Differences in population structure of the anther smut fungus *Microbotryum violaceum* on two closely related host species, *Silene latifolia* and *S. dioica*

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Abstract

We investigated the genetic population structure of the sexually transmitted plant pathogen, the fungus *Microbotryum violaceum*, on the two closely related host species *Silene latifolia* and *S. dioica* using microsatellite markers. We found strong deviations from Hardy–Weinberg expectations, with significant heterozygote deficiency in almost all populations. Fungal strains from the two host species were differentiated, and these host races differed in amount of variation within populations and differentiation among populations. Anther smut from *S. latifolia* harboured significantly less microsatellite diversity and were more genetically differentiated from each other than those from *S. dioica*. Small effective population sizes, rapid population turnover, and less gene flow among populations could lead to this higher population differentiation and lower within population genetic diversity for anther smut populations on *S. latifolia* than on *S. dioica*. These results are in concordance with host ecology because *S. latifolia* grows in more disturbed habitats than *S. dioica* and may provide a shorter-lived host environment.

Keywords: breeding system, host-race differentiation, microsatellites, parasite-host, plant-pathogen, sexually transmitted disease

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Introduction

Knowledge of host and parasite population structure is important for investigating the ecology and evolution of the interacting partners (Thompson 1994; Gandon et al. 1996). In the host-pathogen system of *Silene latifolia* and the anther smut fungus *Microbotryum violaceum* host population structure has been investigated in some detail (Alexander 1989; McCauley 1994; McCauley et al. 1995), while information on pathogen population structure is largely lacking (but see Delmotte et al. 1999). This is due to little fungal variation both in virulence (Alexander & Maltby 1990; Alexander et al. 1993) and at isozyme loci (Antonovics et al. 1995). Because *M. violaceum* from *S. latifolia* was studied mainly in North America, where the host plant was introduced in the early 19th century (McNeill 1977), absence of variation may result from a genetic bottleneck during its introduction (Alexander et al. 1993). In Europe, where both plant and pathogen are native, pathogen population structure and levels of genetic diversity among fungal strains should be higher than in North America. Therefore, in order to investigate genetic population structure of this species we chose to work on populations from the original native range of hosts and pathogens.

The fungus *M. violaceum* parasitizes a range of host plants of the family Caryophyllaceae (Thrall et al. 1993). The sister species *S. latifolia* and *S. dioica* and their respective fungi have been studied for population and metapopulation dynamics (Alexander & Antonovics 1988; Carlsson & Elmqvist 1992; Antonovics et al. 1994; Biere & Honders 1998), host race formation and specialization (Biere & Honders 1996), and local adaptation (Kaltz et al. 1999). Population structure will influence and be influenced by these phenomena, and deviations from Hardy–Weinberg expectations of genotypes within populations can give indirect indications about reproductive system, such as
the amount of inbreeding. Therefore, in this paper we investigate and compare the population structure of native central European populations of the fungus on these two host species using microsatellite markers (Bucheli et al. 1998), which are known to be highly variable (Schlötterer & Pemberton 1994).

Because *S. dioica* and *S. latifolia* are very closely related, hybridizing freely and forming extensive hybrid swarms (Goulson & Jerrim 1997), their anther smut fungi were not assumed to be host-specifically differentiated (Liro 1924). Further, the two anther smuts also hybridize, although no evidence for host-specific differentiation for life history differences and host exploitation ability between *M. violaceum* strains from *S. latifolia* and *S. dioica* (Biere & Honders 1996). Here we use microsatellite variation at five loci to investigate the existence of genetic differentiation between fungal strains from *S. latifolia* and *S. dioica* and differences in their respective population structure to better understand the potential for host specialization between these fungal populations through gene flow and recombination.

**Materials and methods**

**Study organisms**

The anther smut fungus *Microbotryum violaceum* (Pers.) Dem. & Oberwinkler [= *Ustilago violacea* (Pers.) Fuckel] is a sexually transmitted pathogen of many species of the family Caryophyllaceae (Liro 1924). Infected plants bear anthers that contain spores instead of pollen and reduced or aborted ovaries. In dioecious species such as *Silene latifolia* and *S. dioica*, the fungus induces a phenotypic sex change in infected female plants (Baker 1947b). These produce spore-filled anthers, while the ovaries are aborted and the stigmas are reduced as in hermaphrodites. During the vegetative stage of the plant the fungus grows endophytically and remains concealed (Audran & Batcho 1980). It may decrease plant longevity to some degree (Thrall 1998). Alternatively, the initial infection may be of low intensity and for two large populations of *S. latifolia* from which approximately 30 individuals were screened. Diseased individuals are often aggregated within population, and we attempted to collect spores from only one individual per diseased clump to avoid collecting the same genetic individual. The term 'individual' denotes fungus collected from one host plant, the term 'population' denotes the fungi from one host species at one locality. Flower buds were dried and teliospores were shaken into microcentrifuge tubes and stored at room temperature.

DNA was extracted directly from teliospores following the Chelex© extraction method (Walsh et al. 1991). For both methods, teliospores were frozen in liquid nitrogen and stored in microcentrifuge tubes. When extracting with Chelex©, ground spores were immediately mixed with a boiling 5% Chelex© suspension and incubated at 93 °C for 10 min. Then samples were put on ice, spun down for
2 min at 10 000 g and stored at −20 °C. Both extraction methods gave the same results.

Microsatellite analysis

Five microsatellite loci identified from a *M. violaceum* (from *S. latifolia*) library were used (Bucheli et al. 1998). Polymerase chain reactions (PCRs) were set up in 10 μL volumes, each containing 10–50 ng of DNA, 1× Promega reaction buffer, 1.5 mM MgCl₂, 0.6 mM dNTP, 0.4 unit of Promega *Taq* polymerase enzyme, 2 pmol α-32P-end-labelled primer, 20 pmol each forward and reverse primer, and 10 μL sterile distilled H₂O. End-labelling was performed using T4 polynucleotide kinase (Promega) following standard protocols. After a first step of denaturation at 94 °C for 2 min, 30 cycles of amplification were performed (30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C). The last elongation step was lengthened to 2 min. PCR

Table 1: Locations of *Microbotryum violaceum* populations from *Silene dioica* and *S. latifolia*. Elevation is given in m above sea-level. Sample size is the number of individuals collected from one site, while the term ‘individual’ denotes fungus collected from one host plant. Population characteristics were not available for some populations, denoted by a ?, because the samples were provided by colleagues who did not estimate population sizes or infection prevalence.

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products were electrophoresed in sequencing gels (6% polyacrylamide, 5 mM urea). An M13 control sequencing reaction was run next to each locus for sizing of alleles. Visualization was carried out by autoradiography.

**Statistical analyses**

Allele frequencies, observed heterozygosities, unbiased estimates of expected heterozygosities under Hardy–Weinberg assumptions (Levene & Weir 1984) and unbiased estimates of fixation indices (Weir & Cockerham 1984) were calculated for each site and locus using GENOP (Raymond & Rouzé 1995). Means and standard errors of sample sizes, number of alleles, observed and unbiased expected heterozygosities over all loci were calculated for each population using BIOSYS (Swofford & Selander 1989). Deviations from Hardy–Weinberg expectations per locus and means per population were tested by Fisher’s exact test based on the Markov chain method (Guo & Thompson 1992) performed by GENEPOP.

Conventional $F$-statistics were computed using FSTAT (Goudet 1995), a program that calculates Weir & Cockerham’s (1984) unbiased estimators of $F$-statistics. Confidence intervals and tests for departure from zero for $F_{ST}$, $F_{IS}$, and $F_{IT}$ were calculated by permutation. For the estimation of the confidence interval (C.I.) of $F_{ST}$ and $F_{IS}$, and for testing their departure from zero, alleles were permuted within populations and among populations, respectively. For the same statistics of $F_{ST}$, multilocus genotypes were permuted among populations, because $F_{ST}$ was significantly different from zero and alleles could not be considered independent (Goudet 1995). For the estimation of genetic differentiation between *M. violaceum* on *S. dioica* vs. *S. latifolia* ($F_{ST}$ for *S. dioica*) populations were pooled within host species.

$F$-statistics that take allele size differences into account were computed with the help of ARLEQUIN (Schneider et al. 1997), a program that applies analysis of molecular variance (AMOVA) (Excoffier et al. 1992), to microsatellite data to obtain $\Phi_{ST}$, $\Phi_{IS}$ is an analogue of the $K_{ST}$ statistic defined by (Slatkin 1995) as described in Michalakis & Excoffier (1996). The significance of the fixation index is tested by randomly permuting whole haplotypes (Excoffier et al. 1992; Michalakis & Excoffier 1996).

To test for isolation by distance for fungal strains on both of the host plant species separately, a positive correlation between each pairwise $\Phi_{ST}$ matrix with its respective geographical distance matrix was tested using a Mantel Test (Mantel 1967) provided with the GENEPOP package. To test effects of geographical distance, pairwise difference in elevation between populations, and host plant species on genetic differentiation estimated as pairwise $\Phi_{ST}$, a multiple Mantel test employing 1000 permutations were performed. Initially a full model including the three main effects and all interactions was fit to the data, followed by stepwise model simplification, removing first the three-way interaction, then the least significant two-way interactions. Main effects were only removed if they were not included in significant interaction effects. Factors were considered significant if fewer than 2.5% of the permutations revealed values either higher or lower than the real observed value.

Genetic distances were calculated with the help of MICROsat (Minch et al. 1995), a program that calculates $\Delta$ (delta $\mu$) a distance measure especially developed for microsatellite data. $\Delta$ is the squared mean difference between alleles of two populations $\Delta = \mu(A) - \mu(B)$ where $\mu(A)$ is the mean allele size for population A. $\Delta$ has been shown to be linear with time (Goldstein et al. 1995).

**Results**

**Population structure of Microbotryum violaceum within host species**

Table 2 shows mean number of alleles and observed and expected heterozygosities for each population. Mean allelic diversity and expected heterozygosity values were low for *Microbotryum violaceum* from *Silene dioica* and *S. latifolia* (Table 2). Most populations exhibited highly significant heterozygote deficiencies (Table 2), probably indicating introgression, though three anther smut populations from *S. latifolia* lacked variation at all loci and, therefore, could not be tested for deviations from Hardy–Weinberg proportions (Table 2), and the population St. Louis from *S. latifolia* with very little variation did not deviate from Hardy–Weinberg proportions. Nevertheless, $F_{IS}$ values over all populations were high and differed significantly from zero in *M. violaceum* from both host species (Table 3). Two measures of genetic diversity, mean expected heterozygosity and allelic diversity, were significantly higher in *M. violaceum* from *S. dioica* than in those from *S. latifolia* (Table 4).

Genetic differentiation among populations was substantial for *M. violaceum* from both *S. latifolia* and *S. dioica*. Both coefficients of genetic differentiation, conventional $F_{ST}$ (Table 3) and AMOVA based on $\Phi_{ST}$ (Table 5), indicated a significant and large amount of genetic differentiation within the two host-specific groups of populations. However, anther smut populations from *S. latifolia* were more genetically differentiated from each other than those from *S. dioica*, though this comparison is based on only a few loci (Table 3). Neither anther smut populations from *S. dioica* nor those from *S. latifolia* were isolated by distance because there were no correlations of pairwise genetic distances, estimated as $\Phi_{ST}$, with geographical distances (*S. latifolia*: Mantel’s test, $a = 0.799$, $b < 0.001$, one-tailed Spearman rank correlation $P = 0.690$; *S. dioica*: Mantel’s test, $a = 0.100$, $b < 0.001$, one-tailed Spearman rank correlation $P = 0.285$–294

Table 2: Mean sample size, mean number of alleles per locus, and observed and expected heterozygosities averaged over five microsatellite loci of Microbotryum violaceum populations by locality. Data are given with standard errors. Heterozygote deficiencies (HD) were tested on multilocus data using Fisher’s exact tests. Unbiased estimates of P-values are based on the Markov chain method.

Table 3: Conventional F-statistics of anther smut populations from Silene dioica and Silene latifolia. 95% confidence intervals (values in parentheses) and the probability that FST equals zero was calculated; for FST by permuting alleles within populations, for FST by permuting alleles within total, and for FST by permuting multilocus genotypes among populations.

Table 4: Analysis of covariance of allelic diversities and expected multilocus heterozygosities (HET) of Microbotryum violaceum populations from Silene latifolia vs. S. dioica. Mean sample size was used as a covariate. The interaction between mean sample size and host species was far from significant and therefore excluded from the model. Allelic diversity: R = 48%, HET = 58%.

Table 5: Hierarchical AMOVA using the Φ-statistic by Michalakis & Excoffier (1996) for anther smut populations from Silene latifolia and S. dioica. Probabilities that Φ values differed from zero were calculated by permuting whole haplotypes (1000 permutations) by distance. Nevertheless, the tendency for isolation by distance was greater for M. violaceum from S. latifolia than that from S. dioica. Furthermore, because one locus was fixed for the same allele across all populations and another showed low variation, these results, based on fewer than five polymorphic loci, must be interpreted with caution.

Genetic differentiation of fungal populations on S. latifolia vs. S. dioica

We found significant differentiation between anther smut from the two host species S. latifolia and S. dioica (Tables 3 & 5), generated by private alleles and no overlap in allele sizes at two loci. At another locus, only populations from S. dioica at Maderanertal (location 3) and Braunwald (location 4) shared one allele with the population Grosio (location 7) from S. latifolia south of the Alps, while the remaining four populations from S. dioica had alleles that were much larger than those collected from S. latifolia.

These differences in the distribution of alleles between M. violaceum from the two host species are reflected both in conventional F-statistics that estimate population differentiation as deviations from global Hardy–Weinberg expectations (Cockerham 1973) and in the hierarchical AMOVA, which also takes allele size differences, assuming a stepwise mutation model for microsatellite mutations, into account (Michalakis & Excoffier 1996). Indeed, measurements of the distribution of genetic variation as estimated by conventional F-statistics and the AMOVA using FST gave similar results (Tables 3 & 5). Both coefficients of genetic differentiation, respectively, FSL-SD (Table 3) and FSL-SD (Table 5), indicate a significant and large amount of genetic differentiation between the populations from S. latifolia vs. those from S. dioica.

In the neighbour-joining analysis of the pairwise genetic distance Δ (Goldstein et al. 1995) anther smut populations from S. dioica cluster together and are strictly separated from those from S. latifolia (Fig. 3). This separation is confirmed by the multiple Mantel test for the effects of geographical distance, host plant species and elevation of the populations on pairwise FST. After stepwise model simplification, we found a significant main effect of host plant species, with none of the 1000 permutations revealing a value as extreme as the observation. A significant interaction between host plant species and geographical distance between the populations, was also found, with only 13 out of 1000 permutations having a value as extreme as the observation. Elevation was not significant either as main effect or in any interaction effects and was hence removed from the final model. Therefore, although the two host species grow at different elevations (Table 1, Mann–Whitney test, U = 39.5, U0.05(1),6,8 = 38, P < 0.05), elevation does not appear to influence genetic differentiation.
Discussion

Genetic diversity and mating system of Microbotryum violaceum

Similar to the absence of variation in virulence (Alexander & Maltby 1990; Alexander et al. 1993; Biere & Antonovics 1996) and isozymes (Antonovics et al. 1995) within anther smut populations on Silene latifolia in North America, we found very little microsatellite variation within fungal populations on S. latifolia in Switzerland. Indeed, microsatellite variation measured as mean number of alleles per locus (1.9 for populations from S. dioica and 1.3 for those from S. latifolia) (Table 2), was very low compared to that in other eukaryotic organisms (Chapuisat 1996; Chase et al. 1996; Neumann & Wetton 1996; Powell et al. 1996), except endangered species whose populations have experienced bottlenecks (e.g. Gottelli et al. 1994; Taylor et al. 1994). Although data on microsatellite diversity in fungi are scarce, Tenzer (1998) found high allelic diversity in the apple tree pathogen Venturia inaequalis. Therefore, low levels of microsatellite diversity in Microbotryum violaceum may be due, at least in part, to inbreeding as suggested by the high and significant values for $F_{IS}$. Inbreeding reduces genetic diversity by reducing effective population size which increases the risk of allele loss through drift (Abbott & Gomes 1989), and because of hitchhiking of neutral markers with selected loci (Hedrick 1980), or background selection against deleterious mutations (Charlesworth et al. 1993).

Significant $F_{IS}$ and $F_{ST}$ values for anther smut from both S. latifolia and S. dioica (Tables 3 & 5), suggest that M. violaceum is predominantly inbreeding, although other factors, such as structure within sampling units can lead to significant $F_{ST}$. These results corroborate those of (Baird & Garber 1979), who recovered only 6.6% outcrossing events after inoculating plants of S. latifolia with combinations of teliospore genotypes bearing colony colour and auxotrophic markers. Indeed, mixtures of sporidia of opposite mating type conjugate more readily in selfing than outcrossing combinations (Kaltz & Shykoff 1999), and early intrameiotic conjugations within the germinated prothalus commonly occur (Hood & Antonovics 1998), both mechanisms that favour inbreeding. Several populations from S. latifolia exhibited no variation and, thus, $F_{IS}$ could not be calculated (Table 2). These fungi have no opportunity for true outcrossing unless genetically different strains are imported from other populations because intrapopulation outcrossing events would involve only intrACLonal outcrossing (Hebert et al. 1993). In such cases, effective inbreeding is increased by the lack of available genetically different partners.

Differences between the two host races in population structure

M. violaceum from S. latifolia showed less microsatellite variation and higher differentiation among populations than that from S. dioica (Tables 3 & 5; Fig. 2). Ecological differences between the host species may account for these differences in population structure. In this study,
S. latifolia was found on roadsides, waste places and field margins, probably short-lived habitats prone to high extinction and recolonization rates (Antonovics et al. 1994; McCauley 1994), and possible human influence on dispersal via mowing of road verges (McCauley 1997). S. dioica, on the other hand, was found in sparse woodlands, rich meadows and in stands of tall herbaceous perennials and shrubs, and thus may provide a longer-lived habitat with larger, more stable host populations, allowing genetic variation in the pathogen to accumulate over time by mutation and migration. Further, the metapopulation dynamics of S. dioica and its M. violaceum host may be more stable, with fewer extinction and recolonization events than observed for S. latifolia/M. violaceum (Carlsson et al. 1990). The distribution of genetic variation in M. violaceum will also be influenced by activity, behaviour and migration rates of flower visitors. Indeed, the two host species have different pollinator faunas, which certainly differ in behaviour and flight distance, hence the patterns of deposition of fungal spores as well as pollen. S. latifolia is largely moth-pollinated (McNeill 1977), while S. dioica is visited by diurnal pollinators such as bumble bees (Baker 1947a). These differences in patterns of pollen and spore dispersal may account for the significant interaction effect between geographical distance and host plant species on pairwise $F_{ST}$ found by the multiple Mantel test.

The anther smut host races also differed in allele size, with S. dioica fungi harbouring considerably larger alleles than those from S. latifolia at two of the five microsatellite loci studied (Table 2). This is surprising because fungi from S. latifolia served as source organism for microsatellite development, and allele sizes and levels of polymorphism usually decrease with phylogenetic distance from the species used to make the genomic library (Moore et al. 1991; Ellegren et al. 1995). Recently Amos (1997) suggested that microsatellite allele size, like allelic diversity, increases with increasing population size and a history of population expansion. Therefore, longer alleles in the host race from S. dioica may also suggest possible past population expansion or larger effective population sizes than the host race of S. latifolia, as already implied by the higher genetic diversity. However, these population differences were not seen at the time of sampling (Table 1).

**Host race formation on S. dioica and S. latifolia**

Differences in the distribution and sizes of microsatellite alleles between M. violaceum and its M. violaceum appendix S. dioica and S. latifolia indicate separate ‘host races’ on these two host species, as suggested by Zillig (1921) and Biere & Honders (1996). Although fungal strains are able to infect the other host species, they have higher performance on their host species of origin, measured as higher spore production and stronger stimulation of flower production (Biere & Honders 1996). Thus, variation of both neutral markers and selected traits indicate genetic differentiation of M. violaceum on S. latifolia and S. dioica.

In this study the geographical distribution of fungal populations from S. latifolia and S. dioica did not overlap, with infected populations of S. dioica only occurring at high elevation. Thus, we cannot rule out that genetic differentiation was related to elevational differences and was not only host-specific, although the multiple Mantel test revealed a main effect of only host species with no effect of elevation on genetic differentiation (pairwise $F_{ST}$). Geographic differences in infection of S. latifolia and S. dioica were also found by Baker (1947b) in England, with a decrease in infection from south-east to south-west and north in S. latifolia, while infection of S. dioica was mainly restricted to the north and west of England. Assuming that fungal strains are specialized on S. latifolia and S. dioica, Baker suggested that density of the respective host species may be too small for infection to persist in areas where its fungus does not occur. In another smut, Ustilago spinifex, Kirby (1988) observed that the fungus was absent in those parts of its host range where host occurrence was patchy and sparse compared with regions where the host was abundant.

Even though our results indicate the presence of different host races, gene flow may still occur occasionally, especially because strains from S. latifolia and S. dioica can infect S. latifolia and S. dioica plants equally well in cross-inoculation experiments (Liro 1924; Biere & Honders 1996, but see Baker 1947b; Zillig 1921). We found only one allele at one locus in common between fungi from S. dioica and S. latifolia (Table 2). Further studies measuring fungal population structure over a smaller scale within regions where both host species are infected will be necessary to quantify gene flow between the two host races, but at present it appears to be rather small.

**Implications for parasite-host local coevolution**

Low microsatellite variation implies low variation at selected loci, because selected and neutral variation is linked in breeding organisms (Charlesworth et al. 1993; Hedrick 1980). In the outbreeding host plant S. latifolia, on the other hand, there is considerable variation in resistance and isozymes within populations (Alexander et al. 1993; McCauley 1994). Furthermore, host plant populations are far less differentiated than fungal populations (Delmotte et al. 1999), permitting greater exchange of selected alleles among populations. This difference between host and pathogen population differentiation has implications for patterns of coevolution and local adaptation (Gandon et al. 1996; Kaltz et al. 1999). Greater genetic variation and less population differentiation was found for the anther smut from S. dioica, possibly an
indication of more gene flow between populations than that for the fungus from *S. latifolia*. Further investigations are necessary to determine whether this pattern is widespread and whether higher genetic variation in the anther smut from *S. dioica* influences its coevolutionary potential and adaptability to its outbreeding host plant species.

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