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Genotypic diversity of the wheat leaf blotch pathogen *Mycosphaerella graminicola* (anamorph) *Septoria tritici* in Germany

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Abstract

The population structure and genotypic diversity of *Mycosphaerella graminicola* from six natural field populations in Germany were studied with molecular markers. To reveal the potential effects of plant host resistance on the pathogen population, hierarchical samples were taken from susceptible and resistant cultivars. A total of 203 single spore isolates was subjected to molecular marker analysis using the amplified fragment length polymorphism technique (AFLP). Among the 203 isolates analyzed, 142 different multilocus haplotypes (MLH) were identified revealing a high degree of genotypic diversity of the *M. graminicola* population. On average, a F_{ST} value of 0.04 was found, indicating a low genetic differentiation with only 4% of the genetic variation between the local populations but leaving 96% of the genetic variation within the populations. According to the low F_{ST} value, a high migration rate of $Nm \approx 12$ was found. The observed high within-population diversity, and the significant migration between populations, prevented genetic isolation and differentiation of putative geographically separated populations. Furthermore, plant host resistance had no obvious effect on the population structure and diversity of *M. graminicola*. Genotypic variability can be attributed to sexual recombination which appears to have a considerably larger influence on the population structure. Gene flow on this scale could have significant implications for plant breeding and fungicide spraying programmes.

Introduction

The genetic structure of a plant pathogen population refers to the amount and distribution of genetic variation within and between populations (McDermott and McDonald, 1993). Knowledge of the amount and distribution of genetic variation in a plant pathogen population, the potential for gene flow and long-distance dispersal, and the relative contributions of sexual and asexual reproduction have direct implications to agricultural ecosystems. For instance, knowledge of pathogen dispersal provides information about how fast genotypes with novel virulences or fungicide resistances can spread between areas where a crop is cultivated.

Molecular markers are being increasingly used to characterize fungal plant pathogen populations. They have presented versatile and highly informative tools for fungal pathogen identification and diagnosis (Majer et al., 1996) and also for population genetic studies (McDonald and McDermott, 1993; McDonald et al., 1999). They can be used to evaluate levels of genetic diversity and phenetic relationships within and between species, and to identify particular races and pathotypes (Brown, 1996).

The ascomycete *Mycosphaerella graminicola* (Fuckel) Schröter (anamorph *Septoria tritici*), which causes Septoria tritici blotch of wheat occurs in all wheat-growing areas of the world. Substantial yield losses can be attributed to this pathogen,

especially during growing seasons with high rainfall (Verreet, 1995). Few dominant genes conferring to *M. graminicola* resistance in wheat were identified (*Stb1, Stb2, Stb3, Stb4*) (Wilson, 1985; Somasco et al., 1996). However, there are several reports that these resistance sources are not providing the claimed protection when employed against *M. graminicola* populations with wide virulence patterns (Eyal, 1999; Ballantyne and Thomson, 1995).

McDonald et al. (1995) and Chen and McDonald (1996) used RFLP markers to study field populations of *M. graminicola* in the USA. They showed that field populations of *M. graminicola* have a high level of gene and genotype diversity. They concluded that the sexual stage has a major impact on the genetic structure of these populations. Beyond this, they found that populations separated by long distances can be genetically very similar, suggesting significant levels of gene flow among populations.

Our main objective of the study described here was to analyze the genetic structure and diversity of *M. graminicola* populations in Germany by means of molecular markers. We expected to confirm previous findings of population genetics of *M. graminicola* in the USA with a different marker technique.

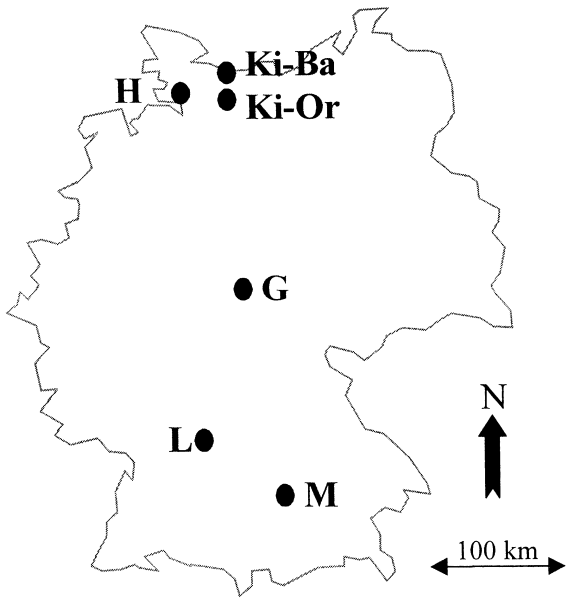

Figure 1. Collection sites of *M. graminicola* isolates in Germany. Abbreviation of locations is as follows: Ki = Kiel, H = Heide, G = Goettingen, L = Ludwigshafen, M = Munich. At each site a field plot of 400 m^2 was planted with the susceptible cultivar Orestis (Or). Additionally, at the Kiel location the resistant cultivar Batis (Ba) was grown.

Materials and methods

Pycnidiospores of *M. graminicola* were obtained from five 400 m^2 field plots in Germany (Figure 1) in 1997. The susceptible wheat cultivar Orestis was planted as a source for the isolate collections. Additionally, the resistant cultivar Batis was grown at Kiel to study the effect of host genotype on the genetic structure of *M. graminicola* populations. At each site, disease severity was estimated by scoring the percentage diseased leaf area (necrosis) damaged by pycnidiospores of *M. graminicola*.

A hierarchical sampling method was used to collect *M. graminicola* isolates from the naturally infected plots. At growth stage (GS) 51/54 (Zadoks et al., 1974), infected leaves were chosen randomly along a transect running across each plot. Samples were taken approximately every 1 m along the transect. To analyze genetic variation among pycnidia within a lesion, single-spore isolations were made from up to three different pycnidia within a discrete, non-overlapping lesion of a leaf. One single-spore isolate was collected from each pycnidium.

The leaves were surface-sterilized by immersion for 30 s in 1% sodium-hypochlorite solution. Excess NaOHCl was removed from the leaf surface with sterile filter paper. Leaves were placed in Petri dishes containing wateragar (1.5% agar) until cirri were visible. Pycnidiospores were streaked onto fresh MYA (0.4% malt, 0.4% yeast, 1.5% agar) Petri dishes to separate the individual spores. Single colonies were isolated for further cultivation and DNA extraction. DNA was extracted from each isolate using a CTAB extraction protocol (Schnieder et al., 1998). AFLP analysis was done basically as described by Vos et al. (1995). For the initial restriction digest with the enzymes MseI and EcoRI 100 ng DNA were used. The selection of biotinylated fragments after ligation was omitted and four primer combinations with each primer having two selective nucleotides (EcoRI-CT/MseI-AT, EcoRI-CT/MseI-AC, EcoRI-AC/MseI-AC and EcoRI-CA/MseI-AG) were used for amplification. Preamplification, labelling reaction, amplification and gel electrophoresis were performed according to Schnieder et al. (1998). Control experiments were done to ensure repeatability of the AFLP results. For this, DNA of 20

isolates was extracted again and the AFLP technique was performed as described before.

Only fragments that could be scored unambiguously were included in each analysis. The fragments were visually scored for presence or absence of bands showing the same mobility in the gel, regardless of their optical density. AFLP fragments were treated as biallelic marker loci with two alleles encoding presence or absence of a band. The fragment data were coded as a binary matrix, where '1' designated presence, and '0' absence, of a particular band. The combined allelic state at all considered AFLP loci was designated as the multilocus haplotype (MLH) of an isolate. Isolates from each field plot and cultivar were treated as a population.

Gene diversity H (Nei, 1972) and unbiased genetic distance D (Nei, 1978) were computed for each locus. In this study, Weir and Cockerham's (1984) methods of calculating Wright's F-statistics are applied to the data. A confidence interval (C.I., 95%) was calculated by bootstrapping over all loci (1000 iterations). Based on Wright's island model of gene flow (Wright, 1951) the average number of individuals that migrate between populations per generation was estimated using the formula $Nm = 1/2((1/F_{ST}) - 1)$.

Results

The four primer combinations yielded 116 polymorphic bands, corresponding to an average of 29 polymorphic bands per primer combination. In the control experiment, identical AFLP fingerprint patterns of the 20 isolates used were obtained.

M. graminicola infections at the field plot sites in Kiel and Heide were favoured by the maritime climate and regular rainfall in this northern part of Germany (Figure 1). The cultivars Orestis and Batis expressed different reaction patterns to *M. graminicola* infections. At the Kiel field plot site, the susceptible cultivar Orestis showed a disease severity of 29% on f-2 (third leaf from the top), whereas Batis had only 11% damaged leaf area (Figure 2). In the more arid climates (Goettingen, Ludwigshafen and Munich), Orestis showed a 15–9% diseased leaf area.

Among the 203 isolates analyzed, 142 multilocus haplotypes (MLHs) were identified. Of those, 94 occurred only once, 35 occurred twice, and 13 were identified three times. Without exception, identical *MLHs* originated from the same lesion. There were 62 cases in which three pycnidia were sampled from a single lesion. In 22 (35%) of these cases, isolates derived

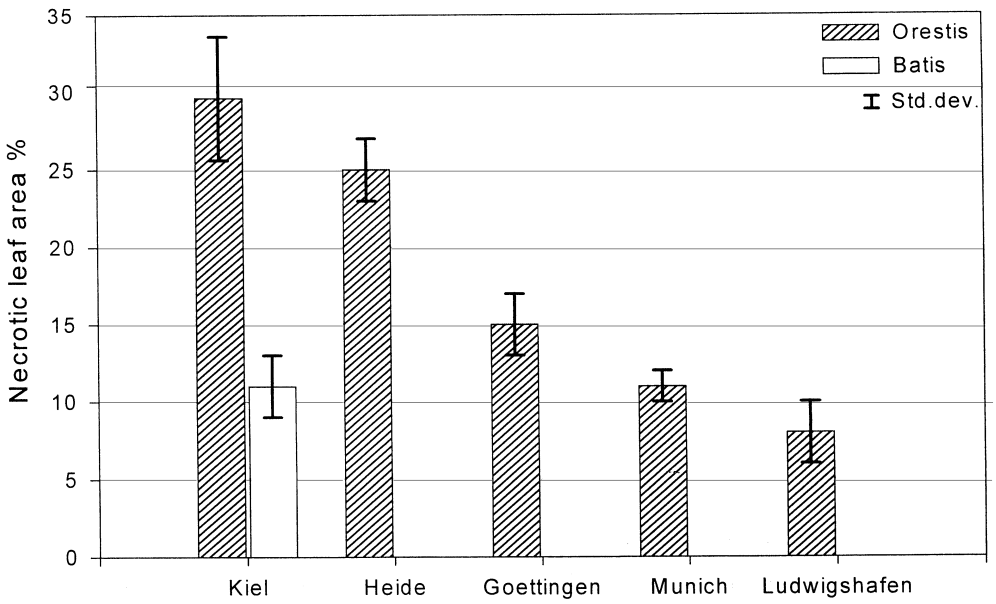

Figure 2. Field response of the susceptible variety Orestis and the resistant variety Batis to natural infections of *M. graminicola* at growth stage (GS) 51/54. Varieties were grown at different collection sites in Germany. Necrotic leaf area on f-2 (third leaf below the top) was estimated from 29 leaves at each sampling site.

from different pycnidia of the same lesion had different haplotypes.

Nei's measure of gene diversity, which was on average $H = 0.27$, across all loci was similar for all populations (Table 1). No significant difference between isolates from the resistant cultivar Batis ($H = 0.26$) or the susceptible cultivar Orestis ($H = 0.28$) was found. Average genetic distance between populations was $D = 0.025$ (standard error 0.002). D ranged from 0.017 (populations from Heide and Kiel, 60 km apart from each other) to 0.039 (populations from Munich and Kiel, 800 km apart from each other) (Table 2).

An indication of the genetic similarity among these populations was the low level of population diversity indicated by F_{ST}, which was on average 0.04 (C.I. $\pm$ 0.014) among all populations (Table 2). The highest F_{ST} value was found between the populations from Munich and Heide (700 km apart from each other), the lowest between the populations from Munich and Ludwigshafen (350 km apart from each other). The estimates of *Nm* under the island model (Wright, 1951) ranged from one to 96 for individual loci. On the basis of an average F_{ST} of 0.04 across all loci a movement of $Nm \approx 12$ individuals (C.I. $\pm$ 5.01) per generation was calculated.

Table 1. Nei's gene diversity *H* of 203 German *M. graminicola* isolates calculated from 116 polymorphic AFLPs

Location	Cultivar	Number of isolates	Average gene diversity *H* (Std. deviation)
Kiel	Orestis	67	0.28 (0.17)
Kiel	Batis	59	0.26 (0.18)
München	Orestis	21	0.24 (0.18)
Goettingen	Orestis	22	0.23 (0.19)
Ludwigshafen	Orestis	17	0.23 (0.19)
Heide	Orestis	17	0.25 (0.19)

Discussion

On the basis of 116 polymorphic AFLP markers, a high degree of genetic diversity in *M. graminicola* in Germany was observed. The haplotype variation between different pycnidia within the same lesion was higher (35% of the comparisons) than in a study by McDonald and Martinez (1990). They found unlike haplotypes in 26% of the comparisons. Beyond this, they found haplotype variation between lesions from the same leaf as we showed in our previous study of a small *M. graminicola* population (Schnieder et al., 1998), indicating that these lesions resulted from independent infection events involving different haplotypes. A high amount of pathogen variation could be found on a single host plant.

In no case was an identical genotype found at another sampling site in the same field plot. Isolates with the same AFLP fingerprint were always sampled from the same lesion. The high degree of genotypic diversity indicates additional evidence for the hypothesis that populations of *M. graminicola* undergo regular sexual cycles. A genetically diverse founding population (ascospores of *M. graminicola*) provides the initial inoculum (Shaw and Royle, 1989; McDonald and Martinez, 1990; Boeger et al., 1993). The reproduction via asexual pycnidiospores of *M. graminicola* over the growing season results in distinct spatial clusters of clones without detectable movement between clusters in the field over the course of a growing season. Despite the high frequencies of migrants concluded from the calculated F_{ST} between spatially separated populations, observation of migration within the relatively large (400 m^2) field plots difficult due to the general high variability of *M. graminicola*.

The cultivars showed different reaction patterns to *M. graminicola* infections as described previously by

Table 2. Genetic distance *D* (below diagonal) and population differentiation F_{ST} (above diagonal) between populations of *M. graminicola* in Germany. Calculations were performed on the basis of 116 polymorphic AFLPs

Location – Cultivar	Kiel Orestis	Kiel Batis	Munich Orestis	Goettingen Orestis	Ludwigshafen Orestis	Heide Orestis
Kiel – Orestis	—	0.029	0.048	0.038	0.036	0.024
Kiel – Batis	0.027	—	0.042	0.023	0.038	0.025
Munich – Orestis	0.039	0.031	—	0.039	0.023	0.049
Goettingen – Orestis	0.029	0.014	0.023	—	0.036	0.030
Ludwigshafen – Orestis	0.027	0.027	0.029	0.019	—	0.033
Heide – Orestis	0.017	0.016	0.034	0.016	0.017	—

Klink (1997). The disease severity of the susceptible cultivar Orestis was, as expected, significantly higher than that of the resistant cultivar Batis. However, genotypic selection for a particular pathogen genotype was not observed. There were no significant differences in gene and genotypic diversity between the populations from the resistant cultivar Batis and the susceptible cultivar Orestis. McDonald et al. (1996) used DNA fingerprinting and RFLP analysis to conduct field experiment with four wheat varieties that differed in resistance to *M. graminicola*. They concluded that there was no evidence that different host genotypes caused selection for particular pathogen genotypes.

The F_{ST} and D values indicated a low genetic differentiation of the populations with very little differentiation between the sampling sites. The average gene differentiation (F_{ST}, ranging from 0.023 to 0.049) and the overall genetic distances (D, ranging from 0.014 to 0.039) between the populations were small. On the basis of an average F_{ST} of 0.04 across all loci, the application of Wright's island model resulted in a gene flow of $Nm \approx 12$ individuals per generation, which would be necessary to account for the high genetic similarity among populations. Wright (1951) pointed out that only one individual per generation is adequate to prevent populations from diverging significantly. They defined populations as a co-evolving unit if more than four genetically diverse individuals per generation are successfully moving between the populations. Complementarily, the temporal scale of the gene flow cannot be determined from this analysis with the indirect measure of *Nm*. These estimates of *Nm* assume constant gene flow over all generations (Slatkin and Barton, 1989).

The high level of gene flow between geographically distant populations merges them into a single, more or less homogenous population which evolves as a co-evolving unit. Species with the potential for widespread, long-distance dispersal will display a greater genetic uniformity across local and distant populations than species with very limited dispersal ability. This appears to be the case with the populations of *M. graminicola* described here. The asexual pycnidiospores are dispersed by rainsplash (Shaw, 1987) and hence have only limited potential for movement over longer distances. However, the sexual ascospores of *M. graminicola* have the potential to move at least several hundred meters (Shaw and Royle, 1989), perhaps over tens of kilometers, indicating their potential as a source of genetic exchange between spatially distant populations.

A human-mediated movement of *M. graminicola*, perhaps in infected seed or straw, could be a reasonable source for the observed high level of gene flow between the far distant sites we sampled. Brokenshire (1975b) showed that *M. graminicola* can infect seeds, which would give it the potential for dispersal over much longer distances. However, he never could prove that infected seeds could give rise to infected seedlings.

Additionally, there may be further, less obvious, mechanisms for the dispersal of ascospores and therefore gene flow among the populations. Brokenshire (1975a) showed that there are some alternate hosts (e.g., *Poa annua, Festuca arundica, Poa pratense, Agropyron elatius, Bromus mollis*) for *M. graminicola*, on which the fungus can produce perithecia and infectious ascospores. Ascospore dispersal over at least less distant populations and the existence of alternate hosts, could together provide a uniform source as the primary ascospore inoculum for infections of winter wheat. But the role of alternate hosts in the epidemiology of *M. graminicola* is yet unresolved and deserves further consideration.

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