Characterization of *Fusarium* Strains Recovered From Wheat With Symptoms of Head Blight in Kentucky

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Abstract

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Fusarium graminearum species complex (FGSC) members cause *Fusarium* head blight (FHB) of wheat (*Triticum aestivum* L.) and small grains in the United States. The U.S. population is diverse and includes several genetically distinct local emergent subpopulations, some more aggressive and toxigenic than the majority population. Kentucky is a transition zone between the Mid-Atlantic and Midwestern wheat production areas. Sixty-eight *Fusarium* strains were isolated from symptomatic wheat heads from central and western Kentucky and southern Indiana in 2007. A multilocus genotyping assay and a variety of additional molecular markers, including some novel markers developed using the *F. graminearum* genome sequence, were used to characterize the pathogen

population. Five of the isolates were identified as members of two non-FGSC species, *F. acuminatum* and *F. cf. reticulatum*, but they did not cause symptoms in greenhouse tests. All the FGSC isolates belonged to the 15-ADON chemotype of *F. graminearum*. Comparative genetic analysis using variable nuclear tandem repeat (VNTR) markers indicated that the population in Kentucky and Indiana belonged to the dominant North American population, with some diversification likely due to local evolution. Telomere and RFLP fingerprinting markers based on repetitive sequences revealed a high degree of genetic diversity within the population, with unique genotypes found at each location, and multiple genotypes isolated from the same head.

Fusarium head blight (FHB) is one of the most economically important diseases of wheat (*Triticum aestivum* L.) and other small grains worldwide (25). In the upper Midwestern United States, losses due to FHB epidemics between 1998 and 2000 totaled \$2.5 billion (16,31,32). In 2003 there was a severe epidemic on soft red winter wheat (SRWW) throughout the southeastern United States, resulting in losses worth approximately \$15 million (6). In addition to reducing grain yields, FHB also results in contamination of the grain with a variety of trichothecene mycotoxins that are harmful to animal and human health (18,49,63). During another severe FHB epidemic that occurred in Kentucky in 2009, deoxynivalenol (DON) levels were reported to be more than 17-fold higher than normal (25).

Isolates of the fungal genus *Fusarium* causing the majority of FHB around the world are classified as members of the *F. graminearum* species complex (FGSC). The FGSC was previously considered to be a single panmictic species; however, multilocus molecular phylogenetic analyses have revealed at least 16 genetically distinct species within the FGSC, including *F. graminearum* Schwabe, identified as the primary cause of FHB in the United States (34,36,44,52). Members of the FGSC produce type B trichothecene mycotoxins, including DON and nivalenol (NIV), and their respective acetylated forms. Three strain-specific profiles of trichothecene metabolites (chemotypes) have been described within the FGSC: 15ADON strains producing primarily DON and 15-acetyl-deoxynivalenol, 3ADON strains producing primarily DON and 3-acetyl-deoxynivalenol, and

NIV strains producing primarily NIV and acetyl-derivatives of NIV (27,61). Most North American (NA) strains produce primarily 15-ADON, but there are regionally defined populations in NA, as well as populations outside of NA, in which 3-ADON and NIV chemotypes dominate (8,9,13,14,21,30,41,42,48,52,62). Chemotype-specific genetic variation has been identified within and near genes that are responsible for chemotype differences, and can be used to predict chemotypes by molecular tests that determine trichothecene genotypes (61,62).

The degree of pathogenically-significant chemotypic and genotypic variation that exists in the NA population of *F. graminearum* has important implications for the durability of FHB management and resistance breeding efforts in the United States. Although it has generally been assumed that new sources of resistance will be broadly effective against diverse isolates of the pathogen from across the country (66,67), this belief has been challenged recently through data that demonstrate the existence of genetically distinct populations in several regions of the United States and Canada, including ones that differ in aggressiveness and toxigenicity from the majority populations (12–14,44,52,62). It has been hypothesized that these subpopulations resulted from localized adaptive shifts, possibly in response to deployment of scab resistant wheat varieties or the introduction and spread of non-native pathogen populations (12–14,44,52,62).

Kentucky represents a transition zone between the Mid-Atlantic and Midwestern wheat production areas. It is potentially a “hot spot” for mixing among *F. graminearum* genotypes adapted to the surrounding wheat- and corn (*Zea mays* L.)-producing regions. The half million acres of SRWW produced annually in Kentucky represent a critical source of capital for grain producers in the state. To assure an efficient cropping system and adequately utilize the available growing season, almost the entire Kentucky winter wheat crop is planted in a rotation following corn. Stalk and ear rot diseases, also caused by members of the FGSC, are common in corn (25), and the pathogen's persistence, combined with the prevalence of no-till or minimal tillage cropping systems, puts Kentucky wheat at a high risk for FHB. There has been only one previous study that included

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Fusarium strains causing FHB on SRWW in Kentucky (48). Results of that study indicated that the pathogen population in the state belonged primarily (>99%) to the 15-ADON chemotype. The purpose of the work described here was to characterize the structure and diversity of the Kentucky pathogen population in more detail, and to compare it with several previously characterized populations from other regions of NA. A variety of molecular markers, including newly developed restriction fragment length polymorphism (RFLP) fingerprinting markers derived from the *F. graminearum* genome sequence, were used to evaluate genetic diversity among a group of *Fusarium* strains isolated from symptomatic wheat heads collected from four locations in central and western Kentucky, and one location in neighboring southern Indiana.

Materials and Methods

Isolation and culture of fungal strains. The fungal isolates used in this study were recovered from SRWW heads displaying typical symptoms of FHB. The samples were collected in the spring of 2007 from four locations across Kentucky: Lexington, in Fayette Co. (1 field); Princeton, in Caldwell Co. (2 fields); Owensboro, in Daviess Co. (1 field); and Logan Co. (1 field). Samples were also collected from one location in southern Indiana (Hartfield, in Spencer Co.), just across the Ohio River from Owensboro. Between four and six symptomatic wheat heads were collected from each location. Samples were disinfested in 10% bleach with agitation for 10 min, followed by three washes in sterile water. Small sections from the edges of lesions were plated on acidified potato dextrose agar (PDA) (1 ml of 85% lactic acid per 1 liter of PDA). All isolates were single-spored and stored on silica at -80°C (57). All strains were routinely cultured on PDA at 23°C under constant white light. Asexual spores for wheat inoculations were produced by growing the isolates for 7 days at 23°C under continuous light on mung bean agar media (1).

DNA extraction. An 8-mm agar plug was taken from the edge of an actively-growing colony of *Fusarium* and placed in 5 ml of YEPD medium (20 g dextrose, 20 g bacto-peptone, 10 g yeast extract/liter). Cultures were incubated at 25°C for 5 to 7 days with shaking at 250 rpm. Mycelia were harvested by decanting cultures onto sterile paper and blotting the excess liquid. The mycelia were flash-frozen in liquid nitrogen and stored at -80°C prior to further manipulation. Frozen mycelia were lyophilized and then pulverized in individual 2-ml Eppendorf tubes using a mini-pestle, or in 96-deep well plates using a 2000 GENO Grinder (Spex Certiprep) (500 strokes/s for 30 s). One milliliter of warm lysis buffer (0.5 M NaCl, 1% SDS, 10 mM Tris HCl, pH 7.5, 10 mM EDTA) was added per 100 to 200 mg of pulverized fungal tissue, and the samples were incubated at 65°C for 30 min, vortexing once during the incubation. After incubation the samples were transferred into individual tubes containing 660 μl phenol-chloroform-isoamyl alcohol (25:24:1), mixed by inverting 4 to 6 times, and then incubated at 65°C for an additional 30 min. The samples were centrifuged in a tabletop centrifuge for 20 min at maximum speed to separate the phases. DNA was precipitated from the aqueous phase by using 1 vol. isopropanol, and the pellet was washed twice with 70% ethanol. The pellet was dissolved in 100 μl of TE, pH 7.9, plus 2 μl of a 5 mg/ml solution of RNase A, at 65°C for 1 h. Between 1 and 5 μg of each DNA sample was digested with appropriate

restriction enzymes (RE) and used in Southern hybridizations (50) for evaluation of transformant and wild-type strains.

Multilocus genotyping (MLGT). Members of the FGSC were simultaneously identified to species and trichothecene genotype via multilocus genotyping (MLGT) using a Luminex 100 flow cytometer as described previously (62). A 48-probe version of the MLGT assay (35,44,62,65) was used to interrogate species and trichothecene chemotype-specific genetic variation in six genes.

Comparative analyses of population structure and diversity. Genetic variation at eight variable number tandem repeat (VNTR) loci (54) was determined for the Kentucky and Indiana *F. graminearum* isolates. VNTR amplifications were performed in three multiplex reactions using fluorescently labeled forward primers as previously described (62). Amplification products were detected and scored using an ABI3100 Genetic Analyzer with GENEMAPPER 3.7 software (Applied Biosystems). To assess population structure and diversity in relation to previously characterized populations from NA, VNTR data from the Kentucky and Indiana isolates were combined with previously published (14,62) data for isolates collected in the Upper Midwest of the United States (UMW, $n = 47$), western Canada (WCAN, $n = 104$), and the Canadian maritime province of Prince Edward Island (PEI, $n = 26$).

Population structure was investigated using the Bayesian model-based clustering procedure implemented in STRUCTURE 2.3 (37), which assigns multilocus genotypes to user-defined K population clusters. Analyses were performed for values of K between 1 and 10 using the admixture model with independent allele frequencies and 100,000 Markov Chain Monte Carlo (MCMC) iterations following a “burn-in” period of 10,000 iterations. The mean number of pairwise differences between haplotypes (π), the average gene diversity across loci (H), and F_{ST} -based genetic distances were estimated in ARLEQUIN 3.5 (10). Pairwise F_{ST} distances were calculated based on the number of different alleles method, and the significance of genetic differentiation was assessed using 1,000 permutations. The F_{ST} distance matrix was used to infer relationships among populations via the neighbor-joining method as implemented in MEGA 6 (55).

Telomere probe. A telomere-specific probe (TTAGGG) $_n$ was generated using the PCR protocol described in Schechtman et al. (45) and Pfu polymerase. Twenty picomoles of each primer were added to the PCR mix along with the manufacturer’s recommended buffer. The thermocycling protocol consisted of 35 cycles of denaturation for 30 s at 94°C , annealing for 30 s at 50°C , and extension for 60 s at 72°C ; followed by a final 2 min extension at 72°C . PCR products were separated on a 1% agarose gel. DNA with a size range of between 1.5 and 2 kb was excised and purified with a gel extraction kit (QIAquick, Qiagen cat. #28704). The purified product (50 to 80 ng) was labeled using the Prime-a-gene labeling system (Promega, cat. #U1100) and used for Southern hybridization analysis.

Repetitive fingerprinting probes. Repetitive motifs present in the genomic DNA sequence of *F. graminearum* strain PH-1 were identified by comparing the whole genome sequence of PH-1 (7) against itself using BLAST. The genome can be downloaded from the *Fusarium* comparative database website, www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html. PCR primers (Table 1) were designed to amplify ~500 bp regions encompassing

Table 1. Primers used to produce molecular probes or markers used for this study

Primer name	Type of marker	RE ^a used	Primer sequences (5'–3')
REP1.52F	RFLP ^b	BsoBI	GGTTCATGAAGACATTGGTC
REP1.52R	RFLP	BsoBI	TGATCCCGGTCTACTACATC
REP1.92F	RFLP	BsoBI	GTCACCAAAGTTTCGGTTCT
REP1.92R	RFLP	BsoBI	CGGTTATGCAATCTGTTCTT
EcoRI3F	CAPS ^c	EcoRI	GGTTCGGTGAGTCTTTAAGCCCC
EcoRI3R	CAPS	EcoRI	CGGCTTGAGGGTTTTCGAGC
PstI2F	CAPS	PstI	TCGACTTGAGAGACAACCGAGCA
PstI2R	CAPS	PstI	TCAAAGGGCTTGAGGAGAGTCA

^a Restriction enzyme

^b Restriction fragment length polymorphism

^c Cleaved amplified polymorphic sequence.

two of the repetitive sequences. The PCR conditions used to create the probes consisted of 3 min of denaturation at 94°C; followed by 35 cycles of denaturation for 30 s at 94°C, 15 s of annealing at 55°C, and 40 s of extension at 72°C; and a final extension of 7 min at 72°C. Amplicons were labeled with ³²P using the Prime-a-gene labeling kit, and used as probes for Southern hybridization analysis. Five hundred nanograms of the genomic DNA isolated from the fungal strains was digested with the RE *Bso*BI. The DNA fragments were separated on a 0.8% agarose gel, transferred to a charged nylon membrane, and probed with the radioactively labeled probes.

Analysis of RFLP data. The RFLP data were coded as dominant markers, either as presence (“1”) or absence (“0”) of the signal, and recorded in a matrix set. The data matrix was analyzed by using the Statistical Analysis Software (SAS Enterprise Guide 5.1) package to generate Jaccard’s similarity coefficients for the construction of a dendrogram using the unweighted pair group method with arithmetic average (UPGMA) procedure. Only a single clone representing each fingerprint pattern was included in the analysis.

Cleaved amplified polymorphic sequences (CAPS) markers. Cleaved amplified polymorphic sequences (CAPS) markers were generated by using a list of single-nucleotide polymorphisms (SNPs) differentiating PH-1 and Gz3639 (7), available from www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html. SNPs within *Eco*RI or *Pst*I restriction sites were identified, and ~500 bp segments spanning two of these SNP loci were amplified using the primer sets listed in Table 1. PCR amplifications contained Phusion polymerase (Finnzymes F-530S) and other components according to the manufacturer’s instructions. The thermocycling protocol consisted of initial denaturation for 1 min at 94°C; followed by 35 cycles of 30 s denaturation at 94°C, 20 s annealing at 68°C, and 45 s extension at 72°C; followed by a final 7 min extension cycle at 72°C. Three microliters of each PCR amplicon was used for each restriction reaction. Restriction reactions used Invitrogen RE *Eco*RI (Invitrogen cat. #15202-015) and *Pst*I (Invitrogen cat. # 15215-015) according to the manufacturer’s instructions. Restriction reactions were separated on a 1% agarose gel for analysis.

Sexual crosses. Strain mat1-1-1KO contains a deletion of the MAT1-1-1 gene in the *F. graminearum* strain Gz3639 background. It was created in Dr. John Leslie’s laboratory (Kansas State University). This strain was kindly provided to us, with permission from Dr. Leslie, by Dr. Frances Trail (Michigan State University). Strain mat1-2-1KO is a MAT1-2-1 deletion mutant produced by split-marker targeted gene deletion in the PH-1 background (SB, unpublished). The parental strains were crossed by using the mycelial plug method (3). Five-millimeter plugs of mycelia taken from the edges of each parental strain, actively growing on PDA, were placed on opposite halves of a 60-mm petri plate. After 4 days of incubation at 23°C, perithecial production was induced by pipetting 500 to 1,000 µl of 2.5% Tween 60 on the surface of each plate, and gently rubbing the aerial mycelium with a sterile glass rod to flatten it. Following induction, the plates were incubated at 23°C with constant fluorescent light until perithecial maturation. Two to three weeks after induction, the mature perithecia extruded cirrhi containing hundreds of ascospores. A sterile glass needle was used to pick up individual cirrhi,

which were then dispersed in water and plated on 2% water agar. After growing on water agar for 10 to 14 h, isolates arising from single ascospores were transferred to individual 6-mm petri plates. LOD scores were calculated using the MapMaker3 program (available for download from <http://www.broadinstitute.org/scientific-community/software>).

FHB pathogenicity assay. The susceptible spring wheat variety “Norm” was used for the pathogenicity assays. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBX growth substrate (3:2) in cone containers and thinned after emergence to one seedling per cone. Plants were grown in the greenhouse with a 14/10-h photoperiod, provided by “Hortilux” LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25 and 28°C. Transplants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation as needed, with the last fertilization at heading. Flowering typically occurred 6 to 7 weeks after planting. At early-to mid-anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µl of a spore suspension as described previously (26). Symptom severity was recorded as the number of symptomatic spikelets per inoculated spike at 7 and 10 days post-inoculation.

Results

Species complex identification using MLGT analysis. Sixty-eight *Fusarium* isolates were recovered from mature heads of SRWW with symptoms of FHB from six production fields across the region (Table 2). MLGT analyses identified all of the isolates, with the exception of five from Owensboro (F-Ow-2, F-Ow-3, F-Ow-4, F-Ow-6, and F-Ow-7), as *F. graminearum* s.s. with the 15-ADON trichothecene genotype. The five Owensboro isolates were negative for all of the species and trichothecene genotype probes included in the MLGT assay, and were also negative for clade probes targeting all members of the FGSC and closely related species within the B-trichothecene-producing clade (B-clade) of *Fusarium*. These results indicated that the five Owensboro isolates were not members of the FGSC or the B-clade of *Fusarium*.

Identification of the Owensboro non-FGSC isolates. A portion of the translation elongation factor 1 (*EF-1α*) gene was sequenced for each of the five Owensboro non-FGSC isolates and used to query *FUSARIUM*-ID, *Fusarium* MLST, and NCBI GenBank employing the BLASTn program. This revealed that all five isolates were members of the *F. tricinctum* species complex (FTSC). The sequences were subsequently analyzed phylogenetically after adding them to an in-house dataset for the FTSC. Three of the isolates (F-Ow-2, F-Ow-3, and F-Ow-4) were identified as *F. acuminatum* based on identical matches of the *EF-1α* sequences to reference strains of this species (Fig. 1, Supplementary Table S1) that were isolated from Canadian wheat (GenBank accessions JX397863.1 and JX397866.1) (33), crown vetch (NRRL 13332 = FRC R-2109), pasture soil (NRRL 13406 = FRC R-6638), pine nursery soil (NRRL 53951 = CBS 119882), and a human bronchial secretion (NRRL 36147 = CBS 109232, GenBank GQ5050420.1). The other two Owensboro strains (F-Ow-6 and F-Ow-7) were tentatively identified as *F. cf. reticulatum* based on a 100% match with *EF-1α* sequences of strains

Table 2. Fungal isolates used in this study, and a summary of results

Location of sample field	Field ID	Symptomatic wheat heads collected	<i>Fusarium graminearum</i> isolates recovered	Isolates of other <i>Fusarium</i> spp. recovered	Unique telomere profiles among the <i>F. graminearum</i> isolates	Wheat heads with >1 telomere profile ^a	Unique RFLP profiles among the <i>F. graminearum</i> isolates	Wheat heads with >1 RFLP profile
Hatfield, IN	F-IN	5	12	0	7	3	9	3
Owensboro, KY	F-Ow	4	2	5	1	2	2	0
Logan Co. KY	F-Lo	4	8	0	2	0	3	0
Lexington, KY	F-Le	6	18	0	13	5	14	6
Princeton, KY	F-Pr	10	23	0	12	4	14	4
TOTAL	NA	29	63	5	35	14	42	13

^a This column also includes the non-*F. graminearum* isolates.

in the NCAUR database (Fig. 1) that were isolated from Danish soil (NRRL 25792 = CBS 618.87) and passion fruit from New Zealand (NRRL 40040 = ICMP 13864).

The FTSC strains did not produce FHB symptoms when inoculated onto heads of susceptible wheat plants. Florets inoculated with the five FTSC strains became brown and discolored, resembling a Type II (25) resistance response (Fig. 2b and c). In contrast, the *F. graminearum* strain PH-1 (Fig. 2a), and the two strains from Owensboro that were identified as members of the FGSC (F-Ow-1, F-Ow-5) (not shown), produced significant bleaching typical of FHB.

FTSC strains produced macroconidia with morphologies that were distinct from that of *F. graminearum* s.s. (Fig. 2d). Macroconidia of the *F. acuminatum* strains (F-Ow-2, F-Ow-3, and F-Ow-4) were slightly more narrow and curved, with a distinct apical cell (Fig. 2e). Macroconidia of the *F. cf. reticulatum* strains (F-Ow-6 and F-Ow-7) were significantly shorter, curved, and had a less distinct apical cell (Fig. 2f).

Comparative analyses of population structure and diversity using VNTR genotypes. Variable number tandem repeat (VNTR) data for the KY/IN FGSC population were combined with previously published data from the Upper Midwest (UMW), western Canada (WCAN), and Prince Edward Island (PEI) populations in order to assess their population structure and diversity in a broader geographic and genetic context. VNTR genotypes were determined for 62 of the 63 KY/IN FGSC isolates. The remaining isolate produced weak amplicons and was excluded from the VNTR analyses.

Analyses with STRUCTURE indicated that the four geographic populations fell into two major genetic clusters. Analyses with $K = 2$ resulted in the greatest improvement in posterior probabilities ($\Delta K = 594.6$), whereas increasing values of K produced much smaller improvements ($\Delta K \leq 48.3$). Isolates were assigned to a specific genetic cluster when the estimate of membership (Q) was ≥ 0.9 . With $K = 2$, 95.4% of the isolates could be assigned to a specific genetic cluster, and these clusters were largely consistent with trichothecene genotype distinctions, as was previously observed in Canada and the Upper Midwest (14,62). Among the isolates assigned to cluster 1, 94.3% were the 3-ADON genotype, whereas 97.1% of the isolates assigned to cluster 2 had the 15-ADON genotype. All of the KY/IN isolates had the 15-ADON trichothecene genotype, and 59 (95.2%) of the 62 KY/IN isolates were assigned to cluster 2. The three remaining unassigned isolates had $Q \geq 0.7$ for cluster 2 indicating that they are more similar to cluster 2 strains than to cluster 1 strains. No KY/IN isolates were assigned to cluster 1. Increasing the number of genetic clusters fragmented the population assignments for most of the 15-ADON isolates from each of the four geographic populations, leaving more than half of the 15-ADON isolates unassigned to a genetic cluster, and further indicating that two genetic clusters captured the major genetic structure in these data.

The isolates were further divided into six groups based on their genetic cluster assignment and geographic origin in order to assess diversity and relatedness among these geographic/genetic populations (Table 3). As the genetic clusters largely corresponded to trichothecene genotype distinctions, these populations are referred to by geographic origin and their predominant trichothecene genotype. Neighbor-joining analyses based on F_{ST} distances recovered two major clades associated with trichothecene genotype differences, with relationships and levels of genetic differentiation within each of these clades reflecting geographic separation (Fig. 3). Genetic differentiation between KY/IN-15 and the 15-ADON populations from the Upper Midwest (UMW-15) and western Canada (WCAN-15) was relatively small ($F_{ST} = 0.06$ to 0.07), but statistically significant ($P < 0.001$) and similar to the level of differentiation ($F_{ST} = 0.03$ to 0.06) between 3-ADON populations in western Canada or the Upper Midwest (WCAN-3 and UMW-3) and the maritime province of Prince Edward Island (PEI-3). This indicates that the KY/IN-15 population is closely related to previously characterized 15-ADON populations from NA, with a low level of genetic differentiation likely resulting from isolation by distance.

In comparison with the other geographic and genetic populations examined, intermediate levels of genetic diversity were observed within the KY/IN-15 population. The mean number of pairwise differences between haplotypes ($\pi = 4.5$), and average gene diversity over loci ($H = 0.56$), were slightly lower than was observed for the other 15-ADON populations, but were substantially higher than in the 3-ADON populations (Table 3).

DNA fingerprinting using the telomere probe. Genomic DNA from each of the strains was restricted and probed with a telomere-specific probe (Fig. 4). The three *F. acuminatum* strains had identical telomere profiles consisting of eight bands, suggesting that they have at least four chromosomes. The two *F. cf. reticulatum* strains had more complex profiles that were similar to one another: F-Ow-07 had 14 bands, and F-Ow-06 had the same 14 bands plus one extra band, suggesting that these strains have seven or eight chromosomes. *F. graminearum* has four chromosomes, and thus we would expect to see eight bands hybridizing to the telomere probe. However, most of the strains had only two bands of varying sizes, probably due to the presence of doublets. At least 35 different telomere fingerprints were discerned among the strains, indicating a large amount of diversity. Patterns were sometimes shared among strains from the same

Fig. 1. Single most-parsimonious tree (MPT) inferred from 16 aligned partial translation elongation factor (*EF-1α*) gene sequences rooted on outgroup sequences of *Fusarium torulosum*. Three of the five non-*F. graminearum* species complex isolates, which are indicated by gray highlight and bold font, were identified as *F. acuminatum* and the other two as *F. cf. reticulatum*. The number above nodes indicates MP bootstrap support based on 1,000 pseudoreplicates of the data. PIC, parsimony informative characters; CI, consistency index; RI, retention index.

locations, but were never shared among strains from different locations. The most diversity, consisting of 13 different hybridization patterns, was found among the 18 isolates collected in Lexington, KY (Table 2). In contrast, isolates from Logan County had relatively low levels of diversity, with only two patterns found among eight isolates (Table 2).

Repetitive fingerprinting probes. The repetitive probe Rep1.92 hybridized to at least 24 polymorphic bands (Fig. 5), while the Rep1.52 probe hybridized to at least two polymorphic bands (Fig. 6). The five FTSC strains from Owensboro (F-Ow-02, F-Ow-03, F-Ow-04, F-Ow-06, and F-Ow-07) did not hybridize to either probe. The repetitive probes revealed genotypic diversity among the *F. graminearum* isolates, with unique RFLP fingerprints in each location ranging from 37.5% of the eight isolates from Logan County, to 100% (both) of the FGSC isolates from Owensboro (Table 2). In addition, multiple genotypes were often isolated from the same wheat head. The number of heads harboring multiple genotypes ranged from zero in the collections from Owensboro and Logan County, to six (100% of the heads) from the Lexington location (Table 2). Three of the six heads from the Lexington collection contained three different genotypes, while the other three harbored two genotypes each. There were no isolates with identical RFLP fingerprints from different locations. Thus, every location appeared to have a unique set of RFLP genotypes. Although some strains with the same telomere banding pattern had different RFLP profiles, there were no strains that had the same RFLP profile that had different telomere banding patterns. Thus, the RFLP fingerprint data gives a better estimate of the actual number of different genotypes recovered from each location.

Each unique genotype was scored for presence or absence of the polymorphic bands, and the output was used to construct an unweighted pair group method with arithmetic mean (UPGMA) distance dendrogram (Fig. 7). Clustering was generally related to the origin of the genotypes. Two major groups, separated by an average distance of 0.98, were resolved. The first group comprised the genotypes from the two fields in Princeton. The second group contained the *F. graminearum* strains from Owensboro; Hartfield, IN; Logan County;

and Lexington. The FGSC F-Ow genotypes were located within a cluster that also contains the F-IN genotypes from just across the Ohio River.

Segregation of repetitive fingerprinting markers in FGSC. The repetitive probes hybridized to multiple polymorphic fragments within the FGSC population from Kentucky. A cross was made between a Δ mat1-1-1 strain (generated in the Gz3639 background) and a Δ mat1-2-1 strain (generated in the PH-1 background). It has been reported previously that deletion of complementary MAT genes in *F. graminearum* results in strains that are no longer homothallic, and can engage only in heterothallic matings (22).

Segregation of the CAPS markers and *MAT1-1-1* and *MAT1-2-1* genes was first evaluated to ensure that perithecia chosen for analysis resulted, as expected, from heterothallic crosses and showed normal segregation patterns. The CAPS markers and MAT genes each segregated in the expected (1:1) Mendelian ratios (Supplementary Figures S1 and S2).

Table 3. Diversity indices for the KY/IN-15 population in relation to five previously defined populations of *Fusarium graminearum* collected from wheat in North America

Population	Isolates	Π^a	H^b
KY/IN-15 ^c	59	4.5	0.56
UMW-15 ^d	24	5.0	0.62
WCAN-15 ^e	53	4.9	0.61
UMW-3 ^f	21	2.1	0.27
WCAN-3 ^g	40	2.7	0.33
PEI-3 ^h	22	3.1	0.39

^a Mean number of pairwise differences between haplotypes.

^b Average gene diversity over loci.

^c Kentucky/Indiana 15-ADON population (this study).

^d Upper Midwest 15-ADON population (14,62).

^e Western Canada 15-ADON population (62).

^f Upper Midwest 3-ADON population (14,62).

^g Western Canada 3-ADON population (62).

^h Maritime province 3-ADON population (62).

Fig. 2. Comparison of macroconidial morphology and aggressiveness (7 dpi) of *Fusarium graminearum* strain PH-1 (A and D), with a representative *F. acuminatum* strain F-Ow-2 (B and E) and a representative *F. cf. reticulatum* strain F-Ow-6 (C and F). The point of inoculation on wheat heads in A to C is indicated by the white arrows. Apical cells of individual macroconidia are indicated in D to F by black arrows.

Four polymorphic bands that hybridized to the repetitive probe Rep1.92 in the two parental strains were scored as dominant markers, for presence or absence, among 20 progeny of the cross (Fig. 8). Statistical analysis of segregation patterns among the four markers using either χ^2 or logarithm of odds (LOD) calculations suggested that they were all single unlinked loci (Table 4). It is important to point out that our sample size of only 20 progeny is relatively small: thus, if weak

linkage actually existed among any of these markers, we might not have been able to detect it. For marker Rep1.92-5, a 3:1 ratio fit the data with a higher significance for the χ^2 calculation, which could imply that this band actually represents two unlinked loci, each of which produces a hybridizing fragment of a similar size. Use of this marker would be expected to result in the underestimation of actual population diversity. Markers Rep1.92-1 and Rep1.92-7 fit a 1:1 segregation model with a relatively low level of statistical confidence. However, a 3:1 model was even less likely for these markers.

Discussion

Genetic and phenotypic diversity in pathogen populations result from migration and gene flow, sexual recombination, and mutation. Selective forces, including host factors such as resistance, act on this diversity to drive shifts in the pathogen population (4). The ability to track diversity in a pathogen population is an important aid to understanding and predicting the role of selective forces in the development of disease epidemics.

A vast majority of *Fusarium* strains causing FHB in North America are members of *F. graminearum* Schwabe (36). The prevalent chemotype is 15-ADON (14,27,62), which dominates worldwide on wheat in temperate zones (59). Recently, an increase in the representation of 3-ADON isolates in the population has been documented in Canada (11,17,61), the upper Midwestern United States (14), and the eastern United States, from North Carolina to New York (25,47,48). It has been reported that these 3-ADON isolates represent a novel and likely introduced population. Moreover, strains from this new population produce higher levels of trichothecenes, grow faster in vitro, have higher fecundity, produce larger macroconidia, are more aggressive (5,12,15,40,58,62), and are more resilient under extreme environmental conditions, suggesting that *F. graminearum* is undergoing an adaptive evolutionary shift in North America (14,62). Although the same studies demonstrated that sexual recombination had occurred between the 3-ADON population and the dominant 15-ADON

Fig. 3. Neighbor-joining phylogram based on F_{ST} genetic distances derived from variable number tandem repeat (VNTR) analyses. Populations are referred to by geographic origin and their predominant trichothecene genotype. 3KY/IN-15 = Kentucky/Indiana 15-ADON population (this study). UMW-15 = Upper Midwest 15-ADON population (14,62). WCAN-15 = Western Canada 15-ADON population (62). UMW-3 = Upper Midwest 3-ADON population (14,62). WCAN-3 = Western Canada 3-ADON population (62). PEI-3 = Maritime province 3-ADON population (62).

Fig. 4. An example of a Southern blot analysis of the *BsoBI* digest of fungal isolates from Hartfield, IN (F-IN); Owensboro, KY (F-Ow); and Logan Co., KY (F-Lo). The digest was probed with a (TTAGGG) $_n$ telomere specific probe.

Fig. 5. Example of Southern blot analysis of the *BsoBI* digest of the sets of fungal isolates from Hartfield, IN (F-IN); Owensboro, KY (F-Ow); and Logan Co., KY (F-Lo) probed with restriction fragment length polymorphism (RFLP) probe Rep1.92.

Fig. 6. Example of Southern blot analysis of the *BsoBI* digest of the sets of fungal isolates from Hartfield, IN (F-IN); Owensboro, KY (F-Ow); and Logan Co., KY (F-Lo) probed with restriction fragment length polymorphism (RFLP) probe Rep1.52.

population, admixture thus far has been insufficient to homogenize the two populations. Spolti et al. (51) reported no differences in several measures of pathogenic and saprophytic fitness between 3-ADON and 15-ADON isolates from New York, but these analyses were not accompanied by genetic analyses of population assignment. A shift in pathogen populations and chemotype frequencies has been observed among *F. asiaticum* affecting wheat in China, where a less aggressive NIV chemotype is being replaced by a more aggressive and more toxigenic 3-ADON chemotype population (68).

NIV-producing subpopulations of *F. graminearum* have been found in Korea (21), China (30,41), Nepal (8,9), New Zealand (28), Argentina (42,43), and Italy (38,39). NIV-producing *F. graminearum* strains are most prevalent on rice and corn in Korea (20,23), and on corn in Nepal (9) and Argentina (42,43). In the United States, the NIV chemotype appears to be common only in southern Louisiana, where 79% of the *F. graminearum* isolates from wheat were NIV producers (13). A smaller proportion of Louisiana *F. graminearum* isolates belonged to the 3-ADON chemotype. Very small numbers of NIV strains have been found in other states including Arkansas, Missouri, North Carolina, New York, and North Dakota. It has been suggested that NIV strains may be particularly well adapted to rice agroecosystems, which are common in southern Louisiana (13,36,52).

Kentucky represents a transition zone between the Mid-Atlantic, Midwestern, and Gulf Coast wheat production areas, so there is potential for introduction and subsequent hybridization among *F. graminearum* genotypes adapted to the surrounding wheat- and corn (*Zea mays*)-producing regions. Population genetic tools can be used to monitor and detect these events. The *F. graminearum* isolates

causing FHB in Kentucky all belonged to the most prevalent North American population of 15-ADON *F. graminearum*. Predominance of the 15-ADON trichothecene genotype in Kentucky is consistent with the findings of an earlier study (48), although one 3-ADON isolate was identified in that study, from Logan County. Additional sampling incorporating strains recovered from additional hosts is needed before we can make firm conclusions about the chemotype diversity (or lack thereof) among the FGSC in Kentucky.

Although the Kentucky population appears to belong to the dominant NA population, a small but significant level of population differentiation was identified between the 15-ADON populations in Kentucky and those of the Upper Midwest and Canada, consistent with isolation by distance. The present study also revealed genetic diversity among the isolates examined. A telomere probe revealed at least 35 different genotypes among the 68 strains in the study. We took advantage of the published sequence of *F. graminearum* (7) to develop some new markers for genetic analysis. By comparing the PH-1 genome sequence against itself, we generated a list of highly repetitive sequences (>20 copies) that had potential as fingerprinting probes. The repetitive fingerprinting probes revealed even more genetic diversity among the isolates in our study: 65 FGSC isolates could be classified into 45 different genotypes with these markers. None of the isolates had fingerprints that were identical to PH-1 from Michigan and Gz3639 from Kansas. A high degree of diversity among patterns of other neutral markers has been reported for other *F. graminearum* populations in North America (13,14,16, 46,66,67). In the current study, isolates from the same location were more similar than isolates from different locations, and isolates with the same pattern were not recovered from more than one location, suggesting that short-distance movement dominates over long-

Fig. 7. Unweighted pair group method with arithmetic average (UPGMA) "dendrogram" of *Fusarium graminearum* genotypes representing isolates collected across Kentucky and Indiana. The samples were collected from one field in Lexington KY [F-Le]; two fields in Princeton, KY [F-Pr]; one field in Owensboro, KY [F-Ow]; one field in Logan Co., KY [F-Lo]; and one field in Hartfield, IN [F-IN]. The UPGMA dendrogram was constructed based on the Jaccard similarity matrix among RFLP profiles from *Bso*BI DNA digests probed with RFLP probe Rep1.92. The scale bar indicates Jaccard similarity values.

distance migration. A similar relationship between genetic distance and geographic distance has also been reported previously (46,66). We recovered an average of 2.07 (± 0.82) genotypes from each blighted wheat head, which is comparable with the average of 1.8 genotypes/head reported in another study (66). Additional sampling will be needed to draw broader conclusions about potential variation in FGSC diversity across the state. In the future, these and additional fingerprinting markers can be used to compare populations in Kentucky with those in other states, and to monitor the occurrence, evolution, and spread of populations and individual clones within the state.

Among the *Fusarium* strains isolated from samples collected from the fields in Owensboro, KY, there were several representing species other than *F. graminearum*. *F. acuminatum* has a broad host range, and is known as a causal agent of FHB on barley (64). On wheat it

is more common as a root and crown rot pathogen (53), although it was recently reported to cause FHB in northern China (60). This species is reported to produce enniatins, moniliformin, and T-2 toxin (24). *F. reticulatum* was found associated with seeds of *Medicago littoralis* and *M. truncatula* (19,56), wheat and barley roots (53), and causing necrosis in English oak (*Quercus robur*) (29) and red staining in box elder (*Acer negundo*) (2). These strains were tested for pathogenicity on the susceptible spring wheat variety 'Norm,' but they did not produce disease symptoms under greenhouse conditions. The strains initially came from a severely symptomatic SRWW heads, so the lack of aggressiveness observed under greenhouse conditions could mean that host or environmental factors were not optimal for pathogenicity of these isolates. Alternatively, these isolates may have been present in combination with a pathogenic *F. graminearum* strain that was actually responsible for the disease symptoms.

Fig. 8. Example of the use of Rep1.92 probe to detect recombination among polymorphic restriction fragment length polymorphism (RFLP) markers in 20 progeny of a cross between $\Delta\text{mat1-1-1}$ (Gz3639) and $\Delta\text{mat1-2-1}$ (PH-1). Bands that are polymorphic between the two parent strains are indicated by the arrows.

Table 4. Segregation analyses for the repetitive RFLP molecular markers among F1 progeny from a cross between $\Delta\text{mat1-1-1}$ (Gz3639) and $\Delta\text{mat1-2-1}$ (PH-1)

Single marker	Expected	Observed	Df	Critical value ($P = 0.05$)	χ^2	P	LOD score
Rep1.92-1	10:10	8:12	1	3.8	0.8	0.37	NA
Rep1.92-5	10:10 (1 locus)	14:6	1	3.8	3.2	0.07	NA
Rep1.92-5	15:5 (2 loci)	14:6	1	3.8	0.3	0.61	NA
Rep1.92-7	10:10	12:8	1	3.8	0.8	0.37	NA
Rep1.92-8	10:10	9:11	1	3.8	0.2	0.66	NA
Marker pairs	Expected	Observed	Df	Critical value ($P = 0.05$)	χ^2	P	LOD score
Rep1.92-1	5:5:5:5	5:3:7:5	3	7.8	1.6	0.66	0.02
Rep1.92-7							
Rep1.92-1	5:5:5:5	4:4:5:7	3	7.8	1.2	0.75	0.58
Rep1.92-8							
Rep1.92-7	5:5:5:5	6:6:3:5	3	7.8	1.2	0.75	0
Rep1.92-8							

Our findings provide an important baseline for future monitoring of population dynamics among FGSC strains causing FHB in Kentucky. A detailed understanding of the population structure, and timely detection of shifts in that population, will be valuable for breeding programs focused on developing wheat varieties with a broad and durable resistance to the targeted pathogen population.

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