers, which will be referred to in the text by their Roman numerals.

I Giles, B. E., Å. Granberg and U. Carlsson-Granér. **Race-specific and spatially variable resistance to *Microbotryum violaceum*, a systemic anther smut disease in *Silene dioica* metapopulations.** *Manuscript*

II Granberg, Å., U. Carlsson-Granér, and B. E. Giles. **Genetic architecture of biochemical resistance to the anther smut *Microbotryum violaceum* in *Silene dioica*.** *Manuscript*

III Granberg, Å., U. Carlsson-Granér, Per Arnqvist and B. E. Giles. **Variation in breeding system traits within and among populations of *Microbotryum violaceum* on *Silene dioica*.** *International Journal of Plant Science (In press)*

IV Granberg, Å., U. Carlsson-Granér, and B. E. Giles. **Neutral gene diversity in the range margins of an obligate fungal pathogen: *Microbotryum violaceum* on *Silene dioica*.** *Manuscript*

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BACKGROUND

Almost all living organisms in nature interact somehow with other living organisms. When differences in fitness between individuals within one species depend on the phenotype of the other species, and vice versa, and when the variation in fitness is heritable, the reciprocal influences might lead to co-evolutionary adaptations. One of the most examined types of co-evolutionary interactions is the one between plants and their fungal pathogens.

Many plant pathogens are of great interest since they attack economically important crops. Plant pathogens are also likely to be present in all natural plant communities (Harper 1990) and local epidemics might develop. However, the total effects of pathogens in natural communities are seldom very drastic. Nevertheless, there are some classical cases where pathogens have caused effects that dramatically changed plant compositions in natural systems. For example, in 1904 the chestnut blight *Cryphonectria parasitica* started to spread in populations of American chestnut (*Castanea dentata*), which at the time was the dominating tree species in eastern North America. Fifty years later the chestnut was a rare species, and the forests were dominated by other tree species. Dutch elm disease (*Ophiostoma ulmi*), which started to spread among elms (*Ulmus ssp.*) in Europe and North America in the beginning of the 20th century had similar effects. Both these cases are examples of situations where the pathogen was introduced and thus non-native to the area where it caused the large-scale epidemic.

The dynamics of a plant-pathogen interaction vary both within and among species. In this thesis, the word "dynamic" is used as a term referring to the temporal and spatial fluctuations within a system. The total dynamic within an interaction includes several components. Numerical fluctuations of the plant and the pathogen, as well as the fluctuations in gene frequencies are included in the total dynamic. But also changes in genetic variability and ability to adaptation, the spatial and temporal changes of where co-evolution takes place within the range of a plant-pathogen interaction, c.f. co-evolutionary hot and cold spots (Gomulkiewicz et al. 2000) and which of the interacting species that has the co-evolutionary overtake at any time and place contributes to the dynamic within a plant-pathogen system.

Today, when climate is changing rather rapidly and movement of both humans and other organisms is increasing, the dynamics of existing plant pathogen system can be expected to change, and novel interactions, with unknown dynamics, are likely to become more common (Parker and Gilbert 2004; Burdon et al. 2006). To be able to predict what might happen in such situations and possibly prevent unwanted epidemics, it is of great importance to acquire knowledge about the factors that affects the interactions and dynamics within plant-pathogen systems.

PLANT PATHOGEN INTERACTIONS IN A SPATIAL CONTEXT

Natural distributions of plants and their pathogens are normally patchy within a landscape. The basic underlying factor restricting the spatial structure of a plant-pathogen interaction is distribution of suitable habitat for the plant. This distribution is a result of the combination of spatial variation in abiotic factors, like climate and nutrients, and biotic factors, like the composition and age of the ecological community (Burdon et al. 1989). Similarly, the presence of the pathogen among the patches suitable for the plant is also controlled by basic abiotic and biotic factors (Burdon et al. 1989). For an interaction to be possible, both players have to have their basic requirements fulfilled. However, this does not mean that all patches suitable for both players are always occupied by them both. Instead, natural populations of most plants are ephemeral and form complex networks interconnected by migration, so called metapopulations, with different local populations being more or less isolated from other local populations. Often the natural pathogens of plants also occur in this scattered, ephemeral pattern, constituting their own metapopulation in the same area as their hosts. Under these circumstances, persistence at a regional scale depends on the balance between extinction of local populations and colonisation of empty patches (Giles and Goudet 1997a) by both players.

The spatial structure of suitable habitat could be thought of as the play-ground on which the game of interaction between the plant and the pathogen takes place. For each interaction and each plant-pathogen system the play-ground looks different. There are several scales of such a play-ground that are important for a plant pathogen interaction. Here I will roughly present three scales, representing the scales that are studied in this thesis. The largest scale is the total global distributions of the plant and the pathogen. These distributions might not always overlap, so that in some parts of the plant's distribution the selective pressure from the pathogen is absent. Likewise, the pathogen might occur on, and adapt to, other host species in parts of its distribution. The intermediate scale is referred to as the regional distribution of populations, i.e. the metapopulation. Here the focus is on the populations and the amount of connection between them due to migration, as well as on the extinction and colonisation dynamics within the metapopulation. The last and smallest scale is the local spatial structure within each population, affecting both the plant and the pathogen.

As mentioned earlier, one of the basic factors characterizing a group of interconnected local populations, i.e. a metapopulation, is frequent local extinctions and colonisation events. This is mainly a result of small populations running a higher risk of extinction compared to larger populations (Hanski and Giplin 1997). However, subdivision into many small populations also brings with it that few extinction events are synchronized between the populations. This suggest that at most times there will still be occupied patches in the surroundings from where individuals could migrate to and (re-)colonize an empty patch. The chance for colonisation increases with the size of the patch, in combination with the distance to the closest occupied patch (Burdon et al. 1989). The extinction/colonization dynamic has both demographic and genetic consequences in a plant-pathogen interaction. Compared to single small isolated populations or to

situations where populations are highly connected, the likelihood of long term persistence of the interaction at a regional scale are higher when local patches are enough isolated to prevent a synchronization of extinctions, but still enough connected to allow relatively frequent migration among them (Carlsson-Granér and Thrall 2002). One further demographic effect of the continuous extinction/colonisation dynamic is that the interaction between the plant and the pathogen might have occurred for different time periods and might be of different intensity at each place and time. The main genetic effect of spatial structuring is the impact it has on the distribution of genetic variation, both neutral and adaptive variation that potentially might be involved in co-evolutionary interactions. Since new populations are mostly founded by a few individuals, only a subset of the total genetic variation present in the metapopulation is represented in a newly established population. Together with drift and selection this can result in low genetic variation within populations, whereas the genetic variation between populations can be high. Giles and Goudet (1997b) showed that the genetic uniqueness of newly founded populations was preserved throughout their existence, despite continuous gene flow from surrounding populations. This effect was further strengthened due to genetic drift as populations became smaller and eventually went extinct in the face of successional changes.

In plant-pathogen interactions where both players experience founder events, the result is a unique combination of the genetic composition for both players at a particular place where they both are present. Furthermore, in plant-pathogen systems the spatial genetic structure of one of the players is likely to contribute to the spatial structure of suitable patches for the other player. In a co-evolutionary system, the genetic structure of one of the players will change in response to the other player and vice versa, contributing to a more or less continuous change in the spatial structure of suitable habitat for both players. Together, all these effects suggest that initial conditions, as well as the trajectory taken, differs considerable between different populations. The local spatial structures within populations might not be a direct effect of extinction/colonisation dynamics, but likewise these local structures can have both demographic and genetic consequences. For example, if there is variation in resistance within a population, resistant individuals may act as physical barriers for the spread of the disease (Garrett and Mundt 1999). Furthermore, the selection pressure exercised by the pathogen on the plant may differ over very small scales, depending on e.g. differences in microclimate. This may result in reciprocal evolution only in some patches of the total population, which in turn drive the local diversity and distribution of resistance (Laine 2006).

SYSTEM SPECIFIC TRAITS AFFECTING CO-EVOLUTIONARY AND NUMERICAL DYNAMICS

If the spatial structuring of a plant-pathogen system is likened to a play-ground, each system also inhibits a unique set of traits that can be thought of as “the rules”, controlling the interaction, and the outcome of the potential co-evolution between the two players. Many “rules” are life history traits of the players involved, while others are physiological or genetic traits. Many of these traits have been examined theoretically,

empirically or both, such as the relative scales of dispersal for the host and the pathogen (Gandon et al. 1996; Thrall and Burdon 1999), the relative generation time of host and pathogen (Gandon and Michalakis 2002), infection type (systemic vs. non-systemic) and severity of disease effects (Thrall and Burdon 1997), population size (Burdon and Thrall 2001), mating system for both organisms (Burdon et al. 2006), variability and spatial structure of co-evolutionary selected traits (e.g. resistance and virulence) (Carlsson-Granér 1997; Garrett and Mundt 1999; Burdon and Thrall 2001; Laine 2004) and race/genotype specificity and underlying genetics of resistance and virulence (Thompson 1994; Burdon et al. 1996; Burdon and Thrall 2001; Gandon 2002).

While it is not fully known how these traits interact, some general patterns have emerged. For instance, in systems where the pathogen is a rust or a mildew and typically disperse further than their hosts, the pathogen tend to be locally adapted, tend to reproduce many times within one host generation and tend to be non-systemic. The genetic interactions between host and pathogen tend to be genotype specific, e.g. of gene-for-gene type, and the pathogen often occurs in epidemics (Burdon et al. 1996; Thrall and Burdon 1997; Kaltz and Shykoff 1998; Gandon 2002).

In the plant-pathogen system of this thesis, *Silene dioica* (L.) Clairv. (Caryophyllaceae) (Red Campion) and its obligate anther smut, in this thesis referred to as the species *Microbotryum violaceum MvSd* (Le Gac et al. 2007) belonging to the species complex *M. violaceum* (Pers.: Pers) Deml & Oberw. (=*Ustilago violaceum* [Pers.] Fuckel) (Ustilaginaceae) (Deml and Oberwinkler 1982), the dispersal ranges are similar for both host and pathogen (Pettersson et al. 2000). The infection is systemic and the fungus sporulates in the flowers of the host, thus sterilizing the plant and resulting in similar generation times for host and pathogen. *S. dioica* is only able to reproduce sexually and since the plant is dioecious it is an obligate outcrosser. *M. violaceum*, which also mainly reproduce sexually, are able to outcross but has been shown to mainly self via automixis.

THIS THESIS

In this thesis I have studied some of the "rules" that influence the interaction and dynamics of the plant-pathogen system *S. dioica* and *M. violaceum*. I looked at the appearance and variation of the traits mainly in the "playground" context of within and among populations in a metapopulation. In two of the included papers the focus is from the plant perspective and two from the fungal perspective of the interaction. From the plant perspective, I have examined different aspects of the biochemical resistance that acts against the fungus once the outer layer of the plant has been broken through. This kind of resistance, also called active resistance (Burdon 1987), is one part of total field resistance, which also includes passive resistance, such as amount of flower display and time of flowering (Burdon 1987). From the pathogen perspective, the main focus has been on the breeding system of *M. violaceum MvSd* and its effects on fitness and distribution of genetic diversity.

In paper (I) I studied the variation and spatial structure of biochemical resistance for *M. violaceum* in *S. dioica* within and among populations in a North Swedish metapopulation, and asked whether this kind of resistance was specific for the attacking fungal strain and also briefly examined how the resistance was genetically controlled.

In paper (II) I more thoroughly examined the genetic architecture of biochemical resistance with the aims to a) confirm what had been suggested by the results in paper (I), namely that biochemical resistance was controlled by few genes and that resistance against the two tested fungal strains was determined by separate genes and b) if possible, determine the number of loci and the allelic interactions of the genes involved in controlling biochemical resistance.

In paper (III) I examined the variability of the breeding system of *M. violaceum* within and between populations, using potentially adaptive traits as markers, which could influence the distribution of genetic variability and pathogen fitness.

In paper (IV), I used neutral genetic markers to study whether the diversity and distribution of genetic variation within and between both regions and populations of *M. violaceum*, were reflecting the breeding system and the historical and on-going demographical processes that could be expected in the Northern parts of *M. violaceum*'s distribution area.

STUDY SYSTEM

SILENE DIOICA is a dioecious, mid-successional, perennial herb that grows in moist, fertile, disturbed habitats. In Sweden, these habitats are commonly found along the coasts, where new land emerges through isostatic land uplift, and in alpine areas just above the tree line, where soil movement creates disturbance. *S. dioica* is insect pollinated, in northern Sweden mainly by bumble-bees (*Bombus* sp.) (Carlsson-Granér et al. 1998), and the plant is predominantly outbreeding (multilocus outcrossing rate 0.996±0.011, Giles and Ingvarsson, in prep) resulting in high within and among population genetic variability both in isozymes and morphology, such as in floral traits (Giles and Goudet 1997b; Ingvarsson and Giles 1999; Giles et al. 2006). The seeds are dispersed by gravity from open capsules, and most end up within a meter of the mother plant (Ingvarsson and Giles 1999). Since the pollen is also dispersed over short distances, with only occasional long distance dispersal events, the populations are structured in highly related family groups, of about 1m in diameter (Ingvarsson and Giles 1999) and in Skeppsvik Archipelago (see below for further description) each group persist for c. three generations (10-12 years) (Giles and Ingvarsson, in prep.).

MICROBOTRYUM VIOLACEUM is a species complex of obligate basidiomycete anther smuts (Le Gac et al. 2007) infecting many species in the Caryophyllaceae family (Thrall et al. 1993). *M. violaceum MvSd* is infecting *S. dioica* and can be found throughout the distribution of its host. In infected plants, diploid teliospores are produced instead of pollen in the anthers of the host, thus sterilizing but not killing its host. If female plants of dioecious hosts are infected they abort the stigmas and produce spore filled anthers (Baker 1947). The fungus is perennial and its only opportunities for reproduction and dispersal are when the plant flowers. For *M. violaceum MvSd* it normally takes at least one year from new infection to sporulation. The fungal teliospores are dispersed by pollinators and host plant and pathogen have approximately the same dispersal ranges (Pettersson et al. 2000). For illustration of the life cycle of *M. violaceum*, see figure 1. When diploid teliospores of *M. violaceum* germinate, they undergo meiosis and give rise to four haploid cells (two of mating type A1 and A2, respectively) (Hood and Antonovics 1998; Hood et al. 2001). Normally three of the cells form a promycelium (basidium) that is initially attached to the teliospore containing the fourth haploid cell. Each promycelial cell can also bud off haploid sporidia (basidiospores) mitotically. Mating compatibility is controlled by a single mating-type locus with two alleles (A1 and A2) (Garber and Day 1985) and conjugation can only occur between haploid cells that differ at this locus. Conjugation is a prerequisite for the formation of the infective dikaryotic hyphae that invade the new host (Fischer and Holton 1957; Cummins and Day 1977) and sexual fusion is therefore required for fungal survival. Conjugation can occur between any two cells of opposite mating types, i.e. between the proximal and the middle cell in the same promycelia (i.e. automixis), or involve free sporidia or promycelia originating from different teliospores. This results in selfing if the teliospores are from the same strain, or in outbreeding if another strain is involved. Some strains of *M. violaceum* from natural populations

produce sporidia of only one mating type (e.g. Kaltz and Shykoff 1997; Oudemans et al. 1998; Hood and Antonovics 2000; Thomas et al. 2003). Teliospores from strains with biased sex ratios are still able to produce four cells during meiosis and thus dikaryotic infectious hyphae by intratetrad conjugation (Oudemans et al. 1998; Hood and Antonovics 2000).

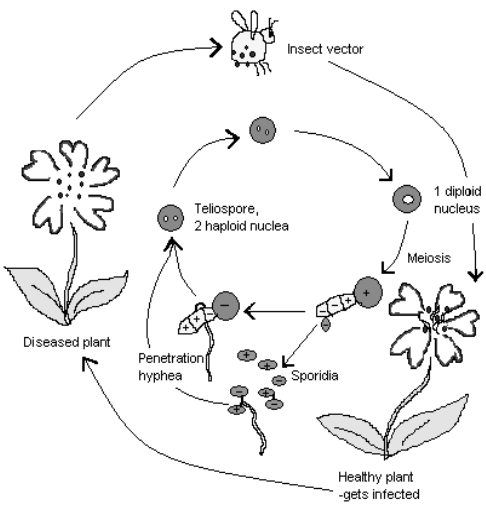

Figure 1. Life cycle of *Microbotryum violaceum.*

HOST-PATHOGEN INTERACTION

Disease prevalence varies between 0 and 60% in different *S. dioica* populations within the same general area (Carlsson et al. 1990) and variation in resistance to *M. violaceum* has been demonstrated, both among genotypes and populations (Carlsson-Granér 1997; Carlsson-Granér and Pettersson 2005; Giles et al. 2006). Field resistance in *S. dioica* for *M. violaceum* is composed of both biochemical resistance, so called active resistance (Burdon 1987), and variation in phenological and morphological traits, so called passive resistance (Burdon 1987), e.g. flowering time (Biere and Honders 1996b) and floral display (Giles et al. 2006). Apart from the differences in resistance, the risk of getting infected increases with the number of spores deposited on a plant (Pettersson et al. 2000). Furthermore, transmission of the disease differ depending on the density of the host plants; when there is a high average density of host plants the risk for a healthy, susceptible host plant to get infected depends on the frequency of infected plants, whereas when the average density of host plants is low the risk of getting infected are more dependent on the number of infected plants per area unit (disease density) (Biere and Honders 1998).

SAMPLES AND STUDY SITES

All *S. dioica* plants used in this study were either collected as seeds from single capsules in the field (I) or were offspring to these plants (II). The plants used in paper I were collected from four islands in the Skeppsviks Archipelago (see Figure 2 and description further down). The fungal strains were collected as teliospores from unopened flower buds. As an infected bud contains teliospores produced by a single fungal strain in *S. latifolia* (Hood 2003) each flower bud was considered to contain only one strain also in *S. dioica*. The fungal strains were collected from 21 populations distributed in three regions in Northern Sweden; Umeå (including the Skeppsviks Archipelago from where all plants originated from), Hemavan and Abisko, and the number of strains and populations used, varied between the four studies (Figure 2). One fungal population was defined as all infected individuals found in a single host population.

THE UMEÅ REGION: Most of the populations in the Umeå region were situated on islands in the Skeppsviks Archipelago, c. 20 km north-east of Umeå (Figure 2), and constitutes a typical metapopulation. In this area we have detailed knowledge about numerical dynamics of host and pathogen populations (Carlsson et al. 1990; Carlsson and Elmqvist 1992; Giles and Goudet 1997b; Giles et al. 1998; V. Pettersson and B. E. Giles unpubl. data). After the glaciation period this area was left under water. Since then, the area has been rising due to isostatic land-uplift, resulting in the constant emergence of new islands. During the primary succession occurring on each island, *S. dioica* is present in the mid-successional phase and during a life time of a host population *M. violaceum* may be present when the plant densities are highest, i.e. in populations of intermediate age. This results in a high population turnover rate, with c. 40 generations (c. 250 years) from founding to extinction and host and pathogen populations seldom occupies the same place more that once (Carlsson 1995). Disease prevalences in the examined populations in Skeppsviks Archipelago were 3 – 36 %. In addition to the populations in the archipelago, two populations from urban environments in Umeå (Ålidhem and Tomtebo) were included in the Umeå region.

THE HEMAVAN REGION is located in a mountain area c. 400 km north-west of Umeå and only *M. violaceum* was studied in this region (Figure 2). One of the populations in this region was situated in the birch forest close to the Hemavan village whereas the other three populations were situated just above the tree line along the south hill side of the mountain Gielestjåhke. Disease prevalence in these populations was estimated to 16 - 40 %.

THE ABISKO REGION is located in a mountain area c. 600 km north of Umeå and also in this region only *M. violaceum* was studied (Figure 2). One of the populations in this region was situated in the birch forest close to Abisko village whereas the other three populations were situated above the tree line on the north hill slope of the mountain Geargečorru. Estimated disease prevalence in these populations was 20 – 28 %.

In both the Hemavan and the Abisko region, all the examined populations within each of the regions were considered to belong to the same metapopulations because potential host populations were distributed continuously between the most distant examined populations, separated by similar distances as in the Skeppsviks archipelago. In the mountain regions we do not have as detailed knowledge about the ages and dynamics of the populations as we do in the Skeppsviks Archipelago. However, populations in the mountain regions are likely to be older (Kullman 1999) and the turnover rate lower than in the Skeppsvik Archipelago, since the habitats are more stable in the mountains. This is a result of regular soil movements that constantly creates new patches to colonise for the host within each locality, while the vegetation at the localities do not change since no primary succession is taking place at these localities.

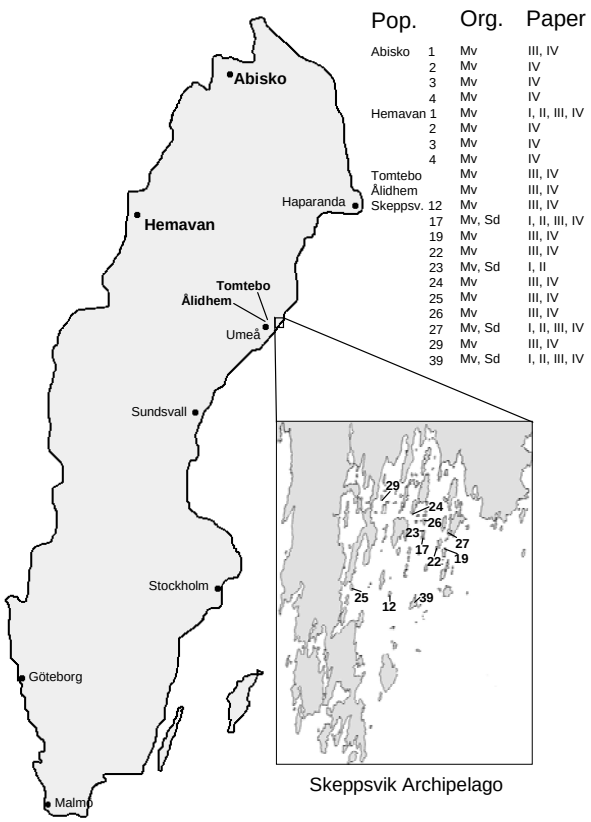

Figure 2. Localities of studied populations. Which of the two organisms, *Silene dioica* (Sd) or *Microbotryum violaceum* (Mv) that was examined and which study the population participated in is reported for each population.

METHODS

Several different methods were used in this thesis and all studies were done under standardized conditions, either in glasshouses or in the lab.

INOCULATIONS (I and II)

Inoculating seedlings or vegetative plants with suspensions containing sporidia of both mating types is a commonly used method to test the biochemical resistance for *M. violaceum* in several host species (e.g. Alexander and Antonovics 1995; Biere and Antonovics 1996; Biere and Honders 1996a; Carlsson-Granér 1997; Kaltz et al. 1999; Carlsson-Granér 2006). In both paper I and II we inoculated 6 weeks old vegetative plants by first repeatedly punctuating them close to the meristems with a needle and then applying 0.2 ml of a sporidia suspension containing approximately 10^7 sporidia /ml H_2O of both mating types. In both studies the same two fungal strains were used; one originated from population 23 in Skeppsviks Archipelago and one originated from population 1 in Hemavan (Figure 2).

CROSSES (II)

The method of using controlled crosses between parents of known phenotype and study the segregation of a trait in the offspring generations to deduce the underlying genetics of relatively simply inherited traits was used already by Gregor Mendel and are still commonly used today, e.g. in determining the genetic control of pathogen resistance in plants (e.g. Vandemark et al. 1992; Burdon 1994; Irzhansky and Cohen 2006; Kniskern and Rausher 2006). Commonly, a crossing design starts with outcrosses between parents which are homozygous for the trait of interest, producing the F1 generation (Griffiths et al. 1998). Secondly, most designs involve selfing in one to several steps, producing the F2-Fx generations. In paper II, we crossed *S. dioica* parents with known disease phenotypes, obtained from the study in paper I, with the aim to examine the genetic architecture of biochemical resistance in *S. dioica* for *M. violaceum MvSd*. However, since *S. dioica* is an obligate outcrosser (Giles and Ingvarsson, in prep.) and successive sib-mating leads to rapid decline in the number of families that produce flowering plants (B. E. Giles, unpublished data), we could not use any of the crossing designs where known homozygous parents are crossed to produce an F1 and a segregating F2. Instead we used a more unconventional approach where the knowledge of the disease phenotype of the parents and the frequency of susceptibles in their families were used in combination with the knowledge of the frequency of susceptibles in the offspring family to determine whether resistance against different fungal strains were controlled by separate genes and to examine gene interactions involved in controlling biochemical resistance in *S. dioica* for *M. violaceum MvSd*. For further description of the approaches used, see materials and methods in paper II.

OBSERVATIONS (III)

In paper III, the germination and mating behaviour of *M. violaceum MvSd* strains from different populations was studied under microscope in low and high nutrient conditions, inspired by the techniques used by Hood and Antonovics (1998; 2000). By counting conjugations and cell types in known volumes of media, we were able to estimate the number of teliospores, cells, promycelia, sporidia, and conjugations (intrapromycelial or involving sporidia) per unit of volume. Using these numbers we could then, per strain, estimate the conjugation propensity, production of infectious dikaryons, propensity to conjugate within the promycelium, propensity to conjugate among sporidia, production of conjugations involving sporidia and sporidia production. We chose these traits because they could influence the distribution of genetic variability and pathogen fitness, through selfing and automixis and they could also potentially be regarded as adaptive traits if they are genetically controlled, and thus could indicate the amounts of adaptive genetic variation (cf. neutral genetic variation, below).

DNA ANALYSIS (IV)

Neutral genetic variation, i.e. the variation that can be found among loci that is not subject to selection (Charlesworth 2003) are well suited to examine breeding system and processes such as gene flow, migration, and drift (e.g. Charlesworth 2003; Holderegger et al. 2006; Ouborg et al. 2006). Commonly used neutral markers are allozymes, AFLP, and microsatellites. Microsatellites, which were used in this thesis, are loci that is composed of varying numbers of dinucleotides repeated in tandem (Griffiths et al. 1998). These loci have relatively high mutation rates, in terms of changes in the number of repeats, because their construction enhances the chances of slippage in these regions during meiosis. Each locus is used as a separate marker, and the studied variation is the length of the microsatellite fragment, i.e. the number of repeats. An advantage with microsatellite markers is that they are co-dominant, i.e. alleles of all gene copies in di- to polyploide organisms are detectable which makes it possible to estimate e.g. heterozygosity and makes them suitable for studying breeding systems. To study microsatellite markers the variable fragments are amplifyed using PCR (polymerase chain reaction) in which specifically designed primers are needed. At the moment, primer pairs for 109 microsatellite loci are developed for *M. violaceum*, of which 60 were published just recently (Bucheli et al. 1998; Giraud et al. 2002; Giraud et al. In press). In this thesis we used four of the microsatellite loci that amplify in *M. violaceum MvSd* to study breeding system and historical and on-going demographical processes within and among in *M. violaceum MvSd* populations in three regions in northern Sweden (IV).

GENERAL RESULTS AND DISCUSSION

BIOCHEMICAL RESISTANCE IN *S. DIOICA* TO *M. VIOLACEUM MVSD*

The results in paper I and II shows that resistance in *S. dioica* to *M. violaceum MvSd* can be specific to the attacking pathogen strain and the results in paper I further demonstrated that the levels of resistance were highly spatially variable, both at the scale of between populations and also at the smaller scale of between family patches. Together, these two major results implies that *M. violaceum MvSd*, faces a highly structured and genetically diverse world of host resistance, and that the structure varies depending the fungal strain, i.e. strains are playing on differently structured "playgrounds".

Compared to systems where resistance is strain non-specific, the establish of a disease in a host-population with strain specific resistance is likely to be delayed because pathogens are more efficiently excluded in such systems (Burdon et al. 1996). However, once a pathogen has established in such system, the spread and amount of the disease within populations are likely to be dramatically reduced if the strain specific resistance also is spatially structured. Most of this effect is probably due to the reduced proportion of plants or tissue that is susceptible to races that can infect it, but also because resistant plants acts as physical barriers for the spread of pathogens (Garrett and Mundt 1999). In addition to these consequences of spatially structured and strain specificity in resistance, chance events will likely play a major roll for the development of an epidemic of *M. violaceum* in a *S. dioica* population, since chance greatly affects which pathogen strain that happens to enter a pathogen population and where in the population it establishes. Because the dispersal range of *M. violaceum* is relatively short in relation to the size of a host family patch, a strain that happens to establish in a patch surrounded by relatively resistant patches probably runs a high risk of becoming extinct before it is able to spread to a more susceptible patch, especially since each patch only persist for c. 3 host generations, while *M. violaceum* is only able to sporulate when the infected plant flowers, which does not occur every year.

The results in paper II also showed that the specific resistances found for each of the two pathogen strains were determined by separate genes, and for both strains resistance were likely to be genetically controlled by a few genes. When few genes of major effect determine resistance, the population response to changes in selection pressures imposed by the pathogen will be more rapid compared to resistance determined by many genes with minor effects (McDonald and Linde 2002). This implies that there could be a potential for relatively rapid response to selection imposed by *M. violaceum* in *S. dioica* populations, especially if populations are highly variable in resistance, which was found in study I. However, when resistance to different strains is controlled by separate genes, selection imposed by one of the strains should not affect the amount or distribution of resistance to the other strain, as long as the genes controlling the different resistances are not connected, e.g. thorough linkage. Thus, changes in amount and structure of

resistance resulting from selection by *M. violaceum* might only be possible to detect if using the pathogen strain that imposed the selection pressure.

BREEDING SYSTEM AND DIVERSITY OF ADAPTIVE AND NEUTRAL GENETIC VARIATION IN M. VIOLACEUM MVSD

Automixis via intratetrad mating has been shown to be common in *M. violaceum MvSl* (the *M. violaceum* species attacking *S. latifolia*) (Kaltz and Shykoff 1997; Hood and Antonovics 1998; Oudemans et al. 1998; Delmotte et al. 1999; Kaltz and Shykoff 1999; Hood and Antonovics 2000; Giraud 2004; Giraud et al. 2005; Zakharov 2005) and this was also found for *M. violaceum MvSd* in both paper III and IV, although we found no fitness reduction, in terms of reduced production of infectious dikaryons, for strains with high rates of intrapromycelial mating (III), as had been predicted (Thomas et al. 2003; Giraud et al. 2006). In paper III, which reflects the mating events that occur prior to plant penetration, we found that intrapromycelial mating seemed to be the dominating conjugation form for *M. violaceum MvSd*, but also that almost all strains in addition produced conjugations involving sporidia, which may improve the chances of outcrossing if the ability and chance is given. In paper IV, we studied the breeding system as well, but used neutral markers which reflect the kind of matings that actually lead to infections in the field. In this study we did not find any signs of outcrossing or selfing, suggesting that even though most strains have the potential for ordinary selfing and outcrossing, it happens very seldom in the field.

There are several aspects of high levels of intratetrad matings that could potentially affect a plant-pathogen interaction. Being able to mate with oneself provides a reproductive assurance in situations where the chances of encountering another individual are low, for example at low population density (Kaltz and Shykoff 1999) and makes it possible for a single teliospore to establish a new population (Charlesworth and Pannell 2001). In such situations intratetrad mating could be advantageous, compared to selfing (matings between the products of different meioses from the same individual), because intratetrad mating preserves heterozygosity in the region linked to the centromers and reduces the rate in which heterozygosity is lost in other regions of the genome (Kirby 1984; Hood and Antonovics 2000, 2004; Johnson et al. 2005; Zakharov 2005; Knop 2006). Furthermore, mating within the tetrad is likely the most rapid way to produce an infectious dikaryon, which might be advantages when individual flowers have a limited lifespan (Kaltz and Shykoff 1999). However, intratetrad mating still is a form of inbreeding, which like all kinds of inbreeding increases isolation between individuals and populations (Charlesworth and Pannell 2001; Charlesworth and Wright 2001; Charlesworth 2003) and the effects are even more pronounced for inbreeding organisms living in metapopulations that experience population turnover (Charlesworth and Pannell 2001; Ingvarsson 2002; Charlesworth 2003). These effects were reflected in the results of paper IV, since almost no variation was found within local populations, whereas variation was found within the region.

In paper IV, we found that the amount of neutral genetic variation was lower in Northern Sweden than what has been found in populations further south in Europe, which could be expected since genetic variation is likely to be lost due to the repeated bottlenecks occurring during colonisation. Neutral genetic diversity should not be expected to reflect the diversity in adaptive traits (e.g. Charlesworth 2003; Holderegger et al. 2006; Ouborg et al. 2006). Nevertheless, the processes that could be traced with neutral markers, such as breeding system, migration, colonisation and on-going processes as metapopulation dynamics are all likely to affect the adaptive genetic diversity in the same way it affect neutral diversity. Thus, also the amount of adaptive variation could potentially be lower in northern Sweden than further down in Europe. However, we did found variation both within and among populations in breeding related traits (III) and between two strains in infection ability (I, II). This variability in adaptive traits found for *M. violaceum MvSd* in northern Sweden suggests that there is a potential for adaption and co-evolution in the area despite the general low variability found in neutral markers.

Together, the studies on which this thesis is based, describes a scenario where *M. violaceum. MvSd* has to constantly colonize to track its host. It has to colonize new patches within existing host populations, newly established host populations within metapopulations with high turnover rates and new areas following its host's post-glacial migration. Furthermore, the colonisation is mediated by pollinating insects, which by their behaviour reduces the chances to encountering another fungal strain (Kaltz and Shykoff 1999). Under these circumstances, selfing could almost be regarded as a necessity for an obligately sexual pathogen, and *M. violaceum* do have the ability to self both by ordinary selfing and automixis. However, our results suggests that the latter alternative is highly favourable since we did not find the proposed fitness disadvantage but automixis has the advantages of slower loss of heterozygosity and also a potentially faster production of infectious dikaryons, compared to ordinary selfing.

SUMMARY AND FUTURE PERSPECTIVES

In this thesis I have demonstrated that resistance to *M. violaceum MvSd* in *S. dioica* can be specific to the attacking pathogen strain and also spatially highly diverse both within and among populations within a metapopulation. Together, these factors are likely to delay the establishment of the disease within host populations and reduce the spread and amount of disease, once it has established. Furthermore, I have also presented results that suggest that the specific resistance expressed against two different *M. violaceum MvSd* strains were determined by separate gene systems and that, in both cases, the resistance were simply inherited. This implies a potential for relatively rapid response to *M. violaceum*-induced selection in *S. dioica* populations variable for resistance.

The results in this thesis indicates that *M. violaceum MvSd* is much influenced by living in metapopulations with typical extinction-colonisation dynamics, leading to that it constantly have to colonize to be able to persist in an area. In such situations, being able to self should be highly favourable. Since we did not find the previously proposed fitness disadvantages associated with high levels of automixis and automixis has the advantages of slower loss of heterozygosity, it was not surprisingly that both studies of the breeding system of *M. violaceum MvSd* showed that automixis is the predominant breeding system. Furthermore, the variability in adaptive traits found for *M. violaceum MvSd* in northern Sweden suggests that there is a potential for adaption and co-evolution in the area despite the general low variability found in neutral markers.

With the results and knowledge gained from working with this thesis, there are two directions in which I would like to continue, if I had the possibility. First, it would be interesting to continue examining the underlying genetics of biochemical resistance in *S. dioica* to *M. violaceum*, much because we have now established a base for more elaborated studies. Secondly, since the breeding system of *M. violaceum* could be expected to differ due to characteristics of the particular host-pathogen system, it would be interesting to further examine the breeding system, either among populations of the same host species differing in e.g. size, turnover rate and pollinator community, or between host species differing in life history characters.

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