

Genetic diversity of *Mycosphaerella graminicola*, the causal agent of Septoria tritici blotch, in Kansas winter wheat

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Abstract

The genetic structure of Kansas populations of *Mycosphaerella graminicola* was evaluated at different spatial scales (micro-plot, macro-plot, and statewide) using amplified fragment length polymorphism (AFLP). Genetic identities among populations were >98%. Tests for population subdivision revealed that 98% of the genetic variability occurred within populations with a corresponding migration rate of 16 to 23 individuals per generation. Little evidence of linkage disequilibrium was observed, on average, only 4.6% of locus pairs were in disequilibrium. These results indicate that Kansas populations of *M. graminicola* are characterized by regular recombination, are genetically diverse, and appear to be homogenous across different spatial scales.

Introduction

Mycosphaerella graminicola (Fuckel) Schröter is a pseudothecial ascomycete that causes Septoria tritici blotch on wheat. The anamorph, *Septoria tritici*, was first described by Desmazières in 1842 (8) but *M. graminicola* was not identified as the sexual stage until over a century later (29). Epidemics are initiated by airborne ascospores produced following meiosis on wheat stubble (11). Asexually produced pycnidiospores that are rainsplash dispersed form the secondary inoculum. Ascospores of *M. graminicola* have two cells of unequal size while pycnidiospores are elongated and enclosed within a pycnidium. Under high humidity ascospores and pycnidiospores germinate to produce hyphae that penetrate the leaf, usually through stomata (7). Symptoms are visible approximately 10 days after infection begins and include chlorotic and necrotic lesions on the host leaf. Dark pycnidia appear within these lesions 2 to 3 weeks after the initial infection. Pycnidia are embedded in the epidermal tissue usually on both sides of the leaf, and are visible in rows alongside the vascular tissue of the leaf (10). The presence of dark pycnidia in a tan lesion is highly diagnostic of the disease.

The Middle East probably is the origin of *M. graminicola* (20), but this fungus is now a worldwide problem affecting wheat-growing areas in Europe (12), Australia (15), Canada(6), and the United States (11, 21). Prior to the 1960s, *M. graminicola* was not perceived as an economically significant pathogen on wheat. However, with the introduction of reduced tillage and new cultivars, this pathogen has become widespread and can reduce yields by as much as 30 to 40%, especially during growing seasons with significant rainfall (25). In Kansas, epidemics are sporadic due to relatively short springs and inconsistent rainfall. The estimated average annual loss in Kansas is 1 % with a range from a trace to 7.4% (2). However, losses in individual fields can exceed 25%.

Knowledge of the genetic structures of a population can play a key role in efforts to manage this pathogen through the understanding of its epidemiology and evolutionary potential. Molecular markers are commonly used to characterize populations of fungal plant pathogens and increasingly more commonly used for identification and diagnostic purposes. *M. graminicola* populations have been studied with anonymous Restricted Fragment Length Polymorphism (RFLP) loci (5,18,19). These studies have found high levels of gene and genotype variability in field populations of *M. graminicola*. It was also concluded that there was strong evidence of regular cycles of sexual reproduction that had a large impact on the genetic structure of the populations, and significant gene flow was occurring as evidenced by comparable

allele frequencies among distant populations.

The genetic structure of field populations of *M. graminicola* is not known in the Central Plains States including Kansas, one of the most important wheat growing areas in the world. In Kansas, there have been significant efforts to develop cultivars resistant to Septoria tritici blotch (3). Knowledge of the genetic structure of *M. graminicola* populations in the region is important to the effective deployment of this resistance. The objectives of this study were: (i) to assess the genetic diversity of natural populations of *M. graminicola* in Kansas winter wheat using AFLP markers; (ii) to determine the structure of these populations at different spatial scales (micro-plot, macro-plot, and statewide); and (iii) to determine if there is any significant genetic differentiation between these populations and those studied previously.

Materials and methods

Isolate collection

Samples were collected from plants in commercial wheat fields naturally infected with Septoria tritici blotch across the state of Kansas during the 2004/05 growing season. All samples were collected in a two day period providing a snap shot of the population at a particular point in time. Samples were placed in paper coin envelopes, dried at room temperature, and stored at 4°C until evaluated.

Collections were made representing three different spatial scales: micro-plot, macro-plot, and statewide. Micro and macro-plot populations were collected in; Cloud, Ellis, and Marion counties. Micro-plots were 1-m² quadrats established ~ 25 m from the edge of a field. All visible lesions within the micro-plot were sampled. Macro-plot samples were taken from the fields in which the micro-plots occurred. Fields varied in size by location but were > 10 ha. At least 60 isolates were obtained from each of the macro-plots with the average distance between isolate collection points of ~ 10 m. An additional 155 isolates was obtained from other randomly selected fields across the state of Kansas. The cultivar present in the Cloud county field was Tomahawk (highly susceptible to Septoria tritici blotch). The wheat cultivar in the fields from which all of the other samples were taken was unknown.

DNA isolation

Wheat leaves with Septoria tritici blotch lesions were placed on wet filter paper at room temperature (20-25°C) for 24 hours. A cirrus from a single pycnidium per lesion was transferred to a Petri dish containing one-fourth strength PDA (0.6% potato dextrose broth, 1.5% agar) and then streaked across the agar surface with a sterile, glass rod to separate individual spores. Plates were incubated for 2 days at room temperature. Colonies originating from a single spore were transferred to liquid YG medium (2% glucose, 0.5% yeast extract) and incubated at 20°C on an orbital shaker (180 rpm) for 5 to 10 days. Isolates did not grow uniformly either in speed or form, e.g. mycelia or budding spores, but all were consistent with *in vitro* cultures of known *S. tritici* isolates. The resulting cultures were concentrated by centrifugation and stored at -80°C until DNA extraction.

DNA was isolated by a cetyltrimethylammonium bromide (CTAB) procedure as described by Murray and Thompson (22) modified by Kerényi *et al.* (13). Extracted DNA was resuspended in 50 to 100 µl of 1× Tris-EDTA buffer and stored at -20°C until used. DNA concentrations were determined by using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

AFLP methodology

AFLPs were generated essentially using the method described by Vos *et al.* (32) as modified by Zeller *et al.* (35). DNA was digested to completion with *EcoRI* and *MseI*. Standard protocols (28) and manufacturers' recommendations were followed in the use of all buffers and DNA modifying enzymes. Ten primer pair combinations were screened for optimal polymorphisms and three of these primer pairs were used to generate AFLPs for all isolates (Table 1). The selected primer pair combinations were; *Eco+AC/Mse+CA*, *Eco+AC/Mse+CC*, and *Eco+AC/Mse+GG*. The *EcoRI* primer used for the selective amplifications was end-labeled with $\gamma^{33}\text{P-ATP}$, and the amplification products separated in denaturing 6%

polyacrylamide (Long Ranger, FMC Scientific, Rockland, ME) gels in 1× Tris-borate EDTA buffer. Gels were run at a constant power of 100 W using a Sequi-Gen GT sequencing cell (Bio-Rad Laboratories, Inc., Hercules, CA), dried, and exposed to autoradiography film (Classic Blue Sensitive, Molecular Technologies, St. Louis) for 2 days. A $\gamma^{33}\text{P}$ -labeled 100-bp molecular ladder was used to estimate band sizes on polyacrylamide gels.

All AFLP bands in the 100-1000 bp range were scored manually for presence or absence and data recorded in a binary matrix. Fragments with the same molecular size were assumed to be homologous. Bands that differed in size were treated as independent loci with two alleles (presence or absence). Occasionally, due to poor amplification, unclear bands were observed and subsequently scored as ambiguous in the ensuing population genetic analyses. In order to ensure the repeatability of the AFLP results, DNAs of four known isolates of *M. graminicola* were extracted and included in all the gels as described previously. Bands generated from these four isolates were also used as reference in determining homology.

Population genetic analyses

The CLUSTER procedure of SAS (SAS Institute, Cary, NC) using the Dice coefficient of similarity and the unweighted pair grouping by mathematical averages (UPGMA) subroutine of PAUP* version 4.10b (31) were used to identify AFLP haplotypes, and to determine genetic similarity among isolates. This analysis was conducted for populations from each of the three locations studied, as well as for the entire pool of isolates. Haplotypes were defined as isolates sharing $\geq 98\%$ of the bands present. Samples were “clone censored” and only one representative of each haplotype was used in the remaining analyses.

The shareware program Popgene version 1.32 (Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada) was used to calculate the fixation index (G_{ST}) (23). The migration rate (Nm) [$Nm = 0.5(1 - G_{ST})/G_{ST}$ (16)], allele frequencies and genetic diversity within and between populations as described by Nei (23), and genetic identity among populations as described by Nei (24) were also calculated using Popgene. Data were analyzed using the haploid, dominant marker subroutines. Estimates of the fixation index (G_{ST}) and migration rate (Nm) were calculated for the complete set of loci and for a subset of loci in which the frequency of both alleles was $\geq 5\%$. This was to determine if rare alleles altered the analyses. Estimates of linkage disequilibrium were calculated with Popgene for the subset of loci in which the frequency of both alleles was $\geq 5\%$.

Results

Population structure and haplotype distribution

126 isolates of *M. graminicola* were recovered from the three micro-plots (Cloud: 43, Ellis: 40, and Marion: 43), 195 isolates from the three macro-plots (Cloud: 69, Ellis: 64, and Marion: 62), and 155 isolates from the rest of the state. There were 174 scorable AFLP markers, 168 of which were polymorphic in at least one population (Table 1). The average similarity of isolates from a micro-plot population was 58%, with a minimum similarity of 29%. Average similarities of isolates from the macro-plot and statewide populations were 62% and 57%, with minimum similarities of 30% and 25%, respectively.

Haplotype diversity was determined from these populations utilizing a pool of 174 loci, and haplotypes were defined as isolates that shared at least 98% UPGMA means similarity in AFLP banding pattern. Genotypic diversity was high (100%) in all populations, with all 476 isolates showing unique AFLP haplotypes. No identical haplotypes were found in any of the populations tested including the micro-plots (Table 2). Identical haplotypes only occurred if isolates were collected within the same lesion (data not shown).

Table 1. Primer-pair combinations, number of amplified bands, and number of polymorphic bands generated for each primer-pair.

Primer-pair combination ¹	Number of amplified bands	Number of polymorphic bands ²
<i>EcoAC/MseCA</i>	72	69
<i>EcoAC/MseCC</i>	47	45
<i>EcoAC/MseGG</i>	55	54
Total	174	168

¹*EcoAC* is *EcoRI* primer (5'-AGACTGCGTACCAATTC-3') followed by the selective base pairs AC. *MseCA*, *CC*, and *GG* are *MseI* primer (5'-GATGAGTCCTGAGTAA-3') followed by the selective base pairs CA, CC, and GG.

²These bands were polymorphic in at least one of the populations studied.

Genetic variability among populations

Of the 174 loci scored, 75% were polymorphic in the micro-plot populations, 80% were polymorphic in the macro-plot populations, and 81% were polymorphic in the statewide population (Table 2). There were 12, 10, and 11 private alleles identified in micro-plot, macro-plot, and statewide populations, respectively. Only three of the private alleles from the micro-plot populations and one from the statewide population were present at frequencies $\geq 5\%$. Gene diversity estimates calculated with Popgene across all loci were similar for all populations (Table 2), whether Nei's gene diversity (~ 0.18) or Shannon's index (~ 0.28) was used for comparison. When loci for which the frequency of the rarer allele was $< 5\%$ were removed, Nei's gene diversity and Shannon's index values increased to ~ 0.24 and ~ 0.37 , respectively.

Table 2. Statistical information related to comparing natural populations of *Mycosphaerella graminicola* collected from three spatial scales (micro-plot, macro-plot, and statewide) in Kansas winter wheat.

Population	Micro-plot ¹	Macro-plot ¹	Statewide
No. of isolates	42	65	155
Unique haplotypes	42	65	155
Polymorphic loci (%)	75	80	81
Private alleles	12	10	11
Nei's gene diversity ^{2,3}			
174 loci	0.182	0.172	0.182
125 loci	0.244	0.231	0.243
Shannon's index ^{2,4}			
174 loci	0.287	0.275	0.288
125 loci	0.380	0.365	0.379

¹Based on an average of three replications corresponding to three locations, these were; Cloud, Ellis, and Marion counties. Micro-plot size was 1 m² and macro-plot size was the entire field (> 10 ha).

²Values were estimated for clone-censored populations. Clones were defined as isolates sharing at least 98% unweighted pair group method with arithmetic means similarity in amplified fragment length

polymorphism banding pattern.

³Nei's gene diversity (23).

⁴Shannon's information index (14).

The overall fixation index, G_{ST} , was estimated among all populations as well as for all possible pairwise combinations of populations (Φ_{ST}); micro-plot/macro-plot, macro-plot/statewide, and micro-plot/statewide (Table 3). The overall fixation index was $G_{ST} = 0.0331$ (min. of 0.0004 for locus GG41, max. of 0.2858 for locus GG26). The migration rate Nm , is a function of G_{ST} (16), and was correspondingly high (>15 individuals per generation) suggesting that significant genetic exchange has occurred among these populations. Φ_{ST} values among the three populations (Table 3) were similar and relatively low (0.021 to 0.030). The results were similar if the 49 loci for which the frequency of the rarer allele was < 5% were removed from the analysis (Table 3).

Table 3. Pairwise comparisons of the three spatial scales (micro-plot, macro-plot, and statewide) of natural populations of *Mycosphaerella graminicola* with all scored loci, and for the 125 loci where the frequency of both alleles was ≥ 0.05 .

	Micro ¹ vs. Macro-plot ¹		Macro ¹ vs. Statewide		Micro ¹ vs. Statewide	
	174 Loci	125 Loci	174 Loci	125 Loci	174 Loci	125 Loci
Fixation index (Φ_{ST})	0.023	0.023	0.021	0.022	0.030	0.031
min. - max.	0-0.36	0-0.33	0-0.36	0-0.36	0-0.33	0-0.33
Migration rate (Nm) ²	21	21	23	22	16	16
min. - max.	0.88-2000	1-2000	0.88-2000	0.88-2000	1-2000	1-2000
Mean gene diversity ³	0.178	0.244	0.180	0.223	0.187	0.252
Std. Deviation	0.174	0.166	0.174	0.172	0.176	0.166

¹Cloud county location was used in this analysis for both micro (1 m²) and macro-plot (entire field > 10 ha) populations.

²McDermott and McDonald(16).

³Nei's gene diversity (23).

The average genetic identity among the populations was 98.8% (Table 4) with the highest value obtained when comparing macro-plot and statewide populations (99%) and the lowest value 98.6% when micro-plot and statewide populations were compared.

Table 4. Genetic identity¹ (above diagonal) and genetic distance¹ (below diagonal) of micro-plot, macro-plot, and statewide populations of *Mycosphaerella graminicola* using all scored loci, and for the 125 loci where the frequency of both alleles was ≥ 0.05 .

Population	Micro-plot ²	Macro-plot ²	Statewide
Micro-plot ²	****	0.989 / 0.985 ³	0.986 / 0.979 ³
Macro-plot ²	0.010 / 0.015 ³	****	0.990 / 0.987 ³
Statewide	0.014 / 0.021 ³	0.009 / 0.012 ³	****

¹Nei's measures of genetic identity and genetic distance (24).

²Cloud county location was used in this analysis for both micro and macro-plot populations.

³Calculated for the 125 loci where the frequency of both alleles was greater or equal to 0.05.

Linkage disequilibrium

Estimates of linkage disequilibrium were calculated by using Popgene for the 125 loci for which the frequency of both alleles was $\geq 5\%$. Of the 7,750 possible pairwise comparisons, 331 (4.3%), 382 (4.9%), and 349 (4.5%) locus pairs were found in disequilibrium in micro-plot, macro-plot, and statewide scales, respectively. The number of significant linkage disequilibria was calculated at $P < 0.05$, and all χ^2 tests had one degree of freedom.

Discussion

The objective of this study was to evaluate the genetic diversity in field populations of *M. graminicola* from Kansas winter wheat collected in a hierarchical manner. Kansas weather conditions are unique and characterized by cold winters followed by short springs where temperatures can fluctuate broadly and rapidly. Septoria tritici blotch epidemics are sporadic in Kansas, and depend on rainfall and temperature, especially in the spring. The strains analyzed were collected at one time (late November) and provide a temporal snapshot of the *M. graminicola* population in the state. Our data suggest that the strains analyzed are members of a larger pathogen pool that is distributed across much of Kansas and probably the central Great Plains. Generally speaking, these results are consistent with previous studies of Septoria tritici blotch populations that used RFLPs (5,18,19), mating type (36) or microsatellite markers (26).

Haplotype distribution

M. graminicola strains were isolated from different lesions, all of which had unique haplotypes, and no identical haplotypes were found outside the confines of a single lesion. Surprisingly, this was true even at the micro-plot level (1 m²) because this fungus is capable of producing large amounts of asexual pycnidiospores that would contribute to clonal dispersion on a small scale (5). These findings may be explained by the fact that sampling was done early enough in the season (late November) before the emergence of the new lesions from secondary inoculum. In Kansas, seed sowing takes place in the first two weeks of October and it takes at least a week for seedlings to emerge. Additionally, under optimum conditions, it takes about three weeks for pycnidia to be produced after inoculation. Therefore, it is likely that the lesions sampled here were all from primary inoculum (ascospores). Ascospores are presumed to be the primary inoculum (5), and our results are consistent with the hypothesis that sexual recombination takes place on a regular basis in *M. graminicola* populations in Kansas.

Sexual recombination in *M. graminicola* populations could alter the management of Septoria tritici blotch since the ready generation of recombinants may quicken the pace at which *M. graminicola* populations adapt to fungicides and resistance genes. For example, a decrease of effectiveness of *Stb4* has been documented in California (1). *Stb4* was effective in the field in California from 1975 to the late 1990s until the appearance of new virulent strains. In Kansas, farmers rely on resistant cultivars to limit losses from Septoria tritici blotch. The current disease resistance has been durable for about 15 years (3), but it is unclear which one(s) of the eight major genes for resistance to Septoria tritici blotch are present in these cultivars. New gene combinations could be dispersed rapidly if they arise in the Great Plains or if sufficient genetic exchange occurs between Kansas and other populations in North America or elsewhere.

Diversity within and among populations

Mean gene diversities (Table 2) were generally very similar, as were allele frequencies (data not shown), suggesting that significant genetic exchange has homogenized these values across these populations. The best evidence that these populations are components of a much larger pool are the low G_{ST} (<0.04) values (Table 3) and genetic identity values near 100% (Table 4). Fixation index values remained low

(0.03) when the micro-plot (1 m²) population was compared with a much larger population representing the entire state (Table 3), indicating that most of the genetic variability is distributed on a small scale rather than between populations. These results are similar to those of McDonald and Martinez (17), who concluded that their population of *M. graminicola* contained a large amount of genetic variation that was distributed on a very fine scale, and those of Schnieder et al. (30), who reported fixation indices ranging from 0.023 to 0.049 (0.021 to 0.030 reported in the present study). Low G_{ST} levels also have been found in populations of other fungal species, e.g. *Gibberella* (34), *Rhizoctonia* (27), and *Cronartium* (9), and in all of these studies the authors concluded that the populations evaluated were components of a much larger, well-mixed pathogen pool.

Migration rate (Nm) is a function of G_{ST} (16). Estimates of Nm were high and ranged from 16 to 23 individuals per generation. Under the island model (33), the movement of as little as one individual per generation is sufficient to prevent significant divergence between populations, although individual genetic traits still may be lost to genetic drift. Under the same model, with a migration rate of four distinct individuals, the populations are said to be co-evolving. Our estimates of migration rate among Kansas populations were high (16 – 23), further strengthening the hypothesis that *M. graminicola* exists as a large, well-mixed pathogen pool that is distributed at least across much of Kansas and probably throughout the central Great Plains.

Linkage disequilibrium

With random mating, alleles at all loci eventually will become randomly associated. Departure from equilibrium estimates may provide insight into the relative importance of sexual and asexual reproduction. Little evidence of linkage disequilibrium was found in the Kansas population with only 4.6% of locus pairs in detectable disequilibrium, based on the 125 loci for which the frequency of both alleles $\geq 5\%$. Our estimates of linkage disequilibria were lower than those of Chen and McDonald (5) in their analysis of California samples. They found significant linkage disequilibrium in 12% of the 66 pairwise combinations, concluding that the great majority of alleles at the RFLP loci they considered were randomly associated. Brown (4) reported that sample sizes of 100 individuals or more may be required to adequately detect disequilibria between loci in natural populations. Our micro-plot and macro-plot populations were not this large; however, linkage disequilibria estimates for these two populations were consistent with those from the statewide population, which was > 100 individuals.

In conclusion, Kansas populations of *M. graminicola* are characterized by regular recombination, are genetically diverse, and appear to be homogenous across various spatial scales. The apparent random mating and high levels of genotypic diversity are a reminder that the risk of adaptation of *M. graminicola* populations to fungicide treatments or resistance genes is high and that the traits could be dispersed quickly, whether the new alleles occur locally through mutation or introduced from other areas by migration. The widespread genetic variation within the population suggests that a variety of sampling methods may be adequate for this pathogen due to the absence of population subdivision and the high levels of genotypic diversity at very small scales.

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