AMPLIFIED FRAGMENT LENGTH POLYMORPHISM IN *MYCOSPHAERELLA GRAMINICOLA*

by

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B.S., Ecole Supérieure d'Agriculture de Purpan, 1999
M.S., Kansas State University, 2001

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Septoria tritici blotch caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*), is an important disease of wheat worldwide capable of reducing yields by as much as 30 to 40%. In Kansas, the disease is widespread and losses in individual fields can exceed 25%. This study examined the genetic structure of Kansas populations of *M. graminicola* at different spatial scales (micro-plot, macro-plot, and statewide) using amplified fragment length polymorphism (AFLP) markers. Three primer pairs were used to resolve 174 polymorphic loci from 476 isolates. The results indicated high levels of genotypic variability, which is consistent with a genetically diverse initial inoculum. Genetic identities among populations representing the three spatial scales were >98%. Tests for differentiation among populations due to population subdivision revealed that on average 97.5% of the genetic variability occurred within populations with a correspondingly high migration rate of 16 to 23 individuals per generation. We observed little evidence of linkage disequilibrium, on average, only 4.6% of locus pairs were in disequilibrium. Our 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. These populations are probably components of a larger pathogen pool that is distributed at least across much of Kansas and probably the central Great Plains. Because of the frequent recombination, the risk of adaptation of Kansas populations of *M. graminicola* to fungicide treatments or resistance genes is high and could be dispersed very quickly, whether these new pathogenic traits occur locally through mutation or by migration from other areas.

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Approved by:

Major Professor
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Abstract

Septoria tritici blotch caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*), is an important disease of wheat worldwide capable of reducing yields by as much as 30 to 40%. In Kansas, the disease is widespread and losses in individual fields can exceed 25%. This study examined the genetic structure of Kansas populations of *M. graminicola* at different spatial scales (micro-plot, macro-plot, and statewide) using amplified fragment length polymorphism (AFLP) markers. Three primer pairs were used to resolve 174 polymorphic loci from 476 isolates. The results indicated high levels of genotypic variability, which is consistent with a genetically diverse initial inoculum. Genetic identities among populations representing the three spatial scales were >98%. Tests for differentiation among populations due to population subdivision revealed that on average 97.5% of the genetic variability occurred within populations with a correspondingly high migration rate of 16 to 23 individuals per generation. We observed little evidence of linkage disequilibrium, on average, only 4.6% of locus pairs were in disequilibrium. Our 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. These populations are probably components of a larger pathogen pool that is distributed at least across much of Kansas and probably the central Great Plains. Because of the frequent recombination, the risk of adaptation of Kansas populations of *M. gramincola* to fungicide treatments or resistance genes is high and could be dispersed very quickly, whether these new pathogenic traits occur locally through mutation or by migration from other areas.

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Dedication

I would like to dedicate this thesis to the loving memory of my mother.

Thanks for everything.

Chapter 1 - Genetic diversity of *Mycosphaerella graminicola*, the causal agent of Septoria tritici blotch, in Kansas winter wheat

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 being the sexual stage until over a century later (29). Epidemics are initiated by airborne sexual ascospores produced on wheat stubble (11) and secondary inoculum is in the form of asexual pycnidiospores that are rainsplash dispersed. Ascospores of *M. graminicola* have two cells that are unequal in size while pycnidiospores formed by *S. tritici* are elongated and enclosed within a pycnidium. Under high humidity conditions, both ascospores and pycnidiospores germinate to produce hyphae that penetrates the leaf mostly through stomata (7). Symptoms are visible approximately 10 days after the infection process is initiated, with the appearance of 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.

Although the Middle East is likely to be the origin of *M. graminicola* (20), it is now a worldwide problem affecting wheat-growing areas in Europe (12), Australia (15), Canada(6), and USA (11, 21). Prior to the 1960s, *M. graminicola* was not perceived as an economically significant pathogen on wheat. However, due to changes in cultural practices, and the introduction of new cultivars, this pathogen has become very pernicious and capable of reducing 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 rainfalls. Estimated average annual losses in Kansas are 1.0% and have ranged from trace to 7.4% (2). However, the disease is widespread and losses in individual fields can

exceed 25%.

Knowledge of population genetic structures can play a key role in the effort to manage this pathogen through the understanding of its epidemiology and evolutionary potential. Molecular markers have been used in characterizing fungal plant pathogens not only for identification and diagnosis purposes, but also as tools for population genetic analyses. *M*. *graminicola* populations have been studied based on the analysis of anonymous RFLP loci (18,19,5). These studies have shown 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* has not been investigated in the Central Plains States including Kansas, one of the most important wheat growing areas in the world. In Kansas, there has been significant effort to develop cultivars with resistance to Septoria tritici blotch (3). Knowledge of the genetic structure of *M. graminicola* in the region is important to the effective deployment of this resistance. The objectives in this study were: (i) assess the genetic diversity of natural populations of *M*. *graminicola* in Kansas winter wheat using AFLP markers; (ii) investigate the structure of these populations at different spatial scales (micro-plot, macro-plot, and statewide); and (iii) determine whether there was significant genetic differentiation between these populations and previously studied ones.

Materials and methods

Isolate collection

Samples were collected from naturally occurring Septoria tritici blotch in commercial wheat fields across the state of Kansas during the 2004/05 growing season. Lesions showing pycnidia of *S. tritici* were collected over a period of two days providing a snap shot of the population at a particular point of time. Samples were placed in paper coin envelopes, dried at room temperature, and stored at 4°C until used.

Collections were made representing three different spatial scales micro-plot, macro-plot, and statewide. Micro and macro-plot populations were collected at three different locations; Cloud, Ellis, and Marion counties. Micro-plots were 1-m^2 quadrats established about 25 m from the edges of the fields. All visible lesions within the quadrat were sampled. Macro-plots were the entire wheat fields where the micro-plots occurred and varied in size from one location to the other but all were greater than 10 ha. At least 60 isolates were obtained from each of the macro-plots with an average distance between isolate collection points of about 10 m. Additionally, a collection of 155 isolates was obtained from other randomly selected fields across the state of Kansas. The wheat cultivars in all the fields were unknown, with the exception of Cloud county where the cultivar Tomahawk (highly susceptible to Septoria tritici blotch) was present.

DNA isolation

Wheat leaves with Septoria tritici blotch lesions were placed on wet filter paper at room temperature (20-25°C) for 24 hours, to allow cirri exudation from pycnidia. Only one isolation was made from each lesion. Cirri were transferred with a sterile, glass needle to petri dishes containing one-fourth strength PDA (0.6% potato dextrose broth, 1.5% agar) and

streaked across the agar surface with a sterile, glass rod to separate individual spores. Spores were allowed to germinate for 2 days at room temperature. Colonies 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. Growth was not always uniform among isolates and some were incubated longer than others. Similarly, the type of fungal tissue produced also varied from one isolate to the other, some grew as mycelia, others as 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 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). The restriction enzymes *Eco*RI and *Mse*I were selected to digest the DNA samples (100-200 ng per reaction). Standard protocols (28) and manufacturer's recommendations were followed in the use of all buffers and DNA modifying enzymes. An initial screening of 10 primer pair combinations for optimal polymorphisms was conducted, 3 were chosen to generate AFLPs for all isolates (Table 1-1). The selected primer pair combinations were; *Eco*+AC/*Mse*+CA, *Eco*+AC/*Mse*+CC, and *Eco*+AC/*Mse*+GG. The *Eco*RI primer used for the selective amplifications was end-labeled with γ^{33}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 γ^{33}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 homologous and represented the same allele. 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 at least 98% using the unweighted pair group method with arithmetic means similarity in amplified fragment length polymorphism banding pattern. Duplicate haplotypes were discarded 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}) in order to measure allelic differences between populations as described by Nei (23). Using the same program, estimates were generated of the migration rate (*Nm*) that measures the average number of migrants between populations from G_{ST} using the formula $Nm = 0.5(1 - G_{ST})/G_{ST}$ (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). Data were analyzed using the haploid, dominant marker subroutines of Popgene. Estimates of fixation index (G_{ST}) and migration rate (*Nm*) were calculated for both the complete pool of loci and for a sub-pool of loci where the frequency of both alleles was greater or equal to 5%. This was to determine if rare alleles altered the analyses. Estimates of linkage disequilibrium were calculated with Popgene for the sub-pool of loci where the frequency of both alleles was greater or equal to 5%.

Results

Population structure and haplotype distribution

126 isolates of *M. graminicola* were isolated and examined from the three micro-plots (Cloud: 43, Ellis: 40, and Marion: 43), 195 isolates were collected from the three macro-plots (Cloud: 69, Ellis: 64, and Marion: 62), and 155 isolates were collected randomly across the state of Kansas. The three primer-pair combinations yielded a total of 174 scorable AFLP markers, 168 of which were polymorphic in at least one population (Table 1-1). The average similarity among isolates within micro-plot populations was 58%, with a minimum similarity of 29%. Average similarities among isolates within 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 2-1). Identical haplotypes only occurred if isolates were collected within the same lesion (data not shown). Ten lesions were randomly selected and tested for haplotype diversity. Four of ten randomly selected lesions had more than one haplotype, but no lesion had more than two. The mean number of haplotypes recovered per lesion was 1.4. Due to the small number of lesions examined, this mean is probably an underestimation of the true mean in the field.

Genetic variability among populations

Among the 174 loci scored, 75% were polymorphic in micro-plot populations, 80% were polymorphic in macro-plot populations, and 81% were polymorphic in the statewide population (Table 2-1). There were 12, 10, and 11 private alleles identified in micro-plot, macro-plot, and statewide populations, respectively. Only three of these alleles in micro-plot populations and one in the statewide population were present at frequencies greater than or equal to 5%. Gene diversity estimates calculated with Popgene across all loci were nearly identical for all populations (Table 2-1). This was true for both Nei's gene diversity (~0.18) and Shannon's index (~0.28). When loci for which the frequency of both alleles was less than 5% were removed, Nei's gene diversity and Shannon's index values increased by 0.06 and 0.09, respectively.

To gauge the genetic exchange among populations, estimates were made of the overall fixation index G_{ST} among all populations as well as for all possible pairwise combinations (Φ_{ST}); micro-plot/macro-plot, macro-plot/statewide, and micro-plot/ statewide (Table 3-1). The overall fixation index calculated with Popgene was G_{ST} = 0.0331 (min. of

0.0004 for locus GG41, max. of 0.2858 for locus GG26). The corresponding migration rate *Nm,* which was calculated as a function of G_{ST} (16), was expectedly high (>15 individuals per generation) suggesting that there has been significant genetic exchange among these populations of *M. graminicola*. Φ_{ST} values among the three populations (Table 3-1) showed high levels of similarity, and were relatively low. These values ranged from 0.021 when comparing macro-plot and statewide populations to 0.030 for micro-plot and statewide populations. Estimates of migration rate were correspondingly high and ranged from 16 to 23 individuals per generation. Similar results were obtained when removing the 49 loci for which the frequency of both alleles (presence or absence) was smaller than 5% (Table 3-1).

Popgene was also used to calculate the genetic identity among the three populations (Table 4-1). The average genetic identity among the populations was 98.8%, the highest value was obtained when comparing macro-plot and statewide populations (99%) and the lowest was established when comparing micro-plot and statewide populations (98.6%).

Linkage disequilibrium

Estimates of linkage disequilibrium were calculated using Popgene for the 125 loci for which the frequency of both alleles was greater or equal to 5%. Of the 7,750 possible pairewise comparisons, 331 (4.3%), 382 (4.9%), and 349 (4.5%) locus pairs were found in disequilibrium in micro-plot, macro-plot, and statewide distributions, respectively. The number of significant linkage disequilibria was calculated at $P < 0.05$, and all Chi-square tests had one degree of freedom.

Discussion

The goal of this study is to examine genetic diversity within field populations of *M. graminicola* collected from different spatial distributions in Kansas winter wheat. Kansas weather conditions are unique and are characterized by cold winters followed by short springs where temperatures can rise very quickly. Septoria tritici blotch epidemics are sporadic in Kansas, and depend on both the amount of rainfall and favorable temperatures, especially in the spring. The collections used here were sampled at a particular point of time (late November) providing a snapshot of *M. graminicola* populations in Kansas, but were distinct from one another in space ranging from 1 m^2 to the entire state. Although these populations were collected from different spatial distributions, they were very similar in their genetic variability. Our data suggest that these populations are components of a larger pathogen pool that is distributed at least across much of Kansas and probably the central Great Plains. Generally speaking, these results are in accordance with previous studies of Septoria tritici blotch populations that used RFLPs (5,18,19), mating type locus (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 m^2) 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). These results provide evidence that sexual recombination takes place on a regular basis in *M. graminicola* populations in Kansas.

The occurrence of sexual recombination among isolates may have repercussions on the management of Septoria tritici blotch. Such information relates to the risk of adaptation of *M. gramincola* populations to fungicide treatments and resistance genes. For example, 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 virulent strains of *M. graminicola*. In Kansas, growers rely on resistant cultivars to limit losses from Septoria tritici blotch. Although this resistance has been durable for about 15 years, 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 California populations. Future research will make a direct comparison of these two populations in order to test for gene flow barriers, if any, between these locations.

Diversity within and among populations

Mean gene diversities (Table 2-1) were generally very similar, so were allele frequencies (data not shown) suggesting that significant genetic exchange has occurred among these populations. Perhaps the best evidence that these populations are components of a much larger pool are the low G_{ST} (<0.04) values (Table 3-1) and genetic identity values near 100% (Table 4-1). Fixation index values remained low (0.03) when the micro-plot (1 m^2) population was compared with a much larger population representing the entire state

(Table 3-1), this is an indication that most of the genetic variability is distributed on a small scale rather than between populations. These were also the findings of McDonald and Martinez (17), they concluded that their population of *M. graminicola* contained a large amount of genetic variation distributed on a very fine scale. This was also consistent with the findings of Schnieder et al. (30) in their analysis of German populations of *M. graminicola*, they reported fixation indices ranging from 0.023 to 0.049 (0.021 to 0.030 reported here in Kansas) concluding that there was very little differentiation between sampling sites. Low G_{ST} levels were associated with other fungal species such as, *Gibberella* (34), *Rhizoctonia* (27), and *Cronartium* (9), and in all of these studies, the authors concluded that these populations are components of a much larger, well-mixed pathogen pool.

Migration rate (*Nm*) was calculated as a function of G_{ST} (16), Estimates of *Nm* were correspondingly high and ranged from 16 to 23 individuals per generation. Under the island model (33), a movement of as little as one individual per generation is sufficient to prevent significant divergence between populations, although genetic traits may be lost at random unless they are selected for. 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, further strengthening the hypothesis of a large, well-mixed pathogen pool that is distributed at least across much of Kansas and probably the Central Great Plains.

Linkage disequilibrium

With random mating, alleles at any locus will rapidly acquire random association. Departure from equilibrium estimates may provide insight on the importance of sexual or asexual reproduction. We found very little evidence of linkage disequilibrium in Kansas populations, on average, only 4.6% of locus pairs were in disequilibrium. All of the

individuals included in this analysis had unique haplotypes, and estimates of linkage disequilibria were based on the 125 loci for which the frequency of both alleles was greater or equal to 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 pairwise combinations, concluding that the great majority of alleles at the RFLP loci they considered were randomly associated. Their measure of linkage disequilibria included fewer loci (12 RFLP loci), which may account for the difference between the two estimates. 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 and macro-plot populations did not meet this criterion; however, linkage disequilibria estimates in these two populations were very consistent with the statewide population that met Brown's requirement of sample size.

Finally, Kansas populations of *M. graminicola* are characterized by regular recombination, are genetically diverse, and appear to be homogenous across different spatial distributions. The frequent recombination is a reminder that the risk of adaptation of *M. gramincola* populations to fungicide treatments or resistance genes is high and could be dispersed very quickly, whether these new pathogenic traits occur locally through mutation, or by migration from other areas. A variety of sampling methods may be adequate for this pathogen due to the absence of population subdivision and the rich genotypic diversity at very small scales.

Table 1-1. 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]
*Eco*AC/*Mse*CA	72	69
*Eco*AC/*Mse*CC	47	45
*Eco*AC/*Mse*GG	55	54
Total	174	168

[1]*Eco*AC is *Eco*RI primer (5'-AGACTGCGTACCAATTC-3') followed by the selective base pairs AC. *Mse*CA, CC, and GG are *Mse*I primer (5'-GATGAGTCCTGAGTAA-3') followed by the selective base pairs CA, CC, and GG.

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

Table 2-1. Statistical information related to comparing natural populations of *Mycosphaerella graminicola* collected from three spatial distributions (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

[1]Based on an average of three replications corresponding to three locations, these were; Cloud, Ellis, and Marion counties. Micro-plot size was 1 m^2 and macro-plot size was the entire field (> 10 ha).

[2]Values 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.

[3]Nei's gene diversity (23).

[4]Shannon's information index (14).

Table 3-1. Pairwise comparisons of the three spatial distributions (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 greater or equal to 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.	0-0.360	0-0.326	0-0.360	0-0.360	0-0.333	0-0.333
Migration rate (*Nm*)[2]	21.3	20.9	22.8	22.4	15.9	15.6
min. - max.	0.88-2000	1.0-2000	0.88-2000	0.88-2000	1.0-2000	1.0-2000
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

[1]Cloud county location was used in this analysis for both micro (1 m^2) and macro-plot (entire field > 10 ha) populations.
[2]McDermott and McDonald(16).
[3]Nei's gene diversity (23).

Table 4-1. Genetic identity[1] (above diagonal) and genetic distance[1] (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 greater or equal to 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]	****

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

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

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

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Chapter 2 - Limited Gene Flow between Natural Populations of *Mycosphaerella graminicola* from Single Fields in Kansas and California

Introduction

Mycosphaerella graminicola (Fuckel) Schröt. in Cohn is a pseudothecial ascomycete that causes Septoria tritici blotch on wheat. The anamorph, *Septoria tritici* Roberge in Desmaz., was first described by Desmazières in 1842 (10) but *M. graminicola* was not identified as being the sexual stage until over a century later (33). Epidemics are initiated by airborne sexual ascospores produced on wheat stubble (13) and secondary inoculum is in the form of asexual pycnidiospores that are rainsplash dispersed. Under high humidity conditions, both ascospores and pycnidiospores germinate to produce hyphae that penetrate the leaf mostly through stomata (9). Symptoms are visible approximately 10 days after the infection process is initiated, with the appearance of 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.

Although the Middle East is likely to be the origin of *M. graminicola* (23), it is now a worldwide problem affecting wheat-growing areas in Europe (12), Australia (18), Canada (8), and the United States (13,24). Prior to the 1960s, *M. graminicola* was not perceived as an economically significant pathogen on wheat. However, due to changes in cultural practices, and the introduction of new cultivars, this pathogen has become very pernicious and capable of reducing yields by as much as 30 to 40%, especially during growing seasons with significant rainfall (29). In Kansas, epidemics are sporadic due to relatively short springs and inconsistent rainfalls. Estimated average annual losses in Kansas are 1.0% ($10 million) and have ranged from trace to 7.4% (2). However, the disease is widespread and losses in individual fields can exceed 25%.

Gene flow as a result of the establishment of migrant individuals sets a limit to how much genetic divergence between populations can occur due to random genetic drift, mutation, or selection. Migration plays a major role in preventing fungal populations from diverging, and has had a significant effect on the genetic structure of other plant pathogenic fungi such as *Phytophthora* (14), *Gibberella* (43), and *Cronartium* (11), but the distinction between current and historic gene flow is usually difficult to discern, if it can be discerned at all. Significant gene flow has been shown to have occurred among distant populations of *M. graminicola* (4,17). At the regional level, air-dispersed ascospores represent an important component of gene flow and likely constitute a significant current evolutionary force. With high levels of gene flow, there is a potential risk of rapid dispersal of mutant alleles that affect pathogen virulence or sensitivity to fungicides.

So far, eight major genes (*Stb1* to *Stb8*) for resistance to Septoria tritici blotch in wheat have been identified (6). 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 virulent strains of *M. graminicola*. In Kansas, there has been significant effort to develop cultivars with resistance to Septoria tritici blotch (3). Knowing how much genetic exchange is occurring between Kansas populations of *M. graminicola* and populations from other regions is important to the effective deployment of this resistance.

Molecular markers have been used in characterizing fungal plant pathogens not only for identification and diagnosis purposes, but also as tools for population genetic analyses. For example, *M. graminicola* populations have been studied based on the analysis of anonymous restriction fragment length polymorphism (RFLP) loci (7,21,22). These studies have shown 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 that

significant gene flow was occurring. The RLFP technique used in these studies has excellent resolution, but is rather labor intensive and the number of loci available is limited. Amplified fragment length polymorphism (AFLP) markers can yield large quantities of information due to, the high resolution of the nearly unlimited number of available markers, replicability, ease of use, and cost efficiency, which make them a popular tool for the differentiation of populations.

Kansas and California have distinct wheat cultivars, climatic differences, crop rotation patterns, and cultural practices. For example, Kansas grows winter wheat that usually is dormant from December through February, while California produces spring wheat that is actively growing during the winter. These elements, could select for different fungal populations. The goal of this study was to use polymorphism at various AFLP loci to assess the genetic diversity of *M. graminicola* populations within single fields in two widely separated (2176 Km), and geographically isolated, sites in Kansas and California. Statistical tests were performed to determine whether there was significant differentiation between the two populations due to the geographic origin of the isolates. Estimates of gene flow were also calculated.

Materials and methods

Isolate collection

Kansas isolates were collected from naturally occurring Septoria tritici blotch in a commercial wheat field in Cloud County on 19 November 2003. The wheat cultivar in this field was Tomahawk, which is highly susceptible to Septoria tritici blotch. Dr. Lee F. Jackson collected the California sample from an experimental wheat field in Colusa County (Erdman Ranch) on the 24th of February 2004. The wheat cultivar in this field was D6301, an

experimental line that has been used in the UC Davis wheat-testing program as an indicator of high susceptibility to Septoria tritici blotch. California isolates were imported to Kansas under APHIS permit number 65750. Sixty seven and 63 lesions showing pycnidia of *S. tritici* were collected from Kansas and California, respectively. Samples were placed in paper envelopes, dried at room temperature, and stored at 4°C until used.

DNA isolation

Wheat leaves with Septoria tritici blotch lesions were placed on wet filter paper at room temperature (20-25°C) for 24 hours, to allow cirri exudation from pycnidia. Only one isolation was made from each lesion. Cirri were transferred with a sterile, glass needle to petri dishes containing one-fourth strength PDA (0.6% potato dextrose broth, 1.5% agar) and streaked across the agar surface with a sterile, glass rod to separate individual spores. Spores were allowed to germinate for 2 days at room temperature. Colonies 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. Growth was not always uniform among isolates and some were incubated longer than others. Similarly, the type of fungal tissue produced also varied from one isolate to the other, some grew as mycelia, others as 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 (26) modified by Kerényi *et al*. (15). 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 using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). At the conclusion of the experiment, all plant materials and remaining cultures from California were autoclaved and disposed of as stipulated in the

APHIS import permit.

AFLP methodology

AFLPs were generated using the method described by Vos *et al*. (32) as modified by Zeller *et al*. (35). The restriction enzymes *Eco*RI and *Mse*I were selected to digest the DNA samples (100-200 ng per reaction). Standard protocols (28) and manufacturer's recommendations were followed in the use of all buffers and DNA modifying enzymes. An initial testing of 10 primer pair combinations for optimal polymorphisms was conducted and 3 were chosen to generate AFLPs for all isolates. The selected primer pair combinations were; *Eco*+AC/*Mse*+CA, *Eco*+AC/*Mse*+CC, and *Eco*+AC/*Mse*+GG. The *Eco*RI primer used for the selective amplifications was end-labeled with γ^{33}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, MO) for 2 days. A γ^{33}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 homologous and represented the same allele. Bands that differed in size were treated as independent loci with two alleles (presence or absence). Occasionally, unclear bands were observed and subsequently scored as ambiguous in the ensuing population genetic analyses. DNA from four known isolates of *M. graminicola* were included in all of the AFLP gels. The bands from these four isolates also served as references 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 (37) were used to identify AFLP haplotypes, and to determine genetic similarity among isolates. This analysis was conducted for both Kansas and California populations, as well as for the entire pool of isolates. Haplotypes were defined as isolates sharing at least 98% unweighted pair group method with arithmetic means similarity in amplified fragment length polymorphism banding pattern. Genotypic diversity in each population was calculated using the formula $\hat{G} = 1 / \sum_{0\text{-N}} fx \times (x/N)^2$ (I), where fx is the number of genotypes observed, x the number of times the genotype is observed, and N is the sample size. Because the two populations had different sample sizes, $\hat{G}$ was divided by N to normalize the diversity measure.

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}) of Nei (27). Popgene was also used to estimate the migration rate (Nm) from G_{ST} using the formula $Nm = 0.5(1 - G_{ST})/G_{ST}$ (20), to estimate allele frequencies and genetic diversity within and between populations as described by Nei (27), and to measure the genetic identity among populations as described by Nei (28). Data were analyzed by using the haploid, dominant marker subroutines. Estimates of fixation index (G_{ST}) and migration rate (Nm) were calculated for clone-censored populations both for the complete pool of loci and for a sub-pool of loci for which the frequency of both alleles was greater or equal to 5%. This was to determine if rare alleles altered the analyses. Estimates of linkage disequilibrium were calculated with Popgene for the sub-pool of loci where the frequency of both alleles was greater or equal to 5%. χ^2 tests were conducted to determine significance.

Exact tests as described by Raymond and Rousset (30) were performed as implemented in the shareware program Tools for Population Genetic Analyses (TFPGA) version 1.3 (25). This test is not biased towards rare alleles, which allowed the inclusion of the entire pool of loci in this analysis. A Markov chain Monte Carlo approach (30) of Fisher's RxC test (35) was used, which provides an accurate and unbiased probability test for population differentiation as well as providing test results for each locus allowing the detection of aberrant loci.

Results

Population structure

AFLPs were run on 130 *M. graminicola* strains isolated from infected wheat leaves. The frequency of *M. graminicola* isolates from individual leaf samples was 100% in both Kansas and California. The three primer-pair combinations yielded 177 scorable bands, 149 of which were polymorphic in at least one of the populations examined (Table 1-2). Average similarity amongst the 63 California isolates was 73% and ranged from 51% to 100%. In Kansas, the average similarity amongst the 67 isolates was 68% with a minimum of 0% and a maximum of 88%. The average similarity for the combined populations was 63%. Haplotype diversity was determined from these populations utilizing the entire pool of loci, and haplotypes were defined as isolates that shared at least 98% UPGMA means similarity in AFLP banding pattern. In all, 118 genetically unique multilocus haplotypes were identified among the 130 isolates (Table 2-2, Fig. 1-2). Genotypic diversity was high ($\hat{G}$ = 67, $\hat{G}/N$ = 100%) in Kansas, with all 67 isolates showing unique AFLP haplotypes. Genotypic diversity was also high in California ($\hat{G}$ = 43.5, $\hat{G}/N$ = 69%), among the 63 isolates sampled, 51 unique haplotypes were identified; 43 of which occurred once, 6 twice, 1 three times, and 1

four times. Not all members of the same multilocus haplotype had identical fingerprints. Among the haplotypes that occurred twice, two pairs of isolates had identical fingerprints. Three isolates with identical fingerprints were identified within the haplotype that occurred four times.

Genetic divergence between populations

Among the 177 scored loci, 79% were polymorphic in Kansas and 53% in California (Table 2-2). Loci with private alleles were identified in both populations, 25 were detected as private alleles in Kansas and 27 in California. 19/25 of the Kansas loci and 21/27 of the California loci had both alleles present at a frequency of ≥5%. Gene diversity estimates calculated with Popgene across all loci for clone-censored populations were slightly higher in the Kansas population (Table 2-2). This was true for both Nei's gene diversity (KS = 0.169, CA = 0.134) and Shannon's index (KS = 0.270, CA = 0.212). When loci with an allele present at <5% were removed, both Nei’s gene diversity and Shannon’s index increased by ~0.065 and ~0.1 respectively.

Differentiation between populations due to population subdivision or fixation index (G_{ST}) was calculated using Popgene. Estimates of G_{ST} were calculated for clone censored populations using all 177 loci as well as for the 119 loci for which the frequency of both alleles (presence and absence) was greater than or equal to 5%. For the full set of loci, the overall value of G_{ST} was 0.211 and was statistically different from zero based on 1000 bootstrap replications. G_{ST} values for individual loci ranged from 0 for locus CA34 to 1.0 for locus CC10. The effective migration rate (Nm) per generation calculated from G_{ST} was 1.87. Similar results (G_{ST} = 0.221, Nm = 1.8) were obtained when removing the 58 loci for which the frequency of both alleles (presence or absence) was smaller than 5% (Table 3-2). Nei’s genetic identity between the two populations was 0.905 if all loci were analyzed, but

decreased to 0.846 when only considering the 119 loci for which the frequency of both alleles was greater ≥5%.

Tests for linkage disequilibrium and population differentiation

Single population estimates of linkage disequilibrium were calculated using Popgene as defined by Weir (40) for the 119 loci for which the frequency of both alleles was ≥5%. There was no evidence of significant linkage disequilibrium in either Kansas or California populations. Of the 7,021 possible pairwise comparisons, 326 (4.6%) locus pairs were found in disequilibrium in Kansas. In California, this figure was slightly higher, and of the 7,021 possible pairwise comparisons, 582 (5.4%) were in disequilibrium. The number of significant linkage disequilibria was calculated at $P < 0.05$, and all Chi-square tests had one degree of freedom.

Exact tests for population differentiation were conducted using TFPGA for the entire pool of loci. This test provides an accurate and unbiased analysis, even on very small samples or low-frequency alleles. 1000 dememorization steps, 20 batches, and 2000 permutations per batch were set in a simultaneous analysis of both populations. Significant ($P < 0.05$) differences in allele frequencies were identified at 53/177 loci. The analysis was not performed on monomorphic loci. Fisher's combined probability test showed an overall significance across loci. The null hypothesis H0 (no differentiation between populations) was rejected with a probability of $P < 0.05$. The Chi-square test had 338 degrees of freedom.

Discussion

Due to the mild winter temperatures, fall seeded white and red spring wheats remain

the predominant classes of wheat used in California and is mainly grown under irrigation. It is more important as a tool for disease control in other crops and soil conservation than it is for economic return. Kansas is the largest wheat producing state, and hard red winter wheat represents 94% of the wheat grown in the state. Kansas wheat is planted and sprouts in the fall, becomes dormant in the winter, grows again in the spring and is harvested in early summer. Kansas and California wheat growing regions differ dramatically in soils and climate, wheat cultivars, crop rotation patterns, and cultural practices, which could select for different fungal populations of *M. graminicola*. The goal of this study was to use polymorphism at various AFLP loci to assess the genetic diversity of *M. graminicola* populations within single fields in two widely separated, and geographically isolated, sites in Kansas and California. Our data show that there are relatively few locus pairs in disequilibrium, and that gene and genotype diversities in both Kansas and California locations were consistent with a genetically diverse initial inoculum source. These results were in accordance with previous studies of *M. graminicola* populations (7,21,22,31). However, our estimate of gene flow ($G_{ST} = 0.215$, $Nm < 2$) was much lower than previously reported (4,17) suggesting that the genetic exchange between these two populations may be decreasing and that these populations are beginning to differentiate from one another.

Haplotype distribution

All 130 *M. graminicola* isolates included in this study were collected from different lesions. In all, 118 genetically unique multilocus haplotypes were identified among the 130 isolates. Genotypic diversity was very high in Kansas, as all of the isolates analyzed had unique haplotypes. No identical haplotypes were found beyond the lesion scale, this was also true for isolates collected from different lesions on the same leaf. In California, 51 unique multilocus haplotypes were identified among the 63 isolates tested.

The early (late November) sampling of the Kansas isolates may have contributed to the lack of clonality observed in this sample. Seed sowing in Cloud county usually takes place in the first week of October and it takes about 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). The combination of low temperatures and limited precipitation in the winter months may postpone any significant pycnidiospore dispersal in Kansas until the spring. In California, however, mild temperatures and abundance of precipitation events are conducive to pycnidiospore dispersal during the fall and winter months, which may explain the recovery of clones in the Califonia sample. In any case, genotypic diversity in the present study was high in both epidemic populations. Comparable genotypic diversity were identified by Chen and McDonald (7), they concluded that sexual reproduction played a major role in their populations of *M. graminicola*. Our data were consistent with frequent sexual recombination in both Kansas and California populations, and randomly mating populations could not be excluded. Regular cycles of sexual recombination may have repercussions on the management of Septoria tritici blotch. Such information relates to the risk of adaptation of *M. gramincola* populations to fungicide treatments and resistance genes.

Population differentiation and evidence for isolation by distance

The mean gene diversity (Table 2-2) was slightly lower in the California population suggesting that the Kansas population may have more genetic variation than the California population. Mean gene diversity increased when both populations were pooled (Table 3-2), meaning that these populations contributed to the combined pool with new alleles. Our estimates of gene diversity were significantly lower than those reported in previous studies of

M. graminicola populations (4,17). However, such a difference is, at least partly, due to the fact that those studies used RFLP loci, which have a larger number of alleles. With AFLPs (two alleles at any locus) the maximum possible value of gene diversity is 0.5. Gene diversities in both epidemic populations were relatively lower than those of Schnieder et al. (34) in their study of German populations of *M. graminicola* based on AFLPs. This may be explained by the fact that German populations are closer to the center of origin of this pathogen, which is believed to be the Middle East (17).

Differentiation between populations due to population subdivision or fixation index (G_{ST}) was calculated for clone censored populations using all 177 loci as well as for the 119 loci for which the frequency of both alleles (presence and absence) was greater than or equal to 5%. The rarer alleles did not have a significant effect on the analysis, and in both cases the calculated G_{ST} was ~0.21 with a corresponding migration rate (Nm) of ~1.8 (Table 3-2). This finding was unexpected as previous estimates of migration rates were significantly higher even among distant populations on different continents. Linde et al. (17) reported an average estimated migration rate of 9 individuals per generation among populations of *M. graminicola* representing Switzerland, Israel, Oregon, and Texas. In the same study, a migration rate of 58 individuals per generation was calculated between the two North American sites in Oregon and Texas. The distance between these two locations was comparable to the two sites examined in this study. Migration rates of 16 – 23 individuals per generation were calculated using AFLPs among various populations within the state of Kansas (Chapter 1). Although the migration rate estimated here is low, under Wright's island model (41), a movement of as little as one individual per generation is sufficient to prevent significant divergence between populations. Under the same model, with a migration rate of four distinct individuals, the populations are said to be co-evolving. A movement of 1 – 2 individuals per generation as estimated in this study may not be low enough to keep these

two populations from diverging significantly, but could be an indication that populations of *M. graminicola* at these two sites may be gradually moving towards equilibrium between genetic drift and gene flow. These results must be interpreted as preliminary in this respect, as bigger sample sizes may be necessary for a more accurate estimate of gene flow. Increased sampling variance due to the allelic dominance of AFLPs can result in biased estimates. Lynch and Milligan (19) reported that more accurate estimates can be achieved by eliminating loci where the frequency of the null phenotype is less than $3/N$, N being the sample size. This adjustment was not applicable to our data set due to the fact that genetic structure parameters were calculated for both the full set of loci, and for the set of loci for which the frequency of both alleles was ≥0.05, the later being above the $3/N$ threshold. Yan et al. (42) compared population genetic parameters calculated from both AFLP and RFLP marker data in their study of *Aedes aegypti* populations. They found that AFLP markers led to a lower F_{ST} and therefore resulting in a higher estimate of gene flow. Differences in mutation rates between AFLP and RFLP loci, if any, could also account for varying estimates of population genetic parameters including gene flow.

Linkage disequilibrium

Linkage disequilibrium estimates can provide useful information on the relative importance of sexual reproduction. Alleles eventually will become indiscriminately associated with random mating. No evidence of linkage disequilibrium was found in either population when associations among the 119 loci for which the frequency of both alleles was ≥0.05 were examined, with only 4.6% of the locus pairs in Kansas, and 5.4% of the locus pairs in California in detectable disequilibrium. These estimates were lower than those reported by Chen and McDonald (7) in their analysis of a California sample, where 12% of the 66 pairwise combinations were found in significant disequilibrium. They concluded that

random association among the RFLP loci they examined could not be excluded. More accurate detection of disequilibria may require sample sizes ≥100 individuals as stipulated by Brown (5). Although our samples were not this large, and the few observed disequilibria may be due to weak linkage or to statistical bias resulting from insufficient sample sizes, the overall pattern suggests that there is little, if any, linkage in either the separate or the combined populations.

In conclusion, both Kansas and California populations of *M. graminicola* are characterized by regular recombination and are genetically diverse. The apparent random mating and the high levels of genotypic diversity may have implications for the management of Septoria tritici blotch due to the high risk of adaptation to fungicide treatments or resistance genes. These traits could be dispersed rapidly, whether they occur locally through mutation or by migration from other regions. Septoria tritici blotch resistance gene *Stb4* has been useful in California for many years (1), however, a recent decreased in effectiveness of this gene has been documented. Although the level of gene flow calculated between Kansas and California populations of *M. graminicola* is low, the transfer of this new pathogenic trait, if it has not occurred already, from California to Kansas cannot be excluded. It is not known which one(s) of the eight major genes for resistance to Septoria tritici blotch are present in the popular resistant Kansas cultivars. If *Stb4* is deployed in this state, there is cause for concern that virulence could migrate from California.

Table 1-2. AFLP analysis of *Mycosphaerella graminicola*; 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]
*Eco*AC/*Mse*CA	72	57
*Eco*AC/*Mse*CC	48	41
*Eco*AC/*Mse*GG	57	51
Total	177	149

[1]*Eco*AC is *Eco*RI primer (5'-AGACTGCGTACCAATTC-3') followed by the selective base pairs AC. *Mse*CA, CC, and GG are *Mse*I primer (5'-GATGAGTCCTGAGTAA-3') followed by the selective base pairs CA, CC, and GG.

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

Table 2-2. Statistical information related to comparing natural populations of *Mycosphaerella graminicola* from Kansas and California

Population	Kansas	California
No. of isolates	67	63
Unique haplotypes	67	51
Polymorphic loci (%)	79	53
Private alleles	24	27
Nei's gene diversity[1,2,4]		
177 loci	0.169	0.134
119 loci	0.240	0.195
Shannon's index[1,3,4]		
177 loci	0.270	0.212
119 loci	0.377	0.307

[1]Values 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.

[2]Nei's gene diversity (27).

[3]Shannon's information index (16).

[4]Calculated for both the full set of loci and for the 119 loci for which the frequency of both alleles was greater or equal than 5%.

Table 3-2. Comparison of Kansas and California populations of *Mycosphaerella Graminicola* using all scored loci, and for the 119 loci where the frequency of both alleles was greater or equal to 0.05.

	177 loci	119 loci
Fixation index (G_{ST})	0.211	0.217
min. – max.	0–1.0	0–1.0
Migration rate (*Nm*)[1]	1.87	1.8
min. – max.	0–2000	0–2000
Nei's gene diversity[2]	0.193	0.279
Std. deviation	0.188	0.169
Genetic identity[3]	0.905	0.846

[1]McDermott and McDonald (20).

[2]Nei's measure of gene diversity (27).

[3]Nei's measures of genetic identity and genetic distance (28).

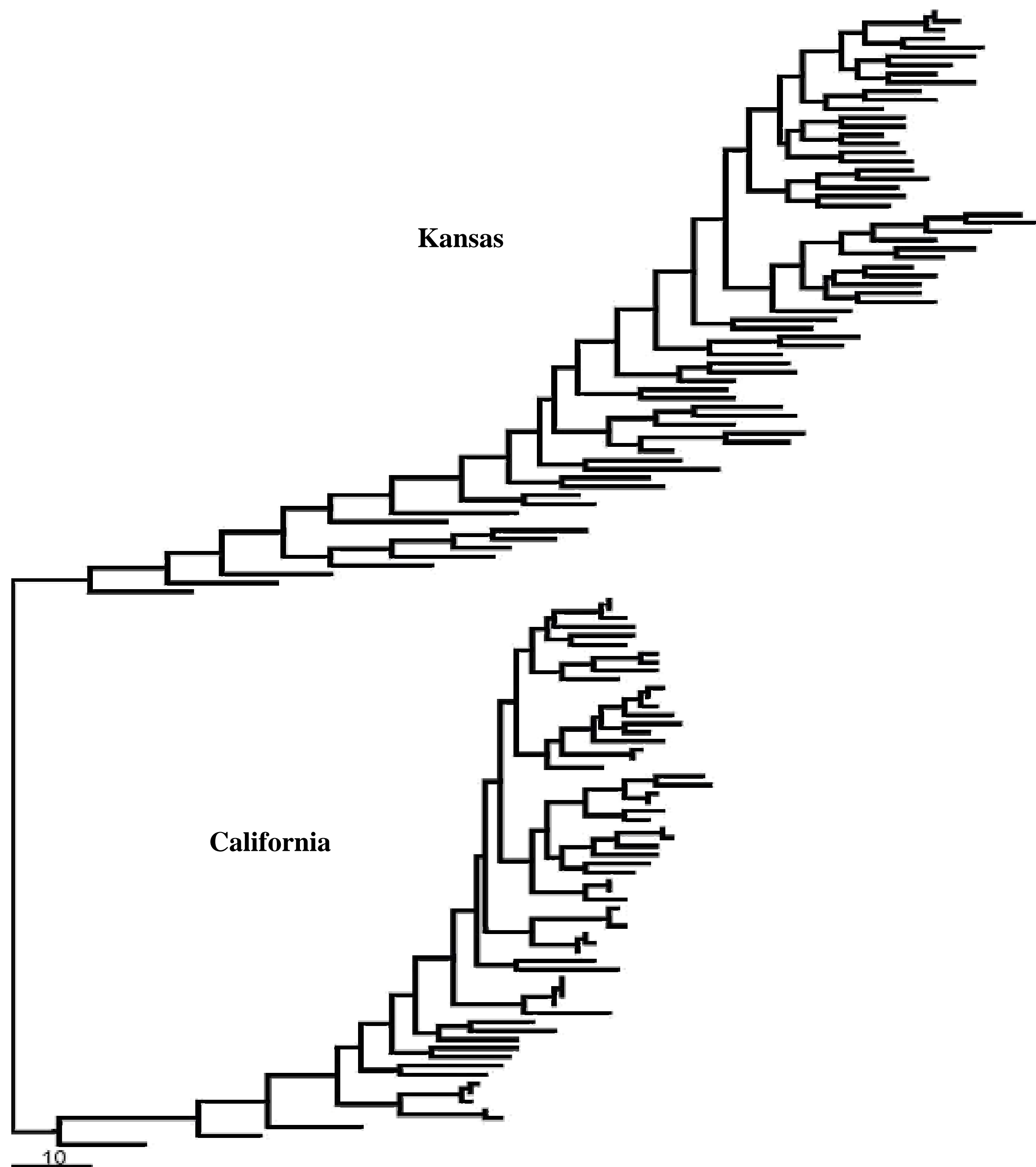

Fig. 1-2. Unweighted pair grouping by mathematical averages (UPGMA) phenogram based on amplified fragment length polymorphism data of *Mycosphaerella graminicola* populations in Kansas and California.

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Chapter 3 - The Role of Sexual Reproduction in the Disease Cycle of Field Populations of *Mycosphaerella graminicola* in Kansas

Introduction

Septoria tritici blotch, caused by *Mycosphaerella graminicola* (Fuckel) J. Schröt. (anamorph *Septoria tritici* Roberge in Desmaz.), is a pseudothecial ascomycete that affects wheat worldwide. Significant losses from this disease have been reported around the globe (5,8,9,15,19) and it is capable of reducing yields by as much as 30 to 40%, especially during growing seasons with significant rainfall (24). In Kansas, epidemics are sporadic due to relatively short springs and inconsistent rainfalls. Estimated average annual losses in Kansas are 1.0% and have ranged from trace to 7.4% (1). However, the disease is widespread and losses in individual fields can exceed 25%. Disease management measures consist of fungicide applications and debris management, but these practices are often not feasible economically (15). Although various levels of host resistance exist (23), no commercial wheat cultivar is immune.

Septoria tritici blotch epidemics are primarily initiated by ascospores produced on wheat stubble (8). Secondary inoculum is in the form of pycnidiospores that are splash dispersed. Ascospores and pycnidiospores germinate under favorable conditions to produce hyphae that penetrate the leaf mostly through stomata (6). Symptoms are visible approximately 10 days after the infection process is initiated, with the appearance of 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 (7).

M. graminicola populations have been studied based on the analysis of anonymous restriction fragment length polymorphism (RFLP) loci (4,17,18). These studies have shown 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.

Although much is known about the sexual stage of Septoria tritici in wheat, the production of ascospores and pseudothecia development are yet to be fully described. Ascospores not only initiate epidemics in the fall, but are thought to cause infections throughout the year in some areas as evidenced by ascospore trapping data (11). Kansas weather conditions are characterized by cold and dry winters followed by short springs where temperatures can rise quickly. Wheat is planted and sprouts in the fall, becomes dormant in the winter, grows again in the spring and is harvested in June. In Kansas, there has been significant effort to develop cultivars with resistance to Septoria trici blotch (2). Repeated sexual cycles during the entire growing season, if they occur in Kansas, would mean that genetic recombination takes place regularly throughout the year, such an occurrence could complicate resistance breeding strategies. The goal of this study was to conduct a field experiment in order to determine the importance of the sexual phase of *M. graminicola* compared with the asexual phase under Kansas conditions. The relative importance of both phases was followed in two successive years. Amplified fragment length polymorphism (AFLP) marker data were used to differentiate sexual and clonal reproduction in fall and spring leaf samples after various fungicide and/or inoculation treatments.

Materials and methods

Field plot establishment

Field plots were established at the Kansas State University Plant Pathology field laboratory near Manhattan, KS during the 2004-05 and 2005-06 growing seasons to evaluate the significance of sexual reproduction of *M. graminicola* in Kansas during the course of a cycle. Due to the similarity of results from the two years, only data from the first year are

presented.

The hard red winter wheat cultivar Tomahawk, which is highly susceptible to Septoria tritici blotch, was used and is commonly grown in Kansas. Wheat had not been grown at this location during at least two years prior to the experiments. The plot area was moldboard plowed, disked, and spike tooth harrowed before planting. Wheat plots were planted during the first week of October in 1.5 m^2 quadrats (1.5 × 1 m); and consisted of five rows planted 25 cm apart. Ten meters of bare ground separated all plots in all directions to avoid splash-dispersal of pycnidiospores among adjacent plots. The nine treatment plots were arranged in a Latin square design with three treatments and three replications per treatment. The three treatments were; 1) no treatment, exposed to natural inoculum; 2) inoculation in the fall with an isolate with a known genotype; an 3) foliage protected in the fall with fungicides then exposed to natural inoculum in the spring. Weed control and fertilization were conducted according to customary commercial practices in Kansas.

Inoculum preparation

M. graminicola isolate MP-22 was used to inoculate the three replicates of treatment number 2. This isolate was collected from a naturally occurring Septoria tritici blotch epidemic in a commercial wheat field in Cloud County Kansas on November 19, 2003. MP-22 was isolated from a single pycnidium after wheat leaves with Septoria tritici blotch lesions were placed on wet filter paper at room temperature (20-25°C) for 24 hours, to allow cirri exudation from pycnidia. Cirri were transferred with a sterile, glass needle to petri dishes containing one-fourth strength PDA (0.6% potato dextrose broth, 1.5% agar) and streaked across the agar surface with a sterile, glass rod to separate individual spores. Spores were allowed to germinate for 2 days at room temperature. A colony from a single spore was transferred to one-fourth strength liquid PDB medium (0.6% potato dextrose broth) and

incubated at room temperature for ~5 days. Spore concentration was adjusted to 1×10^6 /ml and suspended in a 0.25% gelatin solution before inoculation. Inoculations were performed twice beginning at the three-leaf stage and one week apart using a spray bottle at the rate of 150 ml per plot.

Fungicide applications

The systemic foliar fungicide propiconazole (Tilt 3.6E) was used to protect the foliage for plots of treatment 3 throughout the fall. The concentration was 0.7 ml of Tilt per liter of water, and applications were performed using a spray bottle at the rate of 108 ml per plot four times at about 3-week intervals between mid-October (shortly after emergence) through late December (beginning of dormancy).

Lesion sampling

Isolates of *M. graminicola* were collected on November 25, 2004 (55 days after planting) and again on May 7, 2005 (about 21 days prior to the onset of senescence and 44 days before harvest), hereafter referred to as fall and spring samples, respectively. In the fall, all visible lesions within the quadrats were sampled, however, only a small portion from each lesion was collected in order to preserve the original diversity within the plots.

Fall samples comprised 58 and 60 isolates collected from naturally infected (treatment 1) and inoculated (treatment 2) plots, respectively. No visible lesions were present in the fungicide-treated plots (treatment 3) in the fall. Spring samples were collected as high in the canopy as possible, and most of these isolates were recovered from flag minus three and flag minus four leaves. 132 and 169 isolates were collected from the naturally infected (treatment 1) and inoculated (treatment 2) plots, respectively. Very few lesions were

observed in the spring in fungicide-treated plots and a total of only 36 isolates were collected. Samples were placed in paper envelopes, dried at room temperature, and stored at 4°C until used.

DNA extraction and AFLP methodology

Pure cultures of all samples were obtained as described above. Colonies from single spores 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. The resulting cultures were concentrated by centrifugation and stored at -80°C until used. 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.

AFLPs were generated using the method described by Vos *et al*. (32) as modified by Zeller *et al*. (35). The restriction enzymes *Eco*RI and *Mse*I were selected to digest the DNA samples (100-200 ng per reaction). Standard protocols (28) and manufacturer's recommendations were followed in the use of all buffers and DNA modifying enzymes. Isolates from the inoculated plots were analyzed using a single primer pair combination; *Eco*+AC/*Mse*+CA, this was sufficient to identify clonal types. Three primer pair combinations; *Eco*+AC/*Mse*+CA, *Eco*+AC/*Mse*+CC, and *Eco*+AC/*Mse*+GG (Table 1-3), were used to analyze isolates from the naturally infected plots. The *Eco*RI primer used for the selective amplifications was end-labeled with γ^{33}P-ATP, and the amplification products separated in denaturing 6% polyacrylamide (Long Ranger, FMC Scientific, Rockland, ME) gels in 1× Tris-borate EDTA buffer.

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 homologous and represented the same allele. Bands that differed in size were treated as independent loci with two alleles (presence or absence).

Data analysis

The Dice coefficient of similarity of SAS (SAS Institute, Cary, NC) was used to identify AFLP haplotypes. Haplotypes were defined as isolates sharing at least 98% unweighted pair group method with arithmetic means similarity in amplified fragment length polymorphism banding pattern. Genotypic diversity was calculated using the formula $\hat{G} = 1 / \sum_{0\text{-N}} fx \times (x/N)^2$ (I), where fx is the number of genotypes observed, x the number of times the genotype is observed, and N is the sample size. Because populations had different sample sizes, $\hat{G}$ was divided by N to calculate the percentage of diversity.

Additional analyses comparing fall and spring populations of *M. graminicola* were performed using the shareware program Popgene version 1.32 (Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada). Estimates of the fixation index (G_{ST}) were calculated as described by Nei (23), the migration rate (Nm) using the formula $Nm = 0.5(1 - G_{ST})/G_{ST}$ (16), and the genetic identity among populations as described by Nei (24) were calculated. These estimates were calculated for clone-censored populations both for the complete pool of loci and for a sub-pool of loci where the frequency of both alleles was greater or equal to 5%.

Results

Haplotype distribution

A total of 419 isolates of *M. graminicola* were collected in fall and spring from the naturally infected and inoculated treatment plots (Table 2-3). In the plots that were treated

with the fungicide propiconazole, no visible lesions were present during the fall sampling, however, an average of 12 lesions per plot were observed in the spring sampling. In the naturally infected plots, 58 and 132 isolates were collected during fall and spring sampling periods, respectively. It was visually estimated that there were about 1250 Septoria tritici blotch lesions per plot at the spring sampling. Haplotype diversity was determined from these populations utilizing a pool of 159 loci, and haplotypes were defined as isolates that shared at least 98% UPGMA means similarity in AFLP banding pattern. All of the 58 fall isolates had unique haplotypes, and genotypic diversity was at its maximum ($\hat{G}$ = 58, $\hat{G}/N$ = 100%). In the subsequent spring samples, genotypic diversity remained high ($\hat{G}$ = 121, $\hat{G}/N$ = 91.7%), and among the 132 isolates collected, 128 unique haplotypes were identified, 120 of which occurred once, and 6 occurred twice. All members of the same haplotype had identical fingerprints. AFLP profiles of isolates collected in the spring did not match any of those collected in the fall in all three replicates of the naturally infected plots.

In the inoculated plots, *M. graminicola* isolate MP-22 was used to inoculate all three replicate plots of this treatment. A total of 60 and 169 isolates were collected during the fall and spring sampling periods. For this treatment, it was visually estimated that there were 2100 Septoria tritici blotch lesions per plot during spring sampling. The frequency of recovery *M. graminicola* isolate MP-22 was high in both sampling periods. MP-22 was recovered 93% of the time in the fall, and 98% of the time in the corresponding spring samples. Among the 60 isolates collected in the fall, five unique haplotypes were identified, four of which occurred once, and one (MP-22) occurred 56 times. In the corresponding spring samples, four unique haplotypes were identified among the 169 isolate tested, three of which occurred once, and one (MP-22) occurred 166 times. As expected from these data, genotypic diversity was low in both fall ($\hat{G}$ = 1.15, $\hat{G}/N$ = 1.9%) and spring ($\hat{G}$ = 1.03, $\hat{G}/N$ = 0.6%) samples.

Population parameters in the naturally infected plots

Among the 159 loci scored, 74% were polymorphic in fall populations, and 82% were polymorphic in spring populations (Table 3-3). Average similarities among isolates within fall and spring samples were 55% (19-78%), and 57% (23-100%), respectively. There were eight private alleles identified in each of the fall and spring populations of *M. graminicola* in the naturally infected plots. Only six of these alleles in fall populations and seven in spring populations were present at frequencies ≥5%. None of these alleles were fixed in either population. Gene diversity estimates calculated with Popgene across all loci were nearly identical for either population (Table 3-3). This was true for both Nei's gene diversity and Shannon's index. When loci for which the frequency of both alleles was <5% were removed, both estimates of gene diversity increased by ~38%. Fixation index (G_{ST}) estimates were calculated using Popgene. Estimates of G_{ST} were calculated for clone censored populations using all 159 loci as well as for the 112 loci for which the frequency of both alleles (presence and absence) was ≥5%. For the full set of loci, the overall value of G_{ST} was 0.038 and was statistically different from zero based on 1000 bootstrap replications. G_{ST} values for individual loci ranged from 0 for locus CC37 to 0.37 for locus CA40. The effective migration rate (*Nm*) per generation calculated from G_{ST} was 12.36. Similar estimates of fixation index and migration rate (G_{ST} = 0.039, *Nm* = 12.25) were obtained when the 47 loci for which the frequency of both alleles (presence or absence) was <5% were removed from the analysis (Table 4-3). Nei’s genetic identity between the two populations was 0.971, but slightly decreased to 0.956 when only considering the 112 loci for which the frequency of both alleles was ≥5%.

Linkage disequilibrium

Estimates of linkage disequilibrium were calculated as described by Weir (29) for clone censored fall and spring populations collected from the naturally infected plots. Only the 112 AFLP loci for which the frequency of both alleles was ≥5% were used in this analysis. Of the 6,216 possible pairwise comparisons, 228 (3.6%), and 220 (3.5%) locus pairs were found in disequilibrium in fall and spring collections, respectively. The number of significant linkage disequilibria was calculated at $P < 0.05$, and all Chi-square tests had one degree of freedom.

Discussion

Published disease cycles for *M. graminicola* show survival between cropping seasons primarily on wheat residue. Ascospores are discharged from pseudothecia produced on wheat stubble or volunteer plants to initiate infections in the fall, and secondary infections result from splash dispersed pycnidiospores. However, there have been growing reports, at least under some conditions, that ascospores not only originate from pseudothecia on wheat stubble, but also from fruiting bodies within the wheat crop throughout the growing season (11,12). Kansas weather conditions are characterized by cold winters where average low temperatures are below the freezing mark from November through March; precipitations are also at their lowest during this period. These conditions are followed by short springs where temperatures can rise quickly, with harvest often beginning in early June. The goal of this study was to conduct a field experiment in order to determine the importance of the sexual phase of *M. graminicola* during the course of a cycle in winter wheat under Kansas conditions as well as to compare fall and spring populations of *M. graminicola* from natural inoculum. Our AFLP data indicated that sexual reproduction played a major role in the

population structure in the early season (fall), but was not significant during the spring portion of the cycle as evidenced by the observed clonality in the inoculated plots and the limited number of lesions observed in the spring in the plots protected with fungicide in the fall.

Population structure

In the naturally infected plots, all of the visible lesions present in the fall were sampled. Although this fungus is capable of producing large amounts of asexual pycnidiospores that would contribute to the clonal dispersal on a small scale, genotypic diversity was high as all of the 58 isolates collected from these plots had unique haplotypes. The maximum genotypic diversity observed in this early season sample, signifies that the lesions sampled here were all from ascospores. These results were consistent with previous findings (4,17,18,25) concluding that sexual reproduction played a major role in the population structure of *M. graminicola*. The combination of low temperature and the lack of precipitations over the winter months in Kansas may postpone any significant pycnidiospore dispersal until the following spring. However, based on AFLP fingerprints in the corresponding spring samples in the naturally infected plots, genotypic diversity remained high (G/N = 91.7%), and among the 132 genotypes collected from the three replicate plots, 126 were unique. Although the clonal fraction was higher in the spring sample, no particular haplotype occurred at a high frequency, and the most common genotype occurred only twice. The low levels of clonality observed in the spring sample suggest that initial genotypic diversity is so high that the succeeding asexual dispersal has little effect on the genetic structure of the population. Surprisingly, even though only small fractions of the lesions were collected in the fall, none of the genotypes recovered in the spring sample were present in the fall collection. These results suggest that although all visible lesions were sampled in the fall,

our sample only reflected a small portion of the initial diversity. This was probably due to most of the lesions from primary inoculum not yet being visible.

Knowing that splash dispersed asexual pycnidiospores have a limited range < 1m (4), all of our treatment plots were separated so that Septoria tritici infections could only occur through airborne ascospores or by inoculation. In the plots that were treated throughout the fall with the fungicide, no visible lesions were present during the early sampling period, and in the ensuing spring sample, very few lesions were observed (12 lesions per plot) compared to the naturally infected and inoculated treatment plots (Table 2-3). It is difficult to eradicate all of the infection sites inside the plant and often, some pathogens escape the fungicide. In the plots that were inoculated in an attempt to limit the genotypic diversity to a single genotype of *M. graminicola*, the frequency of recovery of this strain was high in both sampling periods. AFLP fingerprints showed that this particular strain was recovered 93% of the time in the fall, and 98% of time in the corresponding spring samples. As expected, the genotypic diversity was low in these plots in the early season sampling ($G/N = 1.9\%$), and even lower in the spring sample ($G/N = 0.6\%$).

If significant spring infections from sexual spores were to occur, one would expect the proportion of infections caused by ascospores to increase throughout the course of the growing cycle. Our inoculated and fungicide plot AFLP data do not support this hypothesis, suggesting that the sexual phase of this fungus does not play a significant role throughout the spring growing season in Kansas. Hunter at al. (11) in their analysis of ascospore trapping data in the UK, found that ascospores can be released throughout the year, which can not entirely be accounted for by ascospores released from the previous year’s stubble, meaning that spores are being released within the developing crop. In Oregon, Zhan et al. (31) reported that the proportion of infections caused by ascospores increased over the growing season, and estimated that by the end of the growing season, 24% of the isolates in their

inoculated plots were sexual recombinants. In the present experiment, we found little evidence to support significant infections from ascospores in the spring under Kansas conditions. Kansas winters are characterized by frequent ground frosts and limited precipitation, which may not be conducive to pseudothecial development. These conditions are followed by short springs where temperatures can rise quickly, wheat senesces during the month of May with harvest often taking place beginning in early June in much of the state. Although ascospore release may occur in late spring and early summer, the early harvest in Kansas may limit its impact on the wheat crop.

Differentiation between fall and spring populations

The data were subdivided based on the sampling period. Population genetic parameters were calculated for clone censored fall and spring populations of *M. graminicola* collected from the naturally infected plots. Estimates were calculated for the full set of loci, and for the 112 loci for which the frequency of both alleles were ≥5%. This was to determine if the rare alleles had a significant effect on the analysis. Mean gene diversities (Table 3-3) were very similar suggesting that significant genetic exchange has occurred among these populations. Perhaps the best evidence that these two populations are mere sub-samples of a larger randomly mating population are the low G_{ST} (<0.04) values and genetic identity values near 100% (Table 4-3). Migration rate (*Nm*) was calculated as a function of G_{ST} (16), estimates of *Nm* were correspondingly high and averaged ~12 individuals per generation. Departure from equilibrium estimates may provide insight on the importance of sexual or asexual reproduction. We found very little evidence of linkage disequilibrium in early and late season populations of *M. graminicola*. On average, only 3.6% of locus pairs in the fall sample, and 3.5% of the locus pairs in the spring sample, were found in disequilibrium. Sample sizes >100 individuals may be required for an adequate detection of disequilibria (3).

Therefore, we could not determine if these estimates were due to weak linkage disequilibrium, or to statistical artifacts resulting from insufficient sample sizes. In any case, there was no evidence of population subdivision between fall and spring samples. These populations are characterized by regular recombination, are genetically diverse, and appear to be homogenous throughout the growing season.

Table 1-3. AFLP primer-pair combinations used in the analysis of *Mycosphaerella graminicola* populations from the naturally infected treatment plots.

Primer-pair combination[1]	Number of amplified bands	Number of polymorphic bands[2]
*Eco*AC/*Mse*CA	57	46
*Eco*AC/*Mse*CC	47	43
*Eco*AC/*Mse*GG	55	53
Total	159	142

[1]*Eco*AC is *Eco*RI primer (5'-AGACTGCGTACCAATTC-3') followed by the selective base pairs AC. *Mse*CA, CC, and GG are *Mse*I primer (5'-GATGAGTCCTGAGTAA-3') followed by the selective base pairs CA, CC, and GG.

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

Table 2-3. Haplotype distribution in fall and spring populations of *Mycosphaerella graminicola* collected from naturally infected and inoculated treatment plots.

	Naturally infected plots		Inoculated plots	
	Fall	Spring	Fall	Spring
Lesions collected (N)	58	132	60	169
Total lesions per plot[1]	58	1250	60	2100
Unique haplotypes[2]	58	126	5	4
Genotypic diversity ($\hat{G}$)[3]	58	121	1.15	1.03
$\hat{G}/N$ (%)	100%	91.7%	1.9%	0.6%

[1]Based on an average of three replications. In the fall all visible lesions were sampled. In the spring sample, the total number of lesions per plot was estimated by counting the number of lesions in one row, and extrapolating to the entire plot.

[2]Identical haplotypes were defined as isolates sharing at least 98% unweighted pair group method with arithmetic means similarity in amplified fragment length polymorphism banding pattern.

[3]Index of genotypic diversity calculated as in Stoddart and Taylor (27).

Table 3-3. Comparison of fall and spring samples of *Mycosphaerella graminicola* populations in the naturally infected treatment plots.

	Fall sample	Spring sample
No. of isolates	58	132
Polymorphic loci (%)	74%	82%
Average similarity (%)[1,6]	55	57
Private alleles	8	8
Nei's gene diversity[2,3,5,6]		
159 loci	0.184	0.176
112 loci	0.253	0.247
Shannon's index[2,4,5,6]		
159 loci	0.285	0.281
112 loci	0.388	0.388

[1]Calculated using the Dice coefficient of similarity in the cluster procedure of SAS (SAS Institute, Cary, NC).

[2]Values 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.

[3]Nei's gene diversity (21).

[4]Shannon's information index (14).

[5]Calculated for both the full set of loci and for the 119 loci for which the frequency of both alleles was greater or equal than 5%.

[5]Based on an average of three replications.

Table 4-3. Population genetic parameters calculated for fall and spring populations of *Mycosphaerella graminicola* from naturally infected plots using all scored loci, and for the 112 loci for which the frequency of both alleles was ≥0.05.

	159 loci	112 loci
Fixation index (G_{ST})[1]	0.038	0.039
min. – max.	0 – 0.37	0 – 0.37
Migration rate (Nm)[1,2]	12.36	12.25
min. – max.	0.84 – 2000	0.84 – 2000
Genetic identity[1,3]	0.971	0.956

[1]Based on an average of three replications.

[2]McDermott and McDonald (16).

[3]Nei's measures of genetic identity and genetic distance (22).

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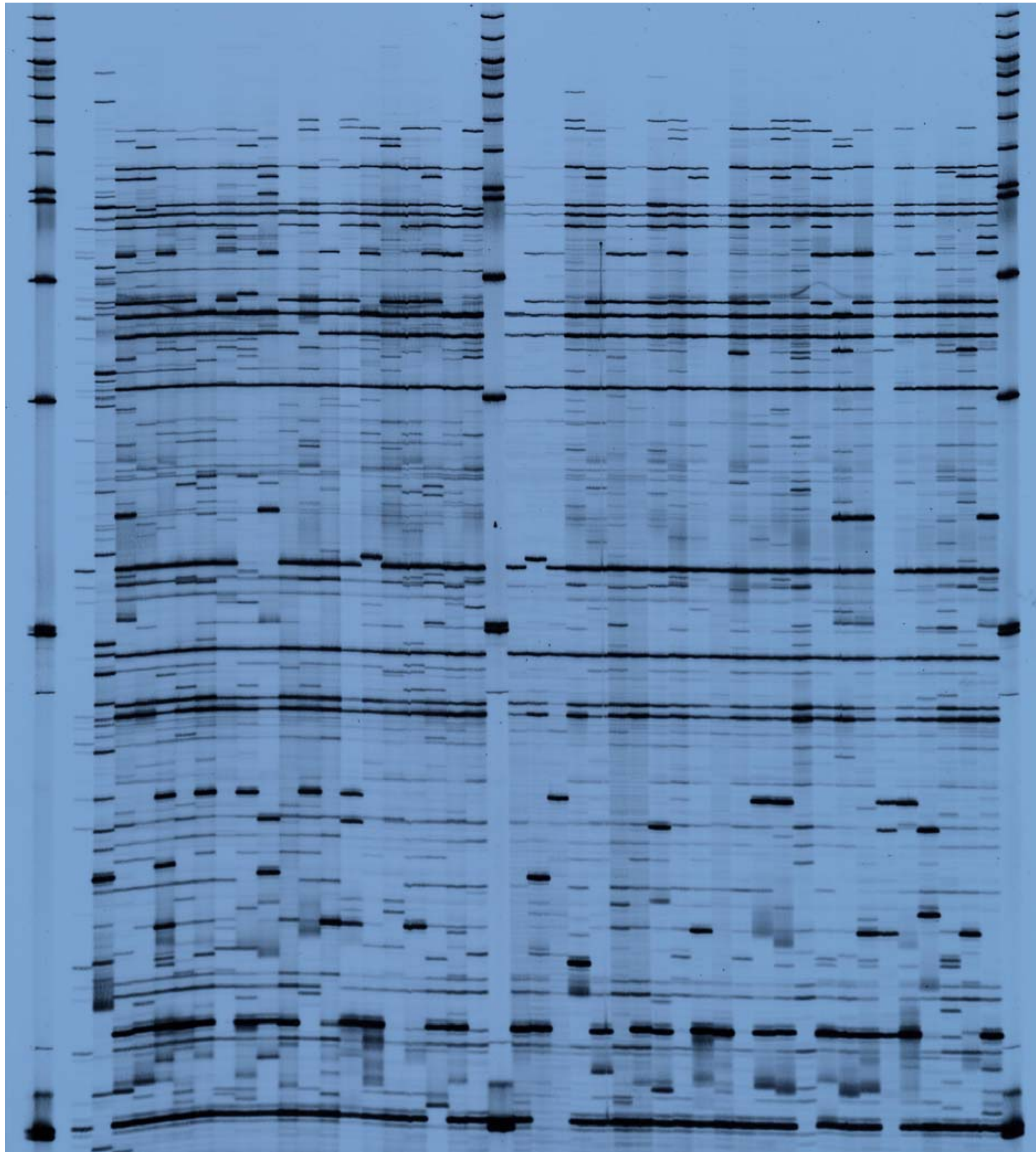
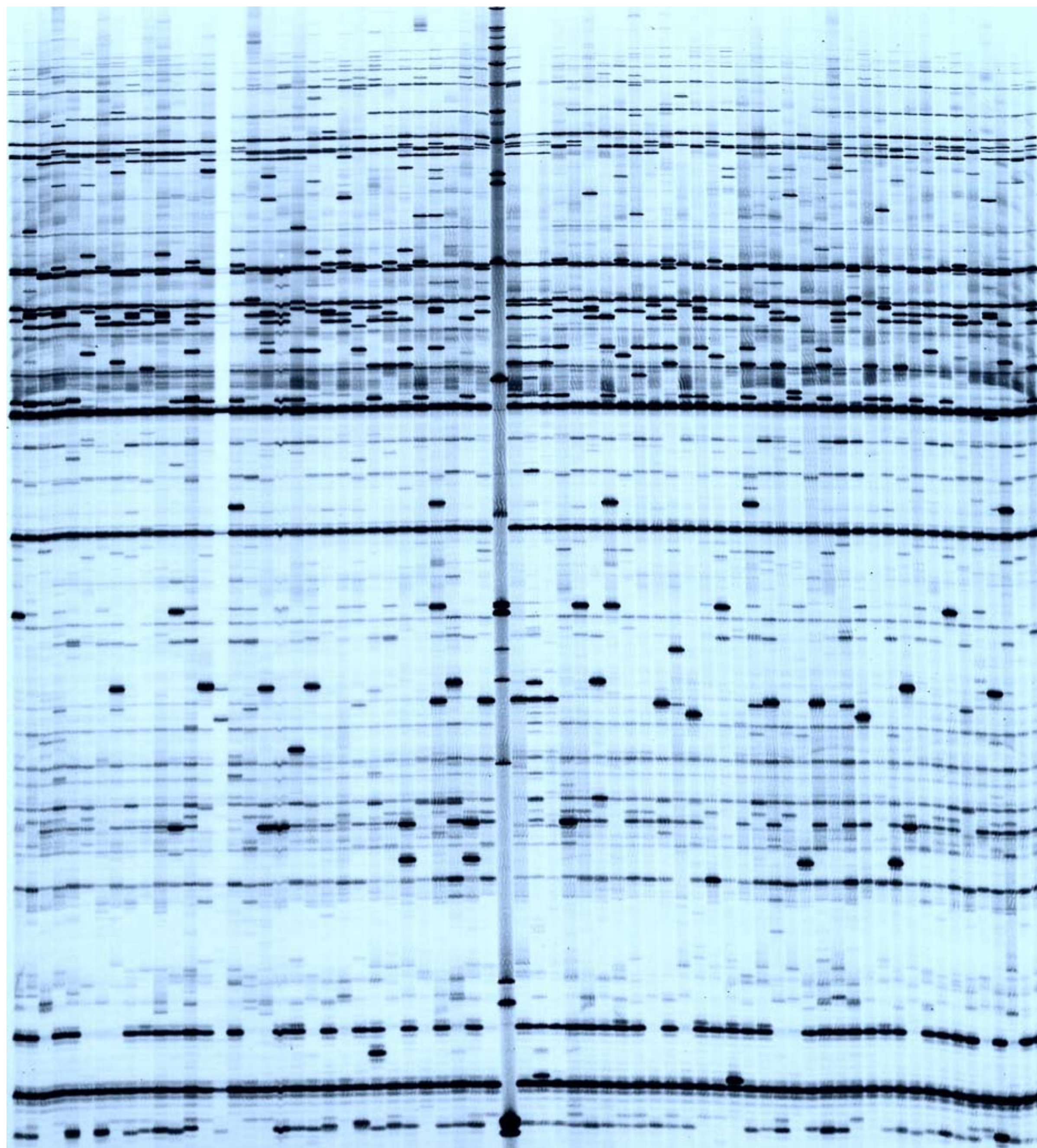
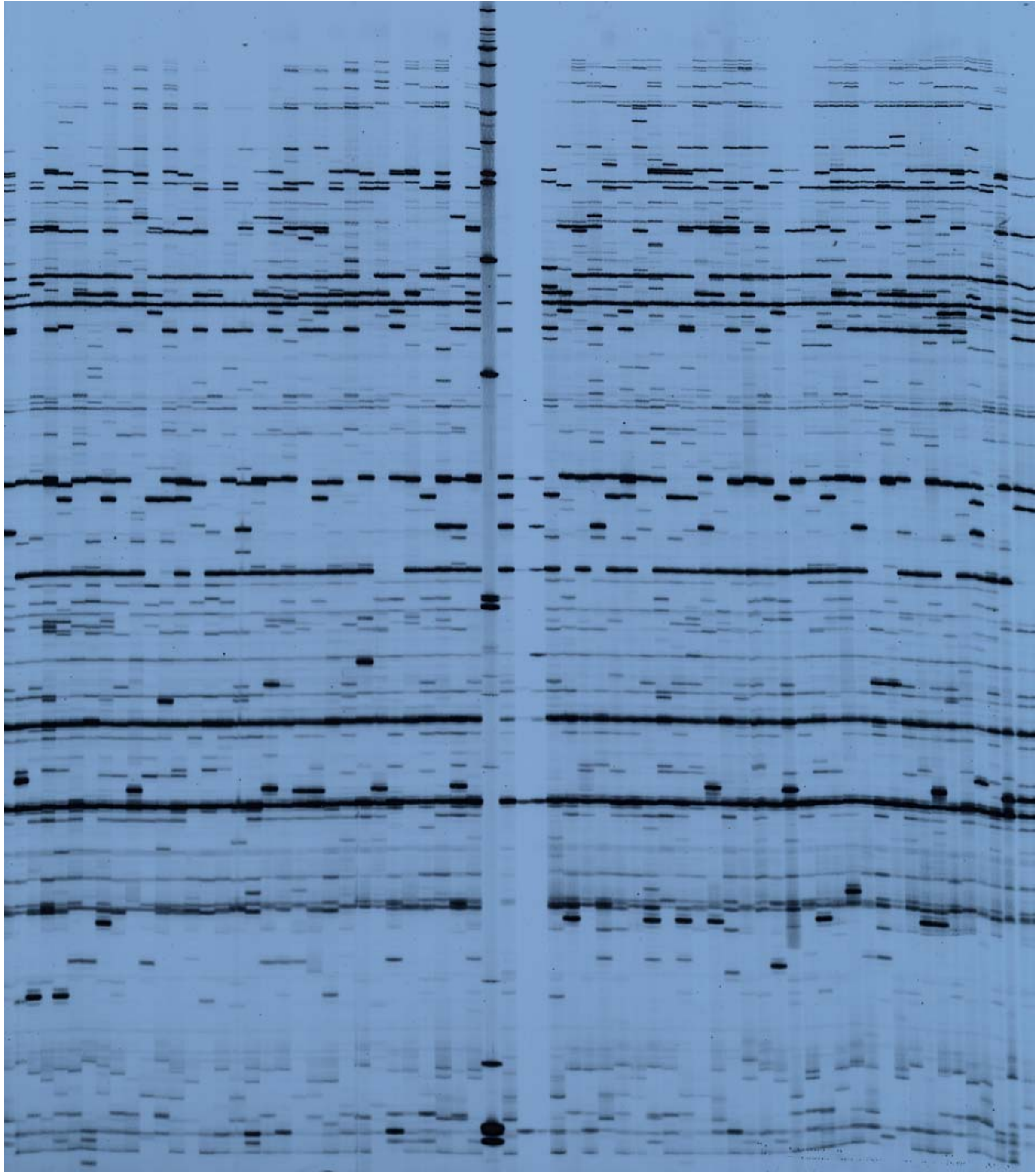
Appendix A – AFLP profile of *M. graminicola* isolates collected at the micro-plot scale in Kansas and generated using the primer pair EcoAC/MseCC



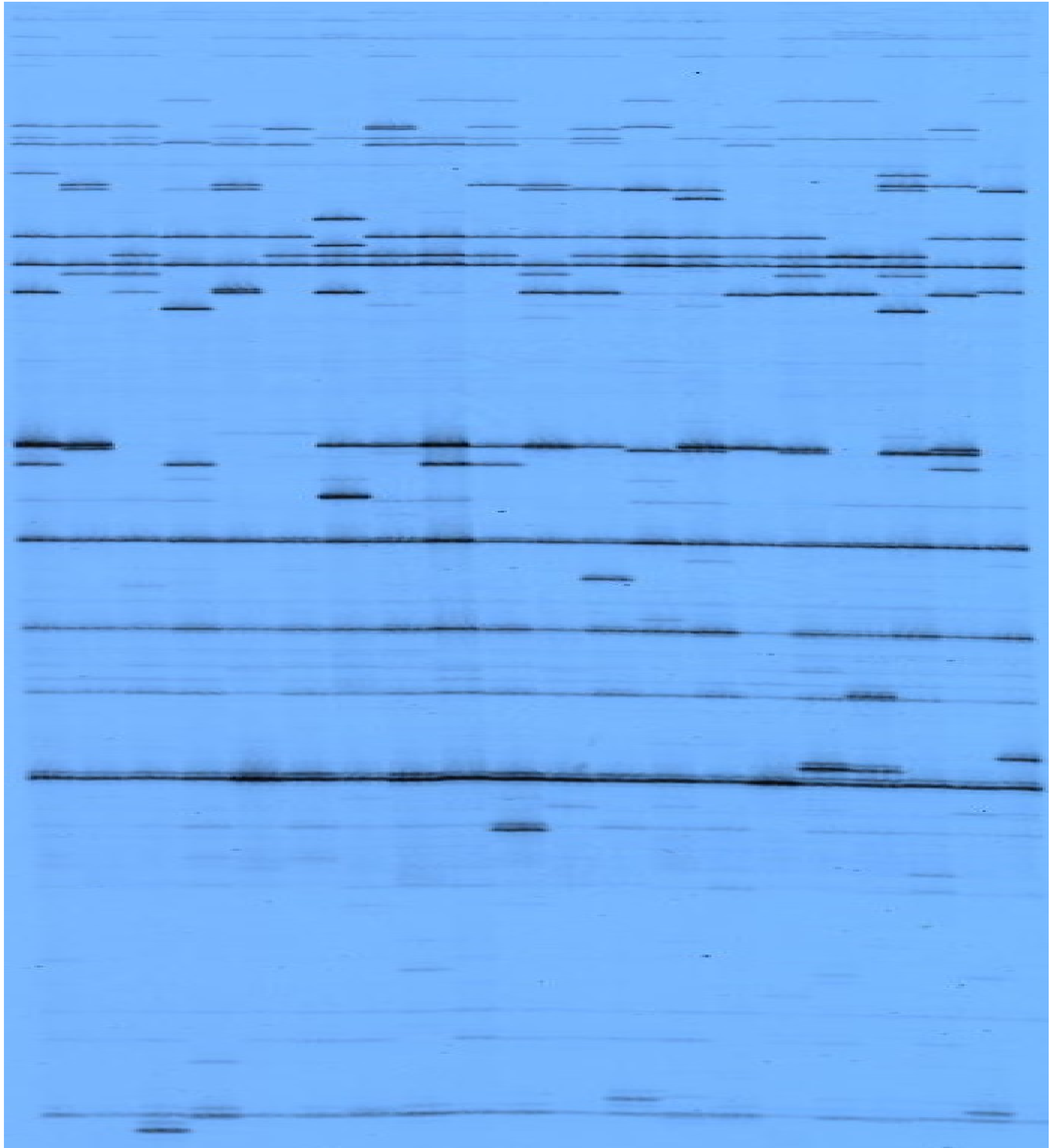
Appendix B – AFLP profile of *M. graminicola* isolates collected at the field scale in Kansas and generated using the primer pair EcoAC/MseGG



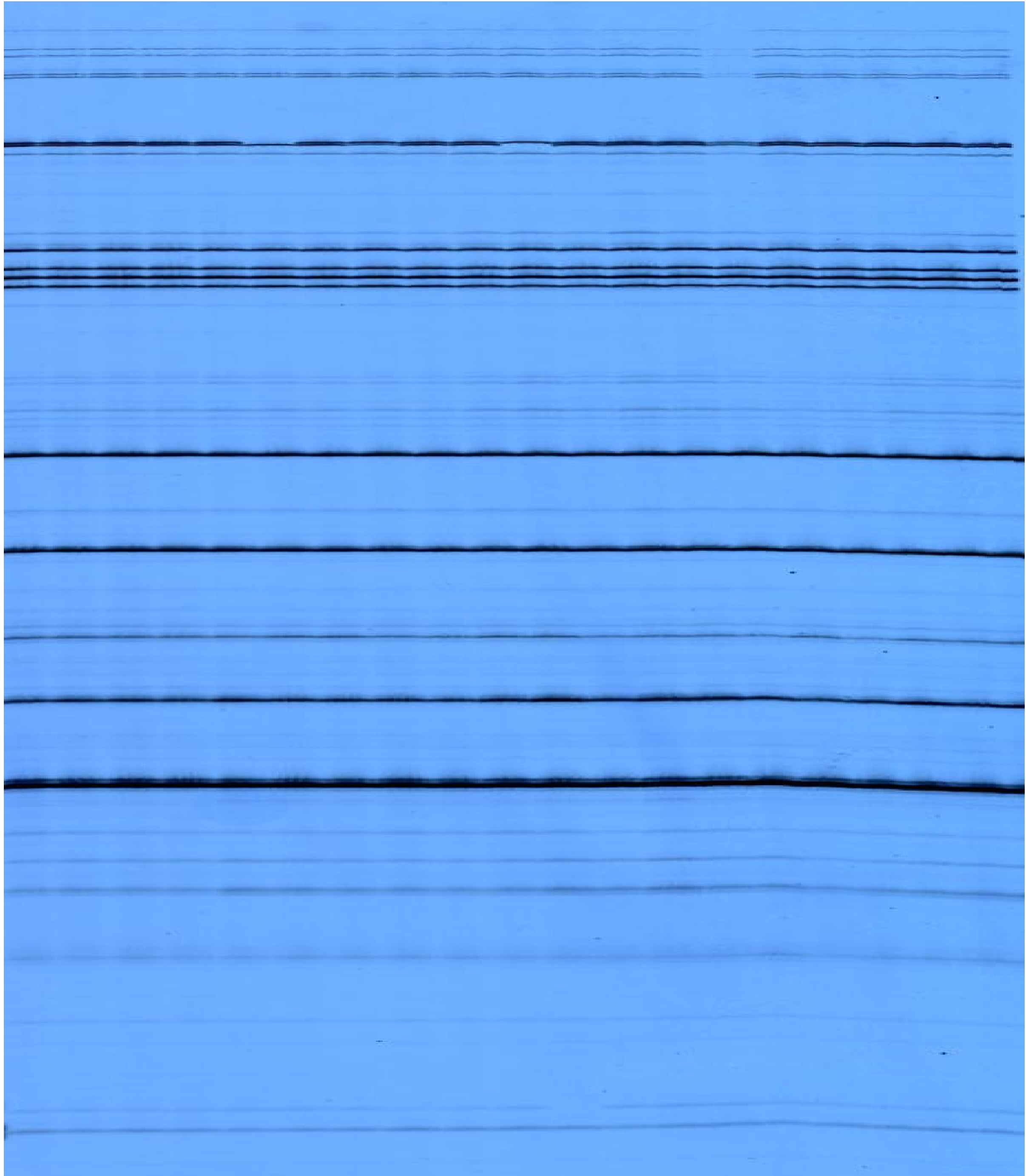
Appendix C – AFLP profile of *M. graminicola* isolates collected statewide and generated using the primer pair EcoAC/MseCA



Appendix C – AFLP profile of *M. graminicola* isolates collected in the fall from naturally infected plots, and generated using the primer pair EcoAC/MseCA



Appendix D – AFLP profile of *M. graminicola* isolates collected in the fall from inoculated plots, and generated using the primer pair EcoAC/MseCA



Appendix E – AFLP profile of *M. graminicola* isolates collected in the spring from inoculated plots, and generated using the primer pair EcoAC/MseCA