# On host race differentiation in smut fungi

Van Putten, Wilhelmus Frederik

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# On host race differentiation in smut fungi

Over waardrasdifferentiatie in brandschimmels

(Met een samenvatting in het Nederlands)

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**Wilhelmus Frederik van Putten**

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PROMOTOR: Prof. dr. J.M.M. van Damme
Faculteit Biologie, Universiteit Utrecht

CO-PROMOTOR: Dr. A. Biere
Nederlands Instituut voor Ecologie
Centrum voor Terrestrische Ecologie, Heteren

Cover: anther smut infected flowers of *Silene latifolia*.

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Voor mijn ouders

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# CHAPTER 1

# General introduction

## HOST RACE FORMATION AND SPECIATION

### Modes of speciation

When discussing different modes of speciation, it is important to know which definition of a species is used. In the broad range of biological studies on speciation, there were, and still are, many different species concepts (*cf.* Harrison 1998). Until the twentieth century, the time of Linnaeus, Darwin and Wallace, a commonly used species concept was a simple one, based on morphology—tigers look like tiger and lions look like lions (Berlocher 1998b). In modern day speciation research, the most well-known of the species concepts, and hence the most frequently used for that matter, is the biological species concept that is defined by Mayr (1963) and Dobzhansky (1970), stating respectively; "*groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups*", and "*systems of populations between which the gene exchange is limited or prevented in nature by a reproductive isolation mechanism or by a combination of such mechanisms*". From these definitions it can be seen that the key mechanisms of speciation should provide some form of reproductive isolation between populations of the same species. This can lead to speciation by natural selection and genetic drift (*cf.* Coyne, 1992). Genetic divergence may increase as populations adapt to their local environments. Prezygotic (e.g. selfing, or positive assortative mating, reducing the production of hybrid offspring) and postzygotic (reduced fitness of hybrid offspring) forms of reproductive isolation will gradually develop between geographically isolated populations. When sub populations have diverged, maladaptive hybridization is often avoided by mate discrimination, a process that is known as reinforcement (*cf.* Noor 1999). Once these processes are completed, speciation has occurred (Rice and Hostert 1993).

Many present day ideas on speciation and possible mechanisms for speciation come from theoretical modeling. The basic allopatric, or geographical models of speciation that have been developed (e.g. Mayr 1963) are often easier to comprehend

than models of sympatric speciation (e.g. Maynard Smith 1966). Virtually all of the older models have postulated that speciation in sympatry is driven by genetic trade-offs in adaptation to different habitats, i.e. by antagonistic pleiotropy of genes that improve fitness in one habitat, and reduce fitness in the other (*cf.* Kawecki 1997). More recent models have shown that other mechanisms, e.g. deleterious mutations (Kawecki 1997), sexual selection (Higashi *et al.* 1999), or disruptive selection and assortative mating (Dieckmann and Doebeli 1999), can provide ecologically realistic conditions as under which sympatric speciation theoretically could occur as well. Empirical data, and good biological examples are still scarce however (Coyne 1992), and are almost exclusively concerned with phytophagous insects and their host plants.

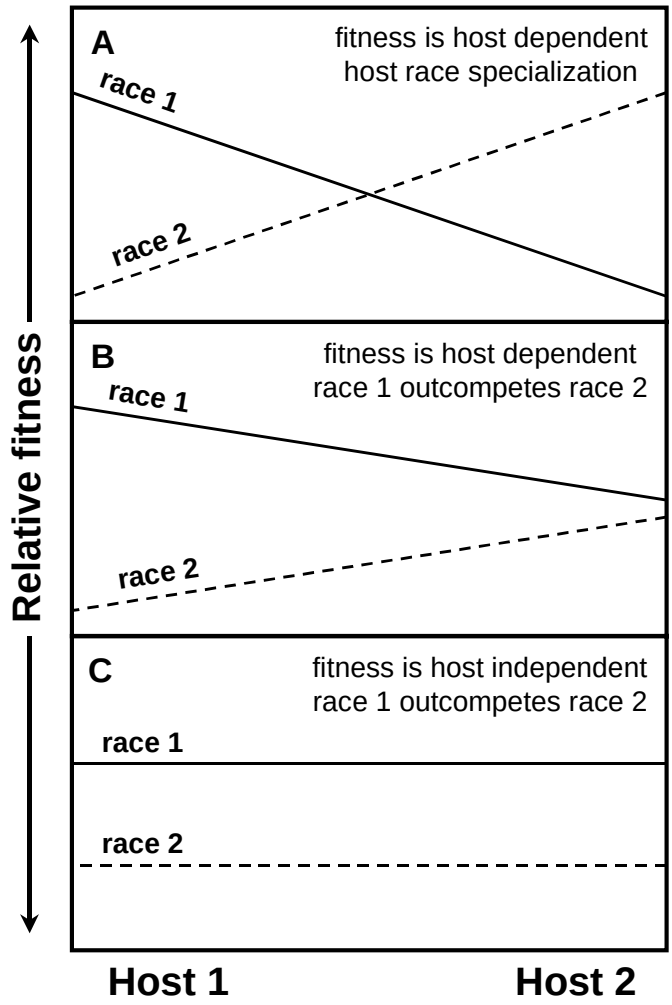


**Figure-1** Schematic representation of pathogen fitness trade-offs on different host species in models of (sympatric) speciation. The scheme displays possible allelic effects on the performance of two host races on two different host species. Formation, specialization or maintenance of host races in sympatry can be achieved actively in panel A, but not in B or C without other mechanisms of reproductive isolation being present (adapted from Kawecki 1997). Note that in the case of host race formation, 'race' in this figure may be referred to as 'deme'.

## Sympatric speciation by host (habitat) choice and fitness trade-offs

Among the first authors presenting empirical evidence for speciation in sympatry is Bush (1969), who investigated frugivorous flies of the genus *Rhagoletis*, the apple maggot fly, and observed strong correlations between mate and host selection in several sympatric groups of sibling species. Together with some other

characteristics of the genus, and evidence that host races had evolved in recent history (reported by Walsh 1867), they suggested that certain groups of sibling species may have evolved in sympatrically as a result of minor alterations in genes associated with host plant selection. The two main pillars on which the Bush' model of sympatric speciation rests; host (habitat) specific mating and host-associated fitness trade-offs (illustrated in figure 1), in fact represent forms of pre- and postzygotic reproductive isolation mechanisms. Sympatric speciation by host- or habitat shift has—since then—gained much more support in a wide range of insect-plant systems (as reviewed in Tauber and Tauber 1989), and more recently for instance in pea aphids (Via *et al.* 1999; 2000), but remains subject of much debate. A frequently used criticism is that most—if not all—cases of sympatric speciation in natural populations could equally well be explained by allopatric mechanisms. Berlocher (1998a) addresses this point and concludes that there are cases that are best explained with sympatric rather than allopatric speciation, pointing to the case of cichlid species in African crater lakes, which due to their size and shape are likely to be colonized only once and consist of several monophyletic flocks (Schliewen *et al.* 1994), that provided even stronger evidence than the huge bulk of data on parasitic insects.

## Host race formation

Berlocher (1998a) gives a clear path of four stages for sympatric speciation via host race formation, with increasing genetic distances between the races. Each of these stages is documented with biological examples on parasitic insects. (1) The first stage is represented by a distinct host shift event. Isolation by host fidelity occurs due to differences in post diapause emergence time and host choice behavior. There are no prezygotic or postzygotic isolation mechanisms independent of host fidelity, and no host-specific allele frequencies in this stage. (2) In the second stage, the species (or races) are still isolated by host fidelity only, without any severe postzygotic isolation mechanism. Allele frequency differences between host races do exist, but do not exclude the possibility of gene flow. There are no species-specific alleles (in allozymes). (3) In the third stage, both pre- and postzygotic forms of reproductive isolation mechanisms that are not related to host fidelity have developed. Species-specific alleles (in allozymes) exist, but are not fixed, and low levels of gene flow are still present. (4) In the fourth and last stage, species are totally isolated, characterized by great genetic divergence, without gene flow, and with a strong postzygotic isolation mechanism that is unrelated to host adaptation.

## From insects to fungi; specialization of fungal host races

Host race formation and specialization of phytopathogenic fungi is much less documented however, let alone sympatric fungal speciation. There are a few examples of fungal speciation which might have evolved in sympatry, for instance in three subspecies of *Phytophtera palmivora* from cocoa (Sansome *et al.* 1979; Brasier and Griffin 1979). However, even in these cases it is feasible that these species arose in allopatry, and that their ranges became overlapping at a later stage (*cf.* Burnett 1983). In these fungi, evidence for (sympatric) speciation points to two major environmental factors (Brasier 1987), (1) (micro-)climate, in which divergence is promoted by locally strong gradients and discontinuities, and (2) substrate, in which divergence is led by opportunistic nutritional strategies of the pathogen. Both pre- and postzygotic mechanisms of reproductive isolation have been reported, ranging from (prezygotic) temporal isolation by asynchronous gamete release (Federici 1982) to 'homing' in basidiomycetes, i.e. active movement of spores towards for instance host produced chemicals (Deacon and Donaldson 1993) and from (postzygotic) hybrid sterility (e.g. Federici 1982) to complex vegetative incompatibility systems, as for instance is found in some ascomycetes (Glass and Kuldau 1992).

However, often these studies are strongly biased towards more economically important organisms (*cf.* Brasier 1987), putting the emphasis unintentionally on the host rather than on the pathogen (but see Giraud *et al.* 1999). This also holds true for the 'physiological specialization' of smut fungi in the review of Fisher and Holton (1957 p.331) that examines susceptibility and resistance of cereal and oat smuts of the *Ustilago* genus. Nevertheless, they represent good examples of host specialization in phytopathogenic fungi, which can be a starting point for host race formation and eventually for speciation. Recent examples often use molecular markers in search of a genetic basis of (sympatric) host races, e.g. studies by Peever and co-workers in *Alternaria* (brown spot fungus of citrus), showing host-related genetic differentiation between citrus fruit cultivars (Peever *et al.* 1999; Peever *et al.* 2000), and in *Macrophomina* (charcoal rot fungus infecting root tissue of some crop species) showing both genetic differentiation between host species, and combine this with data on host preference (Su *et al.* 2001). From the ecological examples, perhaps the most extensively studied organism in this field is the anther smut fungus *Microbotryum violaceum*, phytopathogen of the Caryophyllaceae, and the main subject of this thesis.

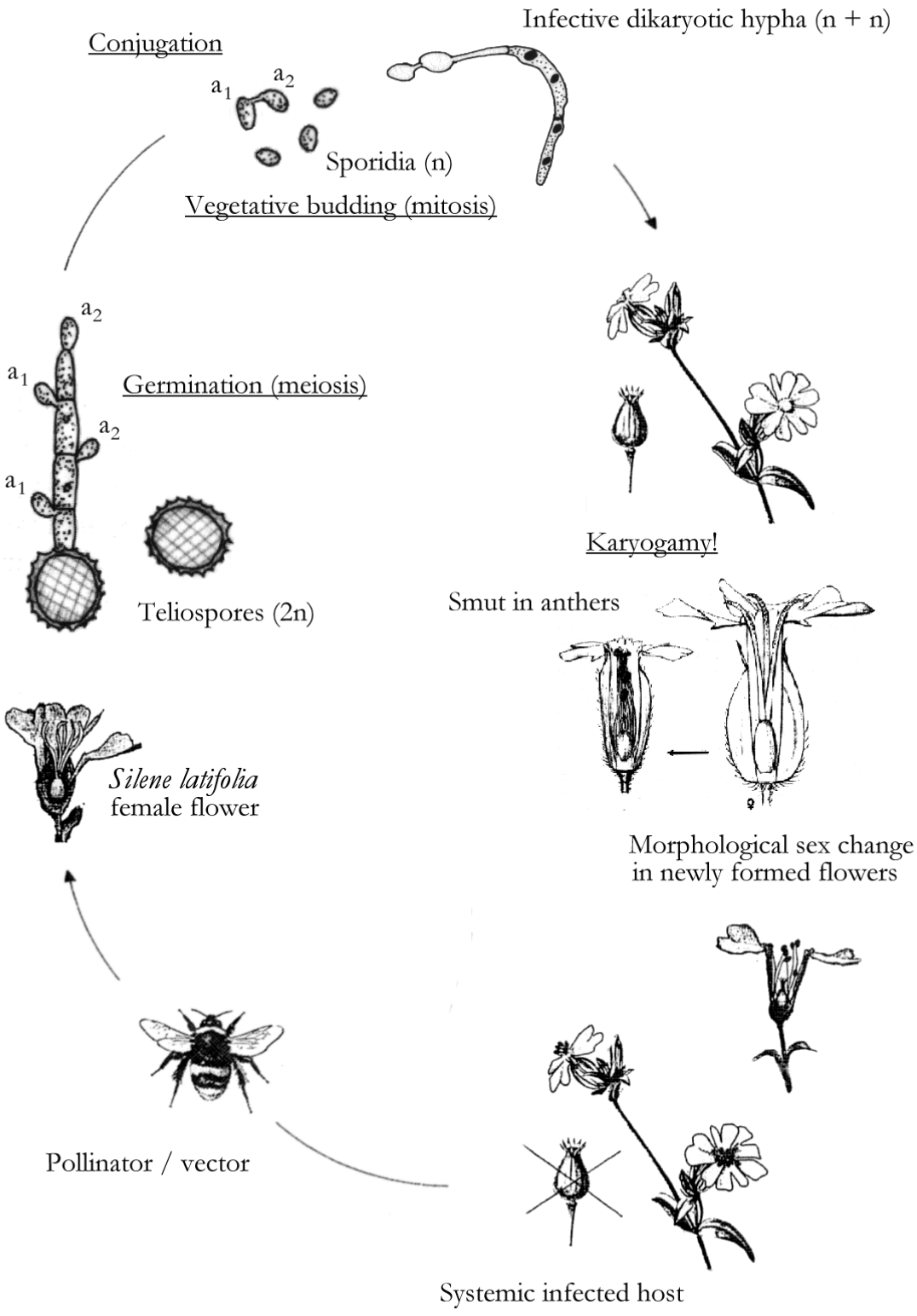


**Figure-2** Lifecycle of *Microbotryum violaceum*. Indicated are the karyotypic phases in the fungal lifecycle, being diploid outside the host plant, and haploid/dikaryotic on/inside the host plant. The schematic picture of morphological sex change has been adapted from Vánky (1994). Note that the scale of each phase is quite different, the size teliospores is 10-12µm, of sporidia 6-8µm, and the corolla of *Silene* host flowers 10-30mm dependent of the host species.

## THE MODEL SYSTEM

### The pathogen

The anther smut fungus *Microbotryum violaceum* (Pers.: Pers) Deml & Oberw. (=*Ustilago violacea* (Pers.) Fuckel) (Ustilaginaceae) (Vánky 1994) is a well-studied example of a heterobasidiomycete fungus that obligatory parasitises susceptible members of the Caryophyllaceae plant family to complete its sexual lifecycle (Thrall *et al.* 1993). The sexual life cycle will be shortly reviewed here, and is presented in figure 2. The lifecycle can be split into two stages, outside the host plant (diplophase) and on/inside the host plant (haplo-/dikaryophase). Starting with the diploid teliospores arriving on a healthy host plant, the sexual life cycle commences with germination. Germination of smut spores by meiosis results in four-celled promycelia from which four haploid cells (sporidia) of two mating types (designated $a_1$ and $a_2$) bud off. However, one or two cells are frequently left behind, or migrate back into the basidium (Ingold 1983; Hood and Antonovics 1998). In principle, the two mating types are produced in a 1:1 ratio. However, biased ratios in both directions have been reported (Kaltz and Shykoff 1997; Oudemans *et al.* 1998), and have been attributed to mating type linked haploid lethal alleles (Oudemans *et al.* 1998), or intratetrad mating (Hood and Antonovics 2000). In nutritious environments haploid cells proliferate asexually by mitosis in a yeastlike manner (Day and Garber 1988). In the absence of nutrients, and at lower temperatures (Cummins and Day 1977) haploid cells of opposite mating types can mate. The mating process and recognition between cells is influenced by pheromones (Bölker and Kahmann 1993; Snetselaar *et al.* 1996), which promote the formation of fungal fimbriae, i.e. microscopic hair like structures composed of collagen, carbohydrates and RNA (Poon and Day 1975 but see Celerin and Day 1998). Hereafter, a conjugation tube between these cells of different mating type is formed (Poon *et al.* 1974), marking the start of the dikaryotic phase in which the infectious hyphae are produced. Other critical factors in the mating process besides low temperature and low nutrient level are the presence of oxygen, and salts (Cummins and Day 1977). The development of the fungus from this point on is regulated by the presence of host plant chemicals (Day *et al.* 1981; Kokontis and Ruddat 1986; 1989), of which α-tocopherol (= vitamin E) has been identified as one of the major factors stimulating hyphal growth (Castle and Day 1984). Indeed, small amounts of synthetic vitamin-E have the ability to induce hyphal growth *in vitro* by itself (personal observation), but it has been argued that it is unlikely that vitamin-E

promotes hyphal growth *in planta* because it is unavailable to the invading hyphae (Ruddat and Kokontis 1988). Infectious hyphae grow intercellularly (Spencer and White 1951; Audran and Batcho 1981) and grow along with the plant's apical meristemic regions (Day 1980). When the dikaryotic parasitic mycelium grows into the stamens of a flower, anthers produce teliospores instead of pollen (Thrall *et al.* 1993). As spores mature in the anther sacs, karyogamy marks the start of the diploid phase (Day and Garber 1988). In dioecious host species, an infection of female plants causes a morphological sex change (Hassan and MacDonald 1971); ovaries are aborted and staminal rudiments develop into stamens that bear spore-filled anthers (Day and Garber 1988; Thrall *et al.* 1993), a process that is induced by the fungus itself (Audran and Batcho 1981; Scutt *et al.* 1997). The teliospores are dispersed by the natural insect visitors of the host plant, which serve the dual role of pollinators of healthy plants and vectors of this sexually transmitted disease (Jennersten 1983; figure 3).

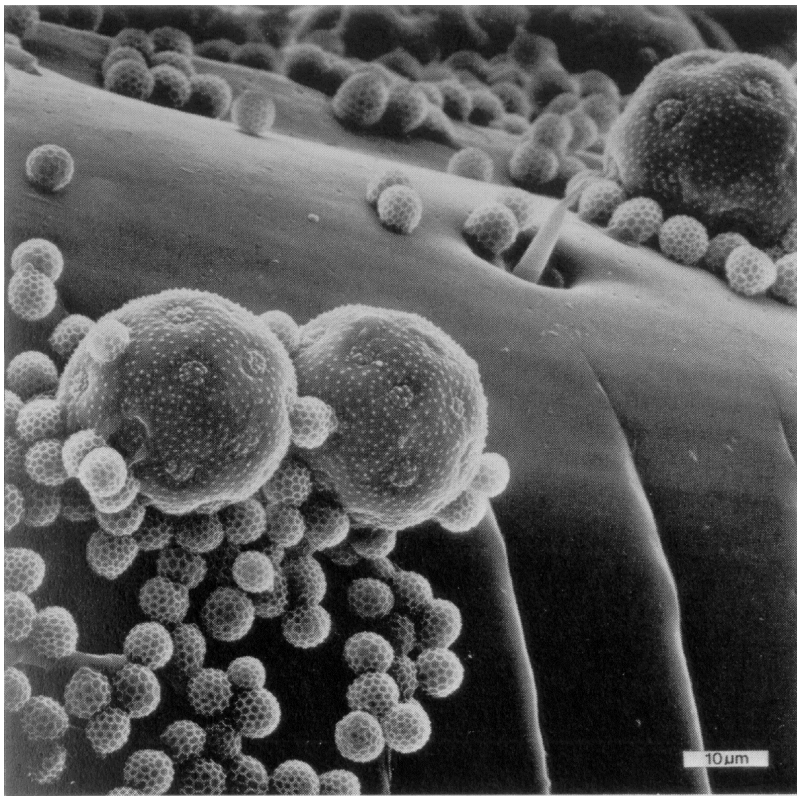


**Figure-3** Electron microscope photograph of pollen grains (the larger spheres) and teliospores of *Microbotryum violaceum* (the smaller spheres) on the proboscis of an insect vector (picture adapted from Jennersten (1983), and used with kind permission of dr. Jennersten).

*M. violaceum* is found to be highly selfing (Baird and Garber 1979), resulting in strong homozygosity in several host races (Bucheli *et al.* 2000; chapter 2). Automixis (mating among products of different meioses from the same diploid origin, i.e. between haploid sporidia from different teliospores from the same infected flower, or plant) as well as autogamy (mating among products of a single meiosis, i.e. within a

single basidium) are more likely to occur (Hood and Antonovics 1998; 2000) than outcrossing, simply due to a closer proximity of cells, thereby limiting the opportunities for outcrossing. Besides this sexual cycle, and the asexual yeastlike growth, the fungus exhibits a parasexual cycle (*sensu* Pontecorvo 1956) in the absence of a suitable host, as can be shown on artificial media (Day and Cummins 1981). In that case the two nuclei of a single conjugate fuse, leading to recombination between vegetative cells by mitotic crossing over. The diploid cells can proliferate by yeastlike growth as well, until haploidization occurs.

## Host races of *M. violaceum*

As early as 1921, when Hermann Zillig published a paper '*Über spezialisierten Formen beim Antherenbrand*', separate host races of this fungus were distinguished (Zillig 1921). In this paper, he found 70 caryophyllaceous host species in literature and herbarium collections that showed a worldwide distribution of this fungus, with the exception of Australia. Furthermore, he compared morphological characteristics of teliospores from a sub set of host species, and performed cross-inoculations between several host races and species. After vainly efforts to infect *Silene dioica* (*Melandrium rubrum* in those days) with smut from *S. latifolia* (*M. album*), and *Saponaria ocimoides* with smut from *S. officinalis*, both closely related host species, he concluded that most host species have their own host races of *M. violaceum* (*Ustilago violacea).* However, Liro (1924) challenged this finding, stating that spores from *S. latifolia* and *S. dioica* could infect either of these host species. This was confirmed by Baker (1947), who stated, "*M. album (S. latifolia) is invading regions once occupied by M. dioicum* (*S. dioica*), *and is producing hybrid swarms. In this way, susceptibility of U. violacea* (*M. violaceum*) *is being introduced in to populations of M. dioicum*" (see also Baker 1948). This is an important statement within the context of this thesis, since the prejudiced reader might interpret this as a possible recent host shift event. With the dawn of molecular marker techniques, however, host races of several of these host species proved to be genetically different as well. Perlin and co-workers showed polymorphisms in both chromosome number and chromosome length (Perlin 1996; Perlin *et al.* 1997) among isolates of *M. violaceum* from different host species. Also, microsatellite analyses revealed strong host-related genetic differentiation between isolates from various caryophyllaceous host species (Shykoff *et al.* 1999; Bucheli *et al.* 2000). In all these studies the existence of a number of genetically different host races, or '*formae speciales*' of anther smut have been demonstrated in allopatric populations of hosts. However, whether this differentiation in sympatric

populations of hosts is of the same magnitude, or is diminished by fungal gene flow is yet unclear.

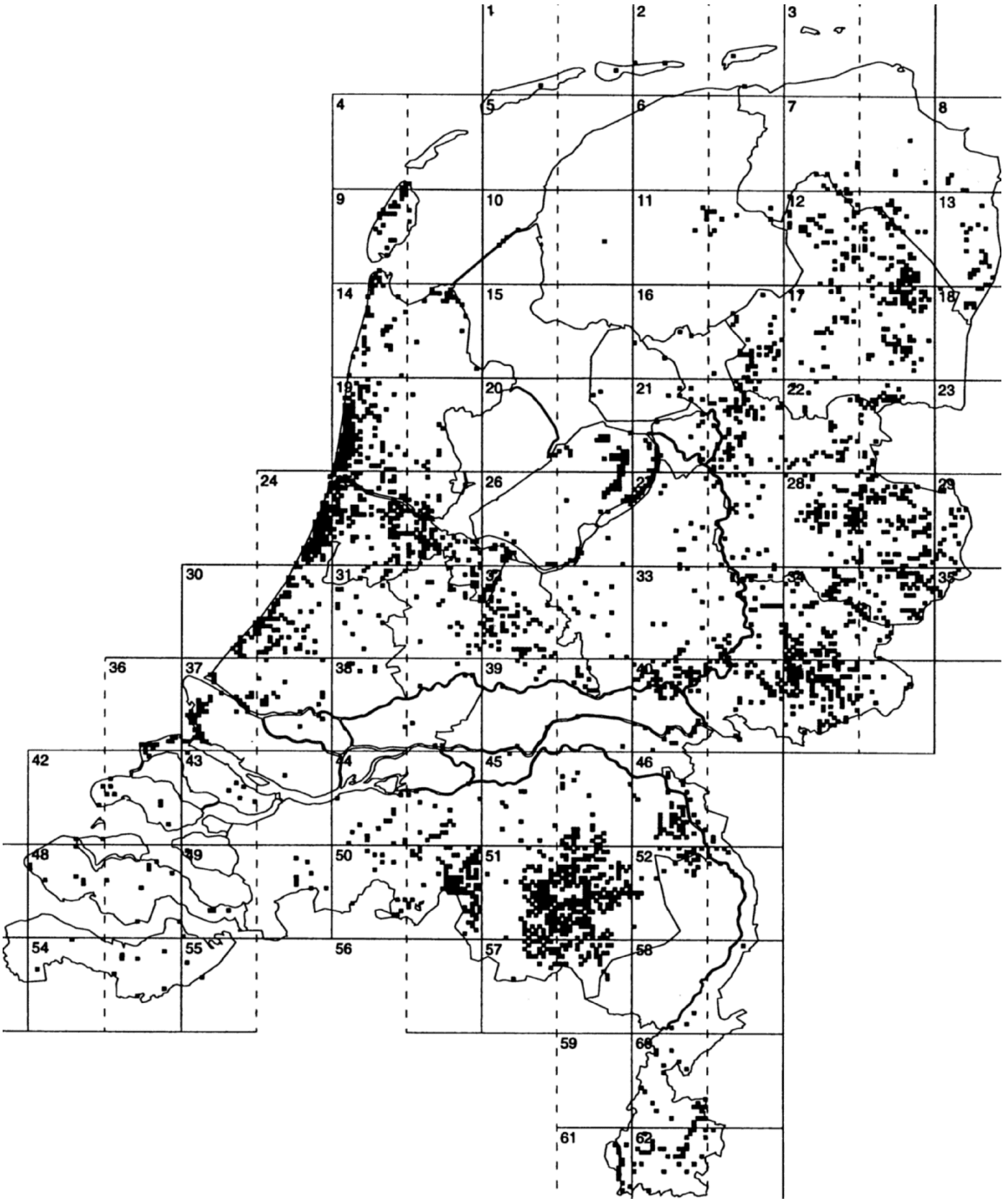


**Figure-4** Distribution of 1-km$^2$ squares with both *Silene latifolia* and *S. dioica* host species present in the Netherlands between 1975 and 1997 (FLORON, Florbase-2d; Leiden The Netherlands). The density of spots is an indication of putative sympatric host populations. Numbers point to numbered topographic maps of scale 1:25000 (Topografische dienst; Emmen The Netherlands).

## The host species *S. latifolia* and *S. dioica*

*Silene latifolia* Poiret (= *S. alba* (Miller) Krause (Caryophyllaceae), the white campion, is a dioecious short-lived perennial from open, disturbed habitats and borders of arable land. *Silene dioica* (L.) Clairv. (Caryophyllaceae), the red campion, is a closely related dioecious perennial that mainly occurs in more shady humid habitats as woodland borders. It is generally agreed that *S. latifolia* and *S. dioica* share a recent common ancestry (Prentice 1979; Desfaux and Lejeune 1996), and they share the same chromosome number (2n=24, *cf.* Prentice 1978). In areas where habitats overlap, or are adjacent, both species frequently occur in sympatry and hybridization is a common phenomenon (Baker 1947; 1948; Goulson and Jerrim 1997). Figure 4 shows the distribution of 1-km$^2$ squares in which both *S. latifolia* and *S. dioica* were present in the Netherlands between 1975 and 1997 (FLORON, Florbase-2d; Leiden The Netherlands). The high density of points in some parts shows that habitats are indeed frequently adjacent, and that parapatric, and possibly also sympatric host populations should be common. Differences in preference to either more shady (*S. dioica*) or sun-exposed habitats (*S. latifolia*) could have evolved by a differential adaptation to light intensity (Willmot and Moore 1973). Besides the differential habitat preferences that structure the population spatially, there are other specific differences that structure sympatric host populations also temporally. *S. dioica* is more a true perennial species than the annual to short-lived perennial *S. latifolia* (Prentice 1979). A consequence of this is that *S. dioica* often does not flower until their second season (Prentice 1978). Moreover, flowering phenology is different between host species and sexes, with *S. dioica* flowering earlier than *S. latifolia*, and males flowering earlier than female hosts (Biere and Honders 1996b). Morphological differences between the two host species include differences in hairiness of the stem and leaves (personal observation), shape of the seeds, and shape and color, size and scent of the flowers (Prentice 1979: Jürgens *et al.* 1996). Furthermore, both species have been reported to have different pollinator guilds in allopatry (*S. dioica*: e.g. Kay *et al.* 1984; Westerbergh and Saura 1994; Carlsson-Granér *et al.* 1998; *S. latifolia*: e.g. Brantjes 1976a; 1976b; 1981; Shykoff and Bucheli 1995; Altizer *et al.* 1998) and in sympatry (e.g. Baker 1947; Biere and Honders 1998; Jürgens *et al.* 1996; Goulson and Jerrim 1997). *S. latifolia* has a typical moth-pollination syndrome (Baker 1961; Baker and Hurd 1968), including heavily scented white flowers that open at dusk (Jürgens *et al.* 2001). *S. dioica*, having flowers that open at dawn and remain open during the day, is primarily pollinated by bumblebees (Kay *et al.* 1984).

## Vectors, pollinators and plant factors affecting transmission

The electron microscope photograph taken from Jennersten (1983), which is displayed in figure 3, clearly shows the relevance of pollinators to this venereal disease. Pollinators carry both teliospores (smaller spheres) and pollen of the host plant (larger spheres), thereby serving the dual role as host pollinator and disease vector. However, some pollinators have been reported to discriminate against infected host plants, for instance bumblebee species preferentially visiting healthy *S. latifolia* over infected ones (Altizer *et al.* 1998). There are many factors influencing the visitation behavior of pollinators, since they visit flowers for different reasons, e.g., foraging for nectar (Willson and Bertin 1978; Waddington 1981; Kay *et al.* 1984; Mitchell and Waser 1992; Pappers *et al.* 1999; Navarro 2000), robbing pollen (Utelli and Roy 2001) or oviposition (Brantjes 1976a; 1976b). Hence, insect visitors exhibit much variation in both efficiency or 'quality' (Herrera 1987), and frequency or 'quantity' (Herrera, 1989) of pollen transfer (Utelli and Roy, 2000). In addition to this, trade-offs between frequency and duration of flower visits have been recorded for bumblebees (Jones *et al.* 1998). Also, there are clear differences in behavior between diurnal and nocturnal pollinator guilds (e.g. Bertin and Willson 1980; Guitan *et al.* 1993; Groman and Pellmyr 1999). A number of plant factors determine and influence the behavior of insect visitors, such as plant density (Schmitt 1983; Bosch and Waser 1999), inter-plant spacing (Bucheli and Shykoff 1999), floral display size (Klinkhamer *et al.* 1989; Goulson et al 1998; Stout 2000) and several morphological characteristics of flowers, e.g. size (Galen and Stanton 1989), color (Kay 1976; Jones and Reithel 2001) and shape (Møller and Eriksson 1995). Although these studies all examine pollen transfer, to some extent many of the factors will also hold true for the transfer of fungal teliospores, since they arise in the anthers of the flower just as pollen does.

## Fungal markers and mimics

In this thesis we use two different types of markers to assess genetic variation in fungal isolates from *S. latifolia* and *S. dioica* (and hybrids). In addition, a third non-genetic marker is used to explore differences in vector visitation patterns between the two host species.

First, we will use variation at one of the sporidial colony color (SCC) loci that is described in Garber *et al.* (1975). Figure 5 shows the biosynthesis pathway of the 'color molecule' β-carotene, starting with phytoene (Porter and Lincoln 1959; Porter and Anderson 1962). In the first part of this pathway phytoene is converted into lycopene by dehydrogenases, in the second part these linear molecules can be

transformed by cyclases, forming rings at the termini of the lycopene molecule. If such a ring is formed at just one terminus, it results in γ-carotene. When rings are formed at both termini, this results in β-carotene, an important precursor of vitamin A (retinol) in higher organisms.

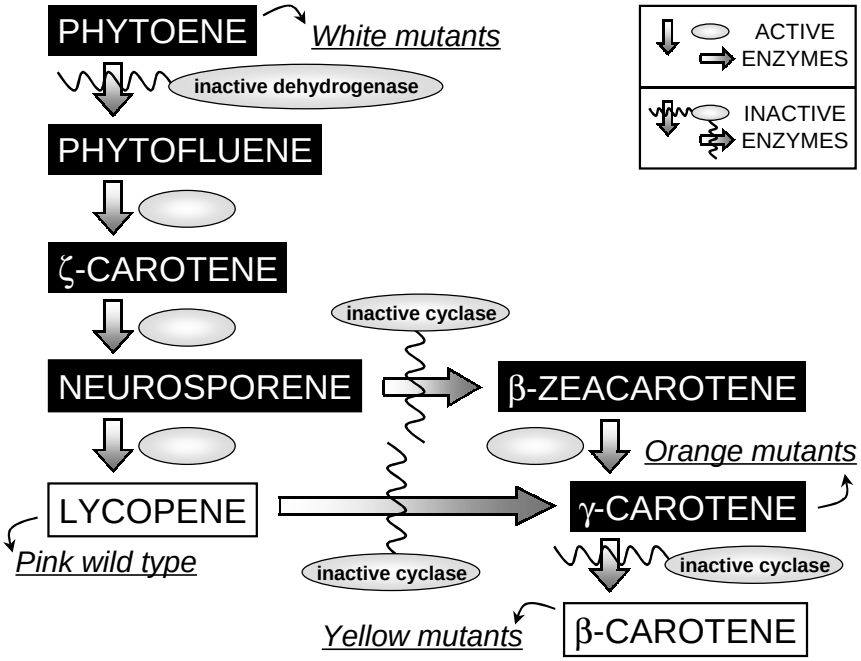


**Figure-5** Carotenogenesis from phytoene to β-carotene, and carotenoid mutants at sporidial colony color (SCC) markers in *Microbotryum violaceum*, as proposed by Garber *et al.* (1975). The enzymes from phytoene to lycopene are dehydrogenases; further down the pathway the enzymes are cyclases. Note that the SCC phenotypes observed and used in this thesis, are the wild type *pink* (in which strains accumulate lycopene) and the mutant *yellow* (in which the cyclases are active, and hence strains accumulate β-carotene).

The exact number of different enzymes that is involved in this pathway is not known. From phytoene to lycopene could require only one type of enzyme (Porter and Anderson 1962). Possibly, only one enzyme is required for the conversion of lycopene to γ-carotene and then to β-carotene as well. Carotenoids give color to many plant fruits, such as the deep orange of carrots and the red in tomatoes. Likewise, these molecules color the sporidia of *M. violaceum* when they are grown on artificial media. The wild type of this fungus does not contain active cyclases, and hence sporidia accumulate lycopene that gives the colonies a pink color. Some natural occurring mutants do contain active cyclases, and result in orange (one active cyclase) and yellow colored colonies (two active cyclases). Other mutants have inactive dehydrogenases and accumulate phytoene, giving the colonies a white color. Biere and Honders (1996a) found that most of the smut isolates from allopatric *S. latifolia* were of the wild type, while most smuts isolated from allopatric *S. dioica* were of the *yellow* mutant phenotype.

The second type of marker that is used in this thesis is a molecular genetic marker, the microsatellite. Microsatellite loci are tandemly repeated short sequence motifs of DNA up to six bases long, that have been detected within the genome of every organisms so far analyzed (Hancock 1999). Microsatellites are co-dominantly

inherited and show high levels of polymorphism (e.g. Tautz 1989), often providing ample resolution for studying population genetic structure within and between populations of a single species. Rates of mutation of microsatellites are high (around $10^{-4}$ to $10^{-5}$ in yeast (Henderson and Petes 1992; Strand *et al.* 1993)) compared to rates of point mutation, which are in the order of $10^{-9}$ to $10^{-10}$. Besides mutation through recombination by unequal crossing-over or gene conversion, the predominant model of the mechanism of mutation is slipped strand mispairing ('slippage') during replication, which occurs due to the repetitiveness of the microsatellite templates (*cf.* Hancock 1999). In that case the nascent strand reanneals out-of-phase, and the resulting strand will be longer or shorter than the template strand. Conventional statistical methods that have been developed to analyze genetic variation, for instance in allozymes, are based on Wright's *infinite allele model* (Wright 1951; Nei 1987; Weir 1996), and yield classic F-statistics such as $F_{ST}$. However, mutations at microsatellite loci satisfy the *stepwise mutation model*, i.e. addition, or deletion of a single repetitive unit (Kimura and Ohta 1978), better than the *infinite allele model*. Therefore, for such data, novel methods based on differences of allele size variances have been developed, yielding R-statistics such as $R_{ST}$ (from the greek P (rho), analogous to F-statistics and Φ (phi)), which is thought to be more appropriate to estimate population differentiation than $F_{ST}$ values (Slatkin 1995). However, population differentiation estimates obtained from microsatellite date should be cautionary evaluated since some constraints on the evolution of microsatellite loci, e.g. constraints on allele size (Nauta and Weissing 1996) might produce biased estimates. Furthermore, Hedrick (1999) showed that the level of genetic divergence between groups, expressed in $F_{ST}$ values, is greatly influenced by the level of heterozygosity due to the method of calculation, and argued that any interpretations from these highly variable loci should therefore be made carefully, since they may yield statistical but not biological significance. Bucheli *et al.* 1998 developed five microsatellite loci for the anther smut *M. violaceum* using isolates from *S. latifolia*. Four of these loci could be successfully transferred with minor modifications to our lab conditions, and were used to investigate genetic divergence between fungal isolates in this thesis. These microsatellite loci showed host-related genetic differentiation among smut samples that were isolated from allopatric populations of a wide range of caryophyllaceous host species (Shykoff *et al.* 1999; Bucheli *et al.* 2000), and specifically between allopatric *S. latifolia* and *S. dioica* host populations in Switzerland (Bucheli *et al.* 2001).

A third non-genetic marker is used to assess differences in visitation patterns between the pollinator guilds of *S. latifolia* and *S. dioica*, that serve as vectors of this fungal disease. This marker is a set of fluorescent dyes that here function as traceable teliospore surrogates. Fluorescent dyes have been successfully used to trace pollen movements across plants in many studies (e.g. Thomson 1981; Waser and Price 1982; Fenster *et al.* 1996; Goulson and Jerrim, 1997), and have proven to be a good predictor of the dispersal of fungal spores as well (Shykoff and Bucheli 1995). The occurrence of dye on a flower is a qualitative measurement of the contact with a pollinator and/or a vector. As Thomson *et al.* (1986) rightly points out, using fluorescent dye as pollen analogue and simply scoring the presence/absence of dye on a flower surely would overestimate the extent of dispersal because stigmas are often more receptive to the much smaller dye particles than they are to pollen grains. Therefore, dye particles may remain longer on a pollinator than do pollen, as was found in the comparative study to the transfer of pollen and fluorescent dye (Thomson *et al.* 1986). However, whereas pollen of *S. latifolia* is size-ranged 35-60μm (Prentice *et al.* 1984), teliospores are much smaller, ranging from 6-9μm (Zogg 1985, see also figure 5). Fluorescent dye particles are more or less of equal size as *M. violaceum* teliospores, and may therefore be better spore-analogues than they are pollen-analogues, although they may be more sticky due to their irregular shape (personal observation). We use different colored dyes to differentiate between dye transmission from *S. dioica*, and from *S. latifolia* plants in our experiments.

## INNOVATIVE ASPECTS, AIM AND OUTLINE OF THIS THESIS

This thesis presents one of the first ecological studies on host race differentiation of a plant pathogenic fungus in host sympatry. Fungal isolates of the anther smut *M. violaceum* from allopatric populations of *S. latifolia* and *S. dioica* have previously shown to be differentiated, which was demonstrated by several authors (e.g. Zillig 1921, Perlin 1996). In sympatric populations of hosts, at least some gene flow is expected, which would act to homogenize the differentiation. On the other hand, if spores from one host species are deposited on the 'alien' host species, this may result in such fitness penalties for the pathogen, that differentiation is maintained, or can even be increased. Differentiation that is observed in allopatry, not necessarily has evolved in allopatry. As the empirical evidence for host race formation and

speciation in sympatry is scarce, and heavily biased towards phytophagous insect systems, it would be interesting to show in the *Silene-Microbotryum* system whether the differentiation between fungal isolates could have evolved in host sympatry. This would strongly contribute to the understanding of the scope for sympatric divergence, host race formation and speciation.

The main aim of this study is to investigate host-specific differentiation between fungal strains from *S. latifolia* and *S. dioica*, as they appear in allopatric, parapatric and sympatric populations of these host species, in the evolutionary context of host race formation and speciation. More specifically, I will try to assess the degree of genetic divergence at different levels of host sympatry, the existence of performance trade-offs, factors affecting reproductive isolation (e.g. vector behavior (fungal gene flow), assortative mating, and reinforcement (inferiority of hybrid dikaryons)), and other processes that may be involved in creating, maintaining, or dissolving genetic divergence between fungal isolates from different host species.

## Outline of this thesis

*Genetic differentiation and degree of sympatry* - In chapter 2, we will address the question to what extent strains from the two host species have diverged and whether the extent of divergence is affected by the degree of host sympatry. For this purpose, a microsatellite study was performed, analyzing the population genetic structure of anther smut populations that were isolated from a number of allopatric, parapatric and sympatric populations of *S. latifolia*, *S. dioica* and their interspecific hybrids, from locations in the Netherlands, France and the United Kingdom.

*Impact of host spatial structure on differentiation* - In chapter 3, the host spatial structure of one of the more sympatric populations from chapter 2 was examined in more detail. Specifically, the impact of host patch structure on the allele frequencies of the sporidial colony color marker was investigated. Furthermore, the multilocus microsatellite genotypes (from chapter 2) were plotted geographically in the population, and we analyzed the randomness of distribution of alleles over the population.

*Mating and competitive ability of strains* - In chapter 4, assortative mating and competitive ability of strains from the two host species were estimated. In a mating experiment, isolates from allopatric hosts from *S. latifolia* and *S. dioica* were crossed *in vitro* in a complete diallel. The conjugation frequencies after 24h in different host extracts and in water were evaluated. Furthermore, fungal isolates from both host species were used in a competition experiment *in vivo* on both *S. latifolia* and *S.*

*dioica*, analyzing their infection success. Also, the amount of multiple infections of a single host was estimated in this experiment. A direct link between the mating experiment and the competition experiment was provided by a sub set of isolates that were used in both experiments. From these experiments, the relative fitness of strains from both host species in different host environments was estimated.

*Host fidelity of vectors* - In chapter 5, the role of vectors in effectuating positive assortative mating between strains from the two host species was studied. We investigated the fidelity of pollinators/vectors for a specific host species in a set of experiments in which fluorescent dye was used to trace vector movements over artificial, and fully mixed plots of *S. latifolia* and *S. dioica*. In these experiments, we distinguished between diurnal and nocturnal pollinator guilds, and varied patch size of the host species. In addition, the distance over which dye was transferred in 24h was compared to the distance of true infection rates of *M. violaceum* in an experiment that examined infection of healthy *S. latifolia* over one flowering season, given a large teliospore source.

*Implications for host races in sympatry* - In chapter 6, the results of the study are summarized and discussed. Special attention is paid to fungal isolates from *S. latifolia* and *S. dioica* in sympatry, addressing the main questions of this study: what factors are involved in creating, maintaining or dissolving of (genetic) variation between fungal isolates from *S. latifolia* and *S. dioica*; to what extent is the degree of sympatry influencing this variation; and is there a balance between migration and selection?

# CHAPTER 2

## Host-related genetic differentiation in the anther smut fungus *Microbotryum violaceum* in sympatric, parapatric and allopatric populations of two host species *Silene latifolia* and *S. dioica*

with Arjen Biere and Jos van Damme

*submitted to Molecular Ecology*


### Abstract

**We investigated genetic diversity in West-European populations of the anther smut *Microbotryum violaceum* in sympatric, parapatric and allopatric populations of the host species *Silene latifolia* and *S. dioica*, using four polymorphic microsatellite loci.**

**Between the allopatric host populations of *S. latifolia* and *S. dioica*, the fungus was highly differentiated, and revealed clear and distinct host races for these host species. In all sympatric and parapatric populations, except for one sympatric population in which the two host species grew truly intermingled, we found significant population subdivision with respect to host species as well, exhibiting high values of $F_{ST}$ and $R_{ST}$. The extent of genetic differentiation between the host races decreased with increasing degree of sympatry, indicating increased levels of gene flow in more sympatric populations. Genetically, fungal isolates from interspecific hybrids resembled isolates from *S. latifolia* more than isolates from *S. dioica*.**

**The mean number of alleles per locus for isolates from each of the host species was significantly higher in sympatric/parapatric than in allopatric populations, suggesting that the nearby presence of strains from other host species can increase the level of genetic variation in each of the demes. Observed levels of heterozygosity were significantly lower than expected under Hardy-Weinberg equilibrium, confirming the selfing nature of this fungus. The overall levels of heterozygosity were found to be significantly lower in samples from *S. dioica* than in samples from *S. latifolia*.**

**The observed host-related genetic differentiation among these geographically spread populations suggest a long-term divergence between these host races of *M. violaceum* that most likely has evolved in allopatry. In sympatric host populations, both host races presumably come in secondary contact, and host-specific alleles are exchanged depending on the degree of sympatry in the population.**

## INTRODUCTION

Plant parasites can often exploit more than one host species, and show intraspecific variation in host use. Different host species can represent different ecological niches to which the parasite can adapt by natural selection. When different host species occur in allopatry and are isolated by distance, pathogen populations are isolated as well, and subject to random processes such as genetic drift, especially when population sizes are small (Wright 1931; Kimura 1955). The process of genetic divergence is strongly enhanced by disruptive selection on habitat preference (Rice and Salt 1990), or on fitness related aspects of the specific combination of host and parasite (e.g. in spider mites: Gotoh *et al.* 1993) where host and mate selection are correlated. As correlations between host (habitat) preference and assortative mating develop (e.g. offspring returning to the parental habitat to mate), host race formation can occur either in allopatry, or when different hosts (habitats) are present within the 'cruising range' of the parasite, in sympatry as well (Berlocher 1998a; Via 1999). In general, host race formation in sympatry is poorly documented in literature (but see Tauber and Tauber 1989; Berlocher 1998a), and almost solely devoted to phytophagous insects (e.g. in frugivorous flies: Bush 1969; in pea aphids: Via 1999; 2000). Many genera of insects exhibit variability in numerous behavioral, physiological and ecological traits that could advance sympatric speciation, including mating in association with the host, or after habitat selection (see Tauber and Tauber 1989 for review).

In phytophagous fungi, there are a few examples of host races, or species that might have evolved in sympatry (for instance in three *Phytophtera palmivora* sub species), but even in these cases it is feasible that they arose in allopatry, and that their ranges became overlapping at a later stage (*cf.* Burnett 1983). The evidence for (sympatric) host race formation and speciation point to two major factors (Brasier 1987), (1) (micro-)climate, in which divergence is promoted by locally strong gradients and discontinuities, and (2) substrate, in which divergence is driven by opportunistic nutritional strategies of the pathogen. However, most of these studies are strongly biased towards more economically important organisms (see Brasier 1987 for review), putting the emphasis unintentionally on the host rather than on the pathogen (but see Giraud *et al.* 1999). This also holds true for the 'physiological specialization' of smut fungi in the review of Fisher and Holton (1957 p.331) that examines susceptibility and resistance of cereal and oat smuts of the *Ustilago* genus.

Nevertheless, they represent good examples of host specialization in phytopathogenic fungi, which can be a starting point for host race formation and, eventually, for speciation. Recent examples often use molecular markers in search of a genetic basis of (sympatric) host races, e.g. studies by Peever and co-workers in *Alternaria* (brown spot fungus of citrus), showing host-related genetic differentiation between citrus fruit cultivars (Peever *et al.* 1999; Peever *et al.* 2000), and in *Macrophomina* (charcoal rot fungus infecting root tissue of some crop species) showing both genetic differentiation between host species, and combine this with data on host preference (Su *et al.* 2001). From the ecological examples, perhaps the most extensively studied organism in this field is the anther smut *Microbotryum violaceum*.

The anther smut fungus *M. violaceum*, phytopathogenic fungus of the Caryophyllaceae (Pinks), provides a good model system to examine sympatric host race formation in natural field populations, since a number of hosts occur in sympatry and hybridize where habitats overlap (Baker 1947; 1948; Goulson and Jerrim 1997). Moreover, strains of this fungus that were isolated from a number of different host species show varying degrees of host differentiation and specialization (Zillig 1921; Biere and Honders 1996a) and genetic differentiation (Perlin 1996; 1997; Shykoff *et al.* 1999; Bucheli *et al.* 2000). Karyotype studies of fungal strains from a wide range of host species within the Caryophyllaceae, examined by Perlin and co-workers, showed polymorphisms in both chromosome number and length (Perlin 1996; Perlin *et al.* 1997). Microsatellite analysis also revealed strong host-related differentiation in various caryophyllaceous host species (Shykoff *et al.* 1999; Bucheli *et al.* 2000). In all these studies the existence of a number of genetically different host races or *formae speciales* of anther smut have been demonstrated in allopatric populations of hosts. In sympatric populations of hosts, gene flow between fungal isolates may be common if prezygotic isolation mechanisms are weak, or absent. However, the amount of fungal gene flow, the impact of this gene flow on the differentiation among fungal isolates, and whether fungal isolates from sympatric populations of hosts show host-related genetic differentiation and are diverged in sympatry, is yet unclear.

In this study we focus on two of the fungus' host species *Silene latifolia* and *S. dioica*, common dioecious herbs in Western Europe with quite different yet frequently adjacent habitats. The occurrence of interspecific hybrids between these plant species, reported to constitute more than 6% of the sympatric population of Norg (Biere and Honders 1996b), is a silent witness of plant gene flow between these host species, and would suggest that there is fungal gene flow as well. Several authors have reported strong divergence between isolated samples from *S. latifolia* and *S. dioica*. Surveys of

allopatric populations of hosts in the Netherlands using one of the sporidial colony color loci (Garber *et al.* 1975) as a marker, showed clear host-specific differentiation between strains isolated from *S. latifolia*, which were almost fixed for the 'pink' allele and strains isolated from *S. dioica*, which were almost fixed for the 'yellow' allele (Biere and Honders 1996a; Van Putten *et al.* chapter 3). Genetic differentiation between strains from allopatric populations of these host species has been observed using RAPD markers (Biere and Honders, unpublished data) as well as in five microsatellite loci (Bucheli *et al.* 2001). In addition, cross inoculation experiments between strains from these hosts have shown that strains had up to three-fold higher spore-production on male plants of their hosts of origin, indicating that fungal strains have adapted to their native host species (Biere and Honders 1996a). However, the same study indicated that infection success of strains was not significantly lower on the non-native host species, indicating that gene flow between strains could occur, depending on the behavior of the pollinators that act as vectors of this phytopathogen.

Here, we investigate genetic differentiation and population sub structuring of anther smuts in sympatric and parapatric populations of hosts in comparison to strains isolated from allopatric populations of hosts. Since no constraints on gene flow in sympatric populations are expected a priori due to frequent hybridization between *S. latifolia* and *S. dioica* (Baker 1947; 1948; Goulson and Jerrim 1997), genetic population subdivision with respect to host species could provide insight into whether the divergence between fungal isolates from these host species could have evolved in sympatry, and whether host-related differentiation could be maintained in the presence of gene flow. Note that the terms sympatry, parapatry and allopatry (*sensu* Kondrashov and Mina 1986) refer to the two closely related, yet different host species rather than to the fungus itself.

Specific questions that will be addressed in this paper are: (1) To what extent are populations of *M. violaceum* genetically differentiated due to host species, and/or due to geographic distance? (2) How does scale of sympatry influence the population structure of this fungus, i.e. is fungal gene flow between anther smuts from different host species, estimated by the genetic differentiation among fungal isolates, dependant of the degree of host sympatry?

# MATERIALS AND METHODS

## The Species

The anther smut fungus, *Microbotryum violaceum* (Pers.:Pers) Deml & Oberw. (= *Ustilago violacea* [Pers.] Fuckel) (Ustilaginaceae) (Deml and Oberwinkler 1982) is a heterobasidiomycete that obligatory parasitises susceptible members of the Caryophyllaceae to complete its sexual life cycle, thereby sterilizing the host plant (Baker 1947). The most striking disease symptom of an infection with this fungus is the overriding of the genetically determined sex expression in dioecious host species by halting the development of female reproductive tissue (Audran and Batcho 1981) and inducing the expression of 'male-specific' genes (Scutt *et al.* 1997) that are also present, yet inactive in female hosts (Matsunaga *et al.* 1996). In female plants, ovaries are reduced and staminal rudiments develop into stamens that contain purple-brownish smut spores. Male flowers also bear teliospores in their anthers instead of pollen. Teliospores are diploid thick-walled heterothallic cells that undergo meiosis when they germinate to produce haploid sporidia of two mating types that proliferate asexually by yeastlike growth. Sporidia of opposite mating type conjugate to produce a dikaryotic infection hyphen that can enter a host plant. Spores are transmitted by the natural pollinators of their hosts, which also serve as vectors of this disease (Jennersten 1983).

*Silene latifolia* Poiret (= *Silene alba* [Miller] Krause), the white campion is a short-lived perennial weed that grows in open, disturbed habitats. The closely related *S. dioica* (L.) Clairv., the red campion, is a perennial weed that mainly occurs on the edges of forests and in open woodland. Both species are dioecious and in areas where habitats overlap hybridization is a frequently occurring phenomenon (Baker 1947; 1948; Goulson and Jerrim 1997). Although both species are common in Western Europe, truly mixed sympatric populations are scarce, or even absent because of differential habitat preferences.

## Collection sites

We have sampled eight populations of anther smut in Western Europe (see figure 1 for their geographical distribution), four from sympatric/parapatric populations of hosts, two from allopatric *S. latifolia* populations and two from allopatric *S. dioica* populations. All populations contained at least a few hundred host plants at the time of sampling. The sympatric/parapatric populations are all patchy

with respect to host species (Table 1). At the Norg sampling site (which has been studied extensively by Biere and Honders 1996b; 1998), patches with predominantly *S. latifolia* and patches with predominantly *S. dioica* are closest together, down to a few decimeters (but on average 10-14 meters).

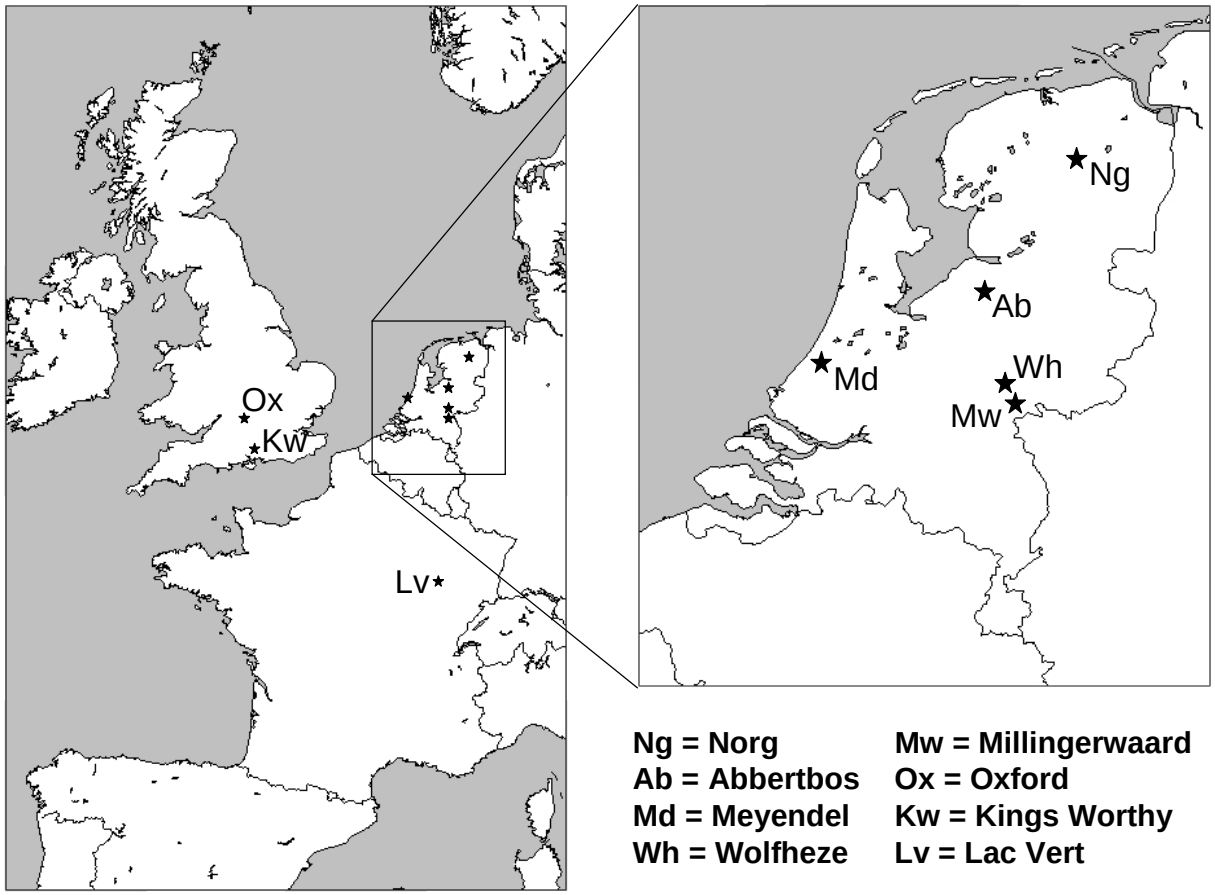


**Figure-1** Geographical locations of the sampled smut populations of *Microbotryum violaceum* in Western Europe. Norg, Abbertbos, Oxford and Kings Worthy are sympatric/parapatric host populations containing *Silene latifolia, S. dioica* and interspecific hybrids, Wolfheze and Millingerwaard are allopatric *S. latifolia* populations, and Meyendel and Lac Vert are allopatric *S. dioica* populations. (Maps created using Online Map Creator; © kk+w - digital cartography, http://www.aquarius.geomar.de/omc/make_map.html).

At the Abbertbos site, patches of both host species are further apart, up to a few decameters at closest, and at the Oxford site they are even more separated, at a distance of a few hectometers from each other. The latter two populations could therefore also be considered parapatric instead of sympatric. In the British population of Kings Worthy, *S. dioica* was present but no infected individuals were found, hence we lack fungal isolates from this host species at this site. The sympatry level of this population was comparable to that of the population at Abbertbos. In all four sympatric populations (Ng, Ab, Ox and Kw) hybrid hosts occurred within *S. latifolia* patches, rather than within *S. dioica* patches. Interspecific *S. latifolia* x *S. dioica*

hybrids could be distinguished from the pure species forms by their intermediate morphology (Goulson and Jerrim 1997), which is expressed in gradients of leaf shapes, flower colors and hairiness of stems (Baker 1951). Hybrids may include both $F_1$ and backcrosses. In populations that were classified allopatric in this study (Lv, Md, Mw and Wh), the other host species was not observed within a range of a few kilometers.

**Table-1** Detailed information about the collection sites of *Microbotryum violaceum* teliospores and their *Silene* host populations (see figure 1 for locations in Europe).

| **Population *Abbrev.*** Host species | **Area description** | **Location** | **N** | **Sympatry status** |
|---|---|---|---|---|
| **Abbertbos *Ab*** | 200m x 800m | 52°26'N | **(39)** | *S. latifolia* and Hybrids are |
| *S. latifolia* | Half-open terrain and | 5°49'E | 17 | mixed in patches, *S. dioica* |
| *S. dioica* | woodland, population | | 12 | are at a discrete distance |
| Hybrids | established > 1960 on | | 10 | (100m - 500m). Sympatric / |
| | newly claimed land. | | | Parapatric. |
| **Norg *Ng*** | 20m x 1 km | 53°06'N | **(38)** | All are mixed in patches, *S.* |
| *S. latifolia* | Rural roadside, along | 6°30'E | 21 | *latifolia* and Hybrids in open |
| *S. dioica* | shrubs (see chapter 3 | | 7 | areas, *S. dioica* under trees. |
| Hybrids | for details). | | 10 | True sympatric. |
| **Oxford *Ox*** | 2km x 4 km | 51°41'N | **(119)** | *S. latifolia* and Hybrids are |
| *S. latifolia* | Rural, open terrain, | 1°23'W | 76 | mixed in patches, in open |
| *S. dioica* | woodland, in between | | 36 | areas, *S. dioica* are at discrete |
| Hybrids | golf courses and | | 6 | distances (100m – few km). |
| | along a pig farm. | | | Parapatric. |
| **Kings Worthy *Kw*** | 500m x 500m | 51°05'N | **(14)** | *S. latifolia* and Hybrids are |
| *S. latifolia* | Open terrain, along | 1°17'W | 12 | mixed in patches, *S. dioica* |
| *S. dioica* | woodland, and high | | - | adjacent in woodland. |
| Hybrids | way. | | 2 | Infected *S. dioica* not |
| | | | | observed. Sympatric / |
| | | | | Parapatric. |
| **Lac Vert *Lv*** | 20m x 200m | 48°06'N | **10** | Allopatric. |
| *S. dioica* | Woodland along lake. | 7°07'E | | |
| **Meyendel *Md*** | 10m x 200m | 52°10'N | **15** | Allopatric. |
| *S. dioica* | Woodland. | 4°30'E | | |
| **Millingerwaard *Mw*** | 2km x 10km | 51°52'N | **15** | Allopatric. |
| *S. latifolia* | Open terrain, along | 6°03'E | | |
| | riverside. | | | |
| **Wolfheze *Wh*** | 15m x 300m | 52°00'N | **9** | Allopatric. |
| *S. latifolia* | Open terrain, rural | 5°48'E | | |
| | roadside. | | | |

## Sampling teliospores and microsatellite analysis

Teliospores were collected from as many infected individual host plants as could be found by browsing through a population, and from both male and female host plants. Where possible, closed flower buds were taken to avoid cross infection. While gently opening the flower buds, spores were collected in 1.5ml eppendorf cups. Since only one dikaryon usually manages to grow into a flower (Day 1980), teliospores from single flower buds are regarded to be identical. Diploid teliospores, from single infections, were plated on standard medium (Cummins and Day 1977) with a sterile inoculation loop. Haploid sporidia, produced after teliospore germination and meiosis, were grown for one week. From the agar medium, cells from many colonies were scraped off the plate, put into an eppendorf cup, and freeze dried for several hours. Freeze dried samples were stored in a dry environment containing silica gel until DNA isolation. DNA was isolated using the PureGene Genomic DNA isolation kit for yeast (Gentra systems, Minneapolis MN, USA). DNA was dissolved in 100 µl DNA hydration solution (Gentra Systems, Minneapolis MN, USA) and stored at –20°C until PCR amplification. DNA from anther smut that is isolated following this procedure contains all genetic material that is present in the original diploid teliospore-parent, thus creating 'pseudo-diploid' DNA samples. We used four microsatellite loci that were developed by Bucheli *et al.* (1998), which are shown in table 2.

**Table-2** Characteristics of the microsatellite loci (adapted from Bucheli *et al.* 1998). $T_a$ = annealing temperature. Standard product lengths (in bp) as reported in Bucheli *et al.* (1998). Allele size range (in bp) as observed in this study and number of alleles in parentheses. Note that locus 17 was omitted from the collection due to amplification difficulties.

| Locus | Primers (5' to 3') | $T_a$ (°C) | Array | Product length (bp) | Size range (bp) (# of alleles) |
|---|---|---|---|---|---|
| 6 | GTAGCCACCTCCCATCCC<br>CGGTGTCGAGTTCCTTGAC | 55 | $(AG)_{15}$ | 134 | 116 – 142<br>(8) |
| 11 | AAAACCCAAGACGACTGACGC<br>TTCCTTCGATGCAGCCTC | 53 | $(AC)_{11}$ | 92 | 96 – 100<br>(3) |
| 14 | GTCGTTCTCGCTCTCTC<br>GGGGCTCGTGAAGCCG | 53 | $(AG)_{15}$ | 60 | 62 – 78<br>(8) |
| 18 | CCCCACAGACGGTATGCTGC<br>CGTGACACCCTTCCTGCCGC | 55 | $(AG)_{15}$ | 146 | 144 – 188<br>(14) |

Conditions for PCR were modified from Bucheli *et al.* (1998) in the following way; reactions were set up in volumes of 15µl, each containing 10-50ng DNA, 1 x reaction buffer (with 1.5mM $MgCl_2$), 0.3mM dNTP, 0.4 units of Expand Taq polymerase (Roche, Indianapolis IN, USA), ±2pmol Cy5-fluorescent labeled forward

primer and ±2pmol reverse primer (loci 6 and 18), and ±20pmol Cy5-labelled forward primer and ±20pmol reverse primer (loci 11 and 14). PCR reactions (40 cycles) were performed in a PTC-200 thermal cycler (MJ Research, Watertown MA, USA). PCR products were visualized using an ALF Express II sequencing system (Amersham Pharmacia Biotech, Uppsala, Sweden). The length of the PCR fragments, relative to three 'internal sizers' (DNA fragments of known length that are close to, but not interfering with the PCR fragment lengths, that were mixed with the loading buffer and hence were running in the same lane of the gel (Ben Vosman, personal communication)) were calculated using the software package ImageMaster Elite v3.00 (Amersham Pharmacia Biotech, Uppsala, Sweden). Repeat numbers of the different alleles were determined from the amplified fragment length relative to the standard product length as described in Bucheli *et al.* (1998).

## Statistical data analysis

Allele frequencies, mean numbers of alleles per locus ($N_A$), number of unique genotypes ($N_G$), observed heterozygosities ($H_O$), and expected heterozygosities (Nei's unbiased estimate of $H_S$, Nei 1987) were determined for all populations with the use of BIOSYS-1 (Swofford and Selander 1989). Deviations from Hardy-Weinberg expectations were tested by Fisher's exact test based on the Markov chain method of Guo and Thompson (1992) in GENEPOP-3.2a (an update of Raymond and Rousset 1995). Linkage disequilibrium was calculated in GENEPOP by creating numerous contingency tables for all pairs of loci, and the independence of these tables was tested with Fisher's exact test. Allelic richness ($R_S$, an estimate of the number of alleles per sample that is not biased by sample size; El Mousadik and Petit 1996), conventional F-statistics (values $F_{ST}$, $F_{IT}$ and $F_{IS}$; Weir and Cockerham 1984), and values for $R_{ST}$ (F-statistic analogues that also take into account the size of the allele (Slatkin 1995) and assume a stepwise mutation model) were calculated with the help of FSTAT-2.9.3 (an update of Goudet 1995). The effective number of migrants per generation *Nm* (Slatkin 1995) between anther smuts within a sympatric and/or parapatric host population was calculated using the Private Allele method of Barton and Slatkin (1986) in GENEPOP, and by hand calculation from derived $F_{ST}$ and $R_{ST}$ values using an adaptation of Wright's infinite island model (1951) by Crow and Aoki (1984). They considered an *n*-island model of population structure with the infinite alleles model of neutral mutation, and showed that, at equilibrium:

$$F_{ST} \approx \frac{1}{(1+4Nm\alpha)} \quad , \qquad \text{(eq. 1)}$$

where

$$\alpha = \left( \frac{n}{n-1} \right)^2 \quad , \qquad \text{(eq. 2)}$$

and *n* the number of islands. In our case each host type (the two parental species and hybrids) is considered an 'island' for the fungus. Therefore, we set n=3, and hence $\alpha$=2.25. To test for isolation by distance, a Mantel test (Mantel 1967), which calculates the correlation between the genetic and the geographical distance matrix (10000 permutations), was performed by GENEPOP. An analysis of molecular variance (AMOVA; Weir and Cockerham; 1984, Excoffier *et al.* 1992; Weir 1996) was performed by ARLEQUIN-2.00 (Schneider *et al.*, 2000). Observed heterozygosities over all loci between pooled host samples were analyzed using the GENMOD procedure in SAS-v8 (The SAS Institute Inc., Cary NC, USA). A consensus Neighbor Joining tree based on Nei's unbiased genetic distance was constructed using PHYLIP-3.5c (Felsenstein, 1993), using a Swiss anther smut population from *S. acaulis* (adapted from Bucheli *et al.* 2000) as an outgroup. Bootstrap values were derived after 10000 resamplings of the data.

## RESULTS

### Population and host structure

Table 3 shows the allele frequencies of the four microsatellite loci for all sampled populations. The heterozygosities, and F and R-statistics for all four microsatellite loci are given in table 4. Table 5 shows these molecular diversity indices for each of the populations averaged over these loci. All four loci contribute to the significant genetic sub structuring with high values of $F_{ST}$ and $R_{ST}$, both among populations and among host species (Table 4). Except for locus 11, the patterns of observed and expected heterozygosities are consistent among loci.

**Table-3** Allele frequencies for four polymorphic microsatellite loci in *Microbotryum violaceum*. Alleles are displayed here as numbers of whole repeats. The underlined frequencies in the bottom part of the table denote the most frequent alleles in the pooled hosts (cumulative % of 75 or more). † = *Silene dioica* plants were present in the population, but no infected specimen were found.

| | **Microsatellite locus** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Population *Abbrev.*** | **6** | | **11** | | **14** | | **18** | |
| Host species (N) | **Allele** | **Freq** | **Allele** | **Freq** | **Allele** | **Freq** | **Allele** | **Freq** |
| **Abbertbos *Ab*** | | | | | | | | |
| *S. latifolia* (17) | 6 | 0.382 | 13 | 0.412 | 19 | 0.412 | 15 | 0.059 |
| | 8 | 0.500 | 14 | 0.088 | 20 | 0.588 | 16 | 0.529 |
| | 18 | 0.118 | 15 | 0.500 | | | 34 | 0.412 |
| *S. dioica* (12) | 6 | 0.042 | 14 | 0.083 | 16 | 0.208 | 15 | 0.167 |
| | 8 | 0.125 | 15 | 0.917 | 17 | 0.333 | 29 | 0.750 |
| | 17 | 0.750 | | | 18 | 0.250 | 34 | 0.083 |
| | 18 | 0.083 | | | 20 | 0.083 | | |
| | | | | | 23 | 0.083 | | |
| | | | | | 24 | 0.042 | | |
| Hybrids (10) | | | | | | | | |
| | 6 | 0.550 | 13 | 0.200 | 17 | 0.100 | 16 | 0.250 |
| | 8 | 0.350 | 14 | 0.250 | 19 | 0.200 | 19 | 0.100 |
| | 17 | 0.100 | 15 | 0.550 | 20 | 0.500 | 28 | 0.100 |
| | | | | | 23 | 0.200 | 34 | 0.550 |
| **Norg *Ng*** | | | | | | | | |
| *S. latifolia* (21) | 6 | 0.905 | 13 | 0.429 | 16 | 0.095 | 16 | 0.024 |
| | 18 | 0.095 | 14 | 0.119 | 19 | 0.333 | 17 | 0.024 |
| | | | 15 | 0.452 | 20 | 0.547 | 28 | 0.619 |
| | | | | | 21 | 0.024 | 29 | 0.095 |
| | | | | | | | 34 | 0.048 |
| | | | | | | | 35 | 0.048 |
| | | | | | | | 36 | 0.143 |
| *S. dioica* (7) | | | | | | | | |
| | 6 | 0.857 | 13 | 0.214 | 19 | 0.286 | 17 | 0.286 |
| | 8 | 0.071 | 14 | 0.214 | 20 | 0.714 | 28 | 0.357 |
| | 18 | 0.071 | 15 | 0.571 | | | 34 | 0.286 |
| Hybrids (10) | | | | | | | 35 | 0.071 |
| | 6 | 1.000 | 13 | 0.150 | 19 | 0.450 | 17 | 0.100 |
| | | | 14 | 0.150 | 20 | 0.450 | 28 | 0.200 |
| | | | 15 | 0.700 | 21 | 0.100 | 31 | 0.250 |
| | | | | | | | 35 | 0.100 |
| | | | | | | | 36 | 0.350 |

*(Table 3 is continued on next page)*

*(Table 3—continued)*

| | **6** | | **11** | | **14** | | **18** | |
|---|---|---|---|---|---|---|---|---|
| **Oxford *Ox*** | | | | | | | | |
| *S. latifolia* (77) | 8 | 0.864 | 13 | 0.428 | 16 | 0.169 | 14 | 0.019 |
| | 9 | 0.104 | 15 | 0.572 | 17 | 0.013 | 15 | 0.396 |
| | 10 | 0.013 | | | 19 | 0.805 | 16 | 0.305 |
| | 17 | 0.013 | | | 20 | 0.013 | 17 | 0.253 |
| | 19 | 0.006 | | | | | 28 | 0.013 |
| | | | | | | | 31 | 0.006 |
| | | | | | | | 34 | 0.006 |
| *S. dioica* (36) | | | | | | | | |
| | 8 | 0.043 | 13 | 0.014 | 16 | 0.114 | 16 | 0.043 |
| | 17 | 0.771 | 15 | 0.986 | 17 | 0.843 | 27 | 0.157 |
| | 18 | 0.171 | | | 19 | 0.043 | 30 | 0.243 |
| | 19 | 0.014 | | | | | 31 | 0.357 |
| Hybrids (6) | | | | | | | 34 | 0.200 |
| | 6 | 0.083 | 13 | 0.500 | 19 | 0.833 | 15 | 0.417 |
| | 8 | 0.750 | 15 | 0.500 | 20 | 0.167 | 16 | 0.333 |
| | 9 | 0.167 | | | | | 17 | 0.250 |
| **Kings Worthy *Kw*** | | | | | | | | |
| *S. latifolia* (12) | 8 | 0.917 | 13 | 0.458 | 16 | 0.042 | 15 | 0.042 |
| | 9 | 0.083 | 15 | 0.542 | 17 | 0.083 | 16 | 0.792 |
| | | | | | 19 | 0.875 | 17 | 0.167 |
| *S. dioica* (-) † | - | - | - | - | - | - | - | - |
| Hybrids (2) | 8 | 1.000 | 13 | 0.500 | 16 | 0.500 | 15 | 1.000 |
| | | | 15 | 0.500 | 19 | 0.500 | | |
| **Lac Vert *Lv*** | | | | | | | | |
| *S. dioica* (10) | 17 | 0.857 | 15 | 1.000 | 16 | 0.600 | 31 | 0.111 |
| | 18 | 0.143 | | | 18 | 0.400 | 34 | 0.333 |
| | | | | | | | 35 | 0.556 |
| **Meyendel *Md*** | | | | | | | | |
| *S. dioica* (15) | 18 | 0.733 | 15 | 1.000 | 18 | 1.000 | 31 | 0.067 |
| | 19 | 0.267 | | | | | 33 | 0.867 |
| | | | | | | | 34 | 0.067 |
| **Millingerwaard *Mw*** | | | | | | | | |
| *S. latifolia* (15) | 7 | 0.033 | 13 | 0.567 | 16 | 0.321 | 15 | 0.067 |
| | 8 | 0.967 | 15 | 0.433 | 17 | 0.071 | 16 | 0.933 |
| | | | | | 19 | 0.214 | | |
| | | | | | 20 | 0.393 | | |
| **Wolfheze *Wh*** | | | | | | | | |
| *S. latifolia* (9) | 6 | 0.438 | 14 | 0.389 | 19 | 0.611 | 16 | 0.188 |
| | 8 | 0.563 | 15 | 0.611 | 20 | 0.389 | 34 | 0.813 |

*(Table 3 is continued on next page)*

*(Table 3—continued)*

| | **6** | | **11** | | **14** | | **18** | |
|---|---|---|---|---|---|---|---|---|
| **Within host species** | | | | | | | | |
| *S. latifolia* (151) | **6** | **0.193** | **13** | **0.417** | 16 | 0.133 | 14 | 0.010 |
| | 7 | 0.003 | 14 | 0.050 | 17 | 0.020 | **15** | **0.220** |
| | **8** | **0.700** | **15** | **0.533** | **19** | **0.633** | **16** | **0.387** |
| | 9 | 0.060 | | | **20** | **0.210** | **17** | **0.147** |
| | 10 | 0.007 | | | 21 | 0.003 | 28 | 0.093 |
| | 17 | 0.007 | | | | | 29 | 0.013 |
| | 18 | 0.027 | | | | | 31 | 0.003 |
| | 19 | 0.003 | | | | | 34 | 0.100 |
| | | | | | | | 35 | 0.007 |
| | | | | | | | 36 | 0.020 |
| *S. dioica* (80) | 6 | 0.086 | 13 | 0.025 | **16** | **0.158** | 15 | 0.026 |
| | 8 | 0.046 | 14 | 0.031 | **17** | **0.424** | 16 | 0.019 |
| | **17** | **0.553** | **15** | **0.944** | **18** | **0.278** | 17 | 0.026 |
| | **18** | **0.257** | | | 19 | 0.044 | 27 | 0.071 |
| | 19 | 0.059 | | | 20 | 0.076 | 28 | 0.032 |
| | | | | | 23 | 0.013 | **29** | **0.115** |
| | | | | | 24 | 0.006 | **30** | **0.109** |
| | | | | | | | **31** | **0.186** |
| | | | | | | | **33** | **0.167** |
| | | | | | | | **34** | **0.179** |
| | | | | | | | 35 | 0.071 |
| Hybrids (28) | **6** | **0.571** | **13** | **0.268** | 16 | 0.036 | **15** | **0.161** |
| | **8** | **0.357** | 14 | 0.143 | 17 | 0.036 | **16** | **0.161** |
| | 9 | 0.036 | **15** | **0.589** | **19** | **0.446** | 17 | 0.089 |
| | 17 | 0.036 | | | **20** | **0.375** | 19 | 0.036 |
| | | | | | 21 | 0.036 | **28** | **0.107** |
| | | | | | 23 | 0.071 | 31 | 0.089 |
| | | | | | | | **34** | **0.196** |
| | | | | | | | 35 | 0.036 |
| | | | | | | | **36** | **0.125** |
| **Different alleles** | **8** | | **3** | | **8** | | **14** | |

**Table-4** Observed and expected heterozygosities ($H_O$ and $H_S$), and F and R-statistics of each of the four microsatellite loci among populations and among isolates of the fungal pathogen *Microbotryum violaceum* from different host species.

| **Hierarchical structure** | **Locus** | **Heterozygosities** | | **F-statistics** | | | **R statistics** |
|---|---|---|---|---|---|---|---|
| | | $H_O$ | $H_S$ | $F_{ST}$ | $F_{IS}$ | $F_{IT}$ | $R_{ST}$ |
| Among populations | 6 | 0.138 | 0.319 | 0.598 | 0.565 | 0.825 | 0.811 |
| | 11 | 0.642 | 0.403 | 0.212 | -0.595 | -0.257 | 0.218 |
| | 14 | 0.113 | 0.498 | 0.459 | 0.706 | 0.841 | 0.417 |
| | 18 | 0.199 | 0.534 | 0.347 | 0.725 | 0.820 | 0.736 |
| | **All** | **0.273** | **0.439** | **0.420** | **0.395** | **0.649** | **0.743** |
| Among host species | 6 | 0.127 | 0.551 | 0.379 | 0.746 | 0.842 | 0.775 |
| | 11 | 0.591 | 0.403 | 0.212 | 0.230 | -0.161 | 0.262 |
| | 14 | 0.134 | 0.643 | 0.273 | 0.795 | 0.851 | 0.326 |
| | 18 | 0.224 | 0.844 | 0.111 | 0.798 | 0.821 | 0.501 |
| | **All** | **0.269** | **0.610** | **0.247** | **0.560** | **0.669** | **0.569** |

**Table-5** Molecular diversity in populations of the fungal pathogen *Microbotryum violaceum*, expressed in mean number of alleles per locus ($N_A$), number of unique genotypes ($N_G$), observed and expected heterozygosities ($H_O$ and $H_S$) and F-statistics and $R_{ST}$ value averaged over four microsatellite loci. † = Significant heterozygote deficiency ($p<0.05$) is denoted by a star (*) in the column of $H_O$.

| **Population** | **Molecular diversity indices averaged over four microsatellite loci** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Host species (N) | $N_A$ | $N_G$ | $H_O$ ± SE † | $H_S$ ± SE | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ | $R_{ST}$ |
| **Abbertbos (39)** | **5.0** | **27** | **0.41 ± 0.10 *** | **0.69 ± 0.05** | **0.262** | **0.456** | **0.264** | **0.204** |
| *S. latifolia* (17) | 2.8 | 12 | 0.56 ± 0.16 | 0.57 ± 0.02 | 0.011 | | | |
| *S. dioica* (12) | 3.8 | 7 | 0.10 ± 0.04 * | 0.45 ± 0.13 | 0.778 | | | |
| Hybrids (10) | 3.5 | 9 | 0.53 ± 0.13 | 0.64 ± 0.02 | 0.189 | | | |
| **Norg (38)** | **4.5** | **25** | **0.31 ± 0.16 *** | **0.51 ± 0.13** | **0.385** | **0.413** | **0.046** | **0.035** |
| *S. latifolia* (21) | 4.0 | 13 | 0.32 ± 0.16 * | 0.49 ± 0.10 | 0.344 | | | |
| *S. dioica* (7) | 3.0 | 6 | 0.32 ± 0.19 | 0.53 ± 0.11 | 0.407 | | | |
| Hybrids (10) | 3.0 | 9 | 0.28 ± 0.14 | 0.48 ± 0.17 | 0.434 | | | |
| **Oxford (119)** | **5.5** | **48** | **0.23 ± 0.12 *** | **0.60 ± 0.08** | **0.455** | **0.708** | **0.464** | **0.904** |
| *S. latifolia (77)* | 4.5 | 28 | 0.28 ± 0.18 * | 0.44 ± 0.10 | 0.365 | | | |
| *S. dioica (36)* | 3.5 | 18 | 0.10 ± 0.03 * | 0.36 ± 0.11 | 0.726 | | | |
| Hybrids (6) | 2.5 | 6 | 0.38 ± 0.22 | 0.50 ± 0.09 | 0.268 | | | |
| **Kings Worthy (14)** | **2.5** | **8** | **0.27 ± 0.22** | **0.37 ± 0.09** | **0.173** | **0.477** | **0.367** | **0.317** |
| *S. latifolia* (12) | 2.5 | 6 | 0.27 ± 0.22 | 0.32 ± 0.08 | 0.154 | | | |
| *S. dioica* (-) | - | - | - | - | - | | | |
| Hybrids (2) | 1.5 | 2 | 0.25 ± 0.25 | 0.33 ± 0.19 | 0.333 | | | |
| **Lac Vert (10)** *S. dioica* | **2.0** | **8** | **0.16 ± 0.11** | **0.34 ± 0.13** | **0.546** | | | |
| **Meyendel (15)** *S. dioica* | **1.8** | **4** | **0 ± 0 *** | **0.16 ± 0.10** | **1.000** | | | |
| **Millingerwaard (15)** *S. latifolia* | **2.5** | **9** | **0.27 ± 0.20** | **0.36 ± 0.16** | **0.250** | | | |
| **Wolfheze (9)** *S. latifolia* | **2.0** | **7** | **0.29 ± 0.16** | **0.46 ± 0.05** | **0.403** | | | |
| **Among host sp (259)** | **8.3** | **122** | **0.27 ± 0.12 *** | **0.61 ± 0.10** | **0.561** | **0.669** | **0.246** | **0.569** |
| *S. latifolia* (151) | 6.5 | 66 | 0.32 ± 0.18 * | 0.58 ± 0.06 | 0.455 | | | |
| *S. dioica* (80) | 6.5 | 43 | 0.11 ± 0.02 * | 0.58 ± 0.17 | 0.814 | | | |
| Hybrids (28) | 5.5 | 25 | 0.38 ± 0.16 * | 0.67 ± 0.08 | 0.429 | | | |
| **Among pops (259)** | **8.3** | **122** | **0.27 ± 0.13 *** | **0.44 ± 0.09** | **0.396** | **0.649** | **0.419** | **0.743** |

Significant deviations from Hardy-Weinberg equilibrium (HW) were observed for all samples from *S. dioica* populations ($p<0.002$), and for populations from allopatric *S. latifolia* ($p<0.05$ and smaller), indicating that one or more of the HW assumptions were violated (results not shown). In most populations, all loci were in linkage equilibrium. In only two of the populations (Ab and Ox), most of the locus pairs showed significant linkage disequilibrium ($p<0.05$ and smaller), except for locus pairs involving locus 11 in the Abbertbos population, and between locus 14 and 18 in the Oxford population. A number of factors could explain this difference between the populations. Most likely, the loci are not physically linked, but have been subject to high selfing rates, founder effects, historical bottlenecks, or differential selection

regimes, but which one of these factors are more important than others goes beyond the scope of this paper.

In the two sympatric/parapatric populations (Ab, Ox), where patches of the two host species were spatially more separated than in the Norg population, significant population differentiation among fungal communities from the host species was observed ($p<0.0001$), with large values for both $F_{ST}$ and $R_{ST}$ (Table 5). The same was observed in a population (Kw) that only consisted of fungal isolates from *S. latifolia* and hybrids (*S. dioica* was present in this host population, but infected specimen were not found). In Norg however, where the absolute geographical distance between different host patches was the smallest, values for $F_{ST}$ and $R_{ST}$ were much lower and their deviation from zero was only marginally significant ($p=0.08$). High values for $F_{IS}$ and $F_{IT}$, which were significantly different from zero, were observed for most fungal populations in allopatric, parapatric and sympatric host populations including Norg, indicating high inbreeding levels both for fungal samples within host species and for fungal samples from different hosts within the total population. Only the fungal strains from Abbertbos *S. latifolia* showed lower values for $F_{IS}$ that were not significantly different from zero. Logically, a similar pattern is observed for heterozygosities (Table 5). In all populations, except samples from Abbertbos *S. latifolia*, observed heterozygosities ($H_O$) were lower than expected (Nei's unbiased estimate for $H_S$), indicating heterozygote deficiency (HD), but this was not in all populations significant (Table 5; populations indicated with * show significant HD, with $p<0.05$ or smaller).

**Table-6** Estimates of the effective number of migrants per generation (*Nm*) of the fungus *Microbotryum violaceum* between the host species *Silene latifolia*, *S. dioica* and the interspecific hybrids, using the Private Allele (PA) method (Barton and Slatkin 1986), and derived directly from $F_{ST}$ and $R_{ST}$ values. Mean distances between different host species are rough estimates.

| **Pop.** | **Distance between different host species** | **Mean N** | **Frequency of private alleles** | **Estimate of *Nm* derived via** | | | |
|---|---|---|---|---|---|---|---|
| | | | | **PA** | **$F_{ST}$** | **$R_{ST}$** | ***Mean*** |
| Norg | < 1m - 10m | 12.7 | 0.11 | 0.86 | 2.30 | 3.01 | *2.06* |
| Abbertbos | < 10m - 100m | 13.0 | 0.24 | 0.16 | 0.31 | 0.43 | *0.30* |
| Oxford | < 100m – 1000m | 39.3 | 0.10 | 0.58 | 0.13 | 0.01 | *0.24* |

In general, values for $R_{ST}$ were comparable to the values for $F_{ST}$, except for the sympatric/parapatric region of Oxford, indicating that in this population frequency differences for larger and smaller alleles were large between the different host categories. In this population the larger alleles from loci 6 and 18 were more frequent in samples from *S. dioica*, and the smaller alleles in these loci were more frequent in samples from *S. latifolia* and hybrids (Table 3). An indirect estimate of the amount of

gene flow is the effective number of migrants per generation (*cf.* McDermott and McDonald 1993). Estimates for this number were derived from $F_{ST}$ and $R_{ST}$ values (Crow and Aoki 1984), and additionally from the number of private alleles (Barton and Slatkin 1986). Table 6 shows that the estimates produced by the different methods were in the same range. If absolute distance between *S. latifolia* and *S. dioica* hosts is a measure for the relative scale of sympatry, the number of migrants based on $F_{ST}$ and $R_{ST}$ values decreased rapidly with decreasing sympatry level, but not for the estimate based on the number of private alleles. However, we found no evidence for isolation by distance; significant correlations between the genetic and the geographical distance between pairs of populations were neither observed using $F_{ST}/(1-F_{ST})$, nor using $R_{ST}/(1-R_{ST})$ as an estimate for the genetic distance between population pairs (Mantel test; $p>0.23$ and larger).

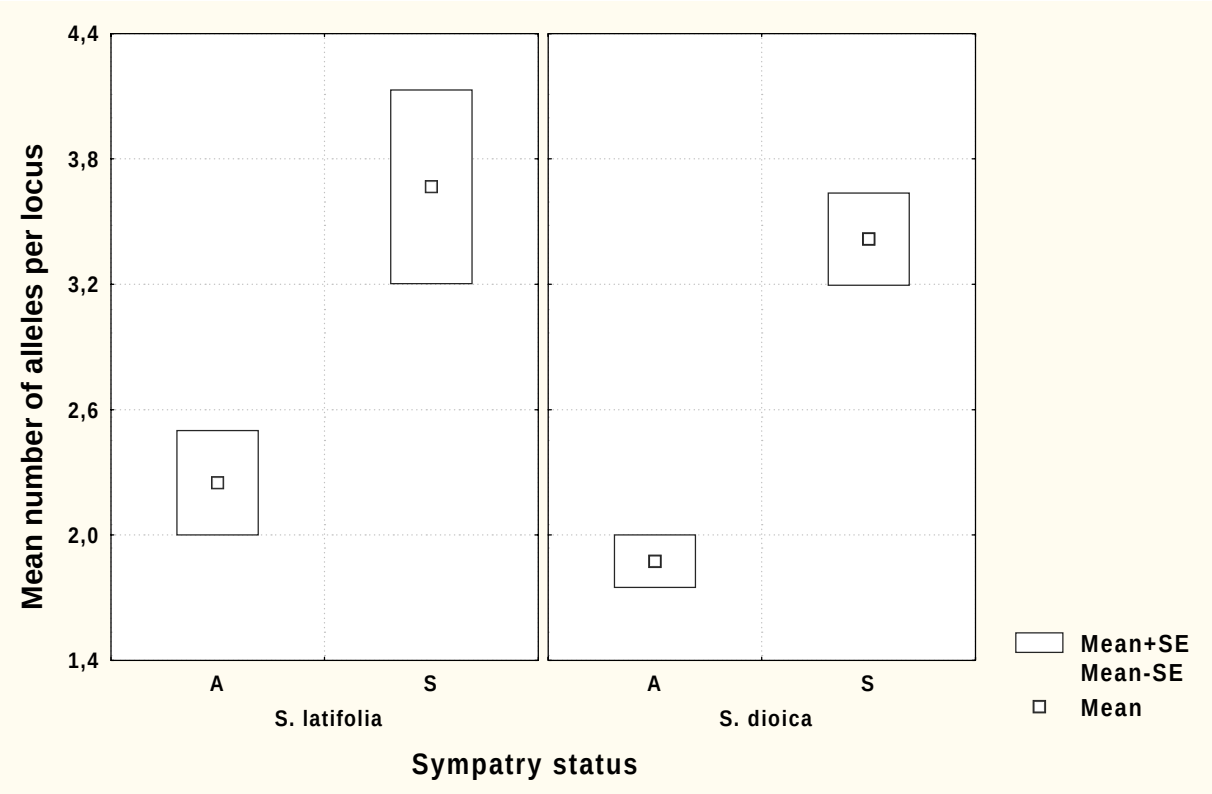


**Figure-2** Mean number of alleles per locus (± SE) in samples of *Microbotryum violaceum* from *Silene latifolia* (left panel) and *S. dioica* (right panel) in allopatric (A), and in sympatric/parapatric (S) host populations.

**Table-7** Source of variation in mean number of alleles per locus in the fungal pathogen *Microbotryum violaceum* in sympatric/parapatric (Ab, Ng and Ox) and in allopatric (Lv, Md, Mw and Wh) populations of its host species *Silene latifolia* and *S. dioica* (ANCOVA with sample size N taken as covariate). See figure 3 for means and standard errors expressing the difference.

| Effect | df | MS | F | P-value |
|---|---|---|---|---|
| Sample size [N] | 1 | 0.62 | 2.8 | n.s. |
| Sympatry status [SYM] | 1 | 3.01 | 13.4 | < 0.015 |
| Host species [HSP] | 1 | 0.06 | 0.3 | n.s. |
| SYM x HSP | 1 | 0.10 | 0.5 | n.s. |
| **Error** | **5** | **0.22** | **-** | **-** |

## Gene diversity of anther smuts in allo- and sympatric host populations

Table 5 shows the gene diversity of the anther smut populations expressed as the mean number of alleles per locus. The mean levels of variation (± SE) of anther smuts in allopatric populations of hosts (2.25 ± 0.3 for *S. latifolia* and 1.88 ± 0.5 for *S. dioica*) are significantly lower (Table 7 and Figure 2; $p<0.015$) than the mean levels of variation (± SE) in sympatric/parapatric host populations of anther smut (3.75 ± 0.8 for *S. latifolia* and 3.42 ± 0.6 for *S. dioica*). Since the mean number of alleles is likely to be highly dependent on sample size, we also calculated allelic richness $R_S$ (El Mousadik and Petit 1996). The difference in allelic richness was also highly significantly different ($p<0.0025$) between samples from allopatric ($R_S=1.93$) and sympatric ($R_S=2.81$) populations of hosts. This pattern holds true for fungal populations in both the Netherlands and the UK, strengthening the suggestion that higher levels of variation in the pathogen can be maintained in the presence of another host species.

**Table-8** Observed heterozygosities in samples of the fungal pathogen *Microbotryum violaceum* from *Silene latifolia*, *S. dioica* and the interspecific hybrids (LR statistics, type III; procedure GENMOD in SAS).

| Main effect | df | $\chi^2$ | P-value |
|---|---|---|---|
| *Contrasts between host species* | | | |
| Host species | 2 | 61.8 | <0.001 |
| *S. latifolia* | | | |
| *vs. Hybrids* | 1 | 2.0 | n.s. |
| *S. dioica* | | | |
| *vs. S. latifolia and Hybrids* | 1 | 57.6 | <0.001 |

## Host-related differentiation

For the analyses of host-related genetic differentiation, data were pooled within host species (Table 3). Besides the heterozygote deficiency at the population level, observed heterozygosities were significantly lower for pooled samples from *S. dioica*

samples than for pooled samples from *S. latifolia* and hybrids (Table 8). Consequently, values for $F_{IS}$ in samples from *S. dioica* were also much larger than in samples from *S. latifolia* and hybrids. The high $F_{ST}$ and $R_{ST}$ values (Tables 4 and 5) showed significant genetic differentiation between smut samples from different host species. Table 3 shows that pooled host samples from *S. dioica* harbored the larger alleles in higher frequencies for loci 6 and 18, whereas pooled host samples from *S. latifolia* carried the smaller alleles in higher frequencies. The other loci, when pooled within host species, also produced host-specific patterns, e.g. samples from *S. dioica* predominantly had allele size 15 for locus 11, whereas samples from *S. latifolia* and hybrids showed both allele sizes 13 and 15 in high frequency. Likewise, at locus 14 samples from *S. dioica* had predominantly allele size 16 to 18, whereas samples from *S. latifolia* and hybrids showed predominantly allele sizes 19 and 20.

**Table-9** Sources of variation in F and R-statistics revealed by Analysis of Molecular Variance (AMOVA), as calculated in ARLEQUIN (Schneider *et al.* 2000).

| **Source of Variation** | | **Sum of Squared Deviancies** | | **Variance components** | | **% Variation** | |
|---|---|---|---|---|---|---|---|
| | **df** | **$F_{ST}$** | **$R_{ST}$** | **$F_{ST}$** | **$R_{ST}$** | **$F_{ST}$** | **$R_{ST}$** |
| Among host species | 2 | 109.7 | 19196.2 | 0.38 | 66.7 | 24.5 | 56.8 |
| Within host species | 515 | 594.4 | 26073.6 | 1.15 | 50.6 | 75.5 | 43.2 |
| **Total** | **517** | **704.1** | **45269.8** | **1.53** | **117.3** | **100** | **100** |

Table 9 shows the results of the analysis of molecular variance (AMOVA, Michalakis and Excoffier 1996). When the AMOVA is based on allele differences (assuming the infinite allele mutation model, $F_{ST}$), 25% of the observed variance is due to differences between host species. However, when alleles size matters in the analysis (assuming the stepwise mutation model, $R_{ST}$), 57% of the observed variance can be attributed to host species. The difference between $F_{ST}$ and $R_{ST}$ based AMOVAs is again accounted for by large frequency differences of the larger (*S. dioica*) and the smaller (*S. latifolia* and hybrids) alleles of loci 6 and 18 (Table 3). As results may be biased by the large sample size of the Oxford population (46% of the samples), we repeated the analysis excluding the Oxford population. This yielded basically the same result. Variance due to host differences is 20% for the AMOVA based on $F_{ST}$ and 38% based on $R_{ST}$.

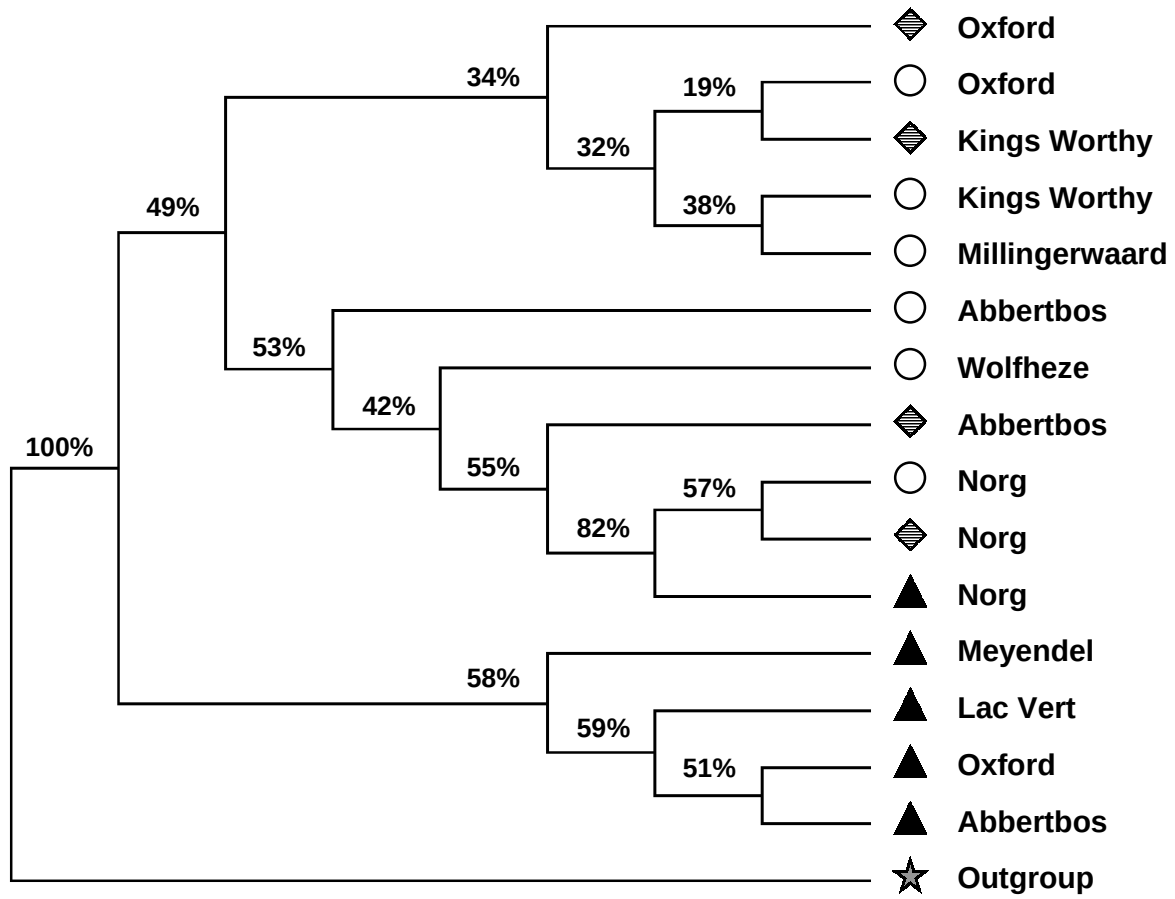


**Figure-3** Majority rule consensus Neighbor Joining tree expressing overall levels of Nei's unbiased genetic distance between the sampled populations of *Microbotryum violaceum*. Distances are based on four microsatellite loci. Open circles denote smut samples from *Silene latifolia*, filled triangles denote smut samples from *S. dioica*, and hatched diamonds denote smut samples from interspecific hybrid hosts. Population locations correspond to those in table 1 and figure 1. Percentages denote bootstrap values after 10000 resamplings. The outgroup (denoted by a gray star) is smut sampled from a Swiss population of *S. acaulis* (adapted from Bucheli *et al.* 2000).

Figure 3 shows the consensus Neighbor Joining tree based on Nei's unbiased genetic distances (Nei 1987) produced after 10000 bootstrap resamplings. Clustering occurs in three major groups, with bootstrap values of 50% and larger. One distinct group is formed by samples from *S. dioica* on the one hand and samples from *S. latifolia* and hybrids in two distinct groups on the other. One of the *S. latifolia*/hybrid groups includes the complete Norg population, which form a separate clade themselves in 82% of all examined trees. The other *S. latifolia*/hybrid group basically consists of the samples from British *S. latifolia* and hybrid hosts together with the sample from Millingerwaard *S. latifolia* hosts. However, bootstrap values within the latter group never exceed 38%, and we therefore do not consider sub clustering within this group. Clear however is that fungal samples from hybrid origin are more similar to samples from *S. latifolia*, than to samples from *S. dioica*.

## DISCUSSION

### Population structure

In the sympatric and parapatric host populations, highly significant genetic differentiation was observed between fungal isolates from *S. dioica* hosts on the one hand and *S. latifolia* and hybrid hosts on the other, resulting in local population structuring of *M. violaceum*. The extent to which host species are mixed in the population, i.e. the degree of sympatry proved to be important for the genetic population structure of the pathogen (Table 7). This is not surprising since spatial scale plays a major role in the evolutionary dynamics of host-pathogen systems (Real and McElhany 1996; Thrall and Burdon 1997; Burdon and Thrall 1999). Only in the Norg population where interspecific plant distance is as close as a few decimeters, gene flow between the two host races, estimated indirectly by numbers of migrants (McDermott and McDonald 1993) was larger than 1, and high enough to keep $F_{ST}$ and $R_{ST}$ values low. When interspecific host plant distances increases, gene flow apparently decreases rapidly. Except for samples from the Abbertbos *S. latifolia* population, in all samples high values for $F_{IS}$ (and $F_{IT}$) were observed. This results in significant heterozygote deficiency in three sympatric, and in one allopatric host population. The insignificance of heterozygote deficiency in the other samples is probably due to the lower sample sizes. It is unclear why the samples from *S. latifolia* in the Abbertbos population have lower $F_{IS}$ (and $F_{IT}$) values than all other populations. Significant heterozygote deficiency can be caused by the presence of null alleles (Pemberton *et al.* 1995). Since only a few of our samples did not amplify at all during the PCR, we do not believe that null alleles caused the heterozygote deficiency in this study. Alternatively, significant heterozygote deficiency can indicate high levels of inbreeding. Selfing in this fungus is thought to be the rule rather than an exception (Baird and Garber 1979; Hood and Antonovics 1998; Kaltz and Shykoff 1999). Therefore, the observed heterozygote deficiency, except for samples from Abbertbos *S. latifolia*, could be explained by high selfing rates, which is consistent with the results of Bucheli *et al.* (2000; 2001). Contrary to what Bucheli and colleagues found, samples from *S. dioica* showed significantly lower heterozygosities than samples isolated from *S. latifolia* or hybrids, indicating that selfing rates are higher in samples from *S. dioica* than in samples from *S. latifolia* and hybrids. A possible explanation could be the difference in mean numbers of flowers produced per plant between the two host species. Diseased *S. dioica* produce more flowers per flowering stalk, and

more flowering stalks per plant than diseased *S. latifolia* (Biere and Honders 1996a). Since all teliospores that are produced in the flowers of a single shoot are almost always the result of a single infectious dikaryon (Day 1980), they will be genetically identical. Many pollinators, serving as vectors of this disease (Jennersten 1983), visit flowers of a single plant often sequentially (personal observation), and will pick up relatively more of the same spores on *S. dioica* hosts than on *S. latifolia* hosts in a geitonogamous manner (Kiang 1972; Morris *et al.* 1994). These spores are likely to be deposited on conspecifics of the host species of origin rather than on heterospecifics (Van Putten *et al.* chapter 5), which should lead to fewer opportunities for outcrossing on *S. dioica* than on *S. latifolia*.

## Gene diversity within host populations

Previous studies of genetic diversity within allopatric populations of the anther smut fungus *M. violaceum* have shown little variation in allozymes (Antonovics *et al.* 1996) in North America, and in microsatellite loci (Bucheli *et al.* 2001) in Switzerland. In allopatric populations of *S. latifolia* and *S. dioica* hosts, we find levels of variation—when variation is expressed in mean numbers of alleles per locus—that are comparable to these studies. Interestingly however, we find significantly higher levels of variation in this fungus in sympatric and parapatric than in allopatric host populations, both in the populations from the Netherlands and in the population from the UK. This leads to the suggestion that higher levels of variation in the pathogen can be maintained in the presence of another host species, even if the levels of gene flow between fungi from one host species to another are low. In a scenario where the two host races have evolved in allopatry, followed by the present situation in which the genetically differentiated fungal populations come in secondary contact with each other in parapatry or sympatry, low levels of gene flow will cause the mutual exchange of alleles and enlarge the variation in both host races, as was observed in a hybrid zone of two chromosome races of the common shrew (Wyttenbach *et al.* 1999). Selectively neutral variation, as variation in microsatellite loci is assumed to be (*cf.* Goldstein and Slötterer 1999), may be maintained for long periods of time. Explanations for a more active maintenance of this higher level of genetic variation are far more speculative. For instance, if these microsatellite loci are not neutral but linked to loci under selection, an explanation could be that natural selection favoring host adaptation keeps fungal isolates from *S. dioica* and *S. latifolia* genetically differentiated, while low levels of gene flow opposes this force, thereby maintaining the variation.

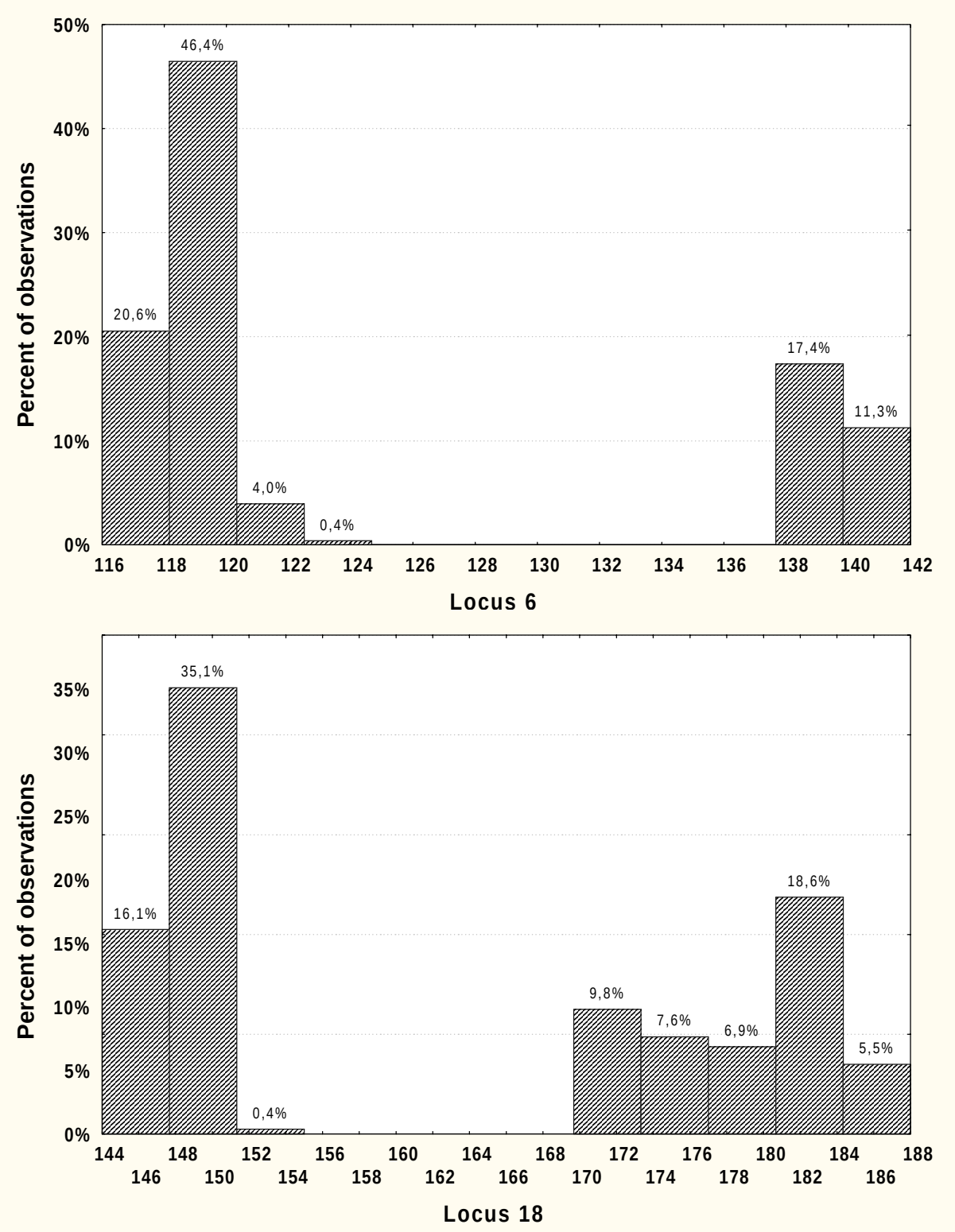


**Figure-4** Histograms of the allele sizes of locus 6 (upper panel) and locus 18 (lower panel) in the fungal populations of *Microbotryum violaceum* sampled in this study (n=259 samples). Note the bimodal distribution at both loci.

## Host-related genetic differentiation

The genetic variation we have observed is strongly host species related, confirming the existence of separate host races for *S. latifolia* and *S. dioica* as

proposed by previous authors (Zillig 1921; Biere and Honders 1996a; Bucheli *et al.* 2001). Allele frequency differences of all four microsatellite loci contributed to the host-related genetic differentiation. However, this was especially apparent in two of the examined loci, showing that samples from *S. dioica* carried the larger alleles in high frequencies, while samples from *S. latifolia* harbored the smaller alleles in high frequencies. A similar difference is observed in populations of anther smut in Switzerland (Bucheli *et al.* 2001). Figure 4 displays the frequency distribution of these host-specific alleles. Clearly these distributions are bimodal, showing gaps of seven repeats (locus 6) and nine repeats (locus 18) in the middle. Obviously, when the fungus would be considered as one species, a more continuous distribution is expected under the stepwise mutation model (Kimura and Ohta 1978). There are at least two different explanations for the occurrence of these gaps. First, mutations (most likely an insertion or a deletion) in one of the flanking regions of the microsatellite locus could explain mutational steps larger than single repeats in the allele size frequency distribution. However, there is evidence that flanking regions of microsatellite loci are highly conserved (e.g. among marine turtles: FitzSimmons et al 1995; among cichlid fish: Rico et al 1996; Zardoya *et al.* 1996) and that mutant alleles generally are non-recombinant for flanking markers (*cf.* Ellegren 2000). Also, at both loci intermediate allele sizes have been reported (Shykoff *et al.* 1999; Bucheli *et al.* 2000), mainly occurring in samples from other Caryophyllaceous host species. Therefore, the second explanation for the observed gaps in allele size distribution, long term divergence between the two separate host races, seems more likely. By assuming a stepwise mutation model, large allele size differences indicate long-term divergence between the host races, at least for anther smut populations in Western Europe. In that case, the host race of *S. latifolia* and the host race of *S. dioica* share a common ancestor with a certain intermediate allele size at each of these loci. In the period subsequent to divergence from this common ancestor, the mean allele lengths at the individual loci have evolved independently, and in different directions in both host races (Ellegren *et al.* 1995). Since we found host-specific alleles of similar sizes throughout the populations in Western Europe, such long-term divergence might have evolved in allopatry. Also, sympatric populations of these host species that contracted an infection with this fungal disease are much less abundant than allopatric populations. Therefore, we speculate that in the sympatric populations that were studied here, both fungal races come into secondary contact with each other.

The consensus Neighbor Joining tree (figure 3) showed that samples from *S. dioica* were clearly clustering together, separate from samples from *S. latifolia* and

hybrid hosts. Smut samples from hybrids clustered within the *S. latifolia* samples. This is consistent with the observation that interspecific host hybrids grow among *S. latifolia* and much less among *S. dioica*, both in populations in the UK (Goulson and Jerrim 1997; personal observation) and in populations in the Netherlands (personal observation). Such a distribution may be caused by similar habitat preferences of *S. latifolia* and hybrid hosts, but might also be caused by asymmetric pollen flow, going predominantly from *S. dioica* towards *S. latifolia* (Goulson and Jerrim 1997), in combination with the limited seed dispersal of these plant species. Such directional pollen flow might be caused by differences in flowering phenology between host species, with *S. dioica* flowering earlier than *S. latifolia* (Biere and Honders 1996b), and between host sexes, with males flowering earlier and over a longer period than females (Purrington and Smitt 1998). Since the same vectors that transmit the pollen transmit the spores, similar arguments may hold for fungal gene flow, explaining the genetic resemblance among fungal isolates from *S. latifolia* and hybrids.

The low bootstrap values within this *S. latifolia* and hybrids group do not really allow for interpretations that go beyond the observation that all four smut samples from the UK, and five out of six smut samples from the Netherlands appeared in two separate clusters.

## Potential for host race formation and speciation in sympatry

The observation that host-specific microsatellite alleles, that are separated by 7-9 repeats between the fungal isolates, occur in populations throughout Western Europe, indicates long-term divergence in the anther smut *M. violaceum* into two separate host races for *S. latifolia* and *S. dioica*. Since the differentiation between fungal isolates in sympatry proved to be significantly lower, this is most likely the result of reproductive isolation in allopatry. If gene flow is high relative to selection, it can prevent divergence and/or break down a situation of reproductive isolation (*cf.* Orr and Smith 1998). In parapatric populations (Ab, Ox) where fungal strains from different hosts presumably come in secondary contact with each other, levels of gene flow are apparently too low, keeping them genetically differentiated. In the only true sympatric population of hosts that we examined (Ng), low values of $F_{ST}$ and $R_{ST}$ indicate that there is little genetic differentiation left at the microsatellite loci across isolates from different host categories, and gene flow must therefore be considerably higher than in parapatric populations of hosts.

The results from this microsatellite study contrast with results of a survey of the allelic distribution of one of the sporidial colony color loci (SCC) in the Norg

population (Van Putten *et al.* chapter 3), that showed clear and significant differentiation between the two host races at this locus, although to a significantly lower degree than what was observed between smut from allopatric host populations of these host species. This suggests that this locus is subject to gene flow but that natural selection counteracts further convergence at this locus. Wild type strains of *M. violaceum* found on allopatric *S. latifolia* produce pink colored colonies on standard yeast-glucose-agar medium due to the accumulation of lycopene. The yellow colored mutant phenotype found on allopatric *S. dioica* converts this lycopene into β-carotene through a cyclase that is inactive in the wild type (Garber *et al.* 1975). Therefore, the selective neutrality of this SCC marker is unclear and there may be host-specific selection on the responsible locus. Assuming that the Norg population of anther smut is in equilibrium, the selectively neutral markers may have converged, while variation in selectively less neutral markers may have maintained some of the divergence that presumably had developed historically in allopatry. In spite of this, we must conclude that we find no direct evidence for active host race formation in pure sympatry in this model system. However, our study clearly presents additional evidence for the existence of separate, genetically diverged host races of the anther smut *M. violaceum* on *S. latifolia* and *S. dioica* in parapatric populations of these host species.

**Acknowledgements**

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# CHAPTER 3

## Effects of spatial structure on host-related differentiation of the anther smut *Microbotryum violaceum* in a natural sympatric host population of S*ilene latifolia* and *S. dioica*

with Arjen Biere, Sonja Honders and Jos van Damme

Abstract

**We have studied the effects of spatial structure of the host species *S. latifolia* and *S. dioica* on the genetic structure of the anther smut *M. violaceum* in a sympatric population of these hosts. For one of the sporidial colony color loci (SCC), divergence among fungal isolates from *S. latifolia* and *S. dioica* was significantly smaller relative to allopatric populations of hosts. Fungal isolates from allopatric populations of *S. latifolia* are almost fixed for the wild type *pink* allele, and isolates from *S. dioica* are almost fixed for the *yellow* allele. However, in contrast to previous studies using microsatellite loci (in which $F_{ST}$ and $R_{ST}$ were not significantly different from zero), convergence between both host races in sympatry was far from complete. Among fungal isolates from *S. dioica*, the frequency of their 'native' *yellow* allele was 56%, but among isolates from *S. latifolia*, their 'native' *pink* allele was close to fixation, as in allopatric populations.**

**The local host structure, consisting of patches that are mostly dominated by either *S. dioica*, or by *S. latifolia*, had a weakly significant impact on the SCC allele frequencies. This suggested that the anther smut population could be divided into a local deme structure, in which selection and migration might be balanced in such a way that the overall variation in this SCC locus is maintained. A closer look at the microsatellite genotypes showed that the more rare alleles were not randomly distributed over the population either, supporting the hypothesis that the patchiness of the host population shapes the genetic structure of the pathogen.**

## INTRODUCTION

Natural populations of pathogens and their hosts tend to be unevenly distributed in space (and time), which is primarily caused by aspects of their dispersal (Burdon *et al.* 1989). Steepness of pathogen dispersal gradients strongly depends on the mode of dispersal, and increases from dispersal through soils, to wind or rain dispersal, or transmission by direct contact. Pathogens carried by vectors show a wide variety of dispersal patterns, largely reflecting the behavior of their vectors (Burdon *et al.* 1989 and references therein). Moreover, the patchiness of pathogen populations is enhanced by the patchy local distributions of the hosts themselves, by organizing the populations of organisms that feed on them into numerous local demes (McCauley 1991). A deme structure of local units within which breeding is random will promote local adaptation of the parasite to the hosts within a deme, as is predicted by the adaptive deme formation hypothesis of plant-herbivore systems (Edmunds and Alstad 1978; Van Zandt and Mopper 1998). Plant pathogens can often employ more than one host species and show intraspecific variation in host use. It has been argued that, to achieve host specialization in sympatry, fitness trade-offs between host species are necessary to compensate for incomplete host fidelity that is likely to occur and would generate gene flow (Feder 1998). If the amount of gene flow is high relative to selection, and/or fitness trade-offs are absent, genetic diversity will homogenize (*cf.* Mopper 1996).

In the anther smut fungus *Microbotryum violaceum*, an obligate parasite of the Caryophyllaceae, host races that each can infect only a limited set of host species have been recognized early in the scientific history of this fungus (Zillig 1921). Recently, fungal isolates from allopatric populations of the closely related host species *Silene latifolia* and *S. dioica* proved to be genetically differentiated. They showed different sporidial colony colors (*sensu* Garber 1975 *et al.*; *cf.* Biere and Honders 1996a), and host-specific microsatellite alleles (Bucheli *et al.* 2001; Van Putten *et al.* chapter 2). This differentiation may partly reflect the adaptation of this pathogen to these two host species. Besides genetic drift that will be important when population sizes are small and mutation, natural selection acting on fitness differences between individual pathogen strains is the driving force creating genetic diversity. Fitness of the pathogen can be divided in infection success, and performance on the host. With respect to infection success, there is no clear evidence for local adaptation of the pathogen to its host at the within species level (*S. dioica*, Carlsson-Granér 1997; *S. latifolia* Kaltz *et*

*al.* 1999), nor at the between species level for this pair of host species (Biere and Honders 1996a). In the *S. latifolia* case, absence of local adaptation might be explained by low migration rates of the pathogen relative to the migration rate of its host species (Gandon *et al.* 1996; Delmotte *et al.* 1999). Notwithstanding the importance of the infection success, or virulence of a pathogen (*sensu* Jarosz and Davelos 1995), it is certainly not the only factor that influences local host adaptation, and hence the genetic structuring of the pathogen population. At the between host species level, Biere and Honders (1996a) performed a cross-inoculation experiment, using fungal isolates from allopatric *S. latifolia* and *S. dioica*, and found a three-fold higher production of smut spores of the 'native' host race in male host plants. This suggested that adaptation of this fungus to its host species might have evolved with respect to aggressiveness (a term used to describe pathogen fitness on a particular host, given that it is virulent to that host, *sensu* Jarosz and Davelos 1995) rather than virulence.

Interestingly, in sympatric populations of these host species, differentiation among fungal isolates from these two host species was found to be significantly smaller, and dependent on the degree of sympatry of the hosts (Van Putten *et al.* chapter 2). In the case that the degree of sympatry reflects the amount of gene flow between the host races, we would expect that in sympatric populations of different host species that have a spatially heterogeneous distribution, differentiation between isolated host patches would be larger than between different host species within a patch. Here, we study the effects of host population structure at a small spatial scale on the genetic population structure of anther smuts in a natural sympatric population of *S. latifolia* and *S. dioica*. In chapter 2 of this thesis, the microsatellite analysis of the Norg population showed that values for $F_{ST}$ and $R_{ST}$, calculated over host species, were not significantly different from zero. This suggested that there was no population subdivision with respect to host species, and supposedly enough gene flow to neutralize the divergence between the host races of anther smut in this population. In this chapter we study this population in more spatial detail, and include the analysis of the allelic distribution at one of the sporidial colony color loci (Garber *et al.* 1975) in this population of anther smuts.

Specific questions that will be addressed are: (1) To what extent are the anther smut host races of *S. dioica* and *S. latifolia* differentiated in this sympatric population of hosts relative to allopatric populations of host species? (2) Does the degree of host differentiation among fungal isolates depend on local host spatial structure? Since the microsatellite study suggested that there was ample gene flow among smut isolates

from different host species, we hypothesize that the differentiation with respect to the SCC marker will be substantially smaller in sympatry than in allopatry. Moreover, since a previous study that quantified the spatial structure in this system stressed the importance of a spatial analysis when host and pathogen populations are subdivided and/or show non-random forms of association that are spatially linked (Real and McElhany 1996 and references therein) as in our case, we hypothesize that the spatial structure of the host population will be reflected in the genetic population structure of this pathogen.

## MATERIALS AND METHODS

### The Species

The anther smut fungus *Microbotryum violaceum* (Pers.:Pers) Deml & Oberw. (= *Ustilago violacea* [Pers.] Fuckel) (Ustilaginaceae) (Deml and Oberwinkler 1982) is a heterobasidiomycete that obligatory parasitises susceptible members of the Caryophyllaceae to complete its sexual lifecycle, thereby sterilizing the host plant (Baker 1947). The most striking disease symptom of an infection with this fungus is the overriding of the genetically determined sex expression in dioecious host species by halting the development of female reproductive tissue (Audran and Batcho 1981) and inducing the expression of 'male-specific' genes (Scutt et al. 1997) that are also present, yet inactive in female plants (Matsunaga et al. 1996). As a consequence, ovaries are reduced, and staminal rudiments develop into stamens that contain purple-brownish smut spores. Male flowers also bear teliospores in their anthers instead of pollen. Teliospores are diploid thick-walled cells, which undergo meiosis when they germinate to produce haploid sporidia of two mating types that proliferate asexually by yeastlike growth. In the presence of a susceptible host, sporidia of opposite mating type conjugate to produce a dikaryotic infection hypha that can enter host tissue. Spores are transmitted by the natural pollinators of their hosts, which also serve as vectors of this disease (Jennersten 1983).

*Silene latifolia* Poiret (= *Silene alba* [Miller] Krause), the White Campion is a short-lived perennial weed that grows in open, disturbed habitats and *S. dioica* (L.) Clairv., the Red Campion, is a perennial weed that mainly occurs on the edges of woodlands. Both species are dioecious and in areas where habitats are adjacent or

overlap, hybridization between these species occurs frequently (Baker 1947; Goulson and Jerrim 1997).

## The Norg sympatric population and smut sampling

Although both species are common in Western Europe, truly mixed sympatric populations of *S. latifolia* and *S. dioica* are scarce, or even absent because of differential habitat preferences. These habitat preferences of the hosts result in the patchy structure of the sympatric population that we have sampled in Norg (The Netherlands, 53°06'N 6°30'E). The Norg population, which has been extensively described and studied by Biere and Honders (1996b; 1998), stretches approximately 900m along a rural, infrequently used sandy road, with a frequently interrupted row of shrubs and small trees on both sides. Vast fields of arable land further surround the population. *S. dioica* host plants grow mainly in the shady humid areas, while *S. latifolia* host plants grow in the more open spots. In 1993, the host population was found to be both spatially and temporally sub structured, and consisted of 1755 flowering *S. dioica* (of which 7.4% was systemically infected) and 1041 flowering *S. latifolia* (of which 17% was systemically infected). Furthermore, the number of putative hybrids was estimated 5.9% of all the *Silene* hosts (Baker 1951) and their systemic disease incidence 18.2%. From these figures, a reliable minimum estimate of the 'population size' (= number of infected host plants) of anther smuts in flowering hosts in the Norg population is approximately 240. This is a minimum estimate because of the vegetative infected host plants and putative multiple infections per hosts (see chapter 4). The distribution of the different hosts along the road is non-random, showing a high frequency of *S. latifolia* and hybrids in the first and last quarter of the road, and high frequency of *S. dioica* in the center part. Individual plants of *S. latifolia*, *S. dioica* and interspecific hybrids incidentally grow as close together as a few decimeters in this population.

Between 1991-2000 we sampled teliospores from 18 geographically spread allopatric host populations of *S. latifolia* (8) and *S. dioica* (10) from the Netherlands, the UK and France (see chapter 2 for details of four of these populations). In Norg, teliospores were collected from both host species in the flowering seasons of 1991, 1992, 1994, 1995, 1996 and 1998. In 1998, teliospores were collected from the interspecific hybrids as well. In the Norg population, for each sample the exact location of the host plant was marked in x-y coordinates. Whenever possible, teliospores were collected from closed flower buds to avoid cross infection. While gently opening the flower buds, spores were transferred to 1.5ml eppendorf cups.

Since only one dikaryon usually infects a shoot and its flower buds (Day 1980), teliospores from single flower buds are assumed to be identical.

Teliospores were plated onto standard yeast-glucose-agar medium (Cummins and Day 1977). Haploid sporidia, produced after spore germination and meiosis were grown at 21°C for one week. Single spore colonies were transferred to new plates containing standard medium. From the samples collected before 1998, single cell colonies were separated and their mating types were determined using reference strains. After a week of growth the sporidial colony color of all strains was determined. Wild type (+) strains of *M. violaceum* produce pink colonies (Sporidial Colony Color; SCC) when growing on standard medium due to the formation of lycopene. The yellow colored mutant converts this lycopene into β-carotene through a cyclase that is inactive in the wild type (Garber *et al.* 1975). When in doubt, strains were replated on standard medium to be certain of their SCC genotype. The samples from 1998 were also freeze-dried, and their DNA was isolated and analyzed for four microsatellite loci (Bucheli *et al.* 1998). This procedure is described in more detail in chapter 2 of this thesis.

## Data analysis

The effect of host species in the allopatric host populations, and the effect of host patch type in the Norg sympatric population on the SCC allele frequencies in a patch were tested in a generalized linear model (procedure GENMOD in SASv8 (The SAS Institute Inc.1999, Cary NC USA). For this purpose, the data from Norg were pooled over years, and plotted according to their x-y coordinates. From these coordinates, we defined host patches using a nearest neighbor joining method. A host plant was considered to be in a patch unless he was more distant than 8m from the nearest other host plant in this patch. The threshold value of 8m to attribute plants into different patches was chosen for the following reasons; first, the linear boundary of spore deposition and infection of a healthy host from an infected source plant was found to be around 10-12m (Alexander 1990; Roche *et al.* 1995; Van Putten *et al.* chapter 5). Second, Biere and Honders (1998) investigated the effects of local density and frequency of diseased plants on the probability of hosts to become infected in the Norg population, and found the 8m scale to be a turning point in the effect of disease frequency on infection probability. Below this value, i.e. at small spatial scales, the effect was significantly positive while above this value the effect was insignificant, suggesting that this and smaller spatial scales are the relevant scales for transmission by insect vectors. This way, the Norg samples were grouped into 28 different patches

of 2 different types; 14 patches in which *S. dioica* was the majority host type (>50%) and 14 patches in which *S. latifolia* (>50%) was the majority host type. We estimated patch size by projecting the ellipse with the maximum x and y differences within the patch, and calculating the surface area. Estimated patch sizes ranged from $1m^2$ (solitary plants) to $330m^2$, with an average size of approximately $55m^2$. Patches were thus defined based on infected host plants only, a classification that is relevant for the spatial structure of the pathogen population and for assessing the majority host species in a patch with respect to spore source. However, since pollinators/vectors will respond to the spatial structure of both healthy and diseased plants, we checked whether the majority host species based on diseased plants only corresponded to the majority host species based on all *Silene* plants, using the extensive studies of this population that were carried out by Biere and co-workers between 1991-93 and involved all (healthy, infected and vegetative) host plants present within a flowering season (Biere and Honders 1996b; 1998). In 1993, out of 57 examined sections covering 16m each, 40 were diseased and in all cases the majority host species based on diseased plants and on both healthy and diseased plants corresponded. Furthermore, since we have pooled data from different years, we make the assumptions that a) the defined patches are reasonably stable with respect to number and density of host plants over these years, and b) smut samples from plants within a certain patch between the different years represent different individuals (otherwise, they would be pseudo-replicates in the analysis). Indeed, patches were found to be rather stable in this population. Between 1991-93, out of 57 examined sections covering 16m each, only two were newly colonized or got extinct, and less than 4% changed in majority host type, mainly due to the few numbers of plants in those sections. Although *S. latifolia* are in general thought to be shorter-lived perennial plants than *S. dioica* (*cf.* Prentice 1979), turnover rates of the flowering population were found to be comparable for both species, up to about 50% per year (A. Biere, personal communication). Finally, none of the smut samples that were sampled shared the exact x-y coordinates, and can safely be regarded to be sampled from different individual host plants.

Attempts to analyze the molecular data from Norg by calculating $F_{ST}$ and $R_{ST}$ values and comparing them over the different hierarchical levels, i.e. host species and patch structure, failed due to low sample size (n=38). Also, it turned out that we have been unfortunate in collecting teliospores in 1998, and sampled for the microsatellite analysis predominantly from patches where *S. latifolia* was the majority host type. Therefore, we plotted the genotypes graphically and analyzed only the randomness of

the distribution of alleles over patches with a simple chi-square test, deriving the expected frequencies from the overall frequencies in the population and comparing them to the observed frequencies in a patch. Since sample sizes within patches were small, we decided to be conservative, and have tested the deviations from a random distribution at a Bonferroni corrected critical probability $\alpha' = \alpha_{0.05} / k$, with k=16 for the 16 examined alleles, yielding a critical $\chi^2_{[1]}$ value of 8.73.

In the flowering season of 2001, the Norg population has been sampled again, collecting teliospores from a much larger number of *S. latifolia*, *S. dioica* and hybrid hosts, which is currently being analyzed for these four microsatellite loci. Unfortunately, these data could not be analyzed within the time span of writing this thesis.

## RESULTS

### Allopatric host populations

Seven out of 10 allopatric *S. dioica* populations and seven out of 8 allopatric *S. latifolia* populations were fixed for their SCC type, resulting overall allele frequencies of >95% of the *yellow (y)* allele in the *S. dioica* hosts and >98% of the *pink (+)* allele in *S. latifolia* hosts, as is visualized in figure 1. This host-specific differentiation of the SCC locus is highly significant ($\chi^2$=153.7; df=17; p<0.0001).

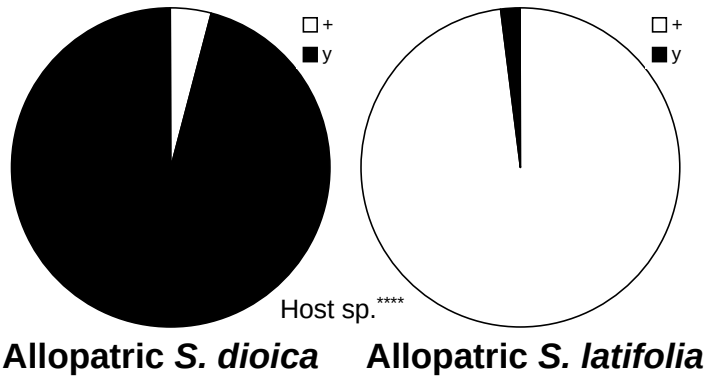


**Figure-1** Host-related differentiation in allopatric populations of host species in the anther smut fungus *Microbotryum violaceum*. Displayed are allele frequencies of the *pink (+)* and *yellow (y)* allele of the Sporidial Colony Color locus in 8 populations of *Silene latifolia* (52 samples), and 10 populations of *S. dioica* (47 samples). The allele frequencies between host species are significantly different with p<0.0001.

### The Norg sympatric host population

Figure 2 shows the geographic distribution of the SCC *pink* and *yellow* alleles in anther smuts of *S. latifolia* and *S. dioica* hosts pooled over six flowering seasons. This picture shows that the host population structure can roughly be described with *S. dioica* occurring midway and *S. latifolia* at both ends of the road.

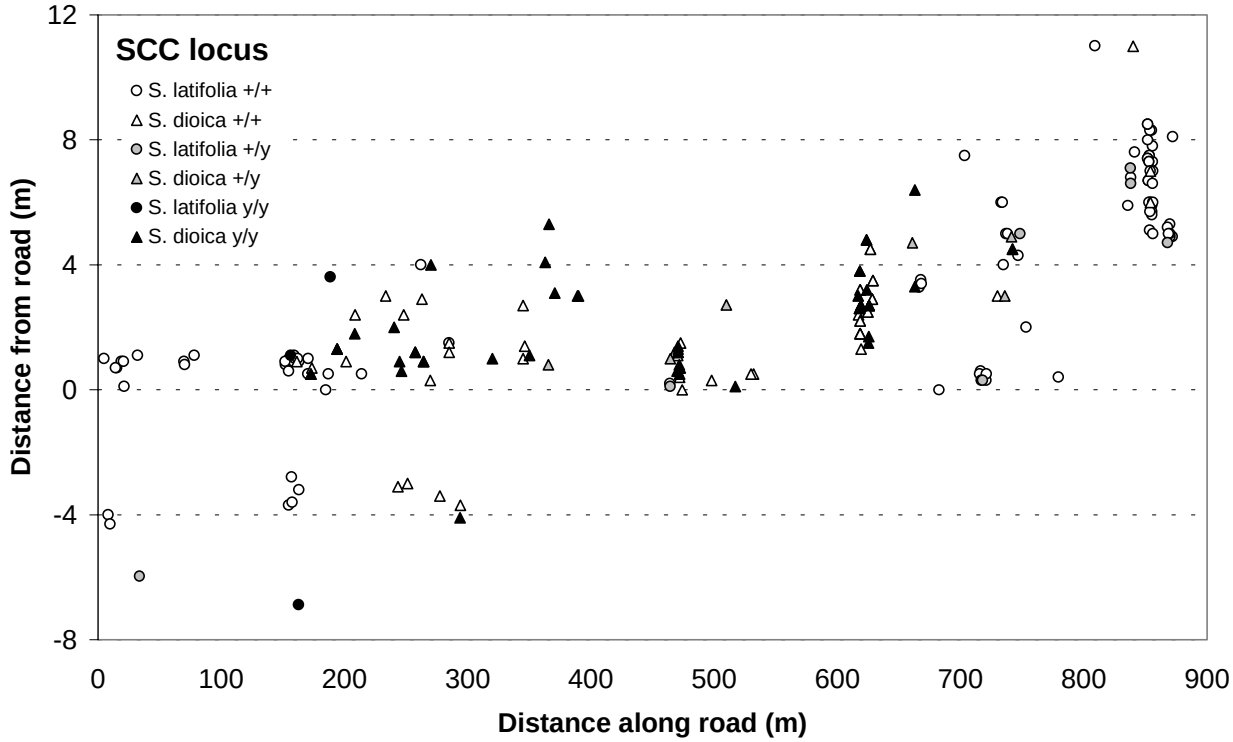


**Figure-2** Spatial and host-related distribution of fungal genotypes of *Microbotryum violaceum* with respect to a Sporidial Colony Color locus in the Norg population. Circles represent *Silene latifolia*, and triangles represent *S. dioica*. Open symbols represent homozygous pink (+/+) genotypes, black symbols represent homozygous yellow (*y/y*) genotypes, and grey symbols represent heterozygous pink/yellow (+/*y*) genotypes. Note that the scaling on the axes is quite different, covering 20m on the y-axis and 900m on the x-axis.

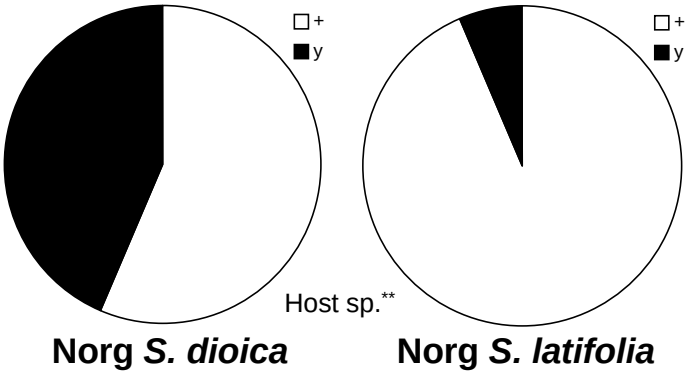


**Figure-3** Host-related differentiation in the anther smut *Microbotryum violaceum* in a sympatric population of the host species *Silene dioica* and *S. latifolia*. Displayed are allele frequencies of the *pink (+)* and *yellow (y)* allele of a Sporidial Colony Color locus in the Norg population of *S. latifolia* (103 smut samples from 6 years), and *S. dioica* (104 samples from 6 years). The allele frequencies between host species are significantly different with $p<0.007$.

Moreover, in smut from *S. dioica* all three genotypes (+/+, *y*/*y*, and +/*y*) were observed frequently, whereas the *y*/*y* genotype was scarce in smut from *S. latifolia* hosts. The host differentiation as observed in allopatric host populations also held true for the Norg population of anther smuts (Figure 3; $\chi^2=7.56$; df=1; $p<0.007$), although the overall frequency of the *pink* allele was significantly lower than in the allopatric samples from *S. latifolia* ($\chi^2=7.60$; df=1; $p<0.006$), and the overall frequency of the *yellow* allele was lower than in the allopatric samples from *S. dioica* ($\chi^2=38.8$; df=1; $p<0.0001$). Also, figures 1 and 3 clearly show that the differences between allopatric

(A) and sympatric (S) populations are much larger in the samples from *S. dioica* (A 95% vs. S 56%) than in samples from *S. latifolia* (A 98% vs. S 94%).

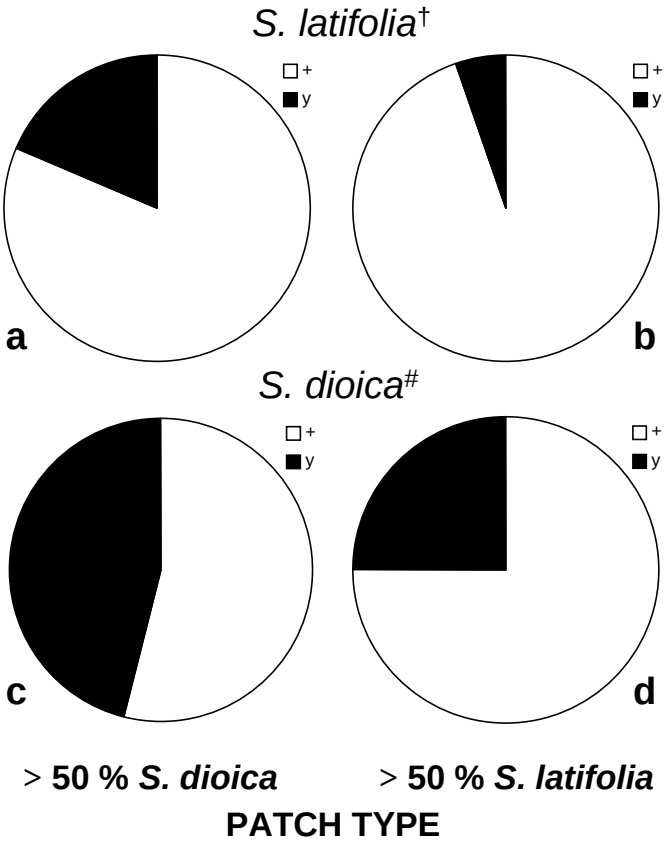


**Figure-4** Effects of local patch structure on the degree of host-specific differentiation in the anther smut fungus *Microbotryum violaceum* in a sympatric population of its host species *Silene dioica* and *S. latifolia*. Displayed are allele frequencies of the *pink (+)* and *yellow (y)* allele of a Sporidial Colony Color locus in the Norg population for *S. latifolia* (a, b), and *S. dioica* (c, d). The left side of the diagram (a, c) represents the patches where *S. dioica* is the majority type (n=14), the right side of the diagram (b, d) represents the patches where *S. latifolia* is the majority type (n=14). In this diagram, pooled data from six years within 1991-98 are shown. For each host species separate tests are given for the effect of PATCH TYPE on the allele frequency of the 'native' allele in that host (+ for *S. latifolia*, and *y* for *S. dioica*). In a combined test for the two host species, the interaction effect PATCH TYPE * SPECIES on the frequencies of the 'native alleles' is significant with $p<0.05$. Significance levels designated in the separate tests; # $p=0.16$; † $p=0.07$.

The degree of host-specific differentiation was significantly affected by local patch composition (the interaction effect PATCH TYPE * HOST SPECIES; $p<0.05$). This interaction is illustrated in figure 4. The frequency of the 'native' allele is higher when host species occur in patches dominated (>50%) by conspecifics (Figure 4b,c) than when host species occur in patches dominated by heterospecifics (Figure 4a,d). In other words, the frequency of the *pink* allele is larger on the 'native' host *S. latifolia* when this host is surrounded by other *S. latifolia* (Figure 4b) than when it is surrounded by *S. dioica* (Figure 4a). Likewise, the frequency of the *yellow* allele is larger on the 'native' host *S. dioica* when this host is surrounded by other *S. dioica* (Figure 4c) than when it is surrounded by *S. latifolia* (Figure 4d).

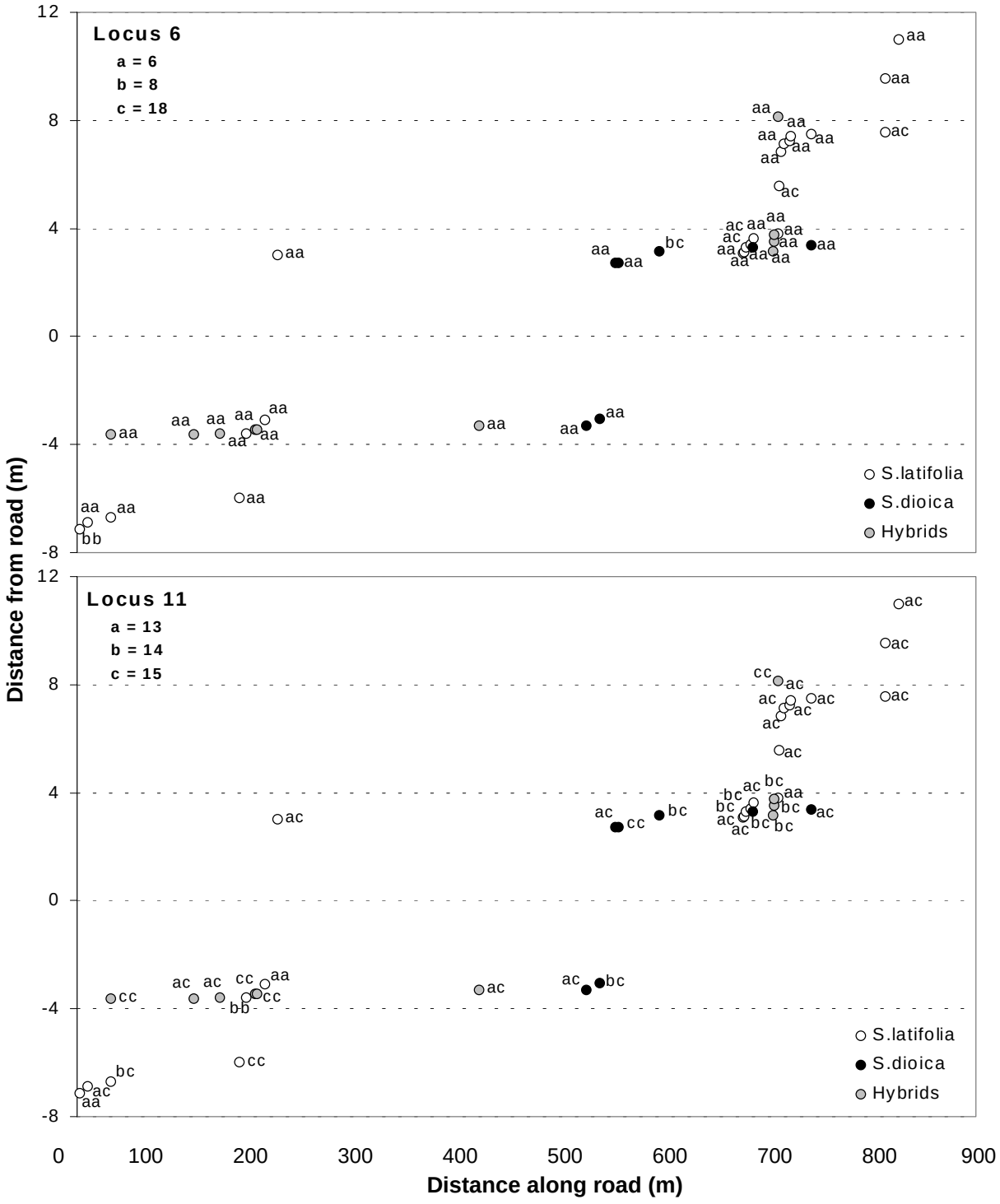


**Figure-5a** Graphical representation of the genetic diversity in the fungal pathogen *Microbotryum violaceum* on microsatellite loci 6 and 11 in the Norg population. Open circles represent fungal isolates from *Silene latifolia*, filled circles isolates from *S. dioica*, and gray circles from interspecific hybrids. Note that the scaling on the axes is quite different, covering 20m on the y-axis and 900m on the x-axis.

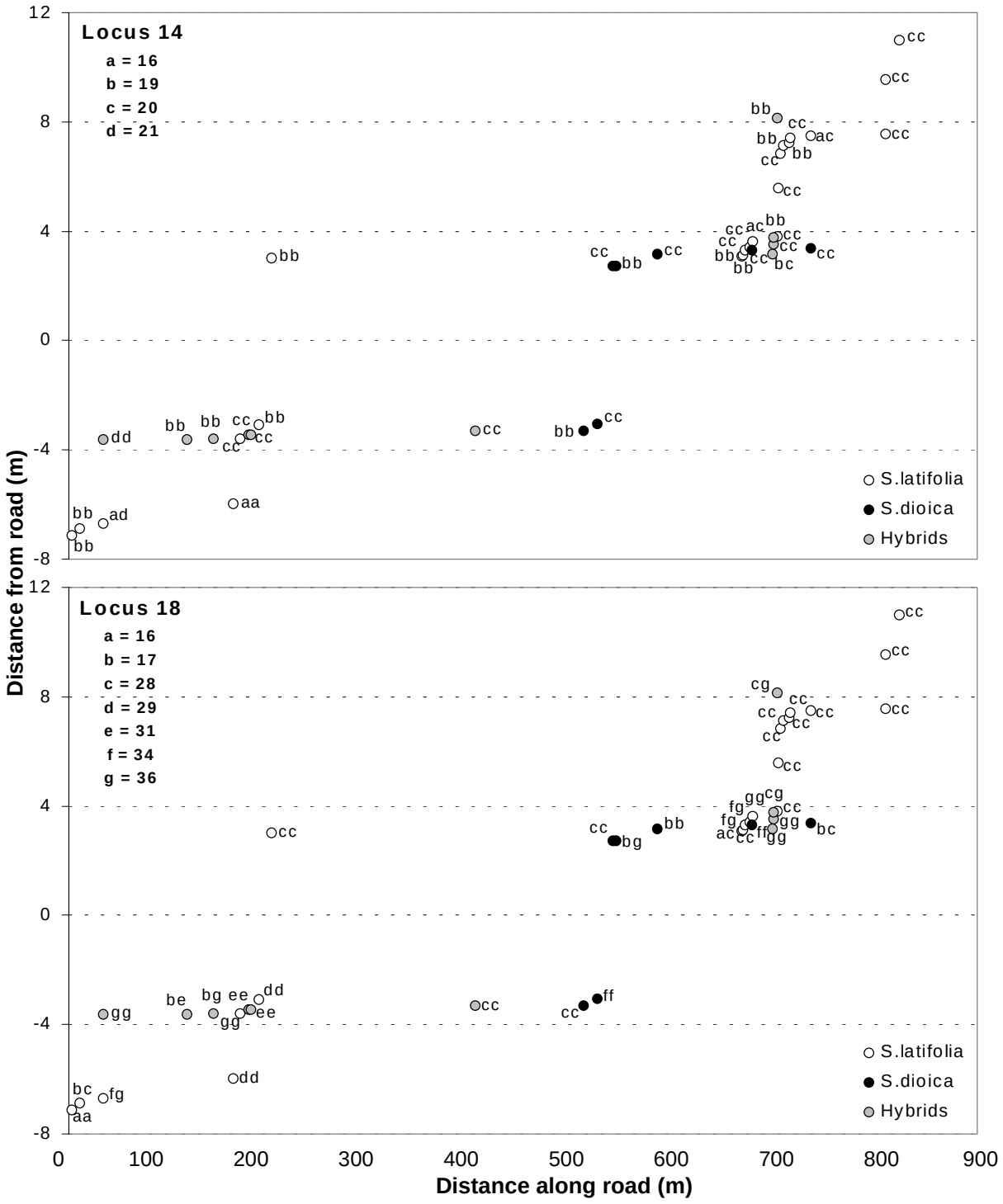


**Figure-5b** Graphical representation of the genetic diversity in the fungal pathogen *Microbotryum violaceum* on microsatellite loci 14 and 18 in the Norg population. Open circles represent fungal isolates from *Silene latifolia*, filled circles isolates from *S. dioica*, and gray circles from interspecific hybrids. Note that the scaling on the axes is quite different, covering 20m on the y-axis and 900m on the x-axis.

**Table-1** Most frequent alleles of four microsatellite loci in the fungal pathogen *Microbotryum violaceum* in host patches of *Silene latifolia* and *S. dioica* with more than one genotyped fungal specimen. Patches are indicated by their x-coordinate range, and patch type is indicated by the dominant host species.
† = Marks the more rare alleles that are locally (co-)dominating a patch. # = Allele was not present in one of these patches, but appeared in a patch with only one genotyped individual.

| **Patch** | **Patch type** | **Locus 6** | | | **Locus 11** | | | **Locus 14** | | | | **Locus 18** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(x-coordinate)** | **(dominant host species)** | **6** | **8 #** | **18** | **13** | **14** | **15** | **16** | **19** | **20** | **21** | **16** | **17** | **28** | **29** | **31** | **34** | **36** |
| 28-43m | *S. latifolia* | • | | | | | • | | | | • † | | | | | | | • |
| 138-178m | *S. latifolia* | • | | | | • † | • | • † | | • | | | | | • † | | | • |
| 539-542m | *S. dioica* | • | | | | | • | | • | • | | | | • | | | | |
| 663-683m | *S. latifolia* | • | | | | | • | | | • | | | | | | | • † | • |
| 696-702m | *S. latifolia* | • | | | • | | • | | • | • | | | | | | | | • |
| 703-708m | *S. latifolia* | • | | | • | | • | | | • | | | | • | | | | |
| 730-753m | *S. latifolia* | • | | | • | | • | | | • | | | | • | | | | |
| 808-810m | *S. latifolia* | • | | | | | • | | | • | | | | • | | | | |
| **Total frequency** | - | **.92** | **.01** | **.07** | **.32** | **.14** | **.54** | **.05** | **.36** | **.55** | **.04** | **.01** | **.09** | **.46** | **.05** | **.07** | **.08** | **.24** |

The microsatellite analysis, as presented previously in chapter 2, revealed no significant population subdivision with respect to the hierarchical level host species (Van Putten *et al.* chapter 2; $F_{ST}$ = 0.046, $R_{ST}$ = 0.035 (the deviancies from zero were only marginally significant, with $0.05<p<0.10$), and the derived effective number of smut migrants per generation between host species was estimated roughly between 1 and 3 in this population). Furthermore, the host-specific alleles of microsatellite loci 6 and 18, with *S. dioica* samples harboring much larger sized alleles in the allopatric populations, did not hold true for samples from the Norg population. Due to the relative small sample size in each patch, we could not calculate $F_{ST}$ and $R_{ST}$ values over the hierarchical level patch structure, nor compare this to the values that were calculated over host species (Van Putten *et al.* chapter 2). Still, the qualitative examination of these four microsatellite loci in relation to their geographical location in the population did provide additional support for the idea that alleles are not spread homogeneously over the population, but are distributed more locally in patches. The multilocus microsatellite genotypes were plotted onto the geographic structure of the population according to their coordinates in the Norg population. Figure 5a shows loci 6 and 11 and figure 5b shows loci 14 and 18. Table 1 shows the most frequent alleles in patches with two or more genotyped specimens (thus, in each of these patches there were four or more alleles examined). Both these figures and the table show that alleles that are relatively scarce in the whole population can be among the most frequent in a certain patch, at least in loci 11, 14 and 18.

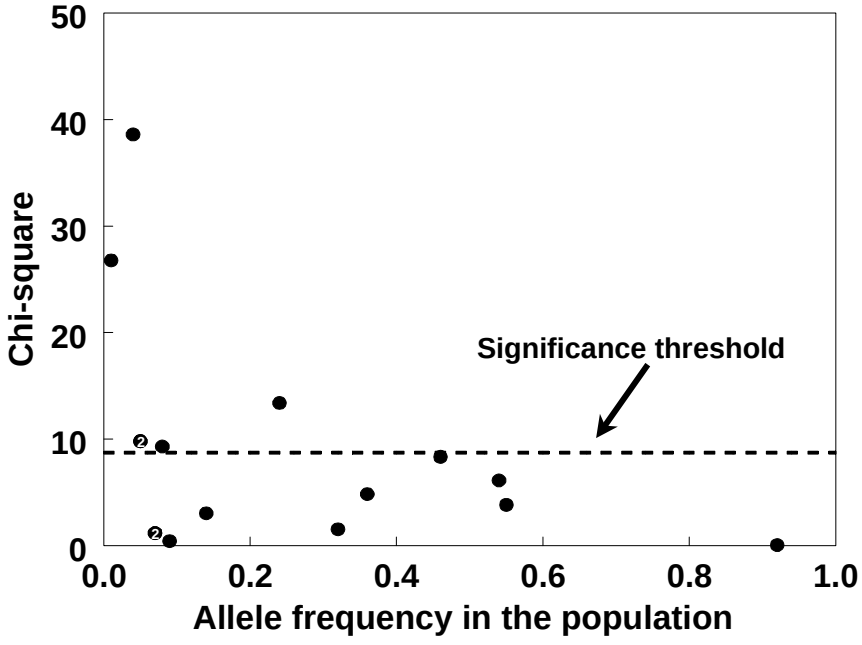


**Figure-6** Results of the Chi-square test for randomness of distribution of alleles over the patches, for each of the 16 alleles from the four microsatellite loci. The significance threshold was Bonferroni adjusted to $\alpha' = \alpha_{0.05}/16$, yielding the conservative critical $\chi^2_{[1]}$ value of 8.73. Six out of the 16 alleles turned out to be not randomly distributed over the population, but were clustered in patches. The '❷'s represent two separate points plotted on top of each other.

Figure 6 provides some statistical support for this rather qualitative statement. In six out of the 16 alleles, the chi-square analysis showed that the distribution of alleles over the eight patches was non-random, especially for the more rare alleles. In this analysis, the distribution of the alleles from loci 6 and 11 did not deviate from randomness at this conservative significance threshold.

## DISCUSSION

### Allopatric host populations

The allelic distribution at one of the sporidial colony loci (SCC) indicated strong divergence of host races in allopatric populations of hosts, at least in populations in Western Europe. Since this divergence was observed in a number of geographically different populations, it may well represent long-term divergence between both host races. The host-specific differentiation observed for in the SCC marker was consistent with the molecular marker studies of Bucheli and colleagues (2001) and chapter 2 of this thesis, confirming once again clear and separated host races of *M. violaceum* (Zillig 1921) on *S. latifolia* and *S. dioica*. Strains from these host species are—in contrast to an early study by Baker (1947)—able to cross-infect each other's host species (Biere and Honders 1996a), without a priori being at a disadvantage on the 'alien' host species (Biere and Honders 1996a; Van Putten *et al.* chapter 4).

### The sympatric host population of Norg

Overall, looking at the SCC distribution of alleles there is significant divergence between the host races in sympatric populations of host species, despite the fact that the microsatellite data indicate there is gene flow between smuts from *S. latifolia* and from *S. dioica* in the range of 1 to 3 migrants per generation (Van Putten *et al.* chapter 2). Gene flow caused significant lower allele frequencies relative to allopatric populations of the *yellow* allele in smut isolated from *S. dioica* hosts in particular, but also of the wild type *pink* allele in smut isolated from *S. latifolia* hosts. Nevertheless, the pattern of variation observed in allopatric and sympatric host populations of the SCC marker is clearly different from what is observed in microsatellites (Van Putten *et al.* 2001), suggesting that the SCC locus might not be as neutral as the microsatellite loci. Presence of the wild type *pink* allele or the *yellow*

allele at the SCC locus results in the production of lycopene and β-carotene respectively, in the carotenogenesis pathway (*cf.* Garber *et al.* 1975). Lycopene is a precursor of β-carotene in carotenogenesis, and its transformation requires an active cyclase that is inactive in the wild type. A study examining carotenoids in rust fungi, a related order of plant parasitic fungi, showed that variation in the amount of carotenes was negatively associated with the amount of pigment in spore-walls (Zwetko and Pfeifhofer 1991). Spores with strongly pigmented walls contained little carotene in their cytoplasm and vice versa. Pigmented spore-walls may provide protection against high light intensities, or UV radiation. Extrapolating this to the anther smut fungus and the variation at the SCC locus, the different genotypes may be subject to host-specific selection pressures that could oppose gene flow and contribute to the observed pattern of variation. However, *S. dioica* is better adapted to low light intensities than *S. latifolia* (Willmot and Moore 1973), hence we would expect just the opposite pattern for the distribution of alleles of the SCC locus for this pair of host species. Nevertheless, if natural selection, acting on the SCC locus itself or on loci linked to this locus, is high relative to gene flow, it might facilitate host specialization. In the traditional view, host specialization is driven by genetic trade-offs in performance, which would be the result of antagonistic pleiotropy. In the *Microbotryum-Silene* system, there is not much evidence for such trade-offs. In a cross inoculation study by Biere and Honders (1996a), strains did not have a higher virulence on conspecifics of the host of origin, but a three-fold higher spore production was observed on infected male plants of the native host species. Recent models have shown that, even without such performance trade-offs for which little evidence is found in the literature (Jeanike 1990), the non-equilibrium frequency dependent cycling of allele frequencies of resistance and virulence loci itself can drive the evolution of host specialization in parasites capable of host choice, given that there is genetic variation in host preference (Kawecki 1998). However, since the pollinators vector the teliospores of this anther smut, host choice is a feature of the vectors rather than of the pathogen. Hence, host specialization of this fungus will largely depend on interactions with its insect vectors, e.g. host fidelity of pollinators (Van Putten *et al.* chapter 5).

## The impact of local structure of the host population on the genetic structure of the smut population

In the Norg population, the host plants provide a heterogeneous environment for the pathogen in three different ways. First and foremost, the host plants are different species. Second and of prime interest to this study, the host plants grow spatially in patches due to differential habitat preferences (Goulson and Jerrim 1997). Third, the flowering phenology between *S. latifolia* and *S. dioica* has been reported to be different, yielding a temporally heterogeneous environment, with *S. dioica* flowering earlier than *S. latifolia*, and males flowering earlier than female hosts (Biere and Honders 1996b). Therefore, we expected that the population of *M. violaceum* in Norg is not panmictic, but consists of a number of demes that genetically structure the population, as often found for phytophagous insects (Mopper 1996). Indeed, we found weak evidence for effects of local host spatial structure on differentiation among fungal isolates within this sympatric host population. Smut samples isolated from *S. latifolia* in patches where *S. latifolia* was the majority type had a higher frequency of the wild type *pink* allele (the *S. latifolia* allele in allopatric host populations) than smut from *S. latifolia* in patches where *S. dioica* was the majority type. Conversely, smut from *S. dioica* in a *S. dioica* patch had a higher frequency of the *yellow* allele (the *S. dioica* allele in allopatric host populations) than smut from *S. dioica* in *S. latifolia* patches. Although sample size of the microsatellite analysis was small (n=38), the results support the hypothesis of a more local distribution of alleles, which was most apparent for the more rare alleles. Unfortunately, smut spores that were collected in 1998 were sampled from seven *S. latifolia* dominating patches and from only one *S. dioica*-dominating patch (Table 1). This might explain why we did not find higher values for $F_{ST}$ and $R_{ST}$ in chapter 2. When adding the 2001 dataset, we expect to gain more statistical power, and clarify the effects of local host spatial structure both on the microsatellite loci and on the SCC marker allele frequencies. Also, host sex can then be included as a factor to be analyzed as well. Pollinators are differentially attracted to *Silene* plants of different sex (Shykoff and Bucheli 1995; Van Putten *et al.* chapter 5). Hence, spatial structuring of smut populations might be further enhanced due to gender and/or gender disease interactions (Real and McElhany 1996).

Assuming a long-term divergence of host races in allopatric host populations, as argued in chapter 2, both the microsatellite data and the SCC marker show signs of fungal gene flow in this sympatric host population. The differentiation between host species, or between patches of host plants can often be a starting point of host specialization, but may here represent a different situation. Presumably, the two host

races have come into secondary contact with each other in this population (Van Putten *et al.* chapter 2). Whereas gene flow opposes the historically evolved differentiation between the host races, and acts to homogenize the genetic diversity, the local deme structure of both host species in this population will favor its maintenance (*cf.* Mopper 1996). Habitat, or host choice is found to be a crucial factor in theoretical models on host specialization (Fry 1996, Kawecki, 1997; 1998). Being the actors of host choice for this fungus, the vector/pollinator guilds of these host species are expected to play a dominant role in the process of maintaining the host-related differentiation among fungal isolates (Van Putten *et al.* chapter 5).

# CHAPTER 4

## Intraspecific competition and mating between fungal isolates of the anther smut *Microbotryum violaceum* from the host plants S*ilene latifolia* and *S. dioica*

with Arjen Biere and Jos van Damme

*submitted to Evolution*


### Abstract

**We have studied intraspecific competition and assortative mating between strains of the anther smut *Microbotryum violaceum* from two of its host species *Silene latifolia* and *S. dioica*. Host differentiation between strains from these two host species is maintained in sympatric host populations despite the presence of gene flow. We studied whether higher competitive ability of strains on their native host species and/or positive assortative mating between host races occurs, which could contribute to the maintenance of such host differentiation.**

**In general, strains isolated from *S. latifolia* outcompeted strains isolated from *S. dioica* on both host species, but in female hosts, heterotypic dikaryons had the largest competition success. Furthermore, latency period was significantly shorter in infections that contained strains from *S. latifolia*, compared to homokaryotic infections with a *S. dioica* origin.**

**The frequencies of conjugation between strains originating from S. latifolia were much higher than conjugation frequencies between strains from *S. dioica*. A significant positive correlation was detected between the relative success rate of strains in competition and in conjugation, suggesting that success of a strain in competition might be partly determined by its swiftness of mating. In addition, reciprocal differences between homotypic and heterotypic crosses revealed a significant effect of fungal mating type, with mating type $a_1$ being the main determinant of mating pace.**

**The observed differences in infection success, conjugation rate and latency period, in favor of strains from *S. latifolia* relative to strains from *S. dioica* on both host species are discussed in an evolutionary context of the maintenance of host race differentiation in sympatric populations of hosts.**

## INTRODUCTION

Plant parasites can often employ more than one host species, and show intraspecific variation in host use. How such variation originates or is maintained, and under which conditions this may lead to host race formation and speciation are central questions in evolutionary biology. Positive assortative mating and differences in performance of pathogens on different host species are expected to play an active role in processes of divergence between strains, or in the maintenance of host-related differentiation. Indeed, host associated fitness trade-offs, i.e. by antagonistic pleiotropy of genes, would lead to increased divergence between strains, and host race formation (Jeanike 1990). Empirical evidence for such genetic correlations across different host species is often found to be ambiguous, or non-negative in studies of phytophagous insects (*cf.* Fry 1996; but see Via *et al.* 2000), and is scant for other organisms including the group of phytopathogenic fungi. From an evolutionary point of view, competition among strains from different host species may be important as well, especially in cases where different physiological races have evolved and occur in sympatry (Day 1980). In such cases competition may enhance differences in the ability of host resource exploitation among strains. Depending on the amount of gene flow between the races, one race might outcompete the other, which could lead to extinction, both races could merge into one race by introgression, or host races might coexist. Empirical examples of phytopathogenic fungi that share the same host species and have to compete for its resources intraspecificly, are scarce (*cf.* Shearer 1995), and often involve saprophytic fungi rather than biotrophic fungi (but see Day 1980; Newton *et al.* 1997; 1999). Besides differences in performance, strains from different host species may show host-specific mating preferences that affect the process of differentiation. In the group of heterothallic basidiomycetes, i.e. fungi that reproduce sexually with physiologically different strains (= mating types), mating involves processes of recognition and conjugation between cells of opposite mating type in order to produce an infectious dikaryon, and precedes entering a host. Positive assortative mating with respect to host species due to faster recognition, or conjugation will promote the divergence between strains, and may lead to (sympatric) host race formation and eventually speciation (Kondrashov and Shpak 1998). Such positive assortative mating in sympatry may be expected between races that have diverged considerably, to avoid maladaptive hybridization between strains, a process that is known as reinforcement (*cf.* Noor 1999).

The anther smut *Microbotryum violaceum*, obligate parasite of a wide host range within the Caryophyllaceae, is a well studied example of a pathogenic fungus, for which different physiological strains have been described on a number of host species (Zillig 1921). Recently, fungal strains from a number of host species have been characterized for a number of molecular markers, revealing strong differentiation (e.g. Perlin 1996; Shykoff *et al.* 1999; Bucheli *et al.* 2000). Two closely related host species, *Silene latifolia* and *S. dioica*, common roadside herbs, frequently occur in sympatry in areas where their preferred habitats overlap (Goulson and Jerrim 1997). Anther smuts from these host species proved to be genetically differentiated in allopatric populations of hosts (Bucheli 2001; Van Putten *et al.* chapter 2), but are still able to cross infect the other host species (Zillig 1921; Baker 1947; Biere and Honders 1996a). In natural sympatric populations of these host species, fungal isolates of anther smut from different host species show significant differentiation (Van Putten *et al.* chapter 3) as well. Gene flow between the host species in these sympatric populations is evidenced by the occurrence of interspecific hybrids, reported to constitute more than 6% of the population (Biere and Honders 1996b). Since this fungus is vectored by the natural pollinators of their hosts (Jennersten 1983), this raises the question how host races remain differentiated in the presence of fungal gene flow (Van Putten *et al.* chapter 2), especially since infection success is not necessarily higher on conspecifics of the host of origin (Biere and Honders 1996a). One factor that could contribute to the maintenance of host differentiation in sympatry is the finding that spore production in male hosts tends to be higher on conspecifics of the host of origin than on heterospecifics in these cross inoculation experiments. Other possibilities that also could play a role include host fidelity of vectors (Van Putten *et al.* chapter 5), a higher competitive ability of strains on their native host species, and/or sufficient host-related positive assortative mating between fungal isolates from the same host species.

The aim of this chapter is to study intraspecific competition and assortative mating between the host races from *S. latifolia* and *S. dioica* of *M. violaceum* in more detail. Specifically, we will address the following questions: (1) Is the success of strains from a host race in competition with strains from a different host race higher on the host species from which they originate, i.e. have strains adapted to 'their own' host species with respect to success in competition? (2) Are there positive assortative mating patterns, i.e. is conjugation between strains of the same host race more frequent and/or faster than between strains of different host races? (3) Do mating frequencies of in*tra*- and in*ter*racial crosses depend on the host species (host extract)

on (in) which mating occurs? (4) How much of the variation in success of strains in competition can be explained by their mating success?

From the observed divergence between these host races in allopatric host populations, that is observed to some degree in sympatry as well (Van Putten *et al.* chapter 2; chapter 3), we hypothesize that strains isolated from allopatric *S. latifolia* and *S. dioica* hosts; (1) have a higher infection success on conspecific hosts than on heterospecific hosts. Heterosis might counteract any host adaptations, but it is difficult to predict the infection success of the heterospecific dikaryons (heterokaryons) relative to conspecific dikaryons in competition; (2) mate assortatively with respect to host species of origin, i.e. conjugate in higher frequencies with strains that were isolated from conspecifics than with strains that were isolated from heterospecific hosts; and (3) that mating (conjugation) frequency is higher in the presence of the 'native' host extract than in water and/or in extracts of the non-native host species.

## MATERIALS AND METHODS

### Anther smuts

The anther smut fungus *Microbotryum violaceum* (Pers.: Pers) Deml & Oberw. (=*Ustilago violacea* (Pers.) Fuckel) (Ustilaginaceae) (Vánky 1994) is a well-studied example of a heterobasidiomycete fungus that obligatory parasitises susceptible members of the Caryophyllaceae plant family to complete its sexual lifecycle (Thrall *et al.* 1993). To achieve a better understanding of the processes involved in competition and conjugation between haploid cells of this fungus, this sexual life cycle will be shortly reviewed here. Starting with diploid teliospores arriving on a healthy host plant, the sexual life cycle commences with germination. Germination of smut spores by meiosis results in four-celled promycelia from which four haploid cells (sporidia) of two mating types (designated $a_1$ and $a_2$) bud off. In principle, the two mating types are produced in a 1:1 ratio. Haploid cells of opposite mating types can then mate on the surface of (flowering) plants. The mating process and recognition between cells is influenced by pheromones (Bölker and Kahmann 1993; Snetselaar *et al.* 1996), which promote the formation of fungal fimbriae, i.e. microscopic hair-like structures composed of collagen, carbohydrates and RNA (Poon and Day 1975 but see Celerin and Day 1998). Hereafter, a conjugation tube between these cells of different mating type is formed (Poon *et al.* 1974), marking the start of the dikaryotic phase in

which the infectious hyphae are produced. Critical factors in the mating process are low temperature and low nutrient level, and the presence of oxygen and salts (Cummins and Day 1977). The development of the fungus from this point on is regulated by the presence of host plant chemicals (Day *et al.* 1981; Kokontis and Ruddat 1986; 1989), of which α-tocopherol (= vitamin E) has been identified as one of the major factors stimulating hyphal growth (Castle and Day 1984). Infectious hyphae grow intercellularly (Spencer and White 1951; Audran and Batcho 1981) and grow along with the plants apical meristemic regions (Day 1980). When the dikaryotic parasitic mycelium grows into the stamens of a flower, anthers produce teliospores instead of pollen (Thrall *et al.* 1993). As spores mature in the anther sacs, karyogamy marks the start of the diploid phase (Day and Garber 1988). In dioecious host species, an infection of female plants causes a morphological sex change; ovaries are aborted and staminal rudiments develop into stamens that bear spore-filled anthers (Day and Garber 1988; Thrall *et al.* 1993), a process that is induced by the fungus (Audran and Batcho 1981; Scutt *et al.* 1997). The teliospores are dispersed by the natural insect visitors of the host plant that serve the dual role of pollinators of healthy plants and vectors of this sexually transmitted disease (Jennersten 1983). *M. violaceum* is found to be highly selfing (Baird and Garber 1979), resulting in strong homozygosity in several host races (Bucheli *et al.* 2000; Van Putten *et al.* chapter 2). Automixis (mating among products of different meioses from the same diploid origin, i.e. between haploid sporidia from different teliospores of the same strain) as well as autogamy (mating among products of a single meiosis, i.e. within a single basidium) are more likely to occur (Hood and Antonovics 1998; 2000) than outcrossing, limiting the opportunities for outcrossing.

## Fungal material

Fungal isolates, hereafter referred to as strains (*sensu* Staley and Krieg 1984; "*a strain is made up of the descendants of a single isolation in pure culture, and usually is made up of a succession of cultures ultimately derived from a single colony*"), were selected out of a collection containing haploid sporidia of single mating type. The original diploid teliospores were collected from several natural allopatric populations of hosts in the Netherlands in 1992 and 1993, and then cultured to separate mating types. Since then, sporidia have been stored at –20°C. Strains were chosen in such a way that both mating types of the original single flower teliospores were present. Wild type (+) strains of *M. violaceum* produce pink colonies (Sporidial Colony Color; SCC) when growing on standard medium due to the formation of

lycopene. The yellow colored mutant converts this lycopene into β-carotene through a cyclase that is inactive in the wild type (Garber *et al.* 1975). Single sporidia colonies that originated from allopatric *S. latifolia* host populations were almost fixed for the 'pink' allele at one of the SCC loci, whereas single sporidia colonies that originated from allopatric *S. dioica* hosts were almost fixed for the 'yellow' allele (*cf.* Biere and Honders 1996a; Van Putten *et al.* chapter 3).

### The host plants

*Silene latifolia* Poiret (= *S. alba* (Miller) Krause (Caryophyllaceae), the white campion is a dioecious short-lived perennial from open, disturbed habitats and borders of arable land. *Silene dioica* (L.) Clairv. (Caryophyllaceae), the red campion is a closely related dioecious perennial that mainly occurs in more shady humid habitats as woodland borders. In areas where habitats overlap, both species frequently occur in sympatry and hybridization is a common phenomenon (Baker 1947; Goulson and Jerrim 1997).

### Plant material

Seeds of *S. latifolia* and *S. dioica* were collected from several geographically spread natural allopatric populations in the Netherlands in 1997 and 1998. Per species, seeds were bulked and then thoroughly mixed to randomize. Seeds were germinated in petridishes on demi-water moistened filter paper at a density of approximately 25 seeds per petridish in a growth cabinet (16/8h light/dark, 21/15°C day/night temperature) after a vernalization period of three days at 4°C that has proven to be sufficient to increase the proportion of flowering plants in previous experiments. Nearly all seeds had germinated after a week.

## THE COMPETITION EXPERIMENT

### Inoculation procedure

For the competition experiment, 24 strains (12 of each mating type) that were isolated from 12 different infected hosts from six allopatric *S. latifolia* populations, and similarly 24 strains that were isolated from 12 different infected hosts from seven allopatric *S. dioica* populations, were used. With these 48 (2x12x2) strains, 64 different inoculation mixtures (one-ninth of all possible combinations) were prepared containing randomly selected strains from each of the four different sporidial types (*S. latifolia* mating type $a_1$ $L_1$; $a_2$ $L_2$; *S. dioica* mating type $a_1$ $D_1$; $a_2$ $D_2$). The number of

times that an individual strain participated in one of the mixtures, ranged between 1 and 12 with median 5 (expected mean = 5.3) and mode 3. Combinations containing two strains originating from a single teliospore were excluded from the experiment. Each of these inoculation mixtures can lead to four different types of conjugates; $L_1L_2$ (yielding teliospores with SCC 'pink' phenotype after a successful infection), $L_1D_2$, or $D_1L_2$ (yielding teliospores with 'pink/yellow' phenotype after a successful infection) and $D_1D_2$ (yielding teliospores with 'yellow' phenotype after a successful infection).

Strains were cultured on a plate containing standard medium. Before the experiment each strain was checked for mating type using reference strains. A large loop of cells was scraped off a plate and suspended in 1.0ml of sterile milliQ water. A CoulterCounter© Z1 (Coulter Electronics Ltd., Luton, England) was used to count the cells. Each sample was diluted to $5.0x10^7$ cells/ml with sterilized demineralized water. An inoculation mixture (inoculum) contained 2.5ml of each of the four standardized strain samples, and was thoroughly mixed overnight in a shaker at 14°C.

Two ml of inoculum was added to petridishes with growing seedlings, which were potted three weeks after inoculation. To check for possible cross-infections, a subset of petridishes with growing seedlings received 2ml demi-water instead of inoculum, and served as control plants in the experiment.

## Greenhouse conditions and procedures

552 *S. latifolia* seedlings (8 per mixture + 40 control plants) and 690 *S. dioica* seedlings (10 per mixture + 50 control plants) were potted and placed in a greenhouse which was kept below 25°C and with a 16/8 hr day/night light regime. In the first week the seedlings were covered with cloth to protect from dehydration. In the second week plant/inoculation mixture combinations were randomized over the greenhouse tables. Plants that started flowering were removed instantly from the greenhouse compartment and were checked for disease status. From infected plants, spores from 5 flowers were collected in separate eppendorf cups, and analyzed for SCC type. Preferably, teliospores were taken from closed flower buds, and from different flowering stalks whenever possible.

## Sporidial Colony Color analysis procedure

Since strains from different host origins have different alleles at one of the SCC loci (*cf.* Garber *et al.* 1975), and strains with the 'pink' allele and strains with the SCC 'yellow' allele were mixed in a 1:1 ratio, the resulting teliospores can be either 'pink', 'pink/yellow', or 'yellow' with the null hypothesis of a 1:2:1 segregation. To

determine the SCC phenotype of each of the infected flowers, a large number of teliospores were transferred with a sterile inoculation loop from the eppendorf cup to a petridish containing standard medium. After one week of growth at 21°C, plates that showed both pink and yellow colonies were scored as heterokaryotic. From plates that showed colonies of only one color, 16 single spore colonies were taken and replated on fresh medium. The sporidial colony color was determined after another week of growth by evaluating the color of these 16 colonies. If all 16 colonies were still of the same color, plates were scored as either homokaryotic pink, or homokaryotic yellow. Note that, following this procedure, heterokaryons originating from $a_1$ *S. latifolia* strains and $a_2$ *S. dioica* strains are not distinguishable from the reciprocal heterokaryon type.

## THE CONJUGATION EXPERIMENT

### Conjugation procedure

For the conjugation experiment, 16 strains (8 of each mating type) that were isolated from 8 different infected hosts from five allopatric *S. latifolia* populations and 16 strains (8 of each mating type) that were isolated from 8 different infected hosts from four allopatric *S. dioica* populations were used. Eight of these 32 strains overlapped with those selected for the previous experiment. This procedure provided 256 (2x8x2x8) possible combinations for conjugation in a complete diallel that were all studied. The procedure that we used for studying the conjugation frequencies between different strains was similar to that described in Kaltz and Shykoff (1999), with the modification that a total volume of 8.0µl was used instead of 50µl, as our pilot studies showed that using smaller volumes leads to higher conjugation frequencies (data not shown). This way, putative differences in conjugation frequency between different combinations of strains may be more pronounced. Conjugation was studied in three different environments, host extract from *S. latifolia*, host extract from *S. dioica*, and sterilized milliQ water. Leaf extracts were used to mimic the different host environments. Studies by Day *et al.* (1981) and Kaltz and Shykoff (1999) indicate that such extracts evoke the same qualitative conjugation behavior as found when placing sporidia directly on the leaf surface, and that conjugation frequencies in host extracts correspond well with conjugation frequencies in more realistic situations where plant architecture is left intact. Host extracts of *S. latifolia* and *S. dioica* were prepared by grinding a few grams of fresh leaf material in a mortar with a pestle after adding 15.0ml/g milliQ water. Host extracts were filtered through a 0.2µm fine mesh

filter before use. MilliQ water was sterilized before use. Haploid cells of single mating type were cultured for one week on a plate containing standard medium at 21°C. Approximately $1x10^8$ cells were scraped off and suspended in 100µl sterile milliQ water. The suspension was mixed thoroughly and cells were counted on a CoulterCounter© Z1 (Coulter Electronics Ltd., Luton, England). Samples were diluted to $2.0x10^8$ cells/ml with sterile milliQ water. Samples were let to conjugate in eppendorf cups containing 2.0µl $a_1$ cell suspension, 2.0µl $a_2$ cell suspension, and 4.0µl host extract of either host species, or a similar volume of sterilized milliQ water. The final cell density in the conjugation mixture was $1.0x10^8$ cells/ml. Samples were placed at 14°C for 24h. After coloring the samples with 8.0µl cotton-blue, conjugation frequencies were determined by counting all conjugated and all non-conjugated cells in 20 small squares of a Cell-Vu© disposable counting chamber (Norwell technologies inc., Marietta, GA USA), under a light microscope (at 400x magnification). The mean number of cells (± SE) that was counted (in 1536 samples) was 198.7 ± 1.5. At two times during the experiment, the mating type of all 32 haploid strains was checked, and confirmed. All 256 possible mating combinations between the $a_1$ and $a_2$ strains were made twice (two replicates). For practical reasons, only 48 experimental crosses for all three extracts could be tested simultaneously. Therefore, the complete experiment was spread out over eleven blocks. Crosses and replicates were randomly divided over blocks with the restriction that replicates of the same cross were not allowed to occur within a single block.

### Time series

A small subset of combinations was selected for a time series experiment. These were six mating combinations with the highest, and six combinations with the lowest conjugation frequencies after 24h (calculated over the three treatments combined). Conjugation frequencies in all three environments were determined after 24, 48, 72 and 168h. They were measured twice in two separate blocks.

## STATISTICAL ANALYSIS

### The competition experiment

The analyses are based on segregation of the SCC marker in successful infections of the 64 inoculation mixtures. Effects of mix, host species and host sex on the frequency of chromosome copies with *S. latifolia* origin (marked by the 'pink'

allele) per plant was analyzed in a generalized linear model using the SAS procedure GENMOD (SAS v8, The SAS Institute Inc., Cary NC USA). The frequency of heterokaryons (marked by the 'pink/yellow' phenotype) per plant, and the frequency of multiple infections (number of different SCC phenotypes per plant) were analyzed the same way. Differences in latency period between different teliospore types (here, the time between inoculation and the release of teliospores in the first infected flower) were tested within host classes non-parametrically using the Kruskal-Wallis test (procedure NPAR1WAY in SASv8, The SAS Institute, Cary NC USA). To correct for multiple comparisons within a host class, significance levels were adjusted using a Bonferroni correction ($\alpha' = \alpha_{0.05} / k$, with k=3 when comparing all combinations of homo- and heterotypic teliospore types; Sokal and Rohlf 1995, p. 703).

## The conjugation experiment

The data were tested for normality (Shapiro-Wilk test for normality) and for heterogeneity of variances (Levene's test for homogeneity of variances). To improve a normal distribution of the data, and homogeneity in the variances among groups, the conjugation frequencies were angular transformed before data analysis. Transformed data were analyzed in a general linear model using SAS procedure GLM (SASv8, The SAS institute Inc., Cary NC USA). We performed two separate analyses; in the first analysis the plant origins of the strains were accounted for, whereas in the second analysis crosses were classified into six cross types (outcross; $L_1xL_2$, $L_1xD_2$, $D_1xL_2$, $D_1xD_2$, and self; $L_1xL_2$, $D_1xD_2$). In both analyses, effects of host extract and cross type (second analysis and time series) were treated as fixed effects. Effects of strain origin (first analysis), and of replicates were treated as random effects.

## Relative success rate of strains

The relative success rate of each strain was calculated for the different experiments in the following way; for strains in the conjugation experiment, the conjugation frequencies of the crosses were first divided by their block means, because of a strong block effect (see results for details). The resulting values were averaged over the three extracts. Finally, in order to normalize the values the relative success rates of individual strains were derived by averaging the values over all combinations they participated in. Note that, due to the unbalanced design of the blocks in the experiment, these estimates of relative success rates have slightly different weights. For strains in the competition experiment, relative success rate was derived by calculating the infection success of individual strains, and dividing these

values by 0.5 to normalize the values (0.5 is the initial chance for an individual strain to successfully infect a flower when competing with the other strain of the same mating type in any inoculation mixture). Four strains with a *S. latifolia* origin and four strains with a *S. dioica* origin were used in both the competition and the conjugation experiment, thereby directly linking the two experiments together.

## RESULTS

### The competition experiment

From all flowering *S. latifolia*, 49.7% (n=437) contracted an infection with *M. violaceum*. A significantly higher proportion of the *S. dioica* (62.5% n=355; $p<0.001$) was infected. None of the flowering plants in the control group (*S. latifolia* n=36; *S. dioica* n=38) became infected. Thus, cross infections with spores coming either from outside the greenhouse, or from relatively late detected infected plants within the experiment can safely be neglected.

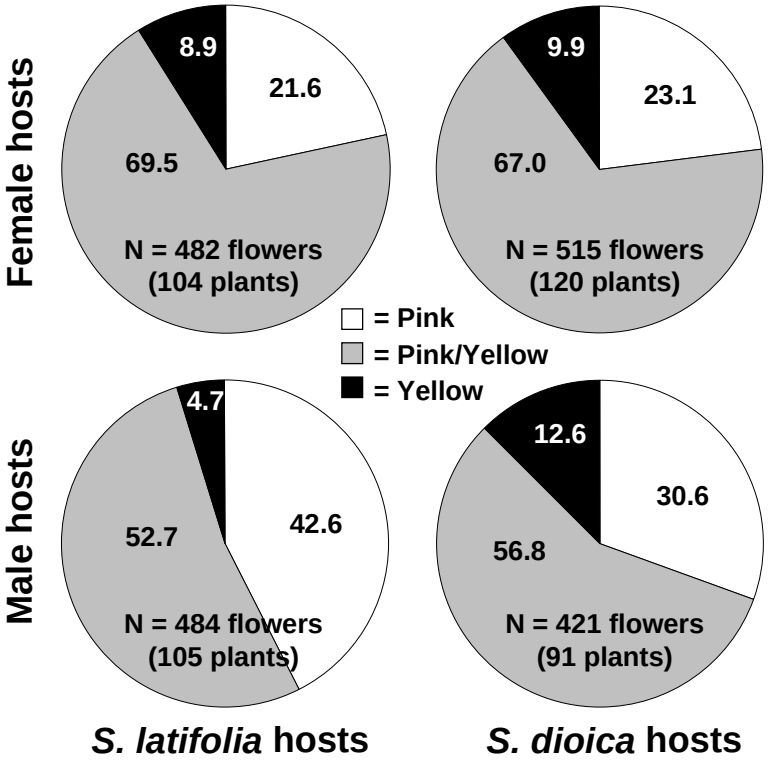


**Figure-1** Segregation of the Sporidial Colony Color marker in germinated teliospores of the fungal pathogen *Microbotryum violaceum* from female and male plants of *Silene latifolia* and *S. dioica* in the competition experiment. The difference in frequency of the pink allele between host sexes was significant ($p<0.02$). Differences between host species, as well as in the interaction between host species and host sex, were marginally significant ($p<0.06$). The difference in the frequency of heterozygotes between host sexes was highly significant ($p<0.001$), but neither the effect of host species, nor the interaction was significant.

Not all of the maximum of 5 flowers (actually 4.6 flowers per *S. latifolia*, and 4.4 flowers per *S. dioica* on average) that were taken from a single infected plant were of the same spore type. Hence, we detected multiple infections in single host plants.

21.5% of the *S. latifolia* was infected with two different types and 0.5% with all three types. 17.5% of the *S. dioica* was infected with two different types and 0.5% with all three types. Since the heterokaryotic $L_1xD_2$ type could not be distinguished from the $D_1xL_2$ type, these values underestimated the true frequency of multiple infections.

**Table-1** Generalized linear model of the frequency of *Silene latifolia*-specific chromosome copies (L) and the frequency of heterokaryotic infections (H) of the fungal pathogen *Microbotryum violaceum*, as a function of inoculation mix, host species and host sex in the competition experiment.

| Source | df | L (Type III $\chi^2$) | P-value | H (Type III $\chi^2$) | P-value |
|---|---|---|---|---|---|
| Inoculation mix | 63 | 79.4 | =0.08 | 62.1 | n.s. |
| Host species | 1 | 3.6 | <0.06 | 0.1 | n.s. |
| Host sex | 1 | 8.5 | <0.02 | 11.4 | <0.001 |
| Species * Sex | 1 | 4.0 | <0.06 | 0.2 | n.s. |

On average, copies of the strains with a *S. latifolia* origin were significantly more successful than strains with a *S. dioica* origin on both host species (testing the deviation from 50:50% that is expected under the null-hypothesis of a 1:2:1 segregation ratio of alleles (one tailed α), overall: 60.2% n=420 p<0.002; in *S. latifolia* hosts: 62.6% n=209 p<0.007; in *S. dioica* hosts: 57.7% n=211 p<0.057). Table 1 shows the effect of mix, host species and host sex on the frequency of the SCC alleles in a generalized linear model, which is visualized in figure 1. Contrary to what we expected beforehand, there was hardly an effect of host species. Strains performed slightly better on conspecifics of the host species of origin, but the effect was only marginally significant (p<0.06). There was a much stronger effect of host sex. First, the frequency of *S. latifolia* strain copies was significantly (p<0.02) higher in male hosts than in female hosts. This difference was expressed in significantly (p<0.001) higher frequencies of heterokaryons in female hosts primarily at the expense of the homokaryotic $L_1L_2$ class rather than the homokaryotic $D_1D_2$ class. A G-test (Sokal and Rohlf 1995, p. 699) showed that in female hosts the ratio of heterokaryons to homokaryons carrying the 'pink' allele was significantly higher than expected on basis of random mating and equal competition success (2:1), at least in *S. latifolia* hosts (*S. latifolia* 76.3: 23.7% n=95 p<0.05; *S. dioica* 74.4: 25.6% n=108 p<0.10; both hosts 75.3: 24.7% n=203 p<0.02). There was no significant effect of the randomly composed inoculation mix at the 95% significance level (Table 1, p=0.08). Latency period of the $D_1D_2$ teliospores was significantly longer than that of the other two teliospores types except in *S. dioica* females (Figure 2).

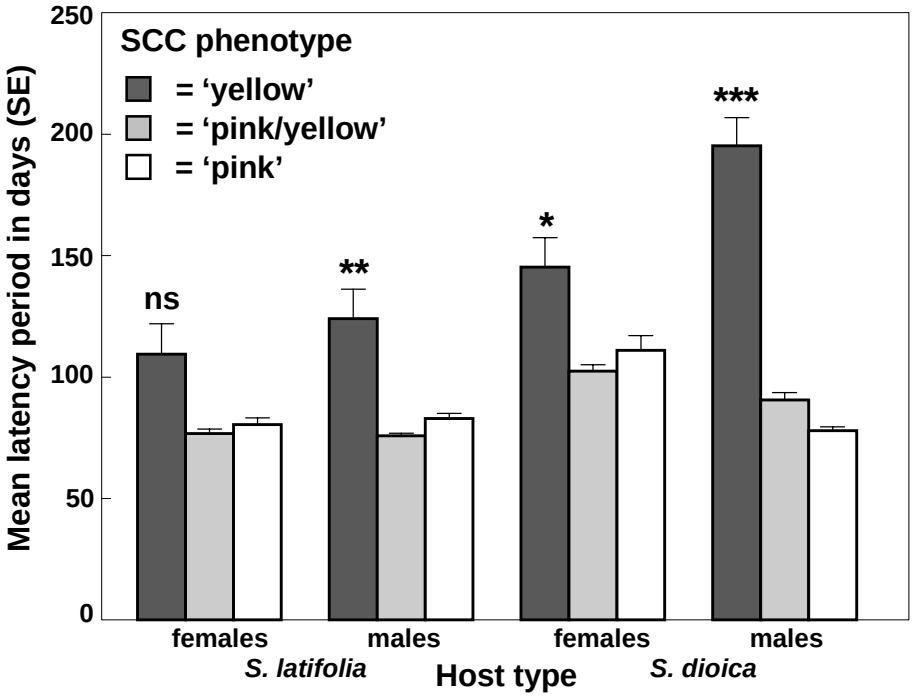


**Figure-2** Mean latency period (in days) of the three different teliospore SCC phenotypes in different host types. The significance of differences between the SCC phenotypes within a host class is shown (Kruskal-Wallis test). Levels of significance after a Bonferroni correction are designated;
ns = not significant;
* = $p<0.05$;
** = $p<0.01$;
*** = $p<0.001$.

**Table-2** Mixed model analyses of variance of angular transformed conjugation frequencies in the fungal pathogen *Microbotryum violaceum* after 24h as a function of host extract and strain origin (analysis 1), and as a function of host extract and cross type (analysis 2).

| Source of variation | df (n, d) | MS | F | P-value |
|---|---|---|---|---|
| ***Analysis 1*** | | | | |
| Block | 10, 758 | 6188.8 | 167.9 | <0.0001 |
| Origin of mt-$a_1$ [$O_1$] | 15, 43 | 4057.3 | 21.8 | <0.0001 |
| Origin of mt-$a_2$ [$O_2$] | 15, 37 | 871.4 | 3.0 | <0.004 |
| Extract [E] | 2, 48 | 21504.0 | 53.8 | <0.0001 |
| $O_1$ x $O_2$ | 225, 471 | 61.8 | 2.1 | <0.0001 |
| $O_1$ x E | 30, 450 | 159.8 | 5.4 | <0.0001 |
| $O_2$ x E | 30, 450 | 269.5 | 9.1 | <0.0001 |
| $O_1$ x $O_2$ x E | 450, 758 | 29.5 | 0.8 | n.s. |
| **Total error** | **758** | **36.9** | **-** | |
| ***Analysis 2*** | | | | |
| Block | 10, 758 | 6188.8 | 167.9 | <0.0001 |
| Extract [E] | 2, 504 | 21504.0 | 529.3 | <0.0001 |
| Cross type [C] | 3, 252 | 18321.0 | 139.3 | <0.0001 |
| Sporidial combination within Cross type [S(C)] | 252, 520 | 131.9 | 3.3 | <0.0001 |
| E x C | 6, 504 | 946.1 | 23.3 | <0.0001 |
| E x S(C) | 504, 758 | 40.6 | 1.1 | n.s. |
| **Total error** | **758** | **36.9** | **-** | |
| ***Contrasts between Cross types*** | | | | |
| *Between both homotypic cross types* | | | | |
| LxL vs DxD | 1, 252 | 39526.0 | 300.5 | <0.0001 |
| *Between both heterotypic cross types* | | | | |
| LxD vs DxL | 1, 252 | 13732.0 | 104.4 | <0.0001 |
| *Between homo- and heterotypic cross types* | | | | |
| LxL, DxD vs LxD, DxL | 1, 252 | 1621.6 | 12.3 | <0.001 |
| **Contrast error** | **252** | **131.5** | **-** | |

## The conjugation experiment

In the conjugation experiment, conjugation frequency after 24h was determined between strains from the two host species in different environments. Two separate analyses were performed, one in which strain origin was accounted for, and the other in which variation due to strain origin was repartitioned in cross type. In both the analyses, the effect of extract type was highly significant as is shown in table 2. Figure 3 shows the magnitude of this difference. Conjugation frequencies in *S. dioica* extracts after 24h was on average ten percent (absolute, or 30% relative) higher than conjugation frequency in *S. latifolia* extract, and 20 percent (absolute, or 79% relative) higher than in the water control. Effects of origins of the $a_1$ and the $a_2$ strains, and their interaction were also significant. Thus, the performance of individual strains did not only depend on their own origin, but also interacted with the origin of the strain of the opposite mating type. This effect was directly analyzed as cross type in the second analysis (Table 2). The selfing cross types ($L_1xL_2$ and $D_1xD_2$, crossing strains that originated from the same single flower) were not significantly different from the outcrossing $L_1xL_2$ and $D_1xD_2$ types, and were therefore pooled.

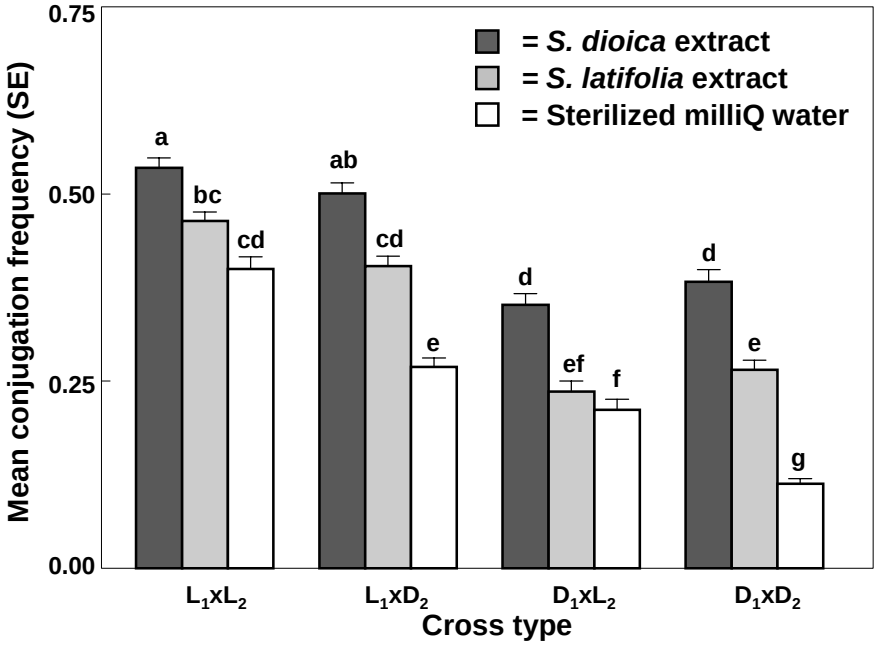


**Figure-3** Mean conjugation frequency of *Microbotryum violaceum* strains in *Silene dioica* leaf extract, *S. latifolia* leaf extract, and in sterilized milliQ water for the different cross-types in the conjugation experiment. Columns with different letters are significantly different from each other with $p<0.05$ (Tukey HSD test).

Figure 3 shows the mean conjugation frequencies of each cross type for each extract. In general, the $L_1xL_2$ cross type had significantly higher conjugation frequencies than other cross types after 24h, immediately followed by the $L_1xD_2$ cross type. The $D_1xL_2$ and $D_1xD_2$ cross types had significantly lower conjugation frequencies than the first two types in all extract types, but were only significantly different from each other in

the water control (Figure 3). Analysis per strain (analysis 1 in Table 2) showed that significantly ($p<0.0001$) more cells from *S. latifolia* had conjugated after 24h than cells from *S. dioica*. This was true for cells of both mating types (overall means ± SE); for mating type $a_1$: *S. latifolia* 42.9 ± 0.6%; *S. dioica* 26.0 ± 0.6 and for mating type $a_2$: *S. latifolia* 36.6 ± 0.7%; *S. dioica* 32.3 ± 0.7%).

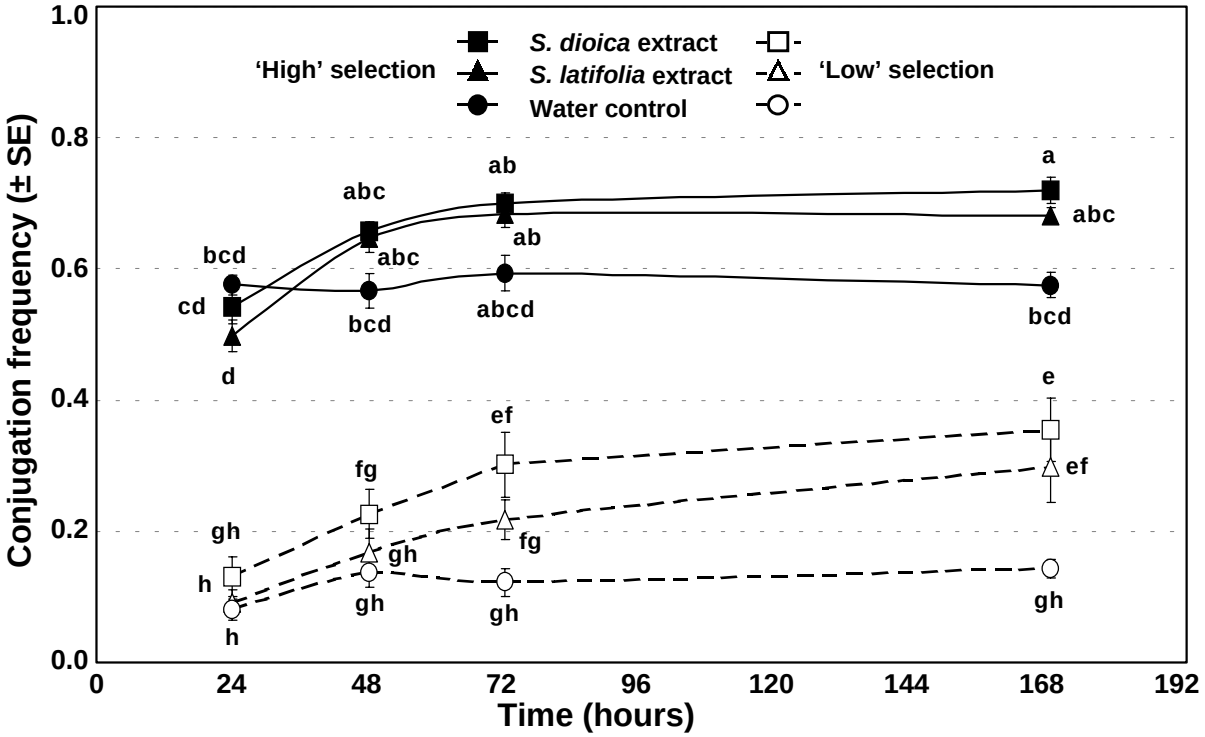


**Figure-4** Time course of mean conjugation frequencies of *Microbotryum violaceum* strains for six selected 'high' (filled symbols), and six selected 'low' (open symbols) combinations out of the 256 combinations in the conjugation experiment. Squares represent samples with *Silene dioica* host extract, triangles represent samples with *S. latifolia* host extract, and circles represent the control samples with sterile milliQ water. Points with different letters are significantly different with $p<0.05$ (Tukey HSD test).

## Time series

Figure 4 shows the results of the time series experiment in which conjugation frequencies were examined after 24, 48, 72 and 168h, of six 'high' and six 'low' combinations that were selected from the previous experiment. Conjugation frequencies reached their maximum after approximately 72h. However, differences between 'high' and 'low' cross combinations were already present after 24h. Significant differences between extracts in this subset of 12 crosses were only found after 72h between the water control and the host extract from *S. dioica* in the 'low' selection ($p<0.0001$), and after 168h between the water control and both host extracts

in the 'low' and the 'high' selection ($p<0.001$), but at no point in time between the extract of *S. latifolia* and the extract of *S. dioica* ($p>0.48$).

**Table-3** Strain origins and relative success rate of strains of the fungal pathogen *Microbotryum violaceum* in the competition experiment (I), and in the conjugation experiment (II). See text for the definitions of relative success rates. Populations are located in the Netherlands, unless stated in bold face. † = Strain was omitted from the experiment due to contamination. # = Reference strain.

| Strain | Origin of host | Host species | Relative success rate per exp. and mating type | | | |
|---|---|---|---|---|---|---|
| | | | Exp. I | | Exp. II | |
| | | | $a_1$ | $a_2$ | $a_1$ | $a_2$ |
| Mi24 | Millingen | *S. latifolia* | 1.26 | 1.19 | - | - |
| Mi30-1 | Millingen | *S. latifolia* | 1.20 | 1.31 | 1.26 | 1.19 |
| Mi33-1 | Millingen | *S. latifolia* | 1.30 | 1.15 | - | - |
| Mi44-1 | Millingen | *S. latifolia* | 1.30 | 1.17 | - | - |
| Mi28 | Millingen | *S. latifolia* | 1.32 | 1.26 | 1.24 | 0.99 |
| Bij1 | Bijland | *S. latifolia* | 1.34 | 1.11 | 1.25 | 1.15 |
| Bij2 | Bijland | *S. latifolia* | 1.09 | 1.21 | 1.37 | 1.31 |
| 023-a | Wolfheze | *S. latifolia* | 0.96 | 0.82 | - | - |
| 023-d | Wolfheze | *S. latifolia* | - | - | 1.37 | 0.82 |
| 016-4 | Wolfheze | *S. latifolia* | - | - | 1.39 | 1.17 |
| 097-a | Goedereede | *S. latifolia* | 1.30 | 1.13 | - | - |
| 097-b | Goedereede | *S. latifolia* | 1.29 | 1.34 | - | - |
| 104-a | Sonniuswijk | *S. latifolia* | - | - | 1.21 | 1.03 |
| 112-a | Eefde | *S. latifolia* | 1.38 | 1.27 | - | - |
| 125-a | Varssel | *S. latifolia* | - | - | 1.13 | 0.90 |
| Sp2-1 | **Spain** | *S. latifolia* | 1.00 | 1.20 | - | - |
| | **Means ± SE** | ***S. latifolia*** | **1.23 ± 0.04** | **1.18 ± 0.04** | **1.28 ± 0.03** | **1.07 ± 0.06** |
| Ca3-1 | Castricum | *S. dioica* | - | - | 0.58 | 0.92 |
| Ca5-1 | Castricum | *S. dioica* | 0.76 | 0.92 | 0.53 | 0.95 |
| Ca7-1 | Castricum | *S. dioica* | 0.70 | 0.76 | - | - |
| Om10-1 | Oude Molen | *S. dioica* | 0.53 | 0.90 | 0.68 | 0.91 |
| Om4-1 | Oude Molen | *S. dioica* | - | - | 0.68 | 0.96 |
| Om7-1 | Oude Molen | *S. dioica* | 0.91 | 0.96 | - | - |
| Pl1-3 | Populierenlaan | *S. dioica* | - | - | 0.77 | 1.10 |
| Pl2-2 | Populierenlaan | *S. dioica* | 0.86 | 0.76 | 0.71 | 0.87 |
| Pl5-3 | Populierenlaan | *S. dioica* | 0.69 | 1.02 | - | - |
| Bb16-1 | Burgvallen | *S. dioica* | 0.76 | 0.85 | - | - |
| Bb3-1 | Burgvallen | *S. dioica* | 0.63 | 0.78 | - | - |
| Bb8-1 | Burgvallen | *S. dioica* | - | - | 1.08 | 0.95 |
| Bb9-1 | Burgvallen | *S. dioica* | 0.80 | 0.65 | 0.77 | 0.78 |
| Vij1-3 | Vijlen | *S. dioica* | - † | 0.66 | - | - |
| Um23 | **Umeå, Sweden** | *S. dioica* | 0.87 | 0.78 | - | - |
| Vs2a-1 | **USA #** | *S. dioica* | 0.76 | 0.82 | - | - |
| | **Means ± SE** | ***S. dioica*** | **0.75 ± 0.03** | **0.82 ± 0.03** | **0.73 ± 0.06** | **0.93 ± 0.03** |

## Conjugation and competition

Table 3 shows the origins of all fungal strains used in the experiments, and the calculated relative success rate of each strain for both mating types. Since four of the strains from *S. latifolia* and four of the strains from *S. dioica* were used in both experiments, relative conjugation success after 24h and relative success in competition

could be compared directly, as is presented in figure 5. There was a significantly positive correlation between the relative success of strains in the conjugation experiment, and the corresponding strains in the competition experiment.

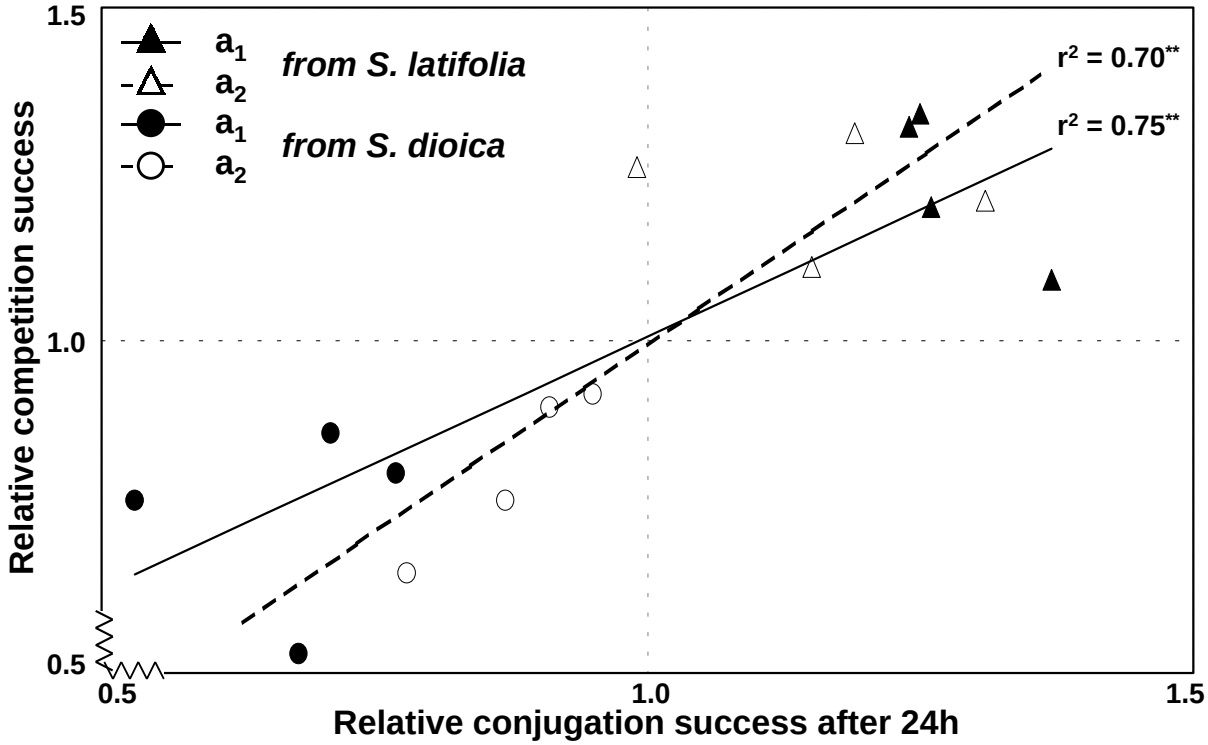


**Figure-5** Relative success rate of strains of the fungal pathogen *Microbotryum violaceum* of mating types $a_1$ (filled symbols) and $a_2$ (open symbols) that were used in both the conjugation experiment and the competition experiment. Strains in the upper right corner of the graph were sampled from *Silene latifolia* (triangles), strains in the lower left corner were sampled from *S. dioica* (circles). De slopes of the $a_1$ and $a_2$ regression lines do not significantly differ from each other (ANCOVA, $p>0.33$; Sokal and Rohlf 1995, p. 499). The combined linear regression function of both mating types together is: $y=0.84x+0.16$ ($r^2 = 0.71$; $p<0.001$; not plotted in the figure).

Strains in the upper right corner of the graph perform better than average in both experiments. Likewise, strains in the lower left corner perform worse than average. All eight strains from *S. latifolia* appear in the upper right group, while all eight strains from *S. dioica* are in the lower left group. The slopes between the regression lines for mating type $a_1$ and mating type $a_2$ were not significantly different (ANCOVA $p>0.33$; Sokal and Rohlf 1995 p. 499). The regression line for the combined strains is $y=0.84x+0.16$ ($r^2=0.71$; $p<0.001$). This line is not significantly different from the identity line ($y=x$), which can be plotted within the 95% confidence interval of the regression line (data not shown).

## DISCUSSION

### Multiple infections of a single host

Day (1980) was among the first authors to mention multiple infections of *M. violaceum* in *S. latifolia* (before the taxonomical debates in the 1990s known as *Ustilago violacea* and *S. alba*). In the competition experiment, examining at maximum five flowers per infected host plant, we could detect that overall 20% of the plants were infected with more than one type of dikaryon, and 1% was infected with all three types. Since we could not distinguish between both heterokaryon types the overall value is most likely underestimated with circa 4% (by simple extrapolation, this is the average of the classes that could be detected). With the two-allele variation at one of the SCC loci in our strains, we could also not detect multiple infections of single flowers like Day occasionally observed in his classic study. The extent of multiple infections in natural populations is poorly studied. If they would occur in natural host populations as well, this would increase the opportunities for outcrossing, because it enhances the chance that non-related teliospores are picked up by a vector on the same host plant, and are transmitted simultaneously to a healthy susceptible host.

In contrast to studies of multiple infections of animals, mainly found in medical literature, ecological studies on multiple infections of single hosts by parasites are scarce, let alone examples involving plants and phytopathogenic fungi. Given the importance of the presence or absence of multiple infections in models of the evolution of virulence (e.g. Van Balen and Sabelis 1995), this is very unfortunate. The few empirical studies that do exist, have investigated mostly viral and bacterial pathogens, e.g. *Wolbachia* in European raspberry beetles (Malloch *et al.* 2000), or pathogenic nematodes in fig wasps (Herre 1993).

### Conjugation, host extracts and mating type

In the conjugation experiment, the highest proportion of conjugates after 24h was observed in crosses between strains that both originated from *S. latifolia* in each of the host extracts. Significantly lower proportions were observed in the interracial crosses, and in crosses between strains from *S. dioica*, respectively. We conclude that strains from *S. latifolia* conjugate faster than strains from *S. dioica*. Furthermore, there was a significant difference between the reciprocal interspecific crosses, with the $L_1xD_2$ crosses showing higher conjugation frequencies than $D_1xL_2$ crosses. In host extract, $L_1xD_2$ resembles $L_1xL_2$, whereas $D_1xL_2$ resembles $D_1xD_2$, suggesting a

predominant role of the $a_1$ mating type in conjugation success. Conjugation tubes grow in a directed manner from cells with mating type $a_2$ towards cells with mating type $a_1$ (Day 1976), as a reaction to the more active signaling of mating type $a_1$ (Day *et al.* 1981). Presumably, cells with mating type $a_1$ are more active in signaling because they have a slower rate of degradation of pheromones that are produced by both mating types, at least in *Ustilago maydis*, a related smut from Maize (Snetselaar *et al.* 1996). Indeed, if mating type $a_1$ is more active in signaling, this may cause reciprocal differences in crosses between strains, i.e. mating type $a_1$ more strongly determining conjugation rate than mating type $a_2$.

Generally, conjugation frequencies in the presence of host extracts were significantly higher than in water, but differences between cross types were almost independent of the type of medium used (Figure 3). Contrary to earlier studies (Ruddat and Kokontis 1988 and references therein), we did not observe any hyphal growth induced by the presence of host extract, not in the large 24h experiment, nor in the 168h time series using pre-selected crosses. Presumably our extracts were either more diluted, or other environmental conditions in our assay were simply unsuitable for hyphal growth. The extract from *S. dioica* leaves induced significantly more conjugated cells than the extract from *S. latifolia* leaves. Assuming, like other studies (Kaltz and Shykoff 1999), that host extracts indeed sufficiently mimic the relevant cues experienced during conjugation *in vivo*, this suggests that mating of these strains may be more stimulated by *S. dioica* than by *S. latifolia* (Figure 3).

The time series showed that conjugation of strains was consistent; differences in conjugation frequency between the selected ‘high’ and ‘low’ combinations could be repeated. Moreover, the results showed that the conjugation process still progressed after 24h, until about 72h. At, and after this point in time, differences between crosses were even more pronounced. This is in contrast to a study by Poon and colleagues (Poon *et al.* 1974) on the kinetics of mating in this fungus, who observed that 70% of the cells had mated after 20h. In our study, this point is reached only in the selected ‘high’ crosses, and not before 72h. This indicates that the conditions for conjugation in our assay were sub optimal. Being one of the most critical factors in mating (Cummins and Day 1977), supply of oxygen was probably sub optimal in the 8µl volumes at the bottom of the eppendorf cups.

## Strains from *S. latifolia* conjugate faster, and outcompete strains from *S. dioica*

In general, strains from *S. latifolia* were more successful in producing infections than their *S. dioica* counterparts in the competition experiment *in vivo*. The conjugation experiment actually represents in detail the initial stages of the competition experiment, in which the infectious dikaryons were produced. From the combined success rates displayed in figure 5, it can be seen that a large part of the infection success can be explained by the swiftness of mating. Thus, although Day (1980) rightly suggests; "*competition may be expressed at the level of growth rate and the ability to keep up with the meristem and/or in the ability to compete for the available space and nutrients in the meristem*", the outcome of competition is initially highly dependent on the time it takes to produce an infectious dikaryon, giving some combinations a decisive head start over other combinations simply by conjugating faster. Interestingly, latency period of the infections involving *S. latifolia* strains was much shorter than infections with only *S. dioica* strains in the competition experiment (Figure 2), which is consistent with this conclusion, and was found in a cross inoculation study by Biere and Honders (1996a) as well. Contrary to our results however, heterotypic combinations in their study showed latency periods that were more comparable to the homotypic *S. dioica* isolates. With a flower-longevity of a few days at most, time for mating, conjugation, producing an infectious dikaryon and entering host tissue is short, especially in male hosts, and conjugation rate is probably an important aspect of infection success. Male plants of *S. latifolia* have been reported to actively drop flowers that contained spores, presumably to avoid becoming infected, shortening the longevity of these flowers with on average 10h (Kaltz and Shykoff 2001). In contrast, female flowers remain much longer on the plant when they are pollinated, in order to produce seed capsules and seeds.

## Implications for host races in sympatric populations of hosts

We found no (positive) assortative mating between the host races. On the contrary, if the supposed gene flow would be large enough, based on its low competitive ability, we would expect the host race of *S. dioica* to decline or go extinct in sympatric populations of these host species. Since both host races do occur in natural sympatric populations of hosts (Biere and Honders 1996b; Van Putten *et al.* chapter 2; chapter 3), the existence of large differences in conjugation rate, infection success and latency period between both host races, presumably representing large fitness differences, are even more puzzling.

Two factors could contribute to the maintenance of host race differentiation in sympatric host populations. First, we observed a highly significant effect of host sex on dikaryon type. Heterotypic dikaryons were found to be the better competitors in female host plants, at least in *S. latifolia*. The fact that this apparent overdominance at one of the SCC loci is primarily found in female hosts might be explained by the more complex requirements for successful infection of female hosts as compared to male hosts. In dioecious *Silene* sex is genetically determined (Ono 1939; Westergaard 1940). The infection of a female host requires a morphological sex change by fungal induction of male specific gene expression in the developing flower (Scutt *et al.* 1997). Subsequently, the development of pollen grains has to be prevented by blocking the formation of microspores (Audran and Batcho 1981). Being more complex and presumably more costly to the fungus, complementation at heterozygote loci may be more important in the infection of female hosts. If this is the case, female hosts could provide a possible 'safe haven' for strains from *S. dioica* in sympatric populations of hosts that might otherwise go extinct. However, the predominantly selfing nature of this fungus (Baird and Garber 1979), resulting in strong observed homozygosity (Bucheli and Shykoff 2001; Van Putten *et al.* chapter 2) opposes this thought. Interestingly in this respect, the production of teliospores per plant in male hosts was found to be significantly higher in conspecifics than in heterospecifics of the host of origin in a cross inoculation experiment, but not in female hosts (Biere and Honders 1996a), which held true both in *S. dioica* and *S. latifolia*.

Second, the natural pollinators of the host plants transmit the fungal spores, and the existence of different pollinator guilds for these two host species (e.g. Jürgens *et al.* 1996; Goulson and Jerrim 1997) could thus reduce fungal gene flow. If pollinators that serve as vectors of smut spores, can prevent, or strongly limit the possibilities for host competition and outcrossing simply by being choosy in host sympatry, both host races might coexist. We have studied the behavior of vectors in artificial, completely mixed plots of *S. latifolia* and *S. dioica* in detail using fluorescent dyes as spore-mimic (Van Putten *et al.* chapter 5). Indeed, results showed that the different pollinator guilds are choosy in their visitation patterns with respect to host species. However, in these experiments there was still a considerable amount of interspecific visitation. Nevertheless, since natural sympatric populations of hosts are spatially (Goulson and Jerrim 1997; Van Putten *et al.* chapter 3) and temporally (Biere and Honders 1996b) much more heterogeneous, the exchange of fluorescent dye between host species that we observed (approximately 30% of total visits), is likely to be an estimate of the absolute maximum exchange possible. Fungal gene flow in natural

sympatric populations of both host species is expected to be considerably lower. Therefore, we expect that host fidelity of vectors will strongly contribute to the reproductive isolation needed to explain the observed host-related differentiation between fungal isolates from different host species.

## Acknowledgements

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# CHAPTER 5

## Host fidelity of the pollinator guilds of S*ilene dioica* and *S. latifolia*; possible consequences for host race differentiation of a venereal disease in sympatry

with Arjen Biere, Jelmer Elzinga and Jos van Damme

*submitted to Oecologia*

Abstract

**We have studied conspecific and heterospecific visitation patterns of the pollinators of *Silene dioica* and *S. latifolia* in experimental, fully mixed plots of these plant species, using fluorescent dyes. Dye particles were used as mimics of teliospores of the pollinator-transmitted fungal pathogen *Microbotryum violaceum*, to estimate the amount of gene flow between the different host races of this fungus from these host species in complete host sympatry.**

**The two host species were visited by different pollinator guilds, with bumblebees preferentially visiting *S. dioica* diurnally and noctuid moths preferentially visiting *S. latifolia* nocturnally. After 24h, we observed a mean rate of interspecific transfer of 26% from *S. latifolia* to *S. dioica*. From *S. dioica* to *S. dioica* interspecific transfer was 34%. These estimates probably represent the absolute maximum of interspecific visitation between these host species, since natural sympatric populations of these host species have found to be spatially and temporally more heterogeneous. Therefore, the observed visitation pattern of pollinators/vectors, in combination with spatial and temporal separation of the host species, might contribute to the maintenance of genetically differentiated host races of the anther smut *M. violaceum* as observed in sympatric and parapatric populations of these host species. Male hosts were found to be preferentially visited over female hosts.**

**In addition, non-linear regression analysis suggested that the range in which the teliospores can be transmitted is probably much larger (20-50+m) than the actual infection range (not much larger than 12-13m) of this venereal disease within a single flowering season.**

## INTRODUCTION

Host fidelity can play an important role in sympatric host race formation by providing a mechanism for prezygotic reproductive isolation, for instance in phytophagous insects (*cf.* Berlocher 1998a). In addition to this, to achieve or maintain host races, it has been argued that host-related fitness trade-offs, i.e. by antagonistic pleiotropy of genes involved in pathogen performance, are needed to overcome any 'leakiness' in host fidelity (Feder 1998). However, empirical evidence for such genetic correlations across different host species is often found to be ambiguous, or non-negative in studies of phytophagous insects (*cf.* Fry 1996; but see Via *et al.* 2000), and is scant for other organisms, including the group of phytopathogenic fungi. Although fungi are usually dispersed by wind, many phytopathogenic fungi are vectored by insects (e.g. by bark beetles in the Dutch elm disease: Ingold 1971; by shining flower beetles in the floral smut *Anthracoidea*: Ingvarsson and Ericson 1998), and some of them can even manipulate their host plants to attract insect vectors that promote their own dispersal (Roy 1994; Pfunder and Roy 2000). In these cases, host fidelity is regulated by the vectors rather than by the pathogen itself, which obviously decouples possible direct linkages between genetic trade-offs of pathogen performance on different host species and host fidelity.

A well-studied example of a pollinator-transmitted disease is the anther smut *Microbotryum violaceum*, which has a wide host range within the Caryophyllaceae (Thrall *et al.* 1993). Teliospores of anther smuts, the stage in which this fungus is dispersed (see Piepenbring *et al.* 1998 for a review) are produced in the anthers of host flowers and are transmitted by the insect visitors of their host plants that serve the dual role as pollinators and vectors of this disease (Baker 1947; Hassan and MacDonald 1971; Jennersten 1983; Alexander and Antonovics 1995). Two of its host species, *Silene dioica* and *S. latifolia*, frequently meet in sympatry (Goulson and Jerrim 1997). *S. latifolia*, the white campion, is a dioecious, short-lived perennial weed from open, disturbed habitats such as roadsides and arable land with a typical moth-pollination syndrome (Baker 1961; Baker and Hurd 1968), including heavily scented white flowers that open at dusk (Jürgens *et al.* 2001). *S. dioica*, the red campion, is a closely related dioecious perennial that mainly occurs in open woodland, and that is primarily pollinated by bumblebees (Kay *et al.* 1984). Flowers open at dawn and remain open during the day (personal observation). Also, flowers of diseased plants of *S. latifolia* emit scent in the evening, and they are still visited by moths (Baker 1947), although

they may be less attractive (Shykoff and Bucheli 1995; Altizer *et al.* 1998). Visitors of *S. dioica* also visit infected flowers, and have found to be carrying teliospores (Hassan and MacDonald 1971).

The presence of interspecific hybrids in areas where their preferred habitats are adjacent or intermixed indicates that there is gene flow between these plant host species, although the extent of this has never been studied in much detail (but see Goulson and Jerrim 1997). Likewise, the amount of fungal gene flow between anther smuts from different host species in sympatric populations of hosts is yet unknown. Early cross-inoculation studies of this fungus have shown that anther smuts from different host species can be grouped into distinct host races (Zillig 1921). Recent studies have demonstrated that smut isolates from allopatric populations of *S. dioica* and *S. latifolia* were differentiated for a number of genetic markers; karyotypes (Perlin 1996; Perlin *et al.* 1997); a sporidial colony color marker (SCC (*cf.* Garber *et al.* 1975) Biere and Honders 1996a; Van Putten *et al.* chapter 3); random amplified polymorphic DNA (RAPDs; Biere and Honders, unpublished results); and microsatellite loci (Bucheli *et al.* 2001; Van Putten *et al.* chapter 2). Microsatellite analysis of smut isolates from sympatric and parapatric populations of *S. latifolia* and *S. dioica* showed that fungal isolates were genetically differentiated in a host-specific manner in parapatric and parapatric/sympatric populations, but not in one true sympatric population where both host species grew truly intermingled (Van Putten *et al.* chapter 2). This suggested that the degree of sympatry is important in maintaining the genetic differentiation. However, even in this true sympatric population, the alleles from two of the four loci were not distributed homogeneously over the population (Van Putten *et al.* chapter 3). At the same time, there was significant host-related differentiation in a sporidial colony color marker, suggesting that there could be strong selection on this locus, and/or that the patchy local structure of hosts in this population could severely limit the amount of gene flow between the host races. A question that arises is how such host-related genetic variation could be maintained in sympatry. One option is that there are trade-offs in performance between different fungal isolates on the different host species that contribute to the maintenance of host-related genetic variation. In a cross inoculation experiment by Biere and Honders (1996a), they showed that the production of teliospores per plant was significantly higher in conspecifics than in heterospecifics of the host of origin, but only in male hosts. This trade-off in performance in male hosts will indeed contribute to some extent to the maintenance of host-specific variation. However, since they found no host-related differences in virulence (*sensu* Jarosz and Davelos 1995) between fungal

isolates from the two host species, this trade-off is presumably not strong enough by itself to entirely explain the observed host-specific genetic variation in sympatry. Also, since a competition experiment has shown that the host race of *S. latifolia* outcompetes the host race from *S. dioica*, presumably due to its higher conjugation rates (Van Putten *et al.* chapter 4), we need to look for other explanatory mechanisms. As this fungus is vectored by pollinators of their host, gene flow between the two host races could highly depend on the visitation behavior of pollinators that visit both host species in order to accomplish substantial interspecific transfer of smut spores. This raises the question, whether host fidelity of the pollinator guilds of *S. dioica* and *S. latifolia* can be an additional factor contributing to the maintenance of these host races in sympatry.

In this chapter we study patterns of spore transfer between and within the host species *S. latifolia* and *S. dioica*. For this purpose, we use fluorescent dyes as traceable teliospore surrogates to explore the visitation patterns of the pollinator community in an artificial sympatric setup of *S. latifolia* and *S. dioica* host plants. Since we expected that different pollinator guilds would be active during day- and nighttime (e.g. Groman and Pellmyr 1999), different experiments were carried out, covering either 24h visitation or daytime only. Furthermore, since patch size may also be an important factor (e.g. Sowig 1989), experiments with different patch sizes were carried out as well. Additionally, to connect the dispersal of fluorescent dye directly to the dispersal of the anther smut disease, the infection rate in relation to the linear distance to a true teliospore source was examined for one of the host species, *S. latifolia*. Specific questions that will be addressed here are: (1) Do different guilds of pollinators discriminate between *S. dioica* and *S. latifolia* in an artificial, completely mixed setup, and show host fidelity? (2) What is the frequency of interspecific visitation between both host species, i.e. to what extent would an assortative visitation pattern with respect to host species provide a basis for the maintenance of host races of *M. violaceum* in sympatric populations of hosts? (3) What is the range of teliospore (fluorescent dye) dispersal after 24h, and of realized infections of *M. violaceum* from a true smut source within a single flowering season?

## MATERIALS AND METHODS

### Study species

*Silene latifolia* Poiret (= *S. alba* (Miller) Krause (Caryophyllaceae), the white campion, is a dioecious short-lived perennial from open, disturbed habitats and borders of arable land. *Silene dioica* (L.) Clairv. (Caryophyllaceae), the red campion, is a closely related dioecious perennial that mainly occurs in more shady humid habitats as woodland borders. In areas where habitats overlap, both species frequently occur in sympatry and hybridization is a common phenomenon (Baker 1947; Goulson and Jerrim 1997).

The anther smut fungus *M. violaceum* (Pers.: Pers) Deml & Oberw. (=*Ustilago violacea* (Pers.) Fuckel) (Ustilaginaceae) (Vánky 1994) is a vector-borne venereal disease that sterilizes its Caryophyllaceous hosts (Thrall *et al.* 1993). Smut spores are produced in the anthers of the host and are transmitted by the natural pollinators of their hosts (Jennersten 1983). In dioecious host species, the ovaries of female plants are reduced and staminal rudiments develop into stamens and anthers that also contain smut spores instead of pollen.

Seeds of *S. latifolia* and *S. dioica* were collected from several geographically spread natural allopatric populations in the Netherlands in 1997 and 1998. Seeds were germinated in petridishes on demi-water moistened filter paper at a density of approximately 25 seeds per petridish in a growth cabinet (16/8h light/dark, 21/15°C day/night temperature) after a vernalization period of three days at 4°C that has proven to be sufficient to increase the proportion of flowering plants in previous experiments. Nearly all seed had germinated after a week. For experiments 1-4, in total 750 *S. dioica* and 500 *S. latifolia* were potted after two weeks from vernalization in round containers (∅12cm), and grown in a acclimatized greenhouse at 21°C and with 16/8h light/dark until flowering. For experiment 5, an additional 1550 *S. latifolia* were potted in 18 cm square containers. From these plants, 200 were inoculated with a suspension of *M. violaceum* conjugates in the seedling stage prior to potting. These conjugates were derived by mixing haploid sporidia of both mating types in an aqueous suspension at 14°C for several hours (Cummins and Day 1977).

### The use of fluorescent dyes

In this study we assess the assortative visitation patterns of pollinator guilds visiting our sympatric plot of *S. latifolia* and *S. dioica* using fluorescent dyes. The

occurrence of dye on a flower is a qualitative measurement of the footsteps of both pollinators and vectors, that has successfully been used to trace pollen movements across plants (e.g. Thomson 1981; Waser and Price 1982; Fenster *et al.* 1996; Goulson and Jerrim, 1997), and has proven to be a good predictor of the dispersal of fungal spores as well (Shykoff and Bucheli 1995). However, as Thomson *et al.* (1986) rightly points out, using fluorescent dye as pollen analogue and simply scoring the presence/absence of dye on a flower surely would overestimate the extent of dispersal because stigmas are often more receptive to the much smaller dye particles than they are to pollen grains. Fortunately, whereas pollen of *S. latifolia* is size-ranged 35-60 µm (Prentice *et al.* 1984), teliospores are much smaller as well, and range from 6-9µm (Zogg 1985). Therefore, spores may remain longer on a pollinator than do pollen, as was found in the comparative study to the transfer of pollen and fluorescent dye (Thomson *et al.* 1986). Fluorescent dye particles are equal in size compared to *M. violaceum* teliospores, and may therefore be better spore-analogues than they are pollen-analogues. However, dye particles are more irregular in shape than teliospores and may be more sticky (personal observation).

## EXPERIMENTAL DESIGN

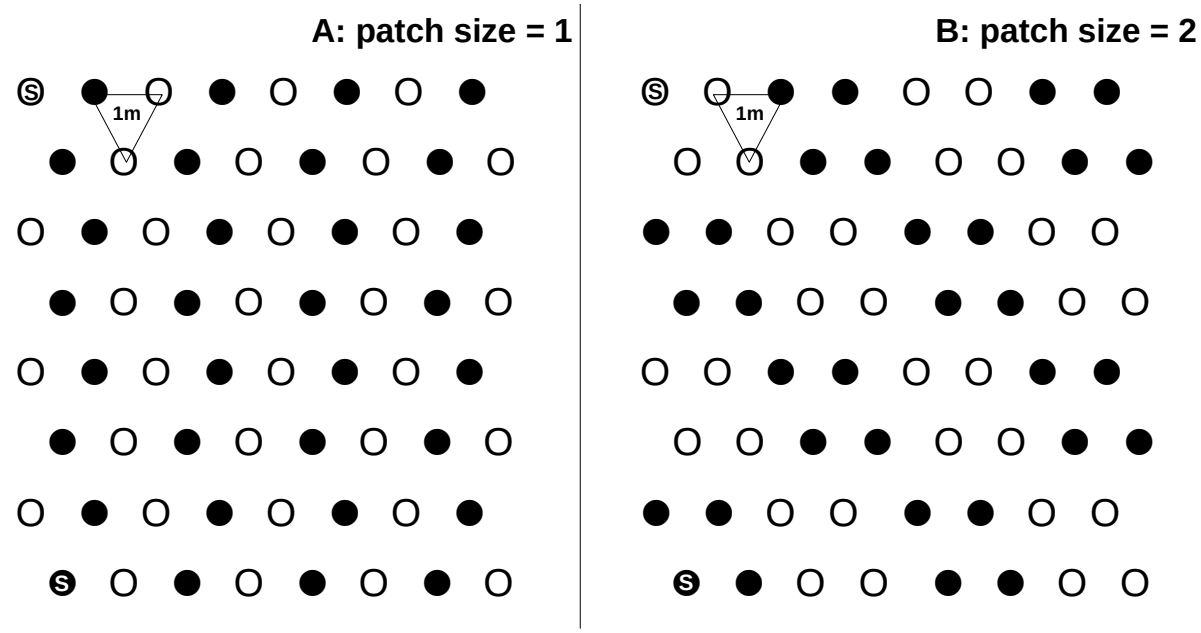


**Figure-1** Schematic set-up of experiments 1-4, which represents plots of 8x8 large containers that each contains one female and one male plant of *Silene latifolia* (open circles), or *S. dioica* (filled circles). Left panel (A) shows the situation when patch size = 1 (DN-P1 and D-P1), right panel (B) represents the situation when patch size = 2 (DN-P2 and D-P2). S = Source of fluorescent dye. The triangles represent the distance between the center points of two adjacent containers, which is 1m. The North-South direction is top-to-bottom in the figure.

## Experiments 1-4

64 containers, each holding one male and one female flowering plant of the same species were placed in a grid of 8 by 8 (Figure 1), so that all neighboring containers were at a distance of 1m. The plants at both corners of one side of the plot served as source plants. A few milligrams of fluorescent dye (Radiant technologies Inc., Richmond CA, USA) was applied with a toothpick to ten open male flowers on the anthers and filaments, and to eight open female flowers on the pistils (because most female flowers bore less than ten flowers). Red fluorescent dye was applied to *S. dioica* and used as *M. violaceum* teliospore mimic of *S. dioica* origin in one corner of the grid, while yellow fluorescent dye was applied to *S. latifolia* and used as *M. violaceum* teliospore mimic of *S. latifolia* origin in the other corner. Both fluorescent dye colors were simultaneously present within a single replicate. Experiments 1-4 were carried out sequentially, separated in time throughout the natural flowering season of the plants. Each experiment was replicated four times (two true replicates, and two replicates with both sources mirrored in the north-south axis to correct for potential dominant wind influences). The experimental setup of experiments 1-2 is schematically represented in panel A of figure 1, and the experimental setup of experiments 3-4 in panel B of figure 1. The four experiments differed in their duration (diurnal versus diurnal plus nocturnal visitation) and in the patch size of host plants of the same species, and were set up sequentially during the season (see below).

### Experiment 1 [DN-P1: Day + Night, Patch size = 1]

20 June-29 June 2000: Containers with *S. latifolia* and *S. dioica* plants were alternated in a checkerboard pattern (patch size = 1x1). Fluorescent dye was applied just before sunset. Dye transmissions were determined using a portable UV-light (UVP Inc., San Gabriel CA, USA) after approximately 24h (after sunset) by counting per plant all clean flowers, all flowers with only red or yellow dye, and all flowers with both red and yellow dye.

### Experiment 2 [DN-P2: Day + Night, Patch size = 2]

18 July-31 July 2000: Containers with *S. latifolia* and *S. dioica* plants are placed in patches of 2x2 containers with the same plant species (alternating *S. latifolia* patches and *S. dioica* patches). Fluorescent dye was applied just before sunset. Dye transmissions were determined after approximately 24h (after sunset).

### Experiment 3 [D-P1: Daytime only, Patch size = 1]

31 August-13 September 2000: As experiment 1, but now with fluorescent dye applied just before sunrise. Source plants were removed at sunset of the same day. Dye transmissions were determined after sunset. For this setup there are only two (mirrored) replicates due to unfavorable weather conditions at the end of the season.

### Experiment 4 [D-P2: Daytime only, Patch size = 2]

9 August-24 August 2000: As experiment 2, but now with fluorescent dye applied just before sunrise. Source plants were removed at sunset of the same day. Dye transmissions were determined after sunset.

### Direct observations

During experiments 1 and 2, on ten separate occasions from about 23:00 till about 1:00, all nightly insect visitors to flowers in the plot were caught, identified and checked for the presence of fluorescent dye, and released again afterwards. During experiments 3 and 4, on six separate occasions in total 215 minutes daily visitors to flowers in the plot were observed and identified. A few of these visitors were also caught and checked for the presence of fluorescent dye. For each experimental day (and night), day length was determined and mean day and night values for temperature, wind, sun radiation and rain were recorded.

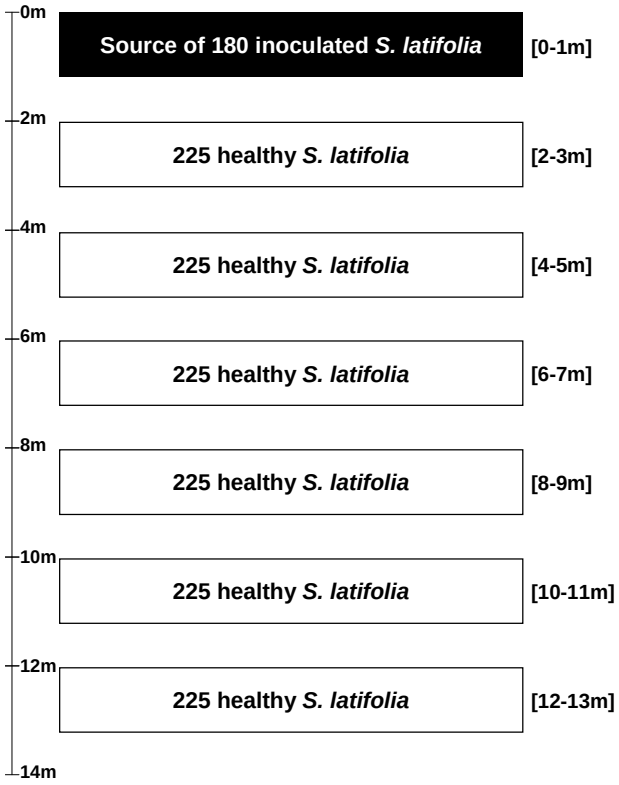


**Figure-2** Schematic set-up of experiment 5, representing seven blocks, of which six consisted of 225 healthy (not inoculated) *Silene latifolia* and one consisted of 180 *S. latifolia*, that were inoculated with conjugates of *Microbotryum violaceum* in the seedling stage prior to potting (of which more than 42% of the flowering plants became infected). The North-South direction is left-to-right in the figure.

## Experiment 5

In experiments 1-4, fluorescent dyes were used as mimic of teliospores. Since we were interested in the fungal disease rather than in fluorescent dyes, we wanted to link dispersal distance of dye in these experiments directly to the dispersal of a true infection with anther smut, using the pollinators guilds from the same area. Therefore, in addition to experiments 1-4, an experiment was carried out to investigate the infection rate as a function of distance to a true teliospore source, for one host species, *S. latifolia*. Containers were placed in seven rectangular blocks at regular distance intervals of 2m (figure 2). The first block contained 180 (placed in four rows of 45 plants) inoculated *S. latifolia* hosts, serving as a source of teliospores. The other six blocks contained 225 (placed in five rows of 45 plants) *S. latifolia* hosts that were not inoculated, serving as recipient host plants. The experiment was set up near the experimental plots of experiments 1-4 (approx. 60m), and started at the end of June 2000. The bulk of plants started flowering at the beginning of August 2000. At the end of October 2000, all flowering plants were checked for sex and disease status.

## Data analysis

Experiment 1-4. Each of the four experiments was analyzed separately. Distances from the source of the fluorescent dye were calculated from the center of the source container to the centers of the receptive containers, neglecting actual positions of plants and flowers inside the container. Analyzed were visitation frequencies (defined as the ratio of number of flowers with dye divided by total flowers per plant) in a logistic regression with backward elimination of the higher order interaction effects until the final model was reached (SAS v8 procedure LOGISTIC, The SAS Institute Inc. Cary, NC USA). Plant species (SP), plant sex (SX) and replicate (R) are categorical factors, distances to the red ($D_r$) and yellow ($D_y$) fluorescent dye sources were regarded as continuous covariates. The fitted models were corrected for overdispersion using the Williams' correction (Williams 1982). Overdispersion was tested for by dividing the Pearson $\chi^2$, and the deviance by their degrees of freedom, which both proved to be significantly larger than 1 in the unscaled model (*cf.* Stokes *et al.* 2000). The effects of host species and host sex on the interspecific visitation rate after 24h, which is defined as the proportion of interspecific visits divided by total number of visits, were tested in a generalized linear model (SAS v8 procedure GENMOD). In experiment 5, the effects of block, i.e. distance from smut source plants, and host sex on the frequency of infection were analyzed using a generalized linear model (SAS v8 procedure GENMOD). To gain

statistical power for a contrast analysis, the six 'recipient' blocks were merged into three new blocks: 2-5m, 6-9m and 10-13m. Contrasts were then made between each pair of adjacent blocks.

## RESULTS

### Experiments 1-4; overall effects in fluorescent dye transmission

Figure 3 shows the mean frequency of flowers with fluorescent dye per plant in relation to the distance to the source plants where the dye was applied. The statistical support for effects that are displayed in this figure comes from the results of the logistic regression analyses shown in table 1. The lines through the replicate means were fitted using an exponential law $y=ae^{-bx}$, which is a commonly used model to describe proportions $y$ that decrease with increasing distance $x$ (see Gregory 1968; Jeger 1990), and details of the fitted functions are shown in table 2. An overall effect in all of the experiments is that the frequencies of fluorescent dye, both red and yellow, significantly decrease with increasing distance from source plants ($p<0.0001$), and hence the frequency of plants with no dye significantly increases with distance ($p<0.0001$). More interesting is the strong host species effect in experiments DN-P1, DN-P2 and D-P2, i.e. the frequency of red fluorescent dye originating from *S. dioica* is significantly higher on *S. dioica* than on *S. latifolia* while the frequency of yellow fluorescent dye originating from *S. latifolia* is significantly higher on *S. latifolia* than on *S. dioica* ($p<0.0001$). This indicates that insect visitors coming from the *S. dioica* source plants tend to visit *S. dioica* more frequently than they visit *S. latifolia*, and vice versa. In other words, there is a significant host species-specific visitation pattern of pollinators/vectors in this artificially mixed sympatric plot. The effect of host species seems to hold true across the whole length of the plot, since the interactions between host species and distance to source were in most cases not significant. In experiment D-P1 however, the species effect is observed only for the yellow fluorescent dye ($p<0.05$), and since the interaction between host species and distance to source was highly significant in this case ($p<0.0001$), this also varied across the plot.

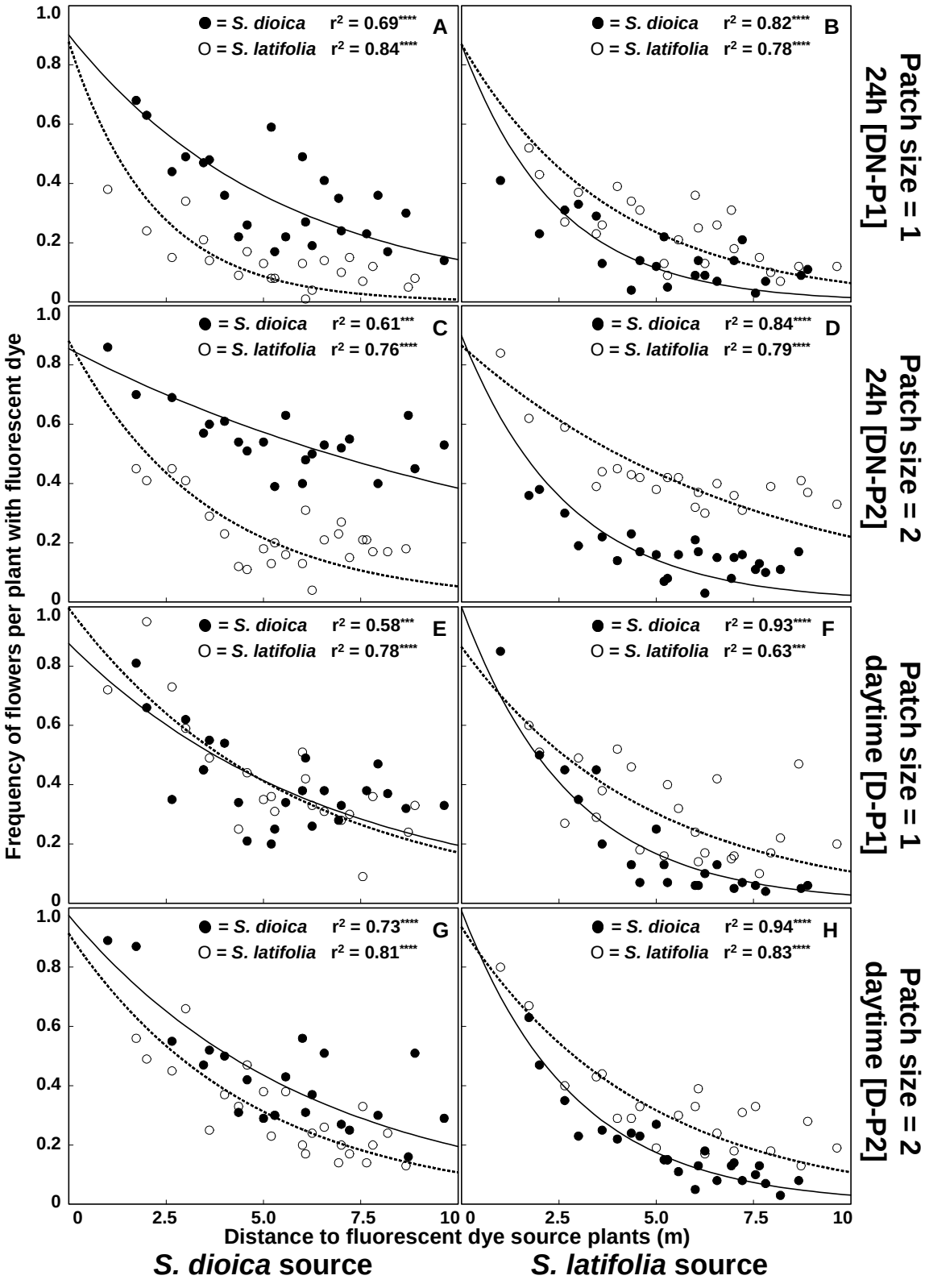


**Figure-3** Inter- and intraspecific movements of fluorescent dye in experiment 1-4. Plotted are the mean frequencies of flowers with fluorescent dye per plant in relation to the distance to the source plant. Open circles represent *Silene latifolia*; closed circles represent *S. dioica*. Left panels (A, C, E and G) represent red dye from a *S. dioica* source plant. Right panels (B, D, F and H) represent yellow dye from a *S. latifolia* source plant. Panels A-B, C-D, E-F and G-H represent experiments 1, 2, 3 and 4 respectively. Non-linear regression curves fit an exponential model of the form $y = ae^{-bx}$ (Gregory 1968; Jeger 1990) where y is the dye frequency, x the distance to the source plant, and a & b are constants with $a \leq 1$. All regression coefficients were highly significant with $p<0.0001$.

**Table-1** Logistic regression model of the frequencies of flowers per plant with either red, yellow, both, or no fluorescent dye in experiments 1-4, with backwards elimination of higher order interactions until the final model was reached. Host species (SP), host sex (SX) and replicate were categorical factors, distances to the red ($D_r$) and yellow ($D_y$) fluorescent dye sources were taken as continuous covariates. Rows with eliminated (E) and/or non-significant effects only, as well as all interactions with replicate were omitted from the table for reasons of clarity, but did appear in some of the models. A (-) indicates that these factors were not in the final model. The indicated levels of significance are: # = p<0.10; * = p<0.05; ** = p<0.005; *** = p<0.0005; **** = p<0.0001.

| Exp. | Source of variation | df | Type III Wald $\chi^2$ per analysis of dye frequency | | | |
|---|---|---|---|---|---|---|
| | | | Red | Yellow | Red and Yellow | No dye |
| 1 | Host species [SP] | 1 | 19.6 **** | 27.2 **** | 0.0 | 26.3 **** |
| Day- and | Host sex [SX] | 1 | 8.9 ** | 2.8 # | 5.4 * | 4.7 * |
| nighttime | Distance red source [$D_r$] | 1 | 57.7 **** | - | 0.0 | 21.2 **** |
| visitation | Dist. yellow source [$D_y$] | 1 | - | 69.5 **** | 11.0 ** | 21.3 **** |
| (24 h) | [SP]*[$D_r$] | 1 | 0.1 | - | 6.2 * | E |
| Patch size | [SP]*[$D_y$] | 1 | - | E | 4.1 * | E |
| = 1 | [SX]*[$D_r$] | 1 | 3.0 # | - | E | E |
| [DN-P1] | [SX]*[$D_y$] | 1 | - | 3.2 # | E | E |
| | Replicate | 3 | 12.0 ** | 8.6 * | 15.8 ** | 9.8 * |
| 2 | Host species [SP] | 1 | 22.2 **** | 23.0 **** | 1.8 | 0.2 |
| Day- and | Host sex [SX] | 1 | 0.1 | 66.0 **** | 39.9 **** | 109.3 **** |
| nighttime | Distance red source [$D_r$] | 1 | 88.7 **** | - | 0.0 | 48.9 **** |
| visitation | Dist. yellow source [$D_y$] | 1 | - | 95.3 **** | 8.9 ** | 14.7 *** |
| (24 h) | [SP]*[SX] | 1 | 1.1 | 3.2 # | E | 17.2 **** |
| Patch size | [SP]*[$D_r$] | 1 | 4.0 * | - | 14.6 *** | E |
| = 2 | [SP]*[$D_y$] | 1 | - | 0.8 | 31.7 **** | 3.9 * |
| [DN-P2] | [SX]*[$D_r$] | 1 | 5.2 * | - | E | E |
| | Replicate | 3 | 2.8 | 7.1 # | 69.9 **** | 3.6 |
| 3 | Host species [SP] | 1 | 0.0 | 5.4 * | 1.2 | 1.0 |
| Daytime | Host sex [SX] | 1 | 6.4 * | 2.9 # | 0.2 | 13.5 *** |
| visitation | Distance red source [$D_r$] | 1 | 43.2 **** | - | 1.2 | 32.8 **** |
| (± 13h) | Dist. yellow source [$D_y$] | 1 | - | 98.3 **** | 35.6 **** | 11.9 ** |
| Patch size | [SP]*[SX] | 1 | 5.2 * | E | E | 7.3 * |
| = 1 | [SP]*[$D_y$] | 1 | - | 15.4 **** | 4.7 * | 3.2 # |
| [D-P1] | [SX]*[$D_r$] | 1 | 3.5 # | - | E | 7.4 * |
| | Replicate | 1 | 1.2 | 0.4 | 13.8 *** | 0.4 |
| 4 | Host species [SP] | 1 | 29.4 **** | 40.0 **** | 0.9 | 4.1 * |
| Daytime | Host sex [SX] | 1 | 1.9 | 3.5 # | 5.8 * | 1.7 |
| visitation | Distance red source [$D_r$] | 1 | 130.3 **** | - | 5.3 * | 103.2 **** |
| (± 14 h) | Dist. yellow source [$D_y$] | 1 | - | 127.6 **** | 38.0 **** | 83.4 **** |
| Patch size | [SP]*[SX] | 1 | 0.0 | 2.9 # | E | E |
| = 2 | [SP]*[$D_y$] | 1 | - | E | E | 2.9 # |
| [D-P2] | [SX]*[$D_r$] | 1 | 3.4 # | - | E | 4.8 * |
| | Replicate | 3 | 27.3 **** | 70.0 **** | 25.0 **** | 12.7 * |

Table 1 shows that there is a frequently significant effect of host sex, which is most apparent for red dye in experiment DN-P1 (p<0.005), and for yellow dye, and red + yellow dye in DN-P2 (both highly significant p<0.0001). Male flowers have a higher chance to receive dye than female flowers (overall a relative higher chance of approx.

12%). However, in experiment D-P1 the effect is reversed, and female hosts receive significantly ($p<0.05$) more red dye than male hosts.

**Table-2** Proportion of fluorescent dye per plant (y) as a function of distance (x) across the experimental plot. Extrapolated are the visitation frequency at the border of the plot (at 10m) and the distance from the source at which the visitation frequency becomes smaller than 1% (in m) and their 95% confidence intervals. R= red; Y= yellow.

| Exp. | Dye color | Plant Species | $f(x): y = ae^{-bx}$ a (SE) | b (SE) | at 10m (in %) (95% CI) | < 1% (in m) (95% CI) |
|---|---|---|---|---|---|---|
| 1 | R | *S. dioica* | 0.90 (0.09) | 0.18 (0.02) | 14.5 (6.9 - 28.6) | 24.7 (18.3 -35.3) |
| 24h | R | *S. latifolia* | 0.88 (0.08) | 0.46 (0.05) | 0.1 (0.0 - 0.3) | 9.7 (7.5 - 13.1) |
| Patch 1 | Y | *S. dioica* | 0.87 (0.08) | 0.41 (0.05) | 1.5 (0.5 - 4.7) | 11.0 (8.5 - 15.0) |
| [DN-P1] | Y | *S. latifolia* | 0.87 (0.08) | 0.26 (0.03) | 6.4 (2.9 -13.7) | 17.2 (13.3 -23.0) |
| 2 | R | *S. dioica* | 0.86 (0.06) | 0.08 (0.01) | 38.4 (25.1 - 57.8) | 55.7 (39.9 - 88.2) |
| 24h | R | *S. latifolia* | 0.88 (0.08) | 0.28 (0.03) | 5.3 (2.4 - 11.5) | 16.0 (12.6 - 21.1) |
| Patch 2 | Y | *S. dioica* | 0.90 (0.07) | 0.37 (0.03) | 2.3 (1.0 - 5.0) | 12.3 (10.0 - 15.4) |
| [DN-P2] | Y | *S. latifolia* | 0.87 (0.06) | 0.14 (0.01) | 22.0 (14.1 - 34.0) | 32.6 (25.9 - 43.4) |
| 3 | R | *S. dioica* | 0.88 (0.09) | 0.15 (0.02) | 19.5 (9.3 - 39.5) | 29.9 (21.2 - 47.3) |
| Daytime | R | *S. latifolia* | 1.00 (0.08) | 0.18 (0.02) | 17.1 (9.3 - 30.7) | 26.2 (20.2 - 35.8) |
| Patch 1 | Y | *S. dioica* | 1.00 (0.06) | 0.36 (0.03) | 2.8 (1.4 - 5.5) | 12.9 (10.8 - 15.7) |
| [D-P1] | Y | *S. latifolia* | 0.87 (0.10) | 0.21 (0.03) | 10.7 (4.2 - 25.7) | 21.4 (15.3 - 32.8) |
| 4 | R | *S. dioica* | 0.97 (0.08) | 0.16 (0.02) | 19.5 (10.5 - 35.1) | 28.5 (21.5 - 40.2) |
| Daytime | R | *S. latifolia* | 0.91 (0.07) | 0.21 (0.02) | 10.7 (6.0 - 18.9) | 21.1 (17.0 - 27.2) |
| Patch 2 | Y | *S. dioica* | 0.99 (0.05) | 0.35 (0.02) | 3.1 (1.9 - 4.8) | 13.3 (11.7 - 15.1) |
| [D-P2] | Y | *S. latifolia* | 0.94 (0.07) | 0.22 (0.02) | 10.8 (6.0 - 19.0) | 21.1 (17.0 - 27.1) |

**Table-3** Seasonal variations in weather conditions during the replicates of experiments 1-4. Day length (D, i.e. the time interval between sunset and sundown), mean temperatures (T), mean wind speed (W), mean sun irradiance (S), and rainfall (R) for each day and night of observation.

| Exp. | Rep. | Date in 2000 | D (h:m) | T (°C) day | T (°C) night | W ($ms^{-1}$) day | W ($ms^{-1}$) night | S ($Wm^{-2}$) day | S ($Wm^{-2}$) night | R (+/-) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 20 Jun. | 16:20 | 31.7 | 22.9 | 1.6 | 0.0 | 362 | 12 | - |
| [DN-P1] | 2 | 21 Jun. | 16:20 | 24.2 | 22.0 | 2.4 | 0.0 | 233 | 13 | + |
| | 3 | 28 Jun. | 16:17 | 16.4 | 9.6 | 1.9 | 0.3 | 307 | 19 | - |
| | 4 | 29 Jun. | 16:16 | 16.9 | 10.9 | 2.2 | 0.5 | 308 | 19 | - |
| 2 | 1 | 18 Jul. | 15:47 | 16.5 | 13.1 | 1.9 | 0.4 | 244 | 16 | + |
| [DN-P2] | 2 | 20 Jul. | 15:42 | 21.0 | 12.9 | 1.7 | 0.0 | 330 | 18 | - |
| | 3 | 26 Jul. | 15:27 | 18.9 | 15.4 | 1.2 | 1.0 | 157 | 16 | - |
| | 4 | 1 Aug. | 15:09 | 26.6 | 17.3 | 1.5 | 0.2 | 302 | 15 | + |
| 3 | 1 | 31 Aug. | 13:20 | 20.2 | 12.4 | 0.8 | 0.5 | 344 | 16 | - |
| [D-P1] | 2 | 13 Sep. | 12:51 | 19.4 | 15.3 | 1.6 | 0.5 | 189 | 14 | - |
| 4 | 1 | 9 Aug. | 14:42 | 23.2 | 13.9 | 0.9 | 0.0 | 310 | 18 | - |
| [D-P2] | 2 | 16 Aug. | 14:16 | 22.2 | 15.1 | 0.3 | 0.3 | 258 | 16 | + |
| | 3 | 22 Aug. | 13:53 | 20.7 | 14.4 | 0.4 | 0.4 | 280 | 15 | - |
| | 4 | 24 Aug. | 13:46 | 22.2 | 13.0 | 1.1 | 1.1 | 350 | 15 | - |

The last overall effect in experiments 1-4 is the frequent occurrence of significant differences between replicates (Table 1), and occasional significant interactions with replicate (interactions with replicate were included in the regression

models, but were omitted from table 1 for reasons of clarity). This suggests that the strong variation in weather conditions between consecutive days and nights of observation within an experiment (shown in Table 3) might have a strong impact on the observed visitation patterns. Especially in experiments DN-P1 and D-P2 the main effect of replicate is significant. Table 3 shows that during experiment DN-P1 the average day and night temperatures are varying considerably across replicates. In experiment D-P2 the day length between replicate 1 and 4 has already shortened almost a full hour. Due to bad weather conditions mid August the replicates could not be performed within a few days as was intended.

**Table-4** Mean interspecific transfer of fluorescent dye after 24h. Shown is both the red dye source towards *Silene latifolia* and the yellow dye source towards *S. dioica*, for patch sizes 1 and 2. Designated are the significance levels of reciprocal differences between the two host species: n.s. = not significant; **** = p<0.0001.

| Exp. | Dye color | → Species | N | Interspecific transfer (± SE) |
|---|---|---|---|---|
| 1 | Red | *S. latifolia* | 194 | 0.346 (± 0.027) n.s. |
| [DN-P1] | Yellow | *S. dioica* | 203 | 0.300 (± 0.023) |
| 2 | Red | *S. latifolia* | 238 | 0.339 (± 0.017) **** |
| [DN-P2] | Yellow | *S. dioica* | 243 | 0.226 (± 0.014) |

Table 4 shows that the rate of interspecific transfer of fluorescent dye after 24h was high in these mixed plots, approximately 30%, and turned out to be higher from *S. dioica* to *S. latifolia* than in the other direction, although this difference was only significant in experiment DN-P2 (p<0.0001).

## Differential pollinator/vector guilds

Table 5 shows the results of the observations of insect visitors to the plants in the experimental plot. The pattern that shows up, is that *S. latifolia* is visited mainly during the night by nocturnal moth species, in particular *Hadena bicruris* and *Autographa gamma* that is also active during daytime. *S. dioica* is mainly visited during daytime by several species of bumblebees (mainly *Bombus terrestris, B. hortorum* and *B. pascuorum*), and hover flies (mainly *Rhingia campestris*). Nearly a third of the insects that were caught contained huge loads of fluorescent dye. Yellow fluorescent dye was detected on five out of 28 caught nocturnal visitors, while none of these insects contained red dye. Red fluorescent dye was detected on eight out of 14 caught diurnal insect visitors to plant in the plot, of which one insect also contained yellow dye.

**Table-5** Results of direct observations of night- and daytime insect visitors to *Silene latifolia* and *S. dioica* in the plots of experiments 1-4, and the presence/absence of fluorescent dye on caught individuals.

| Time of observations | Monitored vector species | | | Visits to | | Dye on vectors (total # caught) |
|---|---|---|---|---|---|---|
| | Group | Latin name | N | *S. latifolia* | *S. dioica* | |
| 23:00–1:00 in total 15-20 hours spread over 10 nights | Noctuid moths | *Hadena bicruris* | 18 | 18 | - | 5 yellow (18) |
| | | *Autographa gamma* | 9 | 7 | 2 | - (9) |
| | | *Plusia* sp. | 1 | 1 | - | - (1) |
| 12:00–17:00 in total 215 minutes spread over 5 days | Bumblebees | *Bombus* spp. | 17 | 3 | 87 | 1 red (2) |
| | Hoverflies | *Rhingia campestris* | 14 | 6 | 31 | 7 red, 1 yellow (10) |
| | | *Episyrphus* sp. | 5 | 2 | 3 | - (1) |
| | | *Syrphus* sp. | 1 | - | 1 | - (-) |
| | Satyrid butterflies | *Satiridae* sp. | 1 | - | 2 | - (-) |
| | Parasitoid wasps | *Microplitis tristis* | 1 | - | 1 | - (1) |
| | Bees | *Apis* sp. | 1 | - | 1 | - (-) |

**Table-6** Infection rate per plant in relation to distance from an inoculum source of the fungal pathogen *Microbotryum violaceum* in male and female plants of *Silene latifolia* (experiment 5). Block[7] represents the original 7 blocks (figure 2 and 4), block[4] represents the original 7 blocks, but with the data from all recipient blocks merged into 3 new blocks, 2-5m, 6-9m and 10-13m, to gain statistical power for the contrast analysis. Designated significance levels; ** = p<0.01; **** = p<0.0001.

| Source of variation in infection rate | df | Type III Wald $\chi^2$ |
|---|---|---|
| Block[7] | 6 | 172.2 **** |
| Host sex | 1 | 35.8 **** |
| Block[7] * Host sex | 6 | 18.2 ** |
| *Contrasts between blocks[4] within sex* | | |
| Male hosts | | |
| *Source vs 2-5m* | 1 | 29.8 **** |
| *2-5m vs 6-9m* | 1 | 8.2 ** |
| *6-9m vs 10-13m* | 1 | 0.7 |
| Female hosts | | |
| *Source vs 2-5m* | 1 | 60.9 **** |
| *2-5m vs 6-9m* | 1 | 0.0 |
| *6-9m vs 10-13m* | 1 | 0.0 |

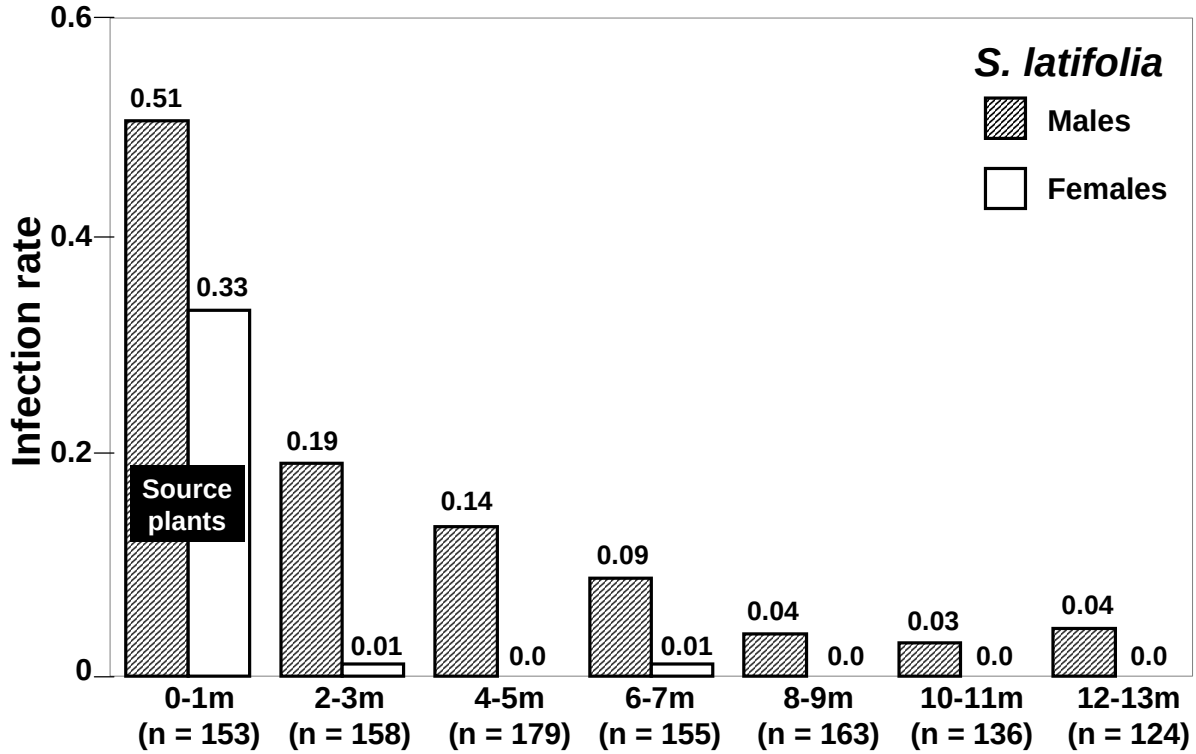


**Figure-4** Decreasing infection rate of flowering host plants of *Silene latifolia* with increasing distance from source plants (experiment 5). Hatched bars represent male plants; open bars represent female plants. See figure 1 for details of the experimental setup.

### Infection of *S. latifolia* in relation to the distance to a true teliospore source

Figure 4 shows a decrease in anther smut infection rate of flowering *S. latifolia* with increasing distance from the infected plants. Furthermore, both figure 4 and table 6 show that mean infection rate in male plants is significantly higher than in female plants across all blocks, including the source. For the contrast analysis, the six 'recipient' blocks were merged two by two into three new blocks, 2-5m, 6-9m and 10-13m to gain statistical power. The contrast analysis between source and the three merged blocks showed a significant decrease of infection rate of male plants between the source and the blocks at 2-5m ($p<0.0001$), and between the blocks at 2-5m and the blocks at 6-9m ($p<0.005$), but not between the blocks at 6-9m and the blocks at 10-13m. For female plants, infection rate was different between the source and the three blocks ($p<0.0001$), but not between any other of the merged blocks. This indicates that the decrease of infection rate with increasing distance from a teliospore source was steep in female hosts, and shallower in male hosts. Moreover, results suggest that an infection of healthy *S. latifolia* within a single flowering season is not likely to occur when the nearest teliospore source is much further away than 13m.

## DISCUSSION

### Host fidelity of different nocturnal and diurnal guilds visiting *S. latifolia* and *S. dioica*

Our data clearly show that visitation of *S. latifolia* and *S. dioica* is highly assortative with respect to host species, even when these host species are placed in an artificial, fully mixed sympatric setup. However, results were influenced by weather conditions that varied between experimental days (Table 3), and this might have influenced both composition and behavior of the pollinator community (e.g. Brantjes 1981; Herrera 1995). Nevertheless, table 2 shows that the dyes are dispersed over a greater distance intraspecificly than by interspecific vectoring, which holds true both for red dye via *S. dioica* and yellow dye via *S. latifolia*, and indicates host fidelity of vectors. A likely explanation for host fidelity could well be the existence of different pollinator guilds for these plant species. This idea is supported by direct observations to both diurnal and nocturnal insect visitors in this study (Table 5). Moreover, the pollinator guilds of *S. dioica* (Kay *et al.* 1984; Westerbergh and Saura 1994; Carlsson-

Granér *et al.* 1998) and *S. latifolia* (Brantjes 1976a; 1976b; Shykoff and Bucheli 1995; Altizer *et al.* 1998) have been described in allopatry, and in sympatry (Baker 1947; Biere and Honders 1998; Jürgens et al 1996; Goulson and Jerrim 1997), pointing consistently to the same genera as the main pollinators, that are different for these two *Silene* species. *S. dioica* is mainly visited during daytime by several bumblebee species and hoverflies of the Syrphidae family, whereas *S. latifolia* is mainly visited nocturnally, by nocturnal moth species of the Noctuidae family. However, interspecific visitation, i.e. pollinators visiting both species, has been described for moths, bumblebees and hoverflies (Goulson and Jerrim 1997). Indeed, interspecific transfer of fluorescent dye after 24h was found to be substantial in both host species. Interestingly, relatively more red dye was transmitted to *S. latifolia* than yellow dye to *S. dioica*, which was only significant in experiment DN-P2, suggesting that interspecific visitation was higher in the direction from *S. dioica* to *S. latifolia* than opposite. This was supported by the direct observations, in which nocturnal moths seemed to be more choosy, and visited more exclusively *S. latifolia* than *S. dioica*, and is consistent with a comparative study by Wirooks and Plassmann (1999) who found that the number of eggs and caterpillars of seven noctuid moth species on *S. latifolia* plants was roughly twenty-fold higher than on *S. dioica*.

*S. latifolia* has a typical moth pollination syndrome (Baker 1961; Baker and Hurd 1968), with flowers that open at dusk and emit an intense scenting fragrance during the night, thereby being more attractive to nightly visitors such as noctuid moths (Brantjes 1978), than *S. dioica* which lacks this phenomenon. Since flowers of *S. latifolia* are often closed during daytime (Jürgens *et al.* 1996) they are less attractive to diurnal pollinators. From the diurnal visitors, that mainly visited *S. dioica* but were recorded to pay occasional visits to *S. latifolia* as well, bumblebees were by far the most abundant. Bumblebees might prefer *S. dioica* over *S. latifolia* for two reasons. First, flowers of *S. dioica* are smaller than *S. latifolia*, specifically the length of the calyx (Jürgens *et al.* 1996). Flowers of *S. dioica* are small enough to make the nectar available to long-tongued bumblebees (*B. hortorum* and *B. pascuorum*) that cannot gain access to the nectar of *S. latifolia* flowers. *B. terrestris*, the third bumblebee species that we observed, has a smaller tongue (Jürgens *et al.* 1996), and can reach nectar of neither *S. latifolia*, nor *S. dioica* flowers. Instead, it pierces tiny holes in the sepal to gain access to the nectar resources of a flower, which is known as ‘nectar robbing’ (*cf.* Heinrich 1976). We observed such bumblebee-inflicted holes frequently in *S. dioica* flowers, as well as in *S. latifolia* flowers. Second, floral display size of *S. dioica* is much larger than of *S. latifolia* in the field, mainly due to the significantly

larger number of flowering stalks (which was up to two times larger for healthy female hosts, and up to 2.5 times larger for healthy male hosts; A. Biere, unpublished results). Foraging bumblebees are more attracted to large floral displays (e.g. Klinkhamer *et al.* 1989; Goulson *et al.* 1998), trying to minimize flight and search times within a foraging bout. Furthermore, bumblebees exhibit flower constancy, i.e. the tendency of experienced pollinators to visit the same plants species or type of flowers regardless of the presence of other potential rewarding flowers nearby (Levin and Anderson 1970; Oster and Heinrich 1976; Waser 1986), promoting a species-specific visitation pattern in a mix of different flower types. Another very active diurnal vector in our study is the hoverfly *Rhingia campestris*. This species has been recorded to discriminate between different colors, favoring violet and blue colored artificial flowers over white ones (Haslett 1989). This is consistent with the *Rhingia campestris* in our study, which favors the pink flowers of *S. dioica* over the white flowers of *S. latifolia*. Flower constancy has been reported for related Syrphid hoverflies as well (Goulson and Wright 1998), which would again strengthen the assortative effect.

In natural sympatric populations of *S. latifolia* and *S. dioica*, species are rarely as mixed as in our experimental plot. Due to habitat differences (Goulson and Jerrim 1997) and differential adaptation to light intensity (Willmot and Moore 1973) populations with co-occurring *S. latifolia* and *S. dioica* are often patchy. This, and the fact that interplant distances between *S. latifolia* and *S. dioica* in the field are usually much larger than 1m, suggests that the interspecific visitation rates as found in this study are absolute maximum estimates of this parameter. Therefore, we expect the host species-specific visitation pattern that we observed in the experimental plot to be much stronger in natural sympatric populations. Nevertheless, the occurrence of hybrids in natural sympatric populations (Baker 1947; Goulson and Jerrim 1997), which has been reported to constitute more than 6% of the sympatric population of Norg (Biere and Honders 1996b), is a silent witness of interspecific visitation of pollinators, and might provide an estimate of exchange between host species in natural sympatry. Moreover, it shows that interspecific visitation in natural field populations is ecologically significant.

### Visitation of *Silene* is host sex specific.

We found in experiments DN-P1, DN-P2 and D-P2, but not in D-P1, that male plants of these dioecious *Silene* species are visited more frequently than female plants, i.e. the frequencies of flowers per plant with either red dye, yellow dye, or both dyes

were significantly higher in male plants than in female plants in most setups. Favoring male hosts over female hosts of pollinators is expected for the following reasons. First, foraging pollinators favor large floral display sizes over smaller ones, as was argued for differences between host species in the previous paragraph. Male plants of *S. latifolia* (e.g. Gross and Soule 1981; Meagher 1992; Delph and Meagher 1995; Carroll and Delph 1996) and *S. dioica* (Hemborg 1998; Hemborg and Karlsson 1999) bear much more flowers than female plants. Second, male flowers of *S. dioica* (Kay *et al.* 1984; Hemborg 1998) and *S. latifolia* (Shykoff and Bucheli 1995; Biere and Honders 1996a; Shykoff and Kaltz, 1998) contain higher concentrations of nectar than female flowers. In a number of studies, male plants of *S. latifolia* (Shykoff and Bucheli 1995; Altizer *et al.* 1998) and *S. dioica* (Carlsson-Granér 1998) have been found to be preferentially visited over female plants by pollinators. Actually, active discrimination against female flowers is suggested to be a common phenomenon in dioecious plants (Bierzychudek 1987 and references therein). These differential sex preferences of pollinators may also contribute to the higher infection rate of the *S. latifolia* males in experiment 5. Indeed, male-biased infection rates, i.e. the number of males that become infected in a flowering season, have frequently been documented in literature (Alexander 1989; Thrall and Jarosz 1994; Alexander and Antonovics 1995; Biere and Antonovics 1996; Biere and Honders 1998). Male plants flower earlier and longer than female plants, which increases the risk of getting infected in their first season (Thrall and Jarosz 1994; Biere and Antonovics 1996). However, disease incidences, i.e. the number of diseased plants in a population at a certain point in time, often do not show biases between host sexes in other studies of these *Silene species* (Zillig 1921; Alexander 1990), or are even female-biased (Lee 1981; Kaltz and Shykoff 2001). This discrepancy between rate of infection and disease incidence might be explained by sexual differences in other parameters that affect disease incidence, such as pre-floral infection rates, recovery and disease-induced mortality (Biere and Honders, unpublished results).

### Differences in transfer distance between dye and disease

Figure 1 showed that the dispersal of dye after 24h (the upper four panels in the figure) has the potential to go beyond the artificial 10m limits of the experimental plot. Indeed, the fitted equations in table 2 clearly show that at the border of the plot dye frequencies are still high, up to 38%. Moreover, if we look at the distances from the source at which the visitation frequency become smaller than 1%, by extrapolating the dataset (Table 2), this yields nearly 56m for red dye on *S. dioica* and nearly 33m

for yellow dye on *S. latifolia* in DN-P2. The estimates for interspecific visitation distances, i.e. red dye on *S. latifolia* or yellow dye on *S. dioica* are significantly smaller. This strongly suggests that teliospores could be dispersed by their insect vectors much further than 10m, especially between hosts of the same species. However, receiving teliospores does not guarantee becoming infected. Empirical studies have shown that the distance range at which infection occurs from a certain inoculum source might be more limited. In a study of by Alexander (1990) spores were transmitted at least 10m from a teliospore source, and infections up to 6m from this source in the following year. Roche *et al.* (1995) detected floral infections of *S. latifolia* up to 11.2m, which was the farthest distance possible in their experiment. The results from experiment 5 are more or less consistent with these studies, and show that the distance of infection is in the same order of magnitude as in Roche's study, with an infection rate of the most distant block (12-13m) still more than 4% of the male hosts. However, we must keep in mind that our teliospore source was much larger, and the density of our plants was much higher than in their studies. We detected no infected female hosts at these distances. Roche and colleagues suggested that the dispersal of teliospores, and the resulting infection of *S. latifolia* are limited to distances close to 12m (Roche *et al.* 1995). In addition to this, our results confirmed that the chances of healthy *S. latifolia* becoming infected with *M. violaceum* within a single flowering season are small at distances much further away then 12-13m from a teliospore source. In contrast to this, studies on metapopulations of the *Silene-Microbotryum* system that examined recolonization rates of the fungus have shown that dispersal of infection further than 40m is likely to occur within a single year (Thrall and Antonovics 1995). Since our experiments were also limited to distances below 13m, we suggest that spatially larger experimental studies are needed to investigate the true dispersal capacity of *M. violaceum* and its infection.

## Implications for the maintenance of host race differentiation in sympatric populations of hosts

Summarizing our results, we have found a host species-specific visitation pattern of pollinators/vectors in an artificial, fully mixed sympatric plot of *S. latifolia* and *S. dioica*. Red campions were visited mostly during daytime by a diurnal guild consisting of several bumblebee species and hoverflies, and white campions were mainly visited during the evening and night by a nocturnal guild consisting of several noctuid moths species. Experiment 5 showed that the range of teliospore dispersal and first season infection was likely to be not much larger than 12-13m. Furthermore,

infection rate of male hosts was significantly higher, and occurred at larger distances from the teliospore source than of female hosts, which could reflect a male-biased visitation pattern, like was found in experiments 1-4. Knowing that natural sympatric populations of *S. latifolia* and *S. dioica* are both spatially and temporally more heterogeneous (*cf.* Van Putten *et al.* chapter 2) than our artificially mixed plot due to habitat differences (Willmot and Moore 1973; Goulson and Jerrim 1996) and differential flowering phenology of both host species (Biere and Honders 1996), we expect the interspecific visitation rates to be much lower in a natural situation than what was estimated in experiments 1-4. Moreover, pollinators/vectors have been recorded to discriminate against *M. violaceum* infected plants, with healthy inflorescences being visited up to three times more frequent than infected ones (Jennersten 1988). This would diminish the amount of teliospore exchange between the host races even further. Therefore, outcrossing between the host races in natural sympatric populations of the two host species is expected to be low, and could provide a basis for the maintenance of the genetic host differentiation, which was observed in a microsatellite study of anther smuts in natural sympatric/parapatric populations of host species (Van Putten *et al.* chapter 2), and in a sporidial colony color marker in a true sympatric host population (Van Putten *et al.* chapter 3). The existence of strong fitness differences between the host races, that were found in a mating and competition experiment (Van Putten *et al.* chapter 4), implies that there must be a mechanism that ensures reproductive isolation between the host races to prevent the host race from *S. dioica* race from getting extinct, and thereby maintain the host-related genetic diversity that was observed in these populations. The host fidelity of the different pollinator guilds of *S. latifolia* and *S. dioica* may well contribute to such a mechanism.

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# CHAPTER 6

# Summarizing discussion and conclusions

In the different chapters the obtained results already have been discussed extensively. In this final chapter the most important results will be summarized and put into perspective with an emphasis on differentiation between anther smuts from different host species in sympatric populations of two host species. First, the following questions will be addressed: Can we consider fungal isolates from different host species in sympatry to be separate host races? To what extent does the degree of sympatry influence the genetic divergence between the host races? And, what is the impact of local host spatial structure on this variation? Secondly, the fitness differences between fungal isolates from different host species that appear in mating and competition, and the implications of these differences for these host races in sympatry are discussed. Thirdly, the host fidelity of vectors in sympatry, and the impact of host spatial structure on the differentiation is discussed, putting forward the plausibility of a balance between gene flow and selection in sympatry that could contribute to the maintenance of host-specific differentiation in host sympatry. Also, some attention will be paid to the role of interspecific hybrid hosts on the amount of fungal gene flow, and the differences between host sexes in the process of maintenance of host-specific genetic variation. Finally, in some concluding remarks, the potential of this model system to investigate sympatric host race formation and speciation of this model system will be evaluated.

## HOST RACE DIVERGENCE AND THE DEGREE OF HOST SYMPATRY

### Host races

In chapter 1, we observed host-specific microsatellite alleles in the allopatric and parapatric host populations of *S. latifolia* and *S. dioica*. For two of the loci, the alleles found in isolates from these two host species were separated in size by a gap of 7-9 repeats each. This was consistent with the work of Bucheli and co-workers (Bucheli *et al.* 2001). Together with the fact that this type of allelic variation was

observed in allopatric host populations throughout Western Europe, this strongly suggests that the observed variation represents long-term divergence between these host races of anther smut, which presumably arose in allopatry. In that scenario, fungal isolates from these host species in sympatry will come into secondary contact with each other whenever there is (fungal) gene flow between isolates from the two host species. A first question that arises is, whether the divergence between strains from *S. latifolia* and *S. dioica* is large enough to consider both races as two sibling species rather than host races. Jeanike (1981) provides distinct criteria to distinguish between host races and sympatric host-associated sibling species, stating that if gene flow among two or more population is restricted solely, or primarily because of differential host preferences, this would constitute host races. Thus, without this basis for reproductive isolation being present, host races would, in an extreme case, fuse into a single panmictic population, whereas sibling species would maintain their separate genetic identities.

On the other side of the spectrum, when is variation among fungal isolates from different host species high enough to consider them as separate host races? The significantly lower genetic divergence in the more sympatric populations of both host species (Chapter 2 and Chapter 3) suggests that these host races could indeed fuse into panmictia, especially since it is known that both races can cross-inoculate each other's host species without being a priori at a disadvantage (Biere and Honders 1996a; Chapter 4). However, chapter 4 also showed that the race from *S. latifolia* often outcompetes the race from *S. dioica*, presumably due to faster conjugation. This indicates that, without a reproductive isolation mechanisms such as temporal (Biere and Honders 1996b) and spatial heterogeneity (Chapter 3), and/or host fidelity of vectors (Chapter 5), this might rather be 'invade and take over' of the host race of *S. latifolia* instead of a merger of host races. Nevertheless, the genetic divergence in allopatry remains rather high, and surely justifies their state of being separate host races.

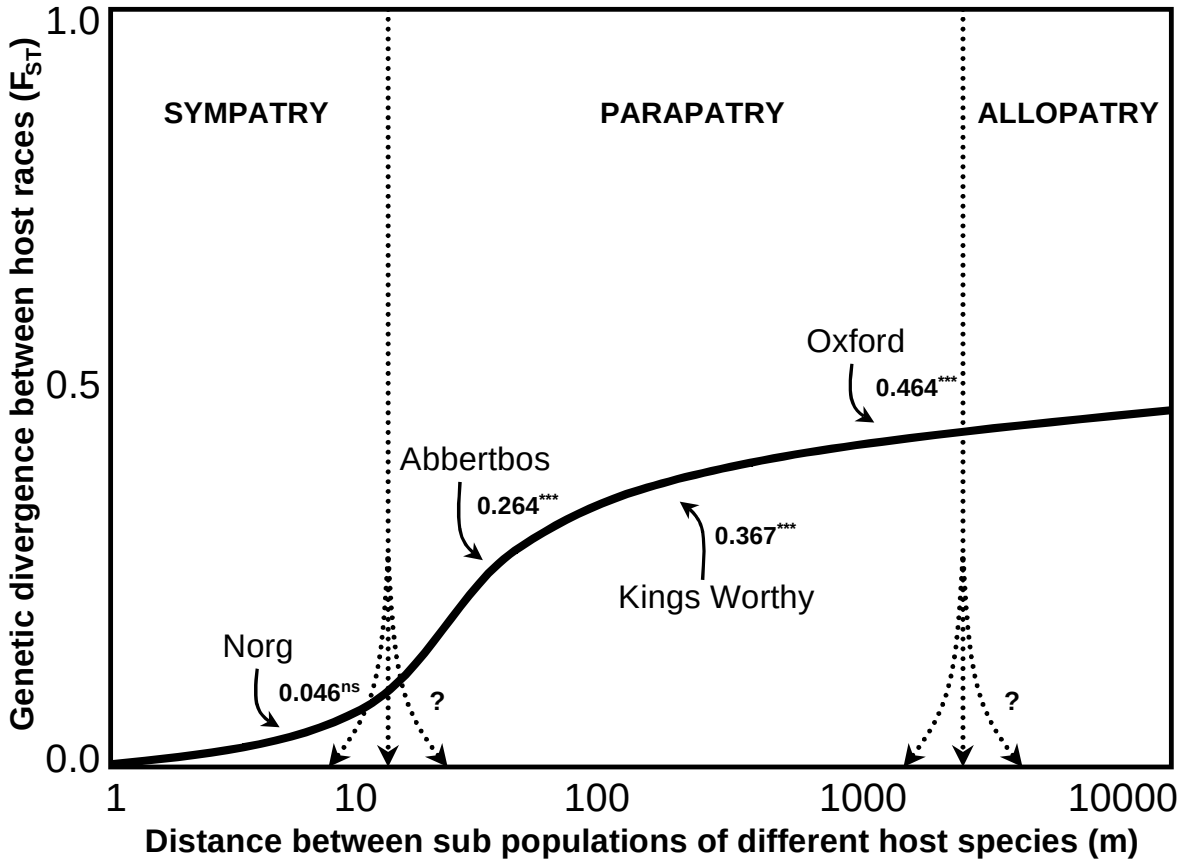


**Figure-1** Hypothetical relationship between the degree of sympatry (approximated by the distance between sub populations of different host species within a metapopulation) and the genetic divergence between host races (estimated by $F_{ST}$ values). The degree of sympatry was roughly estimated 1-10m for Norg, 10-100m for Abbertbos, 100-1000m for Oxford, with the population at Kings Worthy in between Abbertbos and Oxford. The boundaries for sympatry, parapatry and allopatry (*sensu* Kondrashov and Mina 1986) only represent a schematic indication; the exact transition between two types is all but clear. $F_{ST}$ values are based on the four microsatellite loci (Chapter 2).

## The degree of sympatry and host spatial structure

The degree of host sympatry influenced the genetic differentiation between host races (Chapter 2). Results suggested that increasing levels of host mixing increased the amount of gene flow between the host races. The average distance between two sub populations within a metapopulation, dominated each by a different host species, would be a reasonable predictor of genetic divergence between isolates from both host species, i.e. host races, in that fungal metapopulation, which is shown in figure 1. In reality, this picture does not present a one to one relationship with divergence at small spatial scales, as was shown in chapter 3. In that chapter, we showed the impact local of structure of hosts on the genetic population structure of the host races in a sympatric population of hosts. The fungal environment, represented by the host plants of the Norg population, was structured in the following three different ways: First and foremost, the host species belong to two different species. Second and

thirdly, the host populations are both spatially (in distinct patches) and temporally (differential flowering phenology of both host species and sexes, Biere and Honders 1996b) heterogeneous for the pathogen. From this, we expected that the population of *M. violaceum* in Norg might not be panmictic (as the $F_{ST}$ values of Chapter 2 suggested), but actually consists of a number of demes that genetically structures the pathogen population, as has been found for many phytophagous insects (Mopper 1996). The heterogeneity of the environment is often a starting point in host specialization and host race formation (Berlocher 1998a), but may in this population represent a barrier that contributes to the maintenance of pre-existing divergence. Whereas gene flow opposes the historically evolved differentiation between the host races, and acts to homogenize the genetic divergence, the local deme structure of both host species in this population will favor its maintenance (*cf.* Mopper 1996). Habitat, or host choice is found to be a crucial factor in theoretical models on host specialization (Fry 1996, Kawecki, 1997; 1998). Therefore, being the actors of host choice in this model system, a dominant role in the maintenance of the divergence was hypothesized for the pollinator/vector guilds of the host plants (Chapter 5). Among other possible mechanisms that also may contribute to this maintenance of host-specific genetic variation is positive assortative mating between fungal isolates with respect to host species. This latter possibility was investigated and described in chapter 4, in which we performed mating and competition experiments between fungal strains that were isolated from both host species.

## RELATIVE FITNESS DIFFERENCES BETWEEN HOST RACES

### The host race of *S. dioica* seems to be doomed in sympatry

In chapter 4, it was shown that the conjugation frequency *in vitro* after 24h of strains from the host race of *S. latifolia* was significantly higher than strains from the *S. dioica* host race, suggesting that mating between strains from *S. latifolia* occurs faster than between strains from *S. dioica*. Furthermore, strains from *S. latifolia* performed better in competition *in vivo* on both host species, which was shown to be positively correlated to their mating behavior. Also, in this experiment it was shown that strains from the *S. dioica* race had a longer latency period, which was consistent with the study of Biere and Honders (1996a).

As would have been expected beforehand, we did not find (positive) assortative mating between the host races. If the supposed gene flow (Chapter 2) would have been large enough, we would expect the host race of *S. dioica* to decline or go extinct in sympatric populations of these host species, based on conjugation rate, infection success, latency period and competitive ability of strains. Since both host races do occur regularly in natural sympatric populations of these host species (Biere and Honders 1996b; Chapter 2; Chapter 3), other factors must be responsible for the maintenance of host-specific differentiation in sympatry. One factor may be the higher spore production on the native host species under non-competitive conditions, as observed on male plants (Biere and Honders 1996a). In addition, the deme structure due to spatial and temporal heterogeneity of host plants (Chapter 3), the role of vector behavior (Chapter 5), and heterosis, that was observed in female hosts in the competition experiment itself, could be among the other factors that contribute to the maintenance of strains from *S. dioica* and the differentiation in host sympatry (Chapter 4).

## BALANCING MIGRATION AND SELECTION BY HOST FIDELITY OF VECTORS

### *S. latifolia* and *S. dioica* have different pollinator guilds

Pollinators serve a dual role as vectors of anther smut spores (Jennersten 1983). If these vectors can prevent, or strongly limit the amount of fungal gene flow, and thereby the possibilities for competition and outcrossing between fungal isolates from different host species simply by being choosy in host sympatry, host-related genetic differentiation between these host races might be maintained, i.e. both host races might coexist in sympatry. Indeed, the results of chapter 5 showed a significant host species-specific visitation pattern of pollinators/vectors in artificial, fully mixed sympatric plots of *S. latifolia* and *S. dioica*. Red campions were visited mostly during daytime by a diurnal guild consisting of several bumblebee species and hoverflies, and white campions were mainly visited during the evening and night by a nocturnal guild consisting of several noctuid moths species. This reduction of gene flow caused by the existence of different pollinator guilds for these two host species would surely contribute to the maintenance of host-specific genetic variation between the host races in sympatry. However, we estimated that the exchange in the experiments between both host species was still as large as approximately 30%, and occurred mainly from

*S. dioica* towards *S. latifolia*, suggesting that the vector guild of *S. latifolia* (noctuid moths) was choosier than the guild of *S. dioica* (bumblebees). This is a considerable amount of interspecific visitation, possibly high enough to setup the doom scenario that strains of the *S. dioica* host race eventually will go to extinction if this would lead to competition for susceptible hosts with the strains of higher competitive ability from the *S. latifolia* host race. However, the natural sympatric populations of both host species that we have examined turned out to be quite different from the artificially mixed plots in the experiments of chapter 5.

## Impact of host spatial structure on vector behavior

The results of chapter 3 showed that natural sympatric populations of *S. latifolia* and *S. dioica* are spatially more heterogeneous than the fully mixed plots of chapter 5, possibly due to differences in habitat preference of both host species (see also Goulson and Jerrim 1996). Together with the temporal heterogeneity of differences in flowering phenology (Biere and Honders 1996b), we expect the interspecific visitation rates to be much lower in a natural situation than this estimate of 30%, that itself could represent an absolute maximum estimate of 'leakiness' in host fidelity (*cf.* Feder 1998) in this model system. Also, pollinators/vectors have been recorded to discriminate against *M. violaceum* infected plants (Jennersten 1988), which would diminish the amount of teliospore exchange between host species even further. Therefore, outcrossing rates between fungal isolates from different host species in natural sympatric populations of these hosts are expected to be considerably lower, and are presumed to be low enough to hypothesize that host fidelity of vectors plays an important role in the maintenance of host-specific differentiation in sympatry. The occurrence of interspecific hybrids, reported to constitute more than 6% of the sympatric population of Norg (Biere and Honders 1996b), is a silent witness of gene flow between these hosts, and might therefore represent a more accurate estimate of effective interspecific visitation in host sympatry in this population, and hence the exchange of teliospores between the host species. Note that hybrid hosts that are reported in the study of Biere and Honders (1996b) and in our study may include $F_1$'s as well as backcrosses with either parental host species.

## Possible roles of hybrid hosts in the amount of fungal gene flow

Interspecific hybrids can play an interesting role in fungal differentiation, since they could form a 'hybrid bridge' (*cf.* Floate and Witham 1993), i.e. easier shifting between host species via hybrid swarms of intermediate morphology, physiology,

phenology or susceptibility. This could lead to more gene flow than in a situation without hybrid hosts, and would predict that, all else being equal (i.e. similar host composition, degree of sympatry, spatial structure, etc.), genetic divergence between fungal isolates is significantly lower in host populations with a lot of hybrid plants than in host populations that almost lack hybrids. Hybrids between *S. latifolia* and *S. dioica* indeed have an intermediate morphology (Baker 1951), although most morphological characters are often not reliable for identification of hybrids (*cf.* Goulson and Jerrim 1997). Furthermore, Biere and Honders (1996a) showed that interspecific hybrids did not differ in susceptibility from the mid-parent value of the pure parental host species, suggesting that a hybrid bridge may well exist, and is in principle accessible to fungal isolates from both host species. However, hybrid hosts were found to grow predominantly among patches of *S. latifolia* rather than among patches of *S. dioica*, either due to (unknown) habitat preferences of the hybrids or by strong differences in flowering phenology between host species and host sexes (see also Goulson and Jerrrim 1997, and Chapter 2 for a discussion on this issue). This may suggest that a hybrid bridge, if existent at all, is more accessible to fungal isolates from *S. latifolia* host race than for the *S. dioica* host race. Indeed, chapter 2 showed that fungal isolates from hybrid hosts from examined populations resembled the *S. latifolia* host race, rather than the *S. dioica* host race. A rather wild speculation would then be that the *S. latifolia* host race might have pre-adapted to a more '*S. dioica* host-like' environment via the hybrids that grew among them, giving them a head start over strains from *S. dioica* when confronted with the 'alien' host. If this would be true, it could be part of the explanation why we observed strong fitness differences in competitive ability between strains from different host species, the strains from *S. latifolia* having the natural advantage on its own host species, and be competitive on *S. dioica* at the same time. However, except for the microsatellite analysis of chapter 2, we did not include interspecific hybrids in any of the experiments in this study, hence we lack the additional data that would support such an idea.

## THE BALANCE BETWEEN MIGRATION AND SELECTION IN RELATION TO HOST SEX

### A different selection-migration balance for male and female hosts?

In this thesis, we have found a number of differences between the host sexes that suggest that the balance between selection and migration of fungal isolates from

the two host species may be different for male and female hosts. First, differential selection of fungal strains may occur on the different host sexes, and male and female hosts each may contribute to the maintenance of host-specific genetic variation in fungal isolates in a different way. In a cross inoculation experiment, the production of teliospores per plant was found to be significantly higher in conspecifics than in heterospecifics of the host of origin in male host plants, but not in female hosts (Biere and Honders 1996a). This held true for both host species, and provides an argument for the maintenance of host-specific divergence in sympatry by this trade-off in performance in male hosts. In female hosts, a different mechanism may contribute to the maintenance of host-specific genetic variation in fungal isolates. In the competition experiment of chapter 4, we observed a highly significant effect of host sex on the success of dikaryon types. Heterotypic dikaryons were found to be the better competitors in female host plants, at least in *S. latifolia*, which may contribute to the maintenance of the competitively inferior strains from *S. dioica* in host sympatry. The fact that heterosis was observed on female hosts might be explained by the more complex requirements to successfully infect female hosts as compared to male hosts. Infection of a female host requires a morphological sex change by fungal induction of male specific gene expression in the developing flower (Scutt *et al.* 1997) and prevention of pollen grain development (Audran and Batcho 1981) in dioecious *Silene* species. Therefore, being more complex and presumably more costly to the fungus, complementation processes in heterotypic dikaryons may be more important in female hosts than in male hosts. If this is the case, female hosts could contribute to the maintenance of host-specific variation by providing a 'safe haven' for the competitive weaker strains from *S. dioica* in sympatric populations of these host species. However, a number of studies have shown the predominantly selfing nature of this fungus (e.g. Baird and Garber 1979), resulting in strong observed homozygosities in natural host populations even in highly polymorphic microsatellite loci (Bucheli *et al.* 2001; Chapter 2), which could strongly limit the importance the heterosis effects in female hosts.

Second, differential migration rates of fungal strains may occur on different host sexes, since we found significant differences in interspecific host visitation pattern between the two host sexes in the vector experiments of chapter 5. Male hosts were preferentially visited over female hosts, which was consistent with other studies examining pollinator visitation in both these *Silene* species (Shykoff and Bucheli 1995; Altizer *et al.* 1998; Carlsson-Granér 1998). This has also been reported in other dioecious perennial herbs (e.g. Vaughton and Ramsey 1998) and has been proposed to

be a common phenomenon in dioecious plants (Bierzychudek 1987 and references therein). Male biased visitation is expected due to a larger floral display size of males (*S. latifolia* e.g. Gross and Soule 1981; Meagher 1992; Delph and Meagher 1995; Carroll and Delph 1996; and *S. dioica* Hemborg 1998; Hemborg and Karlsson 1999), and higher nectar concentrations of male flowers (*S. latifolia* Shykoff and Bucheli 1995; Biere and Honders 1996a; Shykoff and Kaltz, 1998; *S. dioica* Kay *et al.* 1984; Hemborg 1998). Also, male plants flower earlier and longer than female plants. Both the preferential visitation, the longer flowering, and the large floral display could lead to the male-biased disease incidences that were observed in a number of studies (Alexander 1989; Thrall and Jarosz 1994; Alexander and Antonovics 1995; Biere and Antonovics 1996; Biere and Honders 1998; but see Kaltz and Shykoff 2001 for a discussion).

Adding up these differences between host sexes, the sex ratio of the host population of interest is likely to be an important factor in the overall balance of selection and migration in host sympatry in this model system. Sex-ratio in the genus *Silene* seems to be generally female-biased (e.g. Westergaard 1958; Mulcahy 1967; Lloyd 1974) which is, at least for *S. latifolia*, *S. dioica* and their hybrids, thought to be caused by the presence of sex-ratio distorters and restorers that are linked to the Y-chromosome, which are found to be polymorphic in natural populations (Taylor 1993; 1994; 1999). Since in strongly female-biased host populations the heterotypic crosses between both host races will stand a higher chance to be selected (Chapter 4), the presence of such sex-ratio distorters could indirectly contribute to the maintenance of host-specific strains as well.

## SOME CONCLUDING REMARKS

### The potential for sympatric host race formation and speciation in this model system

Going back to the four stages of sympatric host race formation from Berlocher (1998a) that were mentioned in chapter 1, it seems that our model system could fit a stage 2 model, in which the races are isolated only by host fidelity with allele frequency differences between the host races. We did detect host-specific alleles (in the microsatellite loci; Chapter 2), yet these were most apparent in more parapatric and allopatric populations of hosts, and not in the true sympatric population. Since

there was also no evidence for any mechanism of pre- and/or postzygotic reproductive isolation that is unrelated to host fidelity whatsoever, we can safely conclude that this system has not reached stage 3 yet. However, the most obvious critic to this would be that a much more likely explanation for the evolution of these host races is a more classic allopatric model, in which populations are geographically separated and evolve independent from each other. Especially the strong fitness differences between strains from different host species that we have observed in chapter 4 (*cf.* Figure 1B in Chapter 1), but also the observations that similar (selectively neutral) genetic variation between the host races is widespread (Chapter 2), and that true sympatric populations of these host species infected with this anther smut are probably not so widespread, makes a more geographical model of host race formation much more feasible (i.e. sympatric host race formation much less feasible) in this model system.

Whether these host races in allopatry will evolve to become different sibling species is even less clear. Fact is that within the host range of Caryophyllaceous host species, the host species *S. latifolia* and *S. dioica* are closely related themselves, sharing a recent common ancestor (Prentice 1978; Desfaux and Lejeune 1996). Among other combinations of host species and host races, for instance those that cannot cross-infect each others host species (see e.g. Zillig 1921), and between which the genetic distances are much larger (see e.g. Perlin 1996; Perlin *et al.* 1997; Shykoff *et al.* 1999), one would expect to find examples that approach Jeanike's criteria for belonging to different sibling species, rather than being different host races of the same species (Jeanike 1981). Some studies on host pathogen systems, have shown that the phylogenetic trees of host species and their pathogens were correlated, e.g. in some *Ustilago* species and their graminaceous host species (Bakkeren *et al.* 2000), suggesting coevolution of specialized host-pathogen systems. Future research may reveal matching phylogenies of anther smut fungi and their caryophyllaceous host species as well, since the *Silene-Microbotryum* system seems to be an ideal model system to gather additional evidence for such coevolutionary relationships. Since the relatedness of host species and the relatedness of their smut isolates seem to be related, the future of the host races from *S. latifolia* and *S. dioica* may be tightly connected to the ongoing evolution of these plant species.

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# SUMMARY

Theoretical models have shown that host race formation in plant parasite systems may eventually lead to speciation in sympatric populations of hosts under ecologically realistic conditions (e.g. Dieckmann and Doebeli 1999). The empirical evidence for this however is scarce, and predominantly concerned with phytophagous insect model systems. This thesis presents one of the first ecological studies that investigate host race differentiation of a plant pathogenic fungus in host sympatry.

As a model system, we have used the anther smut fungus *Microbotryum violaceum*, a pathogen that obligatory parasitises susceptible members of the Caryophyllaceae plant family to complete its sexual cycle, thereby sterilizing the host plant. We have studied this fungus in populations of two of its host species, *Silene latifolia*, the white campion, a dioecious short-lived perennial from open, disturbed habitats such as borders of arable land, and *S. dioica*, the red campion, a closely related dioecious perennial that mainly occurs in more shady humid habitats such as woodland borders. This fungus is known to infect 10-67% of the hosts in natural populations of these host species. Spores are produced in the anthers of the host plant. An infection of a female plant causes a morphological sex change in which the ovaries are reduced and staminal rudiments develop into stamens that bear spore-filled anthers. Spores are transmitted by the natural pollinators of their host species, which act as vectors of this disease.

Fungal isolates from allopatric populations of these host species have previously shown to be differentiated. In sympatric populations of hosts some gene flow is expected, which would act to homogenize the differentiation. On the other hand, if spores from one host species are deposited on the 'alien' host species, this may result in such fitness penalties for the pathogen (i.e. 'secondary reinforcement', Ridley 1993 p. 413) that differentiation is maintained, or can even be increased. Aim of this study was to investigate host-specific differentiation between fungal isolates from *S. latifolia* and *S. dioica* as they appear in allopatric, parapatric and sympatric populations of these host species, in the evolutionary context of host race formation and speciation.

## Genetic differentiation and degree of sympatry

We have found that between fungal isolates from four allopatric host populations of *S. latifolia* and *S. dioica* divergence was pronounced, and revealed

clear and distinct host races for these host species (Chapter 2). In nearly all of the sympatric and parapatric populations that were examined, except for one host population in which both host species grew truly intermingled, we found significant population subdivision with respect to host species as well, exhibiting high values of $F_{ST}$ and $R_{ST}$. Genetically, fungal isolates from interspecific hybrid hosts resembled isolates from *S. latifolia* more than isolates from *S. dioica*. Furthermore, the degree of host sympatry to some extent determined the level of gene flow between samples isolated from the different host categories, and thereby the level of genetic differentiation between the host races. The level of variation among isolates from each of the host species was significantly higher in sympatric/parapatric than in allopatric populations. Also, observed levels of heterozygosity were significantly lower than expected under Hardy-Weinberg equilibrium, confirming the selfing nature of this fungus. The overall levels of heterozygosity were found to be significantly lower in samples from *S. dioica* than in samples from *S. latifolia*. The observed host-related genetic differentiation among these geographically spread populations suggest a long-term divergence between these host races of *M. violaceum* that most likely has evolved in allopatry. In sympatric host populations, both host races presumably come into secondary contact with each other, and host-specific alleles are exchanged depending on the degree of sympatry in the population.

## Impact of host spatial structure on differentiation

We have shown that there was an effect of host spatial structure on the genetic structure of anther smuts in a sympatric population of these host species (Chapter 3). For one of the sporidial colony color loci (SCC), divergence among fungal isolates from *S. latifolia* and *S. dioica* was significantly smaller relative to allopatric populations of hosts. Fungal isolates from allopatric populations of *S. latifolia* are almost fixed for the wild type *pink* allele, and isolates from *S. dioica* are almost fixed for the *yellow* allele. However, in contrast to the previous study using microsatellite loci (in which $F_{ST}$ and $R_{ST}$ were not significantly different from zero), convergence between both host races in sympatry was far from complete. Among fungal isolates from *S. dioica*, the frequency of their 'native' *yellow* allele was 56%, but among isolates from *S. latifolia*, their 'native' *pink* allele was close to fixation, as in allopatric populations. The local host structure, consisting of patches that are mostly dominated by either *S. dioica*, or by *S. latifolia*, had a weakly significant impact on the SCC allele frequencies. This suggested that the anther smut population could be divided into a local deme structure, in which selection and migration might be balanced in

such a way that the overall variation in this SCC locus is maintained. A closer look at the microsatellite genotypes from chapter 2 showed that the more rare alleles were not randomly distributed over the population either, supporting the hypothesis that the patchiness of the host population shapes the genetic structure of the pathogen.

## Mating and competitive ability of strains

Intraspecific competition and mating experiments between strains of the anther smut from both host species were performed to investigate the competitive ability of strains and assortative mating (Chapter 4). It was shown that, in general, strains isolated from *S. latifolia* outcompeted strains isolated from *S. dioica* on both host species (Chapter 4). In female hosts, heterotypic dikaryons had the largest competition success. Furthermore, latency period was significantly shorter in infections that contained strains from *S. latifolia*, compared to homokaryotic infections with a *S. dioica* origin. Strains from *S. latifolia* conjugated in much higher frequencies than strains from *S. dioica*. A significant positive correlation was detected between the relative success rate of strains in competition and in conjugation, suggesting that success of a strain in competition might be partly determined by its swiftness of mating. In addition, reciprocal differences between homotypic and heterotypic crosses revealed a significant effect of fungal mating type, with mating type $a_1$ having a dominant effect on the rate of conjugation.

## Host fidelity of vectors

The role of vectors in effectuating positive assortative mating between strains from the two host species by host fidelity of insect vectors was investigated in a set of experiments in which fluorescent dye was used to trace vector movements over artificial, and fully mixed plots of *S. latifolia* and *S. dioica* (Chapter 5). Different pollinator guilds, mainly bumblebees were recorded to visit *S. dioica* diurnally, and noctuid moths were recorded to visit *S. latifolia* nocturnally. Moreover, both guilds were found to be loyal to their preferred host. Mean rates of interspecific transfer after 24h from *S. latifolia* to *S. dioica* were 26%. From *S. dioica* to *S. dioica* interspecific transfer was 34%. These estimates probably represent the absolute maximum of interspecific visitation between these host species, since natural sympatric populations of these host species have found to be spatially and temporally more heterogeneous. Therefore, the observed visitation pattern of pollinators/vectors might contribute to the maintenance of genetically differentiated host races of the anther smut *M. violaceum* that were observed in sympatric and parapatric populations of these host species.

Furthermore, male hosts were found to be preferentially visited over female hosts. In addition non-linear regression analysis suggested that the range in which the teliospores can be transmitted is probably larger (20-50+m) than the actual infection range (not much larger than 12-13m) of this venereal disease within a single flowering season.

## Host race formation in sympatry?

In this thesis special attention is paid to fungal isolates from *S. latifolia* and *S. dioica* in sympatry, and we have seen that fungal isolates from different host species in sympatry were less differentiated than in allopatry, that the host race from *S. latifolia* outcompeted the race from *S. dioica*, and that spatial (and temporal) structure of host plants and host fidelity of vectors contributed to reproductive isolation between both fungal races in sympatry, although this was far from complete. Since sympatric host race formation was one of the starting points of this study, we still have to answer the question to what extent our data provides evidence for the occurrence of this phenomenon in our model system. In an optimistic scenario, following the sympatric host race formation model of Berlocher (1998a), our model system seems to fit a stage 2 model, in which the races are isolated only by host fidelity with allele frequency differences between the host races. We did detect host-specific alleles (in the microsatellite loci; Chapter 2), yet these were most apparent in more parapatric and allopatric populations of hosts, and not in the true sympatric population. Since there was also no evidence for any mechanism of pre- and/or postzygotic reproductive isolation that is unrelated to host fidelity whatsoever, we can safely conclude that this system has not reached stage 3 yet. However, the most obvious critic to this would be that a much more likely explanation for the evolution of these host races is a more classic allopatric model, in which populations are geographically separated and evolve independent from each other. Especially the strong fitness differences between strains from different host species that we have observed in chapter 4, but also the observations that similar (selectively neutral) genetic variation between the host races is widespread (Chapter 2), and that true sympatric populations of these host species infected with this anther smut are probably not so widespread, makes a more geographical model of host race formation much more feasible (i.e. sympatric host race formation much less feasible) in this model system.

# NEDERLANDSE SAMENVATTING

Theoretische modellen hebben aangetoond dat waardrasvorming in systemen van plantparasieten kunnen leiden tot soortsvorming in gemengde populaties van gastheren onder ecologisch realistische omstandigheden (zie bijv. Dieckmann en Doebeli 1999). Er zijn echter maar weinig voorbeelden van studies die dit hebben laten zien in natuurlijke systemen, en de studies die er zijn, gaan voornamelijk over plantenetende insecten. Dit proefschrift is een van de eerste ecologische studies naar gastheerrassen en waardrasvorming van een schimmelziekte van planten in gemengde populaties van verscheidene gastheersoorten.

Als modelsysteem hebben we de brandschimmel *Microbotryum violaceum* gebruikt, een schimmel die obligaat planten uit de familie der Anjerachtigen parasiteert om zich op deze wijze seksueel te kunnen voortplanten. Dit is een proces waarbij de gastheerplant steriel wordt. We hebben deze schimmel bestudeerd in natuurlijke populaties van *Silene latifolia*, de avondkoekoeksbloem, een tweehuizige, kortlevend-meerjarige plant die voorkomt in open, verstoorde leefmilieus zoals aan de randen van landbouwgronden, en *S. dioica*, de dagkoekoeksbloem, een sterk verwante, eveneens tweehuizige meerjarige plant die voorkomt in meer beschaduwde, vochtige leefmilieus zoals aan de randen van bebossing. Deze schimmel kan wel 10-67% (gemiddeld zo'n 25%) van de planten infecteren in natuurlijke populaties van deze gastheersoorten. Schimmelsporen worden geproduceerd in de helmhokjes van hun gastheren. Een infectie van een vrouwelijke gastheer (een gastvrouw) veroorzaakt een morfologische geslachtsverandering van de plant, waarbij de vruchtbeginsels sterk worden gereduceerd en de in beginsel bij beide seksen aanwezige helmdraden zich ontwikkelen tot volgroeide helmdraden, die met schimmelsporen gevulde helmhokjes bevatten. Schimmelsporen worden verspreid door de natuurlijke bestuivers van hun gastheren, die daardoor voor de schimmel functioneren als vectoren. Dit maakt deze plantenziekte in zekere zin tot een seksueel overdraagbare aandoening van planten.

Tussen schimmelisolaten uit populaties van gastheerplanten waar slechts een van de mogelijke plantensoorten groeit, bestaan grote genetische verschillen die specifiek zijn voor de plantensoort waarin zij groeien. We verwachtten dat er in gemengde populaties van deze gastheersoorten uitwisseling van genetisch materiaal zou kunnen zijn, die de genetische verschillen tussen de schimmelisolaten van verschillende plantensoorten zou kunnen opheffen. Maar, als schimmelsporen in zulke populaties terechtkomen op de voor hun vreemde plantensoort, zou dit ook kunnen

leiden tot sterk verminderde overleving op deze plantensoort, waardoor de verschillen zullen blijven gehandhaafd, of zelfs nog zouden kunnen worden vergroot. Het doel van deze studie was om deze gastheersoort-kenmerkende verschillen tussen schimmelisolaten van avond- en dagkoekoeksbloemen te onderzoeken in populaties waarin beide plantensoorten in verschillende mate zijn gemengd, in een evolutionair kader van waardras- en soortsvorming. Daartoe onderscheiden we drie typen gastheerpopulaties; (1) echt gemengde populaties van beide plantensoorten (sympatrisch), (2) populaties waar beide plantensoorten aangrenzend voorkomen (parapatrisch), en (3) populaties waar maar een van beide plantensoorten voorkomt binnen een straal van enkele kilometers (allopatrisch).

## Genetische verschillen tussen schimmels en de mate van menging

We hebben gevonden dat tussen schimmelisolaten uit vier allopatrische populaties van gastheren de genetische verschillen tussen de isolaten van verschillende gastheren erg groot waren voor een aantal selectief neutrale genetische merkers (in dit geval microsatellieten, stukjes repeterend DNA die op veel plaatsen in het DNA voorkomen, en die opmerkelijk veel variatie vertonen die veelal niet voor- of nadelig is voor het organisme). Dit bevestigde dat we op grond van de genetische verschillen met recht kunnen spreken van verschillende gastheerrassen voor de brandschimmels van beide soorten planten (Hoofdstuk 2). In bijna alle sympatrische en parapatrische populaties van planten die wij onderzochten, behalve in één populatie waarin beide plantensoorten op korte afstand van elkaar, en echt door elkaar groeiden, vonden we eveneens genetische verschillen met betrekking tot gastheersoort, die echter afnam met de mate van menging van beide gastheersoorten. Gezien hun genetische samenstelling, leken de schimmels die werden geïsoleerd uit hybride planten (dit zijn kruisingen tussen avond- en dagkoekoeksbloemen) meer op de schimmels die waren geïsoleerd uit de avondkoekoeksbloem dan op de schimmels die waren geïsoleerd uit de dagkoekoeksbloem. Tevens was de mate van heterozygotie (dit is de mate waarin binnen een individu verschillende vormen van een gen voorkomen in het DNA (maximaal twee in een diploïd organisme)) veel lager dan zou kunnen worden verwacht op grond van de hoeveelheid genetische variatie die in een populatie aanwezig was. Hiermee werd nog eens bevestigd dat deze schimmel zich voornamelijk door zelfbevruchting voortplant, iets dat al bekend was uit andere studies over deze schimmel. De mate van heterozygotie in schimmelisolaten uit dagkoekoeksbloemen was lager dan in isolaten uit avondkoekoeksbloemen.

We hebben deze gastheerspecifieke genetische verschillen tussen isolaten uit de beide gastheren gevonden in een aantal populaties, die geografisch gezien nogal ver uit elkaar liggen. Dit deed ons vermoeden dat deze verschillen tussen de gastheerrassen reeds lange tijd zouden kunnen bestaan, en dat deze rassen het meest waarschijnlijk zijn ontstaan in allopatrische populaties, de populaties van planten van maar één gastheersoort. We veronderstellen dat in de meer gemengde populaties van de twee gastheersoorten de beide gastheerrassen opnieuw met elkaar in contact komen, nadat ze eerst in allopatrische populaties genetisch uit elkaar zijn gegroeid. In deze meer gemengde populaties kunnen dan de gastheerspecifieke allelen (dit zijn vormen van een gen, of van een specifieke plaats in het DNA van een individu) worden uitgewisseld, wat afhankelijk lijkt te zijn van de mate van menging.

## De invloed van ruimtelijke structuur op de genetische structuur

We tonen aan dat de ruimtelijke structuur van de gastheerpopulatie een effect heeft op de genetische structuur van de schimmelpopulatie (Hoofdstuk 3). Van een bepaald gen, dat een rol speelt bij synthese van bètacaroteen, een molecuul dat bijvoorbeeld kleur geeft aan tomaten en wortels en een rol speelt bij de synthese van vitamine A (retinol) in mensen, bestaan in deze schimmel verscheidene vormen. Schimmelisolaten uit allopatrische gastheerpopulaties van avondkoekoeksbloemen, kleuren bijna allemaal roze wanneer deze groeien op een kunstmatig voedingsmedium in een plastic schaaltje, terwijl schimmelisolaten uit allopatrische gastheerpopulaties van dagkoekoeksbloemen bijna allemaal geel kleuren. Echter, in tegenstelling tot hetgeen we zagen met betrekking tot de genetische verschillen in selectief neutrale merkers waar er bijna geen verschillen meer waren tussen schimmelisolaten van verschillende gastheren in de meest gemengde populatie van gastheren, zien we voor deze kleurmerker nog wel gastheerspecifieke variatie. Isolaten van avondkoekoeksbloemen waren weliswaar nog steeds bijna helemaal roze, maar grofweg de ene helft van de isolaten van dagkoekoeksbloemen bleek roze, de andere helft geel. De locale ruimtelijke structuur van gastheerplanten, bestaand uit groepjes planten met voornamelijk avondkoekoeksbloemen en groepjes planten met voornamelijk dagkoekoeksbloemen, bleek van invloed op de genetische structuur van schimmelisolaten. Isolaten afkomstig uit dagkoekoeksbloemen waren meer ‘geel’ in groepjes planten met voornamelijk dagkoekoeksbloemen dan in groepjes met voornamelijk avondkoekoeksbloemen. Omgekeerd, schimmelisolaten afkomstig uit avondkoekoeksbloemen waren meer ‘roze’ in groepjes met voornamelijk avondkoekoeksbloemen dan in groepjes met voornamelijk dagkoekoeksbloemen.

Hieruit kunnen we concluderen dat de totale populatie schimmels waarschijnlijk niet bestaat uit één grote panmictische populatie waarbinnen paring tussen individuele schimmelstammen willekeurig plaatsvindt, maar uit een aantal kleinere subpopulaties die ieder worden gevormd door een groepje gastheerplanten die bij elkaar staan. Binnen deze subpopulaties wisselen brandschimmels frequent genetische informatie uit door kruising, maar tussen deze sub populaties gebeurt dit veel minder. De variatie in selectief neutrale merkers uit hoofdstuk 2 is in deze populatie van gastheren tevens in kaart gebracht, waarbij duidelijk werd dat de verdeling van allelen niet geheel willekeurig was. Dit ondersteunt de hypothese dat de genetische structuur van deze schimmels wordt beïnvloed door de ruimtelijke structuur van deze twee gastheersoorten.

## Kruising en competitieve sterkte van schimmelstammen

We hebben competitie- en kruisingsexperimenten tussen schimmelstammen van beide gastheersoorten uitgevoerd, om te onderzoeken of kruisingen tussen stammen afkomstig van dezelfde gastheersoort wellicht hun eigen plantensoort beter kunnen infecteren dan kruisingen tussen stammen afkomstig van verschillende gastheersoorten (Hoofdstuk 4). De resultaten hiervan zijn dat in het algemeen de stammen afkomstig van avondkoekoeksbloemen het veel beter doen in competitie dan de stammen afkomstig van dagkoekoeksbloemen, ongeacht op welke gastheersoort zij zich bevonden. Verder zien we dat kruisingen waarbij stammen afkomstig van avondkoekoeksbloemen bij betrokken zijn in veel hogere frequenties tot paring blijken te komen in één etmaal, dan kruisingen met stammen die louter afkomstig zijn van dagkoekoeksbloemen. Dit suggereert dat het paringsproces tussen stammen afkomstig van avondkoekoeksbloemen wellicht veel sneller verloopt dan tussen stammen afkomstig van dagkoekoeksbloemen. Echter, in competitie in vrouwelijke gastheren, doen kruisingen tussen stammen afkomstig van verschillende gastheersoorten het juist beter dan kruisingen tussen stammen van dezelfde gastheersoort, iets dat voornamelijk in avondkoekoeksbloemen naar voren komt. De latentietijd (dit is de tijd tussen het in contact komen van de schimmel met de plant tot aan het produceren van een nieuwe generatie schimmelsporen) is tevens veel langer voor schimmelstammen afkomstig van dagkoekoeksbloemen, ongeacht de gastheersoort. Er blijkt een verband te bestaan tussen de snelheid van paring van een schimmelstam in het paringsexperiment en zijn succes in het competitie-experiment. Dit suggereert dat het succes van een stam in competitie grotendeels zou kunnen worden verklaard door zijn snelheid van paren. Dit grote verschil in competitieve sterkte tussen stammen impliceert eveneens, dat als er

veel uitwisseling zou bestaan tussen stammen van beide gastheren in gemengde populaties van beide gastheersoorten, de gastheerspecifieke genetische variatie van dagkoekoeksbloemen wel eens snel zou kunnen verdwijnen.

## Gastheergetrouwheid van bestuivers

Deze schimmel wordt verspreid door de natuurlijke bestuivers van zijn gastheren, wat deze schimmelziekte in wezen maakt tot een seksueel overdraagbare aandoening van planten. Gastheergetrouwheid van deze bestuivers, of vectoren, kunnen een belangrijke rol spelen bij het genetisch verschillend zijn van schimmelisolaten van verschillende gastheersoorten, en de handhaving hiervan. Dit is onderzocht in een aantal experimenten waarbij beide gastheren kunstmatig door elkaar zijn gezet, en waarin met fluorescerend poeder de bewegingen van bestuivers / vectoren is gevolgd gedurende een etmaal, of gedurende de dag (Hoofdstuk 5). Het blijkt dat beide gastheersoorten hun eigen bestuiversgilde kennen, bestaand uit nachtvlinders (in het bijzonder nachtuiltjes) die in de schemer (en in minder mate ook ’s nachts) voornamelijk avondkoekoeksbloemen bezoeken, en bestaand uit hommels en zweefvliegen die overdag voornamelijk dagkoekoeksbloemen bezoeken. Bovendien blijken deze vectoren vrij kieskeurig in het bezoeken van beide plantensoorten, zelfs als deze volledig door elkaar staan zoals in onze experimenten. Het aantal bezoeken tussen gastheersoorten was echter nog wel dusdanig hoog, zo’n 26% van avondkoekoeksbloemen naar dagkoekoeksbloemen en zo’n 34% in de tegengestelde richting, dat dit op zichzelf niet verklaart waarom schimmelstammen met het voor dagkoekoeksbloemen kenmerkende genetische profiel nog bestaan in gemengde populaties, noch waarom stammen afkomstig van beide gastheersoorten in gemengde populaties genetisch nog van elkaar verschillen. Natuurlijke gemengde populaties van beide gastheersoorten blijken echter niet zo mooi homogeen gemengd als in deze experimenten, maar zijn gestructureerd, zowel ruimtelijk (Hoofdstuk 3) als in de tijd. Er bestaan namelijk verschillen in bloeitijd tussen beide gastheersoorten, en ook tussen beide gastheergeslachten, waarbij dagkoekoeksbloemen eerder bloeien dan avondkoekoeksbloemen, en mannelijke planten eerder en langer dan vrouwelijke. Hierdoor verwachten we dat in dergelijke populaties het aantal bezoeken tussen de gastheersoorten veel kleiner is dan werd geschat in deze experimenten, en is wellicht klein genoeg om beide schimmelisolaten effectief gescheiden te houden. Hierdoor zouden gastheerspecifieke genetische verschillen kunnen blijven gehandhaafd.

## Waardrasvorming in gemengde populaties?

In dit proefschrift wordt de meeste aandacht besteed aan schimmelisolaten afkomstig uit avondkoekoeksbloemen en dagkoekoeksbloemen in gemengde populaties van gastheren. We hebben gezien dat schimmelisolaten afkomstig uit verschillende gastheersoorten in gemengde populaties genetisch minder van elkaar verschillen dan in allopatrisch populaties, dat de schimmelstammen afkomstig van avondkoekoeksbloemen competitief beter presteren dan schimmelstammen van dagkoekoeksbloemen, en dat de ruimtelijke (en de temporele) structuur van gastheer populaties en de gastheergetrouwheid van vectoren bijdragen aan de reproductieve isolatie tussen beide gastheerrassen, al blijkt dit laatste verre van volledig. Een van de oorspronkelijke doelstellingen van deze studie was het onderzoeken van waardrasvorming in gemengde populaties van gastheren. We hebben echter nog niet beantwoord in welke mate de verkregen data nu aanwijzingen leveren voor het voorkomen van dit fenomeen in dit modelsysteem. In een optimistisch scenario, het vier-stappenplan voor sympatrische waardrasvorming van Berlocher (1998a) volgend, zou ons modelsysteem een plaats kunnen krijgen in stap 2. In deze fase zijn beide waardrassen gescheiden door getrouwheid van vectoren (Hoofdstuk 5), en bestaan er genetische verschillen tussen de rassen, inclusief het bestaan van voor het gastheerras kenmerkende allelen (Hoofdstukken 2 en 3). Deze verschillen worden echter voornamelijk gevonden in de meer parapatrische (minder gemengde) populaties van gastheerplanten, en niet in de echt gemengde natuurlijke populatie. Vanwege het feit dat we ook geen mechanismen hebben kunnen aantonen die bijvoorbeeld het kruisen van beide rassen onmogelijk zouden maken, die anders zijn dan de getrouwheid van vectoren, bevindt de waardrasvorming zich zeker niet in een volgende fase volgens dit stappenplan. De meest voor de hand liggende kritiek op bovenstaand scenario is echter dat het ontstaan van deze waardrassen veel eenvoudiger is te bewerkstelligen in allopatrie. Zeker gezien de grote verschillen in competitieve sterkte van beide gastheerrassen (Hoofdstuk 4), en de wijdverbreide variatie in selectief neutrale microsatelliet merkers, waarbij steeds dezelfde allelen worden gevonden voor elk van de beide gastheersoorten in geografisch nogal verspreide populaties in West Europa (Hoofdstuk 2), terwijl geïnfecteerde, gemengde populaties van beide gastheersoorten helemaal niet zo wijdverbreid zijn. Dit maakt een meer geografisch gescheiden model voor de evolutie van deze gastheerrassen in dit modelsysteem aannemelijker.

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# CURRICULUM VITAE

Pim van Putten werd geboren 24 december 1971 te Lelystad. Van 1979-84 doorliep hij christelijke basisschool 'Prins Willem Alexander' in Elburg. Hierna bezocht hij protestants christelijke scholengemeenschap 'Lambert Franckens College' in Elburg, alwaar hij in mei 1990 eindexamen VWO B deed. Aansluitend werd begonnen met de studie Biologie aan de Rijksuniversiteit Groningen. In augustus 1996 studeerde hij af als bioloog met als afstudeerrichting populatiegenetica. Gedurende deze afstudeerfase verrichtte hij onderzoek aan de effecten van homozygotie op de stressgevoeligheid in *Drosophila melanogaster*, onder supervisie van dr. R. Bijlsma, en aan breedtegraadvariatie in vleugellengte in *D. melanogaster*, onder supervisie van dr. J. van 't Land. Voor deze laatste studie werden in februari en maart 1995 door hem en dr. Van 't Land fruitvliegen verzameld in Chili, in samenwerking met dr. H. Villarroel en de Universiteit van Playa Ancha te Valparaíso, Chili. In een derde afstudeeronderwerp in Groningen werd door hem de hoeveelheid genetische variatie in deze Zuid-Amerikaanse populaties fruitvliegen onderzocht met behulp van RAPDs, onder supervisie van dr. L. van de Zande.

Vanaf maart 1997 was hij werkzaam als onderzoeker in opleiding bij de werkgroep Populatiebiologie van Planten van het Nederlands Instituut voor Ecologisch Onderzoek, centrum voor Terrestrische Ecologie (NIOO-CTO) te Heteren, alwaar het promotieonderzoek werd uitgevoerd dat is beschreven in dit proefschrift.

Vanaf december 2001 is hij werkzaam als postdoc bij de vakgroep populatiegenetica van de Rijksuniversiteit Groningen, alwaar hij werkt aan het evolutionair aanpassingsvermogen van genetisch verarmde (ingeteelde) populaties van *D. melanogaster* onder veranderende omgevingsomstandigheden.

# LIST OF PUBLICATIONS

## Pre-thesis work:


**BIJLSMA R, J BUNDGAARD, AC BOEREMA and WF VAN PUTTEN** (1997) Genetic and environmental stress, and the persistence of populations, pp. 193-208 in *Environmental Stress, Adaptation and Evolution*, edited by R. Bijlsma and V. Loeschcke. Birkhäuser, Basel.

**BIJLSMA R, J BUNDGAARD and WF VAN PUTTEN** (1999) Environmental dependence of inbreeding depression and purging in *Drosophila melanogaster*. Journal of Evolutionary Biology **12:** 1125-1137.

**VAN 'T LAND J, P VAN PUTTEN, B ZWAAN, A KAMPING and W VAN DELDEN** (1999) Latitudinal variation in wild populations of *Drosophila melanogaster*: heritabilities and reaction norms. Journal of Evolutionary Biology **12:** 222-232.

**VAN 'T LAND J, WF VAN PUTTEN, H VILLARROEL, A KAMPING and W VAN DELDEN** (2000) Latitudinal variation for two enzyme loci and an inversion polymorphism in *Drosophila melanogaster* from Central and South America. Evolution **54:** 201-209.


## Submitted for publication:


**VAN PUTTEN WF, A BIERE and JMM VAN DAMME** (2002a) Host-related genetic differentiation in the anther smut fungus *Microbotryum violaceum* in sympatric, parapatric and allopatric populations of two host species *Silene latifolia* and *S. dioica*. *Submitted to Molecular Ecology*. **(Chapter 1)**

**VAN PUTTEN WF, A BIERE and JMM VAN DAMME** (2002b) Intraspecific competition and mating between fungal isolates of the anther smut *Microbotryum violaceum* from the host plant *Silene latifolia* and *S. dioica*. *Submitted to Evolution*. **(Chapter 3)**

**VAN PUTTEN WF, JA ELZINGA, A BIERE and JMM VAN DAMME** (2002c) Host fidelity of the pollinator guilds of *Silene dioica* and *S. latifolia*; possible consequences for host race differentiation of a venereal disease in sympatry. *Submitted to Oecologia*. **(Chapter 4)**

The research described in this thesis, NIOO-thesis number 13, was conducted at the department of Plant Population Biology, Center for Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-CTO) in Heteren, The Netherlands. The Netherlands Institute of Ecology is one of the institutes of the Royal Netherlands Academy of Arts and Sciences (KNAW). This study was financially supported by the Earth and Life Science Foundation of the Netherlands Organization for Scientific Research (NWO-ALW); grant 805-36-391.