Population Subdivision of *Fusarium graminearum* from Barley and Wheat in the Upper Midwestern United States at the Turn of the Century

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ABSTRACT

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Fusarium graminearum, the causal agent of Fusarium head blight (FHB) in wheat and barley, is one of the most economically destructive pathogens of these grains worldwide. Recent population genetic studies of the pathogen obtained from wheat in North America supported population subdivision in part correlated with the spectrum of trichothecene mycotoxins (chemotype) produced by individuals within each population. In contrast, a recent study of *F. graminearum* obtained from diseased barley in the upper Midwestern United States concluded that only a single population was present, consisting of individuals with various chemotypes. To test whether strains derived from different hosts potentially have different population dynamics, we obtained the barley strains used in the previous study and compared them with wheat strains isolated at a similar time and geographic origin. A total of 247 *F. graminearum* isolates from barley were assigned firmly into two clusters using a Bayesian clustering method. Subdivision within the barley population corresponded to the previously described NA1 (correlated with the 15ADON chemotype) and NA2 (correlated with the 3ADON chemotype) populations from wheat. However, in both sampling periods the barley population exhibited a higher level of genetic differentiation between NA1 and NA2 populations, fewer admixed individuals and evidence of unidirectional gene introgression (15ADON strains with NA2 genetic backgrounds). These results suggest less recombination between NA1 and NA2 populations on barley compared with wheat. The frequency of 3ADON chemotype strains in the most recently surveyed barley population suggests a latitudinal cline from the northern (49%), central (40%) to the southern (29%) sampling area. The potential to produce a novel trichothecene, 3α-acetoxy,7α,15-dihydroxy-12,13-epoxytrichothe-9-ene (NX-2), was not detected in the barley population but occurred at a low rate (2.4%) in the wheat population.

Fusarium graminearum sensu stricto (O'Donnell et al. 2004), the principal cause of Fusarium head blight (FHB) of wheat and barley, contaminates grains with several mycotoxins which pose a threat to human and animal health and create serious yield reduction and economic losses worldwide (Láday et al. 2004; McMullen et al. 1997; Qu et al. 2008; Ramirez et al. 2007; Steffenson 2003; Tóth et al. 2005; Zhang et al. 2010). North Dakota and Minnesota are among the most important barley and wheat production regions in the United States. Between 1993 and 1998, FHB epidemics led to barley yield decreases of 38% in North Dakota and 21% in Minnesota. Wheat yields dropped in these two states by 48 and 39%, respectively (Anonymous 2015).

In worldwide surveys conducted to date, *F. graminearum* is widely distributed and has been reported in Asia, Africa, America, Europe, and Oceania (O'Donnell et al. 2000) and is the predominant species associated with FHB in the United States. Usually, *F. graminearum* produces in planta type B trichothecenes having a 7-hydroxy, 8-keto-trichothecene core structure. Based on this core structure and the acetylation position, four common trichothecenes have been identified: deoxynivalenol (DON), 3-acetyl, deoxynivalenol (3ADON), 15-acetyl, deoxynivalenol (15ADON), and nivalenol (NIV). Additionally, a novel trichothecene (3α-acetoxy,7α,15-dihydroxy-12,13-epoxytrichothe-9-ene, called NX-2) recently was identified from *F. graminearum* in the United States (Liang et al. 2014; Varga et al. 2015). NX-2 lacks the keto group at C-8 and thus is considered a type A trichothecene but otherwise resembles 3ADON. Individual isolates of *F. graminearum* generally produce a limited spectrum of trichothecenes (known as chemotype) which is under genetic control at several trichothecene biosynthetic loci (Seong et al. 2009).

Previous studies indicated that the 15ADON chemotype was predominant in the North American *F. graminearum* population (Abramson et al. 2001; Gale et al. 2007). However, a temporal shift toward the 3ADON chemotype occurred in western Canada from 1997 to 2004 (Ward et al. 2008). A similar increase in the frequency of strains with a 3ADON chemotype was also observed in the upper Midwestern United States *F. graminearum* population from barley (Burlakoti et al. 2011) and wheat (Liang et al. 2014) and in the southern China *F. asiaticum* population from barley (Zhang et al. 2010).

The genetic structure of *F. graminearum* populations from wheat in the United States has been studied extensively. Zeller et al. (2004) reported low levels of population subdivision within *F. graminearum* and a homogeneous population in North America. However, subsequent studies reported substantial population subdivision correlated with trichothecene chemotype differences in the upper Midwestern United States and elsewhere in North America (Gale et al. 2007, 2011; Puri and Zhong 2010; Ward et al. 2008). Few studies have focused on the population genetic structure of *F. graminearum* from barley. Recently, isolates of *F. graminearum* from barley fields in

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North Dakota and Minnesota were collected and analyzed using variable number tandem repeat (VNTR) markers (Burlakoti et al. 2011). Low F_{ST} between samples grouped according to the 3ADON or 15ADON chemotype suggested a minor influence of the chemotype on population subdivision. A similar study of the FHB population of *F. asiaticum* from barley in southern China did however present evidence for subdivision corresponding to chemotype composition (Zhang et al. 2010).

The objectives in this study were to (i) compare the population genetic structure of *F. graminearum* from barley and wheat in contemporaneous populations from the upper Midwestern United States, (ii) test the population subdivision of *Fusarium graminearum* from a barley-only population, and (iii) investigate whether isolates producing the novel trichothecene NX-2 are found in the barley population of *F. graminearum*.

MATERIALS AND METHODS

Fungal strains from barley and wheat. The strains of *F. graminearum* from barley used in a previous study (Burlakoti et al. 2011) were cultured from infected barley leaves obtained from North Dakota State University. A total of 247 *F. graminearum* isolates were recovered (n = 110 for the older population isolated from 1997 to 2000 and n = 137 for the newer population isolated in 2008) and isolates were grouped by geographic location as Northern region, Central region, and Southern region (Table 1). Of the 262 *F. graminearum* isolates used in the previous study, 5 and 10 isolates were lost from the older and newer barley collections, respectively, due to missing or nonviable samples.

To compare the population genetic structure of *F. graminearum* from barley and wheat, contemporaneous strains of *F. graminearum* from wheat were also obtained from a previous study (Liang et al. 2014). For the older population (n = 110), *F. graminearum* isolates were mainly collected from 1999 to 2000 and also were grouped as northern region (older wheat population from northern region, OldWN), central region (OldWC), and southern region (OldWS). However, for the newer population (n = 56) only isolates collected in southern region (NewWS) were available for 2006. The details of sampled counties and the number of isolates from each county are shown in Table 1. To balance sample size between wheat and barley isolates, only the counties in bold (n = 146, Table 1) from the barley collection were used to compare with all isolates from wheat. These isolates from barley were also grouped according to three geographical regions: older northern region (older barley population from northern region, OldBN), older central region (OldBC), older southern region (OldBS), and newer southern region (NewBS). Culture conditions, preparation of mycelium, and DNA extraction of each isolate were performed as described previously (Gale et al. 2002).

Trichothecene profiling of *F. graminearum* populations from wheat and barley. The trichothecene chemotype of every *F. graminearum* isolate was determined using previously validated trichothecene chemotype polymerase chain reaction (PCR) assays. *TRI3* and *TRI12* multiplexes were used to distinguish 3ADON, 15ADON, and NIV chemotypes (Starkey et al. 2007; Ward et al. 2002). The *TRI1*-ApoI enzyme combination was used to detect the novel trichothecene type, NX-2 (Liang et al. 2014). The PCR reactions, annealing temperature and program cycle for every primer set followed previous studies (Liang et al. 2014; Ward et al. 2008).

PCR-restriction fragment length polymorphism (RFLP) molecular genotyping. Ten pairs of PCR-RFLP primers designed in a previous study (Gale et al. 2011) were used to generate molecular datasets for population genetic analysis (Table 2). The amplification reactions were performed in 20-µl volumes with 2 µl of 10× r*Taq* buffer (Takara Biotechnology, Dalian), 1.6 µl of dNTP (2.5 mM/µl), 20 pmol of each primer, 0.5 U of r*Taq* (Takara Biotechnology), and 20 ng of genomic DNA. Amplification was carried out in a Bio-Rad Peltier Thermal Cycler using the following program with different annealing temperatures (Table 2): initial denature for 2 min at 94°C, followed by 30 cycles of denature at 94°C for 1 min, different annealing temperature for 1 min, extension at 72°C for 2 min and an additional extension at 72°C for 10 min. Enzyme digestions were performed in 15-µl volumes using 10 µl of the PCR end product above, 0.15 µl of 5U/µl of every restriction enzyme, 0.15 µl of 100× BSA (if necessary, see Table 2), and sterile water added to bring the final volume to 15 µl. All restriction enzymes were incubated at 37°C for 2 h except *Taq*aI, which was incubated at 65°C for 2 h. PCR reactions were conducted in 96-well plates with a negative control (primer and water) and a positive control (primer plus DNA from strain PH-1 [NRRL31084]) in each

TABLE 1. Strains of *Fusarium graminearum* from barley and wheat fields in North Dakota and Minnesota

Population[a]			Barley fields[b]	Wheat fields[b]
Older population	Northern	MN	Kittson (4), Roseau (1)	
		ND	**Bottineau (1), Burke (2), Cavalier (4), Divide (1) Pembina (5), Renville (3), Rolette (1), Towner (3)**	**Bottineau (6), Cavalier (16), Divide (3), Rolette (1), Towner (5)**
	Central	MN	Marshall (5), Pennington (2), Red Lake (3)	
		ND	**Benson (4), Grand Forks (3), McHenry (5), Mountrail (2), Nelson (3), Pierce (3), Ramsey (2), Walsh (3), Ward (2)**	**Grand Forks (3), McHenry (2), Pierce (2), Walsh (1), Ward (6)**
	Southern	MN	**Clay (2), Mahnomen (3), Norman (4), Polk (2)**	**Clay (5), Norman (12), Polk (22)**
		ND	**Barnes (3), Cass (2), Dickey (2), Eddy (2), Forster (2), Griggs (3), Kidder (2), La Moure (2), Logan (1), McIntosh (2), Morton (1), Richland (1), Sargent (3), Sheridan (2),Steele (3), Trail (1), Wells (5)**	**Barnes (9), Dickey (4), La Moure (2), Richland (1), Wells (10)**
Newer population	Northern	MN	Kittson (3), Roseau (4)	
		ND	Renville (12), Bottineau (2), Cavalier (13), Towner (1), Pembina (5), Rolette (3)	
	Central	MN	Marshall (14), Pennington (2), Red Lake (2)	
		ND	Ward (1), Benson (3), McHenry (1), Pierce (4), Walsh (7), Grand Forks (2), Nelson (7)	
	Southern	MN	**Polk (10), Clay (10)**	**Big Stone (1), Douglas (23), Grant (11), Pope (1)**
		ND	**Barnes (5), Eddy (6), Forster (6), Griggs (10), Ransom (2), Trail (1), Sheridan (1)**	**Dickey (15), La Moure (2), Richland (2), Sargent (1)**

[a] Older populations: *F. graminearum* isolates from barley were collected in 1997 to 2000 and those from wheat were collected in 1999 to 2000. Newer populations: *F. graminearum* isolates from barley were collected in 2008 and those from wheat were collected in 2006.

[b] Numbers in parentheses indicate number of isolates recovered in each county. Only the isolates of counties in bold were used in the comparison of *F. graminearum* between wheat and barley populations.

plate. Scoring was described in a previous study (Gale et al. 2011). Each specific pattern per locus was considered to be a different allele.

Population genetic analysis. For balancing the sample size and locations, only the strains collected from regions including both wheat and barley isolates (isolates in bold in Table 1) were used to compare genetic structure between collections arising from wheat and barley. The resulting collections were as follows: OldWN (n = 31), OldWC (n = 14), OldWS (n = 65), NewWS (n = 56), OldBN (n = 95), OldBC (n = 26), OldBS (n = 48), and NewBS (n = 51). The frequencies of distinct alleles at each locus and gene diversity were calculated in POPGENE 1.32 (Yeh et al. 1997). Distinct multilocus genotype (G) and genotypic diversity (GD) were calculated in each population using MULTILOCUS 1.3 software (Agapow and Burt 2001). Linkage disequilibrium (LD) was assessed in MULTILOCUS 1.3 using the loci-corrected index, rD. Genetic differentiations (Φpt) among populations were estimated using Genalex 6.2 (Peakall and Smouse 2006). The statistical significance of rD and Φpt were assessed using 1,000 random permutations.

A Bayesian clustering method was used to test the population subdivision of *F. graminearum* strains from wheat and barley in STRUCTURE 2.1 software (Pritchard et al. 2000). All *F. graminearum* isolates from wheat (n = 166) and barley (n = 146) were assigned into K populations with K ranging from 1 to 10 based on their multilocus haplotypes generated from 10 pairs of PCR-RFLP primers. The admixture model and correlated alleles were used. Each K was run for 10,000 iterations of the Monte Carlo Markov Chain (MCMC) scheme with a 1,000 burn-in period. Other settings were as recommended by default. The number of populations (K) best representing the observed data was evaluated by ΔK as proposed by Evanno et al. (2005). In order to test the potential of population subdivision in the barley-only collection, all recovered *F. graminearum* strains from barley in previous study (Burlakoti et al. 2011) (n = 247) were rerun in STRUCTURE software using the same settings as above.

In order to compare values for pair-wise genetic identity (I) and gene flow (Nm) estimated in the previous study (Burlakoti et al. 2011) in which subpopulation was grouped based on trichothecene chemotype (3ADON and 15ADON), values of I and Nm were recalculated for the current study using populations classified based on chemotype alone as in the previous study (Burlakoti et al. 2011) or based on Bayesian assignment obtained using the STRUCTURE software. POPGENE 1.32 (Yeh et al. 1997) was used to calculate I, and Nm was estimated by Genalex 6.2 (Peakall and Smouse 2006).

TABLE 2. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) primer annealing temperature, relevant restriction enzymes, number of alleles, and allele frequency in *Fusarium graminearum* isolates from wheat and barley

Primers	T (°C)	RE[a]	AW[b]	FW[c]	AB[d]	FB[e]
1389/1890	57	*Hae*III/ *Bam*HI	2	0.271–0.723	2	0.288–0.651
1401/1402	57	*Hin*fI	5	0.006–0.608	4	0.007–0.712
1403/1404	56	*Hae*III	5	0.012–0.482	4	0.007–0.548
1407/1407	56	*Hin*dIII/ *Msp*I	4	0.006–0.620	3	0.089–0.596
1155/1156	58	*Hae*III	6	0.006–0.705	4	0.007–0.774
1415/1416	57	*Hae*III	5	0.024–0.741	5	0.014–0.651
1439/1440	52	*Hin*fI	6	0.012–0.253	8	0.007–0.295
1441/1442	57	*Taqa*I	4	0.006–0.572	5	0.007–0.582
1449/1450	57	*Hin*fI	3	0.241–0.392	5	0.007–0.438
1457/1458	58	*Hae*III	4	0.078–0.620	4	0.075–0.582
Total			44		44	

[a] Restriction enzyme(s) used to detect polymorphisms in this amplicon.
[b] Number of distinct alleles for this locus in *F. graminearum* isolates from wheat.
[c] Allele frequencies for this PCR/RFLP locus in *F. graminearum* isolates from wheat.
[d] Number of distinct alleles for this locus in *F. graminearum* isolates from barley.
[e] Allele frequencies for this PCR/RFLP locus in *F. graminearum* isolates from barley.

RESULTS

Trichothecene profiling. The frequency of 3ADON producing strains was compared between isolations obtained from wheat and barley. Strains were chosen to reflect similar spatial and temporal collections; only isolates obtained from sampled counties indicated in bold in Table 1 were used. Four 3ADON producing strains were detected in the older barley population (n = 95) with a frequency of 4.2%, which was not significantly different (P = 0.52) from the frequency (5.5%) of 3ADON producing strains in the older wheat population (n = 110) (Table 3). However, the frequency of 3ADON producing strains in the newer barley population

TABLE 3. Trichothecene profile, genetic diversity, distinct genotypes, gene diversity, and linkage disequilibrium analysis of eight geographic and temporal subpopulations of *Fusarium graminearum* from wheat and barley

		Trichothecene profile							
Population[a]	N[b]	15ADON	3ADON	NX-2	G[c]	GD[d]	H[e]	rD[f]	Range[g]
OldBN	20	20	0	0	20	1.000	0.471 ± 0.166	0.037, P = 0.028	–0.040–0.067
OldBC	27	26	1	0	27	1.000	0.526 ± 0.125	0.029, P = 0.022	–0.034–0.048
OldBS	48	45	3	0	46	0.998	0.517 ± 0.134	0.021, P = 0.014	–0.023–0.043
NewBS	51	36	15	0	46	0.996	0.537 ± 0.114	0.073, P < 0.001	–0.024–0.026
Total	146	127	19	0	124	0.997	0.542 ± 0.119		
OldWN	31	29	2	0	29	0.994	0.507 ± 0.130	0.034, P = 0.012	–0.033–0.051
OldWC	14	14	0	0	13	0.989	0.514 ± 0.164	0.010, P = 0.029	–0.045–0.090
OldWS	65	59	4	2	62	0.998	0.513 ± 0.118	0.015, P = 0.046	–0.002–0.031
NewWS	56	30	24	2	50	0.994	0.533 ± 0.156	0.055, P < 0.001	–0.017–0.022
Total	166	132	30	4	148	0.998	0.556 ± 0.118		

[a] OldB = isolates collected from the older barley population; OldW = isolates collected from the older wheat population; NewB = isolates collected from the newer barley population; NewW = isolates collected from the newer wheat population. The last letters (N, C, and S) indicate isolates representing northern, central, or southern region, respectively (Fig. 1).
[b] Number of isolates.
[c] Number of distinct genotypes (G).
[d] Genotypic diversity (GD) in each population calculated as $GD = (n/n - 1)(1 - \Sigma pi^2)$, where pi is the frequency of the ith genotype and n is the number of individuals sampled.
[e] Mean ± standard error of the mean gene diversity within each population, calculated based on Nei's genetic distance (Nei 1973).
[f] Measure of linkage disequilibrium from observed dataset.
[g] rD range for 1,000 random permutations in MULTILOCUS 1.31 (Agapow and Burt 2001).

(NewBS) (29.4%) ($n = 51$) was significantly lower ($P = 0.001$) than the frequency (42.9%) in the newer wheat population (NewWS) ($n = 56$). No NX-2 producing strains were detected among the 247 isolates from barley. In contrast, two NX-2 producing strains were isolated from wheat in the older collection (Clay County, Minnesota) and another two were isolated from the newer wheat collection (Douglas County, Minnesota).

The geographic distribution of 3ADON producing strains in older wheat and barley collections was similar, with 3ADON strains mainly isolated from the border counties between Minnesota and North Dakota except one strain (KB1228, in McHenry County) isolated from the central region in the older barley collections (Fig. 1). Due to the low number of 3ADON isolates in both the older wheat ($n = 6$) and barley ($n = 4$) (Table 3) collections as well as the limited sampling of counties (Table 1) in the newer wheat population, the geographic distribution of 3ADON strains was analyzed only in the newer barley collection. A latitudinal cline was identified in which the frequency of 3ADON producing strains was highest in the northern region (49%), decreasing to 40% in the central region ($P = 0.068$, Fisher's test), and significantly decreasing to 29% in the southern region ($P = 0.003$) (Fig. 1).

Population genetic analysis with PCR-RFLP markers. Ten pairs of PCR-RFLP primers used in this study generated the same total number of alleles ($n = 44$) in wheat and barley populations (Table 2). The number of alleles identified at these loci ranged from two to eight with a slight variation between wheat and barley populations. Allele frequencies at each locus ranged from 0.006 to 0.774 (Table 2). A total of 122 distinct genotypes were detected in the combined older and newer barley population ($G = 0.85$), which was similar to the genotypic diversity in the wheat population ($G = 0.89$). High *GD* and similar *H* were observed in both barley and wheat populations (Table 3). Inference from *rD* values suggested random mating within all older wheat and barley subpopulations (all *P* values > 0.05) but not within newer wheat and barley subpopulations (both *P* values < 0.001) based on 1,000 random permutations in linkage disequilibrium tests (Table 3).

Comparison of genetic structure between wheat and barley populations. In total, genotypes of 146 *F. graminearum* isolates from barley and 166 isolates from wheat were combined to test for population structure. In previous studies, two *F. graminearum* populations were identified from wheat isolates in the upper Midwest United States (Gale et al. 2007; Liang et al. 2014): NA1 (previously called the MW 15ADON population) and NA2 (previously called the UMW 3ADON population). Using STRUCTURE to test population models for the combined genotypes from barley and wheat, the log likelihood was maximized when $K = 2$ (LnP(D) = –2735.13) (Supplementary Table S1). Most 3ADON strains (84.5%) from wheat and all 3ADON strains (100%) from barley were placed together into the previously reported NA2 population with high membership fraction $Q = 0.97 \pm 0.03$ (Fig. 2). Similarly, 81.3% of 15ADON strains from wheat and 91.3% 15ADON strains from barley were assigned together to the NA1 population with $Q = 0.96 \pm 0.04$ (Fig. 2). These results indicate the barley population of *F. graminearum* is characterized by population subdivision and these subdivisions correspond to the previously observed NA1 and NA2 populations. NA1 contains the largest proportion of 15ADON isolates and NA2 contains the largest proportion of 3ADON isolates, regardless of host origin.

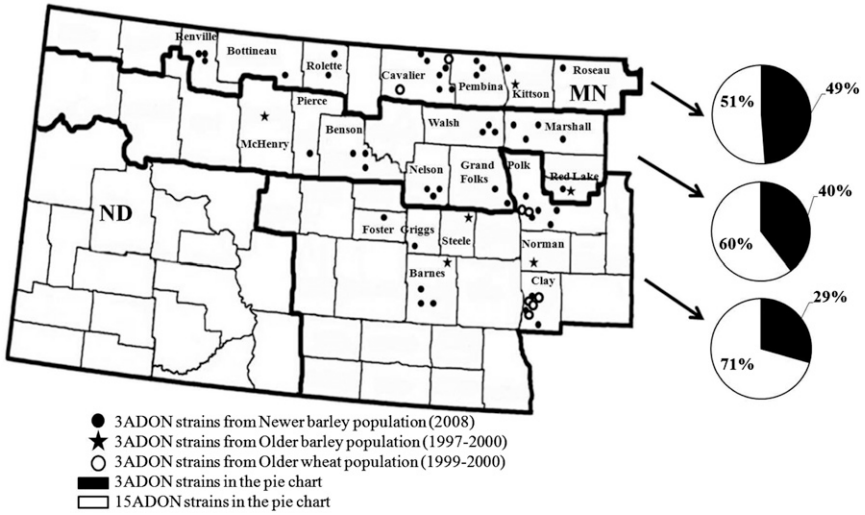

Fig. 1. Distribution of 3ADON chemotype isolates from the barley and wheat collections from the previously defined northern, central, and southern regions (Burlakoti et al. 2011). Only frequencies of 3ADON strains from the Newer barley population are shown in the pie charts because few 3ADON isolates were found in the Older barley ($n = 4$) and Older wheat ($n = 6$) collections and only limited sampling locations among these regions were done for the Newer wheat collections.

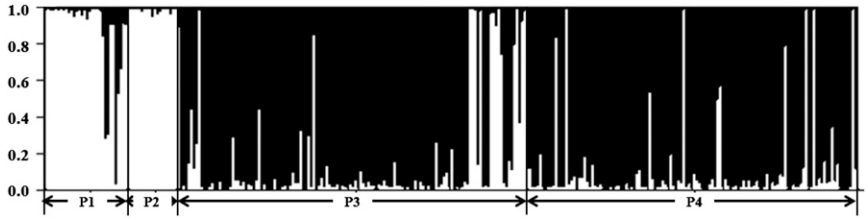

Fig. 2. Population structure of *Fusarium graminearum* based on 10 polymerase chain reaction (PCR)-restriction fragment length polymorphism data for 166 isolates from wheat and 146 isolates from barley. All isolates were assigned into two populations, NA1 population (black) and NA2 population (white). Isolates within P1 and P2 ranges are 3ADON producing strains of *F. graminearum* based on *TRI*-based multiplex PCR from wheat and barley, respectively. Isolates within P3 and P4 ranges are 15ADON producing strains from wheat and barley, respectively.

When analyses were limited to the barley isolates recovered from the previous study, 247 *F. graminearum* isolates (n = 110 for the older barley population and n = 137 for the newer barley population) were also assigned into two genetic groups in STRUCTURE (K = 2, LnP(D) = −1929.04) (Supplementary Table S2). In addition, this subdivision was also correlated to trichothecene chemotype with 98.3% of 3ADON producing strains having high membership fraction (Q) > 0.9 (average Q = 0.97 ± 0.04 [standard deviation]) assigned into the white partition (NA2 population) illustrated in Figure 3, and 85.1% of 15ADON strains with Q > 0.9 (average Q = 0.97 ± 0.02) assigned into the black partition (NA1 population) in Figure 3. These results indicate that the population subdivision among the barley isolates wasn't an artifact owing to the inclusion of the wheat isolates in the Bayesian analyses described above, but reflect a similar subdivision of *F. graminearum* populations across the two hosts.

To determine whether potential differences occur for the NA1 and NA2 partitions between wheat and barley populations, pair-wise genetic differentiation (Φpt) among NA1 and NA2 subpopulations in two sampling periods within wheat and within barley were compared. All pair-wise Φpt subpopulations combinations were significant except Older NA2 and Newer NA2 (Φpt = 0.018, P = 0.269) (Table 4), demonstrating that subpopulations defined based on Bayesian assignment results represented genetically differentiated populations. Interestingly, the Φpt values between NA1 and NA2 subpopulations were higher among barley isolates (Older: Φpt = 0.244 and Newer: Φpt = 0.378) than those from wheat (Older: Φpt = 0.141 and Newer: Φpt = 0.249) (Table 4). These results suggest that the NA1 and NA2 populations on barley are less genetically integrated than on wheat.

Analysis of potential recombinant genotypes. The Bayesian assignment result in STRUCTURE suggested population subdivision was closely related with chemotype, but two 15ADON strains from barley, nine 15ADON and two 3ADON strains from wheat were weakly (0.5 < Q < 0.8) assigned to NA1 population or NA2 population. In addition, 9 and 16 15ADON strains with Q > 0.5, from barley and wheat, respectively, were assigned to the NA2 population and three 3ADON strains from wheat were assigned to the NA1 population. These assignments suggested introgression between the NA1 and NA2 populations in both wheat and barley populations. Isolates with 0.5 < Q < 0.8 and Q > 0.5 but having a trichothecene type contrary to the predominant chemotype of the genetic subpopulation assigned were defined as admixed strains. For example, KB1307 is a 15ADON producing strain and although at Q = 0.989, it was assigned into NA2 which predominantly consists of 3ADON chemotype strains (Table 4). Thus, 41 admixed isolates were obtained from 312 *F. graminearum* isolates of wheat and barley populations (Table 4). Among 41 admixed isolates, 30 isolates (18.1% of wheat strains) were from the wheat population while 11 isolates (7.5% of barley strains) were from the barley population. For the Old period, the admixture proportion (the frequency of admixed isolates among all isolates) in the wheat population (10.9%) was not even twice as much as in the barley population (6.3%). However, in the New period the gap increased, with the admixture proportion of the wheat population (32.1%) being more than three times that in the barley population (9.8%) (Table 4).

All 11 admixed isolates from the barley population were 15ADON producing strains, and their membership fraction (Q) of the genetic materials derived from NA2 population ranged from 0.345 to 0.989 with six (55%) isolates assigned into NA2 at Q > 0.8 (Table 5). No 3ADON admixed isolates in the barley population were detected suggesting unidirectional gene introgression between NA1 and NA2 in the barley population. In contrast, five 3ADON admixed isolates (16.7% of total admixed isolates in wheat population) were detected in NA1 of the wheat population with Q from 0.342 to 0.967. Nevertheless, most of the admixed isolates in wheat population were 15ADON strains, and their proportion of genetic material derived from the NA2 population ranged from 0.250 to 0.990 with 14 isolates (56%) assigned into NA2 with Q > 0.8. Thus, the presumptive gene introgression between NA1 and NA2 in wheat the population appears bidirectional. Meanwhile, these results suggest a potential bias in the direction of gene introgression, as 15ADON isolates with a NA2 genetic background were significantly more common (F test; P = 3.8×10^{-5} for the barley population and $P = 1 \times 10^{-5}$ for the wheat population) than the 3ADON isolates with a NA1 genetic background.

Population level analysis in the barley population. Burlakoti et al. (2011) found high *Nm* (*Nm* = 23.26) and low genetic differentiation (F_{ST} = 0.02) between 3ADON and 15ADON populations using VNTR markers suggesting minor influence of chemotypes on *F. graminearum* population subdivision among barley isolates. Based on PCR-RFLP markers, we recalculated overall gene flow (*Nm*) and genetic differentiation (Φpt, equivalent to F_{ST}) between 3ADON (n = 59) and 15ADON (n = 188) strains as in Burlakoti et al. (2011). Contrary to the previous study, we found that *Nm* was only 0.63 and Φpt was high and significant (0.44, P = 0.001). These results suggest limited gene flow and high genetic differentiation between 3ADON and 15ADON isolates. Correspondingly, analysis of molecular variance revealed 44% genetic differentiation between isolates grouped by chemotype, which was much higher than the value (3%) reported previously (Burlakoti et al. 2011).

Due to gene introgression, isolates grouped solely by chemotype do not accurately reflect the population structure of the barley isolates. Subpopulations as based on time of strain collection and Bayesian assignment resulted in the following: Newer NA2 (n = 61), Newer NA1 (n = 76), Older NA2 (n = 8), and Older NA1 (n = 102). Pair-wise *Nm* and I were calculated for these partitions to depict the picture of population structure after some level of introgression. To compare with the results of Burlakoti et al. (2011), pair-wise *Nm* and I were also recalculated for subpopulations grouped by chemotype alone as in the previous study (Burlakoti et al. 2011): Newer 3ADON (n = 53), Newer 15ADON (n = 84), Older 3ADON (n = 6), and Older 15ADON (n = 104). Calculations for the Older NA2 and the Older 3ADON were not conducted due to small sample size. Compared with the previous study (Burlakoti et al.

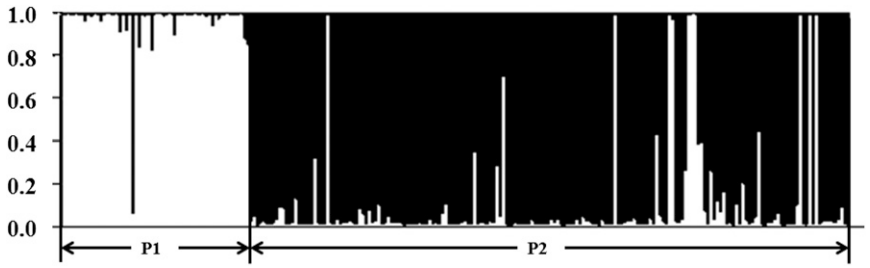

Fig. 3. Assignment of *Fusarium graminearum* isolates (n = 247) from barley based on polymerase chain reaction (PCR)-restriction fragment length polymorphism molecular data using STRUCTURE software. Vertical axis denotes membership fraction in one of two populations (denoted by black or white portions of bars). Vertical bars on the horizontal axis represent each of the 247 *F. graminearum* isolates. Isolates within the P1 range are 3ADON producing strains while isolates within the P2 range are 15ADON producing strains as determined by the *TRI*-based multiplex PCR test.

2011), our calculations of pair-wise *I* and *Nm* between subpopulations grouped by chemotype were significantly reduced between Newer 3ADON and Newer 15ADON as well as between Newer 3ADON and Older 15ADON subpopulations ($P = 0.014$ for *I* and $P = 0.036$ for *Nm*). Meanwhile, our values for *I* (0.98) and *Nm* (36.42) between Newer 15ADON and Older 15ADON subpopulations were higher than previously reported (0.87 for *I* and 28.71 for *Nm*) (Table 6). When subpopulations were grouped based on Bayesian assignment, pair-wise *I* values were similar to analogous comparisons based on chemotype and *Nm* values were greater between Newer NA1 and Older NA1 (Table 6).

DISCUSSION

A previous study of *F. graminearum* strains from barley in the upper Midwestern United States concluded that population subdivision was not evident among the collected strains (Burlakoti et al. 2011). These findings were noteworthy because other studies consisting largely of strains derived from wheat in the northern United States and Canada found clear evidence of persistent population subdivision strongly, but not absolutely, correlated with trichothecene chemotype (Gale et al. 2007; Liang et al. 2014; Ward et al. 2008). The findings of Burlakoti et al. (2011) thus suggested that population dynamics of the pathogen could be quite different between barley and wheat. To test for differences in population structure between isolates obtained from wheat and from barley, we tested all available (247 of 262) barley isolates utilized in the previous study (Burlakoti et al. 2011). Of those, 146 isolates from barley were matched with 166 isolates obtained from wheat at similar times and locations (Table 1).

As Burlakoti et al. (2011) previously observed, the current study also detected a greatly increased frequency of 3ADON strains from the older period (1997 to 2000) to the newer period (2008). We additionally observed an increased occurrence of 3ADON strains in the wheat population compared with the barley population. However, unlike the previous study (2011), we found evidence for significant population subdivision associated with trichothecene chemotype differences among both barley and wheat populations, which is consistent with most previous analyses of *F. graminearum* populations from wheat in this region (Abramson et al. 2001; Gale et al. 2007; Liang et al. 2014; Ward et al. 2008). Because Burlakoti et al. (2011) and the current study used the same barley isolates, what is the basis for our different findings?

The difference between our work, which uses PCR-RFLP genetic markers, and the work of Burlakoti et al. (2011), which uses VNTR markers, apparently does not result from differences in the markers used. Ward et al. (2008), Puri and Zhong (2010), and Bec et al. (2014) all documented significant population structure associated with chemotype differences on the basis of VNTR markers. Puri and Zhong (2010) also found significant structure using amplified fragment length polymorphic markers, though F_{ST} was lower than with VNTR. Therefore, we believe our differences with the previous work are not attributable to the different genetic markers used. Because Burlakoti et al. (2011) do not adequately detail the method by which F_{ST} was calculated for their study, it is impossible to determine whether they have used an appropriate metric for determining F_{ST} from microsatellite data.

One other contemporary study fails to find population subdivision associated with chemotype. Guo et al. (2008) used sequence related amplified polymorphism (SRAP) markers for analyzing *F. graminearum* collections from Manitoba. SRAP markers have shortcomings if they have not been unambiguously assigned to specific genetic loci and due to the fact that they may produce both dominant and codominant markers, making interpretation of data challenging (Wang et al. 2011). Further, Guo et al. (2008) did not use F_{ST} to evaluate population level differences and so their results are not directly comparable to other contemporary studies of population structure in Canada. In contrast, Ward et al. (2008) used VNTR markers to document significant population structure associated with trichothecene chemotype in Manitoba using isolates collected from roughly the same time period as Guo et al. (2008). Ward et al. (2008) included an evaluation of F_{ST} as well as a Bayesian assessment of admixture and population assignment.

Comparison of chemotype profiles of *F. graminearum* strains revealed that 15ADON was the most frequently isolated chemotype from both barley and wheat. However, 3ADON producing strains may have a higher rate of dissemination and spread on wheat than on

TABLE 4. Admixed isolates from wheat and barley populations based on Bayesian assignment result

Population	Admixture proportion[b]	Isolate name	Trichothecene type	Membership fraction (Q)[a] NA1 population	Membership fraction (Q)[a] NA2 population
Older barley population (n = 95)	6.3%	KB1187	15ADON	0.504	0.496
		KB1282	15ADON	0.470	0.530
		KB1319	15ADON	0.429	0.571
		KB1305	15ADON	0.165	0.835
		KB1233	15ADON	0.012	0.988
		KB1307	15ADON	0.011	0.989
Newer barley population (n = 51)	9.8%	253ND6	15ADON	0.655	0.345
		22MN1	15ADON	0.209	0.791
		212ND5	15ADON	0.013	0.987
		215ND5	15ADON	0.012	0.988
		231ND5	15ADON	0.010	0.990
Subtotal	7.5%				
Older wheat population (n = 110)	10.9%	99-185	15ADON	0.708	0.292
		00-836	15ADON	0.750	0.250
		00-800	15ADON	0.739	0.261
		00-544	15ADON	0.701	0.299
		00-677	15ADON	0.675	0.325
		00-781	15ADON	0.564	0.436
		00-834	15ADON	0.561	0.439
		00-619	15ADON	0.155	0.845
		00-810	15ADON	0.101	0.899
		00-795	15ADON	0.018	0.982
		00-556	3ADON	0.695	0.305
		00-552	3ADON	0.719	0.281
Newer wheat population (n = 56)	32.1%	06-217	15ADON	0.776	0.224
		06-180	15ADON	0.635	0.365
		06-158	15ADON	0.259	0.741
		06-176	15ADON	0.205	0.795
		06-151	15ADON	0.106	0.894
		06-191	15ADON	0.077	0.923
		06-138	15ADON	0.035	0.965
		06-148	15ADON	0.035	0.965
		06-128	15ADON	0.021	0.979
		06-198	15ADON	0.021	0.979
		06-122	15ADON	0.017	0.983
		06-246	15ADON	0.011	0.989
		06-247	15ADON	0.011	0.989
		06-179	15ADON	0.011	0.989
		06-154	15ADON	0.010	0.990
		06-163	3ADON	0.341	0.659
		06-142	3ADON	0.476	0.524
		06-140	3ADON	0.967	0.033
Subtotal	18.1%				

[a] Membership fraction (Q) denotes the percent of possibility to each population corresponding to NA1 and NA2 as defined previously (Liang et al. 2014).

[b] Admixture proportion = number of admixed isolates / the total number of isolates in this population. Admixed isolates are those with 15ADON and 3ADON chemotypes corresponding to NA1 and NA2 at $0.5 < Q < 0.8$ respectively and those with 15ADON and 3ADON chemotypes assigned into NA2 and NA1 populations at $Q > 0.5$, respectively.

barley during both the old and new sampling periods. One possible explanation for the differential ability to spread could be the differences in the infection processes of *F. graminearum* on wheat and barley. On wheat, after initial infection the fungus readily spreads within the spike but such spread rarely occurs in barley (Jansen et al. 2005). Additionally, differences in sampling locations should also be considered. Sixteen isolates out of twenty-four 3ADON strains were collected from Douglas and Grant Counties, which were not included in the barley population sampling. Although we attempted to match temporal and geographic samplings from the two hosts, this was not always possible given the retrospective nature of the analyses, and additional studies of closely matched isolate collections are needed to further evaluate these potential host-specific differences in trichothecene chemotype frequencies.

The novel trichothecene type, NX-2, was detected among isolates from wheat but not from barley collected over a similar period of time. The absence of NX-2 isolates from the barley collection could reflect some difference in host-specific adaptation, either directly associated with this chemotype or with the NA2 genetic background in which the NX-2 chemotype was observed. However, the absence of NX-2 isolates among the barley population is not inconsistent with the very low frequency (2.4%) of such isolates among the collection from wheat, and may simply reflect sampling error. However, the fact that NX-2 isolates were detected both in older and newer collections suggests that the NX-2 chemotype has persisted for some time and that strains capable of producing other unique toxins may remain undetected at low frequency.

In accord with previous studies, an increase in frequency of 3ADON isolates of *F. graminearum* was detected in current wheat and barley collections. The increasing frequency of 3ADON strains was first reported for isolations from wheat in Canada (Ward et al. 2008). Based on observations implying greater fitness for 3ADON isolates associated with NA2 including higher virulence, significantly more trichothecene accumulation, higher growth rates, and greater conidia production (Burlakoti et al. 2011; Foroud et al. 2012; Gilbert et al. 2010; Puri and Zhong 2010; Von der Ohe et al. 2010; Vujanovic et al. 2012; Ward et al. 2008), authors have speculated that such enhanced fitness may have caused the increasing frequency of 3ADON strains in North America. Recently however, Spolti et al. (2014) noted that for isolates in New York State, no fitness attributes of 3ADON isolates over 15ADON isolates were detected; these strains however were not assigned to genetic population. The gradient of decreasing frequency of 3ADON isolates from east to west in Canada was interpreted as migration of a newly introduced 3ADON population (corresponding to the NA2 population described here) (Liang et al. 2014). Indeed, the frequency of 3ADON chemotype strains increased greatly in the western provinces of Manitoba and Saskatchewan between the years of 1998 and 2004 (Ward et al. 2008). Schmale et al. (2011) evaluated the trichothecene type of 998 *F. graminearum* isolates collected in the eastern United States in 2006 and found an increasing frequency of 3ADON strains from south to north with highest frequency (15.4%) found in New York State and the lowest (0.5%) in North Carolina. This latitudinal cline in the eastern United States combined with a similar higher frequency of 3ADON chemotype strains toward the northern upper Midwestern United States suggest a national trend perhaps indicating movement of the NA2 population from Canada into the United States.

To compare the genetic structure detected within *F. graminearum* populations in the current study with the previous study, isolates

TABLE 5. Pair-wise genetic differentiation (Φpt[a], below diagonal) and their probability (above diagonal) estimates among NA1 and NA2 subpopulations of *Fusarium graminearum* based on STRUCTURE 2.1 from wheat (n = 166) and barley (n = 146) populations

	Wheat population[b]				Barley population[b]			
Population	Older NA1 (n = 101)	Older NA2 (n = 9)	Newer NA1 (n = 20)	Newer NA2 (n = 36)	Older NA1 (n = 86)	Older NA2 (n = 9)	Newer NA1 (n = 32)	Newer NA2 (n = 19)
Older NA1	...	0.001	0.001	0.001	...	0.001	0.048	0.001
Older NA2	0.141	...	0.001	0.001	0.244	...	0.001	0.269
Newer NA1	0.242	0.307	...	0.001	0.014	0.243	...	0.001
Newer NA2	0.311	0.314	0.249	...	0.361	0.018	0.378	...

[a] Φpt, an analog of F_{ST}, is also the estimate of population genetic differentiation when binary and haploid data are analyzed.
[b] Older denotes the isolates collected from the barley population in 1997 to 2000 and from the wheat population in 1999 to 2000; Newer denotes the isolates collected from the barley population in 2008 and from the wheat population in 2006. NA1 and NA2, formerly known as MW15ADON and UMW3ADON populations (Gale et al. 2007), respectively, based on STRUCTURE 2.1. Isolates were placed in the NA2 or NA1 populations with membership fraction Q > 0.5 regardless of trichothecene type. Probability of Φpt value was determined by 1,000 random permutations.

TABLE 6. Pair-wise comparisons of gene flow (above diagonal) and genetic identity (below diagonal) between barley subpopulations grouped solely by trichothecene type or by Bayesian assignment

	Populations grouped by chemotype							Populations grouped by Bayesian assignment		
	Previous study[a]			Current study[b]						
Subpopulations[c]	Newer 3ADON	Older 15ADON	Newer 15ADON	Newer 3ADON	Older 15ADON	Newer 15ADON	Subpopulations[d]	Newer NA2	Older NA1	Newer NA1
Newer, 3ADON	…	11.24[e]	16.47	…	0.78	1.05	Newer, NA2	…	0.72	0.76
Older, 15ADON	0.72[f]	…	28.71	0.49	…	36.42	Older, NA1	0.49	…	84.19
Newer, 15ADON	0.80	0.87	…	0.60	0.98	…	Newer, NA1	0.53	0.98	…

[a] Results from Burlakoti et al. (2011) in which isolates were amplified using variable number tandem repeat markers and grouped by chemotype alone.
[b] Isolates recovered from Burlakoti et al. (2011) independently genotyped using polymerase chain reaction-restriction fragment length polymorphism primers and grouped by chemotype as in Burlakoti et al. (2011).
[c] Newer and Older represent the barley populations collected from 2008 or from 1997 to 2000, respectively.
[d] Isolates were grouped into NA1 and NA2 populations based on Bayesian assignment resulting from STRUCTURE analysis. NA1, previously called the MW population, in which 15ADON producing strains are predominant and NA2, previously called the UMW population in which 3ADON producing strains are predominant.
[e] Gene flow (Nm) was calculated as $Nm = 0.5[(1/F_{ST}) - 1]$ using GENALEX 6.2 (Peakall and Smouse 2006), where F_{ST} was calculated as the proportion of the variance among populations in total variance.
[f] Genetic identity was estimated based on Nei's unbiased genetic distance (Nei 1978) in POPGENE 1.32 (Yeh et al. 1997).

were grouped based on sampled region and year as in Burlakoti et al. (2011). Consistent with previous results, high genotypic diversity and low linkage disequilibrium were observed for strains from barley and wheat (Table 3). However, unlike the previous study, we found evidence for population subdivision within the most recently sampled isolates. While Burlakoti et al. (2011) found the frequency of 3ADON strains increased rapidly between their sampling time points, no evidence for population subdivision was inferred due to the high *Nm* and *I* values between sampling periods. However, in the current study, lower linkage disequilibrium values were obtained for the older populations, which was consistent with Burlakoti et al. (2011) as well as Zeller et al. (2004), but *rD* values in the newer barley and wheat collection suggested increased disequilibrium, apparently due to increased population structure arising from increasing frequency of NA2 population genotypes. Thus, our data indicated a pattern of population subdivision in the newer barley population.

In an effort to better understand the admixture potential and dynamics between these populations from wheat and barley, 41 putatively admixed isolates were intensively studied (Table 4). Higher admixture frequencies (Table 4) and lower pair-wise genetic variation between NA1 and NA2 (Table 6) in the wheat population compared with the barley population suggested more extensive gene flow within the wheat population. In addition, the ratio of admixture frequencies from wheat relative to barley increased from 1.73 (10.9%/6.3%) to 3.28 (32.1%/9.8%) (Table 4) over the surveyed 8 to 10 years, which suggested faster dissemination of 3ADON genetic backgrounds and faster integration between NA1 and NA2 populations on wheat. Among wheat isolates, the frequency (16/30) of 15ADON isolates with NA2 genetic backgrounds ($Q > 0.5$) was remarkably higher than the frequency (1/30) of 3ADON isolates with NA1 genetic backgrounds (Table 4). This is consistent with previous studies that found a bias favoring the genetic background of the NA2 population among admixed isolates from western Canadian wheat (Ward et al. 2008). A similar finding was also reported in an *F. asiaticum* population from barley, where the genetic background typical of a 3ADON population was found among admixed isolates with the NIV chemotype more frequently than the opposite combination (Zhang et al. 2010). Although a predominant directionality of introgression existed, both directions could be detected in the above two examples. Interestingly, 4.5% (11/247) of 15ADON strains with NA2 genetic material was observed in the barley population of current study, but only one 3ADON strain (146 ND3) with NA1 genetic material was identified. Because NA2 strains, and the accompanying 3ADON chemotype, likely represent recent introductions into western Canada and the Upper Midwest (Burlakoti et al. 2011; Ward et al. 2008), we speculate that unidirectional gene flow in the barley population could be due to the selection for the NA2 genetic background, consistent with the increased frequency of the NA2 population as a whole. Moreover, slower dissemination of 3ADON strains on barley may reflect the additional time required for gene introgression in the barley population, leading to a strong signal of unidirectional gene flow. Overall, however, the exact mechanisms by which the NA1 and NA2 populations have remained largely distinct over the decade sampled in this study remain uncertain and worthy of further study.

As previously reported, the U.S. and Canadian populations of *F. graminearum* were essentially homogenous and almost exclusively of the 15ADON chemotype prior to the 1990s. However, population structure and diversity has increased dramatically over the last 10 to 20 years, as a novel genetic population (NA2) dominated by the 3ADON chemotype spread across Canada and the United States, bringing with it a variety of traits that may impart adaptive advantages over the previously dominant NA1 population (Burlakoti et al. 2011; Liang et al. 2014; Puri and Zhong 2010; Ward et al. 2008). Here, we document for the first time that this population heterogeneity extends to barley isolates from the Upper Midwest, contradicting a previous report suggesting that trichothecene chemotype polymorphism among barley isolates was not connected to population structure. Additional studies will be required to monitor the spread of the NA2 population as well as the 3ADON and NX2 chemotypes, and to evaluate potential differences in the relative competitive abilities of the different populations and chemotype on different hosts.

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