Fungal Genetics and Biology 73 (2014) 83–92

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regular Articles

Temporal dynamics and population genetic structure of *Fusarium graminearum* in the upper Midwestern United States

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ARTICLE INFO

Article history:
Received 10 July 2014
Accepted 1 October 2014
Available online 13 October 2014

Keyword:
Fusarium graminearum
Population genetic structure
Trichothecene mycotoxins
NX-2

ABSTRACT

Fusarium graminearum sensu stricto causes Fusarium head blight (FHB) in wheat and barley, and contaminates grains with several trichothecene mycotoxins, causing destructive yield losses and economic impact in the United States. Recently, a *F. graminearum* strain collected from Minnesota (MN) was determined to produce a novel trichothecene toxin, called NX-2. In order to determine the spatial and temporal dynamics of NX-2 producing strains in MN, North Dakota (ND) and South Dakota (SD), a total of 463 *F. graminearum* strains were collected from three sampling periods, 1999–2000, 2006–2007 and 2011–2013. A PCR-RFLP based diagnostic test was developed and validated for NX-2 producing strains based on polymorphisms in the *Tri1* gene. Trichothecene biosynthesis gene (*Tri gene*)-based polymerase chain reaction (PCR) assays and ten PCR-restriction fragment length polymorphism (RFLP) markers were used to genotype all strains. NX-2 strains were detected in each sampling period but with a very low overall frequency (2.8%) and were mainly collected near the borders of MN, ND and SD. Strains with the 3ADON chemotype were relatively infrequent in 1999–2000 (4.5%) but increased to 29.4% in 2006–2007 and 17.2% in 2011–2013. The distribution of 3ADON producing strains also expanded from a few border counties between ND and MN in 1999–2000, southward toward the border between SD and MN in 2006–2007 and westward in 2011–2013. Genetic differentiation between 2006–2007 and 2011–2013 populations (3%) was much lower than that between 1999–2000 and 2006–2007 (22%) or 1999–2000 and 2011–2013 (20%) suggesting that most change to population genetic structure of *F. graminearum* occurred between 1999–2000 and 2006–2007. This change was associated with the emergence of a new population consisting largely of individuals with a 3ADON chemotype. A Bayesian clustering analysis suggested that NX-2 chemotype strains are part of a previously described Upper Midwestern population. However, these analyses also suggest that the NX-2 isolates could represent a distinct population, but that interpretations of population assignment are influenced by the small number of NX-2 strains available for analysis.

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1. Introduction

Fusarium graminearum sensu stricto (O'Donnell et al., 2004) causes Fusarium head blight (FHB) in wheat, barley, and other small cereal grains, and is an economically harmful cereal pathogen worldwide (Yang et al., 2008; Ward et al., 2008; Miedaner et al., 2008). Considerable annual losses have occurred in the United States since 1993 (McMullen et al., 2012). In the Northern Great Plains and Central United States, the direct and secondary economic impact of FHB for 3 years (1998–2000) were $870 million and $1.8 billion, respectively (Nganje et al., 2004) and upper Midwestern states of Minnesota (MN) and North Dakota (ND) suffered almost half of all losses (McMullen, 2003; Windels, 2000). In addition, the pathogen contaminates grains by producing trichothecene mycotoxins which pose a health threat to human and animals as most induce toxic effects such as emesis, oral lesions, dermatitis and hemorrhaging (D'Mello et al., 1999; Pestka and Smolinski, 2005).

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http://dx.doi.org/10.1016/j.fgb.2014.10.002
1087-1845/Published by Elsevier Inc.

In the United States, *F. graminearum* mainly produces the type B trichothecene mycotoxins, deoxynivalenol (DON) (Fig. 1a), and its acetylated derivatives, 15-acetyldeoxynivalenol (15ADON) (Fig. 1c) and 3-acetyldeoxynivalenol (3ADON) (Fig. 1d), and to a lesser extent, the related trichothecene nivalenol (NIV) (Fig. 1b). A survey between 1999 and 2000 in the Midwestern United States revealed that 15ADON trichothecene chemotype strains were the most frequently observed (94.7%) whereas the 3ADON trichothecene type was only detected in a small sampling area in MN and ND. Based on neutral genetic markers, at least two *F. graminearum* populations were described within the US. One was predominant within the Midwest (MW) and consisted largely of strains with a 15ADON chemotype and thus was named as the "MW15ADON" population. The second population was found exclusively in the upper midwest (UMW) and consisted largely of 3ADON strains and so was named the "UMW3ADON" population (Gale et al., 2007). The NIV trichothecene type is rare in United States but, a recent study (Gale et al., 2011) showed field collections from south-central Louisiana segregated for all three trichothecene chemotypes with the majority (79%) being the NIV chemotype. Since all strains were nearly exclusively found in Louisiana, this population was named the southern Louisiana population.

The current study is based in part on initial results from a pathogen survey conducted from 2003 to 2006. An isolate (02-264) from MN was genotyped as a 3ADON producer but in chemical analysis, neither deoxynivalenol, nivalenol nor their acetylated derivatives could be detected (Gale et al., 2010). Very recently, the product of this isolate grown in toxin inducing medium was determined to be the novel trichothecene 3α-acetoxy, 7α, 15-dihydroxy-12, 13-epoxytrichothe-9-ene, also named as NX-2 (Fig. 1e) (Varga et al., 2014) which is nearly identical to the B type trichothecene 3ADON (Fig. 1c) but which lacks the keto group at C-8. Due to the association between trichothecene chemotype and population subdivision described above, we hypothesized that NX-2 producing strains may form a new population in the upper Midwestern United States.

Previous studies revealed that the *Tri1* gene in *Fusarium sporotrichioides* (*FsTri1*) and *F. graminearum* (*FgTri1*) encodes an enzyme catalyzing the hydroxylation of trichothecenes (McCormick et al., 2006). *FsTri1* encodes a cytochrome P450 monooxygenase that results in C-8 trichothecene hydroxylation leading to the synthesis of type A trichothecenes whereas *FgTri1* encodes a cytochrome P450 oxygenase required for hydroxylation at both the C-7 and C-8 positions and giving rise to 7-hydroxy, 8-keto-trichothecenes such as DON and NIV (type B trichothecenes) (McCormick et al., 2004; Alexander et al., 2011). Thus McCormick et al. (2006) suggested that *FgTri1* leads to C-7 and C-8 hydroxylation but *FsTri1* results only in C-8 hydroxylation. The discovery of the novel toxin NX-2 having only C-7 hydroxylation led us to postulate that the *FgTri1*allele in NX-2 producing strains may encode a cytochrome P450 monooxygenase that hydroxylates only at the C-7 position. In this study, the sequence of the *Tri1* gene from NX-2 producing strains was determined and compared with a DON producing strain in order to identify DNA polymorphisms unique to NX-2 producers. A PCR-RFLP assay was developed by which polymorphisms specific to the *Tri1* gene of NX-2 producers could be rapidly identified in order to assess their distribution and frequency.

To understand the temporal dynamics of *F. graminearum* populations in the upper Midwestern United States and the distribution of NX-2 producing strains, genetic diversity among isolates collected in MN, ND and SD from three sampling periods (1999–2000, 2006–2007 and 2011–2013) was assessed using 10 neutral RFLP markers, as well as the *Tri1* PCR-RFLP assay designed in current study, and PCR assays targeting *Tri3* and *Tri12*. The specific objectives of this study were to (1) establish trichothecene chemotype profiles of *F. graminearum* populations from each sampling period; (2) determine temporal and spatial dynamics of the *F. graminearum* populations in the upper Midwestern United States, and (3) test the hypothesis that NX-2 strains represent a new *F. graminearum* population which is divergent from the two currently known populations in the upper Midwestern United States.

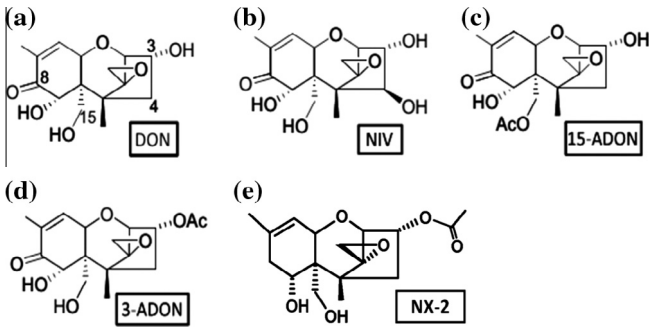

Fig. 1. Chemical structures of deoxynivalenol (DON), nivalenol (NIV), acetylated derivatives, 15-acetyl deoxynivalenol (15ADON) and 3-acetyl deoxynivalenol (3ADON) and 3α-acetoxy, 7α, 15-dihydroxy-12, 13-epoxytrichothe-9-ene (NX-2) (Varga et al., 2014).

2. Materials and methods

2.1. Isolate collection

FHB-infected wheat heads were collected from three sampling periods, 1999–2000, 2006–2007 and 2011–2013, from MN, SD and ND during the course of wheat rust surveys conducted annually by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Cereal Disease Laboratory, St. Paul, MN. The isolates from 1999 to 2000 and 2006 to 2007 collections were chosen randomly from previous population studies of *Fusarium graminearum* (Gale et al., 2007, 2011). Samples from 2011 to 2013 were isolated specifically for this study. To isolate *F. graminearum*, six seeds were picked from each infected head. Usually, multiple seeds from the same wheat spike yield the same *F. graminearum* genotype, so only one seed per spike was used for analysis. Species identification was conducted by visual assessment of typically pink colonies and conidial characteristics during single-sporing, using an Olympus dissecting SZX 12 microscope (Olympus America, Melville, NY). Details of isolation, culturing and single-sporing followed the protocol described previously (Gale et al., 2002). The sampling locations included 29 counties, 20 counties and 22 counties in the 1999–2000, 2006–2007 and 2011–2013 sampling periods, respectively (Table 1). In total, 463 isolates of *Fusarium graminearum* were analyzed in this study.

2.2. DNA extraction

Preparation of mycelium culture was performed as described previously (Gale et al., 2002, 2011) and genomic DNA extraction of each isolate followed the CTAB protocol (Fredlund et al., 2008). DNA samples were quantified using a Hoefer fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA) and working DNA concentrations were adjusted to 10 ng/μl by adding TE buffer (pH 8.0).

2.3. DNA sequencing of the Tri1 locus

The *Tri1* locus of three NX-2 producing strains (02-264, 03-348, and 06-204) was sequenced and compared to the *Tri1* locus from strains producing DON (GenBank Accession numbers KM999941–KM999943). Amplification of the *Tri1* region was performed in

Table 1
Sampled counties and number of isolates from each county for the three sampling periods in this study.

Sampling period	State	No. of isolates	County
1999–2000	MN	77	Dakota (5), Redwood (5), Clay (5), Polk (47), Norman (13), Stearns (1), Stevens (1)
	SD	24	Bookings (11), Union (1), Roberts (1), Aurora (3), Day (5), Brown (1)
	ND	76	Lamoure (2), Cavalier (16), Dickey (4), Grand (3), McHenry (2), Pierce (2), Wells (10), Walsh (1), Barnes (9), Richland (2), Towner (5), Bottineau (6), Williams (4), Ward (6), Rolette (1), Divide (3)
	Subtotal	177	
2006–2007	MN	84	Big Stone (1), Douglas (60), Grant (12), Kandiyohi (1), Lac Que Parl (2), Traverse (4), Pope (1), Wilkin (1), Yellow medicine (2)
	SD	54	Codington (2), Day (3), Grant (2), Hamlin (16), Marshall (24), Hugles (6), Kingsbury (1)
	ND	42	Dickey (36), Lamoure (6), Richland (2), Sargent (1)
	Subtotal	180	
2011–2013	MN	29	Stevens (6), Waseca (7), Lamberton (4), Crookston (3), Morris (2), Redwood (4), Todd (1) Polk (1), Traverse (1)
	SD	54	Codington (4), Brooking (19), Walworth (2), South shores (6), Highmore (5), Sissiton (7), Ipsrrich (5), Audra (6)
	ND	23	Cavalier (4), Wells (2), Bottineau (3), Cass (1), Ward (13)
	Subtotal	106	
	In total	463	

Note: The numbers in parentheses denote the number of isolates sampled from each county.

25 μl volumes with 1X High Fidelity PCR buffer (Invitrogen Life Technologies, Carlsbad, CA), 2 mM MgSO4, 0.2 mM concentration of each deoxynucleoside triphosphate, 0.6 μM concentrations of primers *Tri16*-IF1 (5′-GCCTSATAGCGACGATCTTGC-3′) and Fg_*Tri1*-R1 (5′-AACAAGTGGCGAGATCAAACC-3′), 1.0 Units of Platinum Taq DNA Polymerase High Fidelity (Invitrogen Life Technologies), and 200 ng of genomic DNA. PCR consisted of an initial denaturation of 120 s at 96 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C, and 180 s at 68 °C. PCR products were purified using Montage PCR96 Cleanup filter plates (Millipore Corp., Billerica, MA).

Sequencing reactions were carried out according to the method of Platt et al. (2007) using internal primers. Sequencing reaction products were purified using BigDye XTerminator (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI 3730 DNA Analyzer (Applied Biosystems). DNA sequences were assembled and edited with Sequencher (version 4.10, Gene Codes, Ann Arbor, MI).

2.4. Determination of trichothecene genotype

Two *Tri*-based multiplex PCR assays (*Tri3* and *Tri12*) were performed to differentiate isolates with 15ADON, 3ADON and NIV genotypes within the main trichothecene biosynthetic gene cluster (Starkey et al., 2007; Ward et al., 2002). A PCR-RFLP assay was designed to target variation in the *Tri1* gene that differentiated NX-2 producers from other *F. graminearum* strains. The *Tri1* gene was amplified using conserved primers (*Tri*1F: 5′-ATGGCTCTCATCACCAG-3′ and *Tri*1R: 5′CAATTCCAATCGCAGACAA-3′), which resulted in an amplicon of approximately 1740 bp (Fig. 2a). The amplification reactions were performed in 20 μl volumes with 2 μl 10 × rTaq buffer (Takara Biotechnology, Dalian), 1.6 μl dNTP (2.5 mM/μl), 20 pmol of each primer, 0.5 U r*Taq* (Takara Biotechnology, Dalian) and 20 ng of genomic DNA. Amplification was carried out in a Bio-Rad Peltier Thermal Cycler using the following program: an initial denature for 2 min at 94 °C, followed by 25 cycles of denature at 94 °C for 1 min, primer annealing at 50.3 °C for 1 min, extension at 72 °C for 1:45 min and an additional extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in 1% agarose gel with 0.5 × TBE buffer and visualized under ultraviolet (UV) light following SYBR@ safe DNA gel staining (Life Technologies – Invitrogen). A 1 kb DNA Ladder from New England Bio Labs Inc. was used as a molecular size standard for the PCR product of the *Tri1* gene.

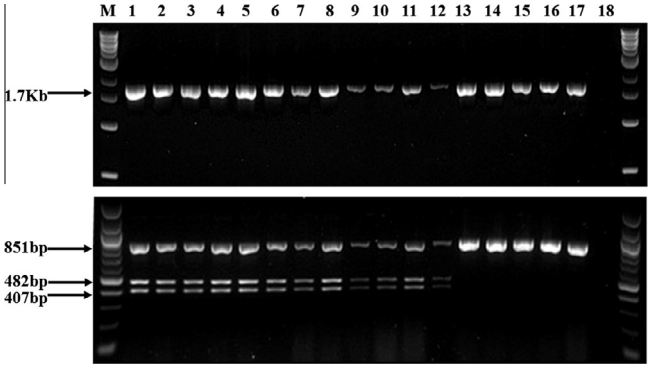

Fig. 2. Amplification of PCR products of 17 selected *F. graminearum* strains with NX-2 and DON chemotypes using primer set *Tri1*-F/*Tri1*-R (upper panel) and ApoI restriction enzyme digestion products (lower panel). M, 1 Kb marker in upper panel and 100 bp ladder marker in lower panel. Lane 1 to Lane 12, NX-2 strains: 00-556, 02-264, 02-267, 03-279, 3-348, 04-188, 04-308, 04-322, 06-146, 06-171, 06-190, 06-205, 12MN1-3; Lane 13, PH-1 (15ADON); Lane 14, 03-313 (15ADON); Lane 15, 00-500 (3ADON); Lane 16, 06-163 (3ADON); Lane 17, 02-15 (NIV); Lane 18, H_2O.

Following PCR amplification, the *Tri1* amplicon was digested with the restriction endonuclease ApoI (New England BioLab Inc), which produced two restriction fragments of 888 bp and 851 bp for isolates with 15ADON, 3ADON and NIV chemotypes (Fig. 2 lower panel) and three fragments of 407 bp, 482 bp, and 851 bp for isolates with a NX-2 chemotype (Fig. 2 lower panel). The *Tri1* amplicon digestion was performed in 15 μl volumes with 10 μl of amplicon, 1.5 μl 10 × NEB buffer 3, 0.15 μl ApoI (5 U/μl), and 0.15 μl 100 × BSA. The enzyme digestion was carried out in a Bio-Rad Peltier Thermal Cycler using the following program: 1 cycle of digestion at 50 °C for 2 h. PCR products were separated by electrophoresis as described above.

2.5. Determination of the trichothecene chemotype

A wheat spikelet inoculated with the fungus was weighed and placed into a 1 dram glass vial. A 2 ml volume of acetonitrile/water

(84/16, v/v) was added, and the vial was shaken on an Eberbach reciprocal shaker (Ann Arbor, MI) at room temperature for 24 h. The extract was passed through a minicolumn packed with C18 and aluminum oxide (1/3, w/w). A 1.5 ml volume of the filtrate was transferred to a 1/2 dram glass vial and evaporated to dryness under nitrogen. A 25 μl amount of TMS reagent (TMSI/TMCS = 100/1, v/v) was added to a 1/2 dram vial containing dried extract. The vial was rotated to ensure that the TMS reagent was in contact with all extract in the vial. The vial was then shaken on an Eberbach shaker for 10 min. A 200 μl volume of isooctane containing 4 mg/l of mirex was added and shaken gently, after which 200 μl of HPLC water was added. The vial was shaken on a vortex mixer, and the clear upper isooctane layer was transferred to a GC vial with a 200 μl glass insert.

GC–MS analysis was performed on a Shimadzu single quadrupole GCMS-QP2010 gas chromatograph–mass spectrometer (Shimadzu Corp., Kyoto, Japan). Perfluorotributylamine (PFTBA) was used to tune mass spectrometer, and a J&W DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in GC system. A high-pressure injection method (300.0 kPa, 1.00 min) was used in the splitless injection system. Linear velocity of flow control mode was used with the following oven temperature program: 150 °C for 1 min and the 30 °C/min to 280 °C holding 5 min. Injection, ion source and interface temperatures were kept at 260, 250 and 280 °C, respectively. Injection volume was 1 μl. The data were collected from *m/z* 50 to 550 in electron ionization (EI) mode with electron ionization energy of 70 eV. Under these conditions, the de-acetylated NX-2 (NX-3) was detected at 6.1 min as a major product in the inoculated wheat spikelet, and NX-2 was detected at 6.7 min as a minor product.

High-resolution accurate mass measurements were performed in EI and positive chemical ionization (PCI) modes using a Finnigan MAT 95 double-focusing mass spectrometer instrument coupled with a HP5890 Series II gas chromatograph with a J&W DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The instrument resolution was set at 4000 (10% valley). Perfluorokerosene (PFK) was used as internal reference for EI. The reagent gas for PCI was 4% ammonia in methane. Identification of individual trichothecene molecular species was as previously described (Varga et al., 2014).

Thirteen isolates were screened for chemotype using a two-stage media protocol, modified from Miller and Blackwell (1986). Each strain was grown on V8 plates initiated with spores from a glycerol stock solution. Mycelia were washed from the V8 plates with 3.5 ml water and used to inoculate 50 ml of first stage media (GYEP: 3 g NH_4CL, 2 g $MgSO_4{\cdot}7H_2O$, 0.2 g of $FeSO_4{\cdot}7H_2O$, 2 g KH_2PO_4, 2 g peptone, 2 g yeast extract, 2 g malt extract, 20 g glucose in 1 L distilled water) in 250 ml Erlenmeyer flasks. The cultures were grown at 25 °C on a rotary shaker at 200 rpm in the dark. After three days, first stage cultures were transferred to a 250 ml beaker and dispersed with a stick blender. The macerated culture was transferred to a 50 ml conical tube and centrifuged 5 min at 460*g*. Half of the medium was removed and the remaining fungal mass and medium were mixed well. Second stage cultures were initiated by adding 1.5 ml of the concentrated first stage culture to 20 ml of second stage production medium, (1% yeast extract–1% peptone–10% sucrose) in a 50 ml flask. After seven days, 5 ml aliquots (fungal biomass and medium) were removed from second stage cultures and extracted with 2 ml ethyl acetate. The extracts were dried under a nitrogen stream and re-suspended in 200 μl ethyl acetate. GC–MS analysis was performed on a Hewlett Packard 6890 gas chromatograph fitted with a HP-5MS column (30 m × 0.25 mm × 0.25 μm) and a 5973 mass detector. The carrier gas was helium with a 20:1 split ratio and a 20 ml/min split flow. The column was held at 120 °C at injection, heated to 260 °C at 20 °C/min and held for 5.4 min. Under these conditions, NX-2 was detected at 9.1 min.

2.6. Population genetic analyses

Ten pairs of PCR-RFLP primers distributed among unlinked loci on all four *F. graminearum* chromosomes were applied to generate molecular haplotype datasets. Primer sequences, annealing temperature and restriction enzymes were described previously (Gale et al., 2011). Restriction patterns were captured as line drawings, which were sequentially numbered and considered to be alleles at a genetic locus. DNA from the strain PH-1 was analyzed with every 96-well plate as a positive control to avoid potential scoring bias due to isolate origin. Genes to diagnose trichothecene chemotype were not used to generate haplotypes used for population analysis.

To analyze population genetic structure, isolates were grouped into 3 temporal populations and 9 subpopulations based on sampling period and geographic state of origin (Table 2). Multilocus 1.3 (Agapow and Burt, 2001) was used to calculate distinct multilocus genotypes (*G*), genetic diversity (*GD*) and multilocus disequilibrium (*LD*). Gene diversity (*H*) was calculated in the program POPGENE version 1.32 (Yeh et al., 1997). *GD* is defined as the probability that two individuals taken at random have different genotypes and was calculated by $(n/n-1)\,(1-\Sigma pi^2)$, where *pi* is the frequency of the *i*th genotype and *n* is the number of individuals sampled (Agapow and Burt 2001). *LD* was assessed using the index of *rD* which is virtually a standardized measure of index of association (I_A) ranging from 0 (panmixia) to 1 (absolute linkage disequilibrium) (Agapow and Burt, 2001). *rD* is independent of the number of loci analyzed, as encountered with I_A, and makes comparison among studies possible. The statistical significance was also inferred by using 1000 randomized datasets under a null hypothesis of panmixia. *H* was estimated in the total population (H_T) and within population (H_S). Genetic variation among three temporal populations was evaluated by analysis of molecular variance (AMOVA) in GENALEX 6.2 (Peakall and Smouse, 2006). *Φpt*, an analogue of *Fst* but more suitable for binary and haploid data,

Table 2
Genetic diversity and multilocus linkage disequilibrium (*LD*) analysis of *Fusarium graminearum* isolates from the nine subpopulations in this study.

	N[a]	*G*[b]	*GD*[c]	*H*[d]	*rD*[e]
1999–2000					
MN	77	71	0.997	0.5278 ± 0.1187	0.015**
SD	24	22	0.993	0.4937 ± 0.1888	0.001***
ND	76	71	0.998	0.5054 ± 0.1305	0.012***
Subtotal	177	154	0.998		...
2006–2007					
MN	84	70	0.989	0.5075 ± 0.1559	0.061
SD	41	39	0.970	0.5495 ± 0.1539	0.025**
ND	55	51	0.997	0.5798 ± 0.1137	0.057
Subtotal	180	152	0.996		...
2011–2013					
MN	29	29	1.000	0.5333 ± 0.1582	0.031**
SD	54	52	0.999	0.5267 ± 0.1464	0.009***
ND	23	20	0.980	0.4818 ± 0.1713	0.152
Subtotal	106	100	0.999		...
Total	463	375	...		...

[a] Number of isolates in each subpopulation.
[b] Number of distinct genotypes.
[c] Genetic diversity (*GD*) within subpopulation, calculated as $GD = (n/n-1)\,(1-\Sigma pi^2)$, where *pi* is the frequency of the *i*th genotype and *n* is the number of individuals sampled.
[d] Gene diversity within populations.
[e] Measure of multilocus linkage disequilibrium.
** $P < 0.01$.
*** $P < 0.001$.

was used to estimate genetic differentiation between temporal populations as well as among groups having different chemotype.

A Bayesian clustering method was used to investigate the subdivision of *F. graminearum* in the upper Midwestern area and to evaluate the hypothesis of a new population with the NX-2 trichothecene type. Multilocus haplotypes of 463 isolates generated from 10 PCR-RFLP loci were assigned by STRUCTURE version 2.3.1 (Pritchard et al., 2000) which implements a Monte Carlo Markov Chain (MCMC) approach in a Bayesian framework to cluster individuals into K clusters. The model is based on maximizing linkage equilibrium within clusters and disequilibrium between them. The MCMC scheme was run for 10,000 iterations with a 10,000 burn-in period. We used the admixture model that allows for the possibility that each individual may have mixed ancestry and allele frequencies were assumed to be correlated between populations. Other settings were as recommended by default, with *K* ranging from 1 to 10 with 10 repetitions to check the convergence of likelihood value for each value of *K*. The number of population best representing the observed data was determined by plotting the graph of estimated values of Ln likelihood for each *K*, and selecting the value which maximized the Ln likelihood of the data, as proposed by Evanno et al. (2005).

3. Results

3.1. Trichothecene profiling

Polymorphisms in the enzyme encoded by *Tri1* are known to result in differences in the oxygenation patterns of the trichothecene molecule at the C-7 and C-8 positions (McCormick et al., 2004, 2006). Since NX-2 differs from other trichothecene molecules by its unique oxygenation at position C-7 but not C-8 (Fig. 1), it was suspected that polymorphisms with the *Tri1* gene would be correlated with NX-2 producing strains. Therefore a PCR-RFLP method for detecting NX-2 producing strains was developed based on a polymorphism in the *Tri1* gene discovered by comparing the DNA sequence of this locus in the 15ADON strain PH-1 with the sequence in a previously identified NX-2 producer 02-264 (previously called WG-9). A 1.7 kb *Tri1* amplicon digested with restriction enzyme ApoI distinguishes the alleles found in PH-1 as well as in other DON or NIV producers from the allele found in 02-264 (Fig 2).

To validate the detection system, the DNA of 463 strains collected in MN, ND and SD from 1999 to 2013 (Table 1) were tested for the presence of the *Tri1* allele found in 02-264. A total of 13 strains were found to have the 02-264 allele and each of these was confirmed to produce NX-2 based on GC–MS detection. Ten strains having the PH-1 type allele and ten strains having the 3ADON type allele were tested and all produced DON and corresponding acetylated derivatives (data not shown) (see Fig. 3).

Therefore we infer that a total of thirteen NX-2 producing strains (00-552, 00-556, 06-132, 06-146, 06-156, 06-167, 06-171, 06-188, 06-190, 06-204, 06-205, 11-64 and 12-1.3) were found from three time periods sampled in this study making their frequency (2.8%) fairly low overall. All 13 NX-2 producing strains were isolated from wheat grown near the borders of MN, SD and ND (Fig. 4). The 13 NX-2 producing strains were found in five counties in MN (Polk, Grant, Douglas, Stevens and Yellow Medicine) and one county in SD (Brookings). Combined with multiplex PCR-tests based on *Tri3* and *Tri12*, most strains (82.3%) of *F. graminearum* have a genotype consistent with a 15ADON trichothecene chemotype and 14.9% of strains consistent with a 3ADON trichothecene type. No NIV producing strains were detected in this study. It should be noted that all strains having a NX-2 chemotype were scored as "3ADON" based on the *Tri3* and *Tri12* genotype tests and this is consistent with the fact that, like 3ADON, NX-2 has an acetyl group at the C-3 position and lacks a hydroxyl group at C-4. NX-2 chemotype strains thus must be distinguished from authentic 3ADON chemotype strains by the *Tri1* polymorphism.

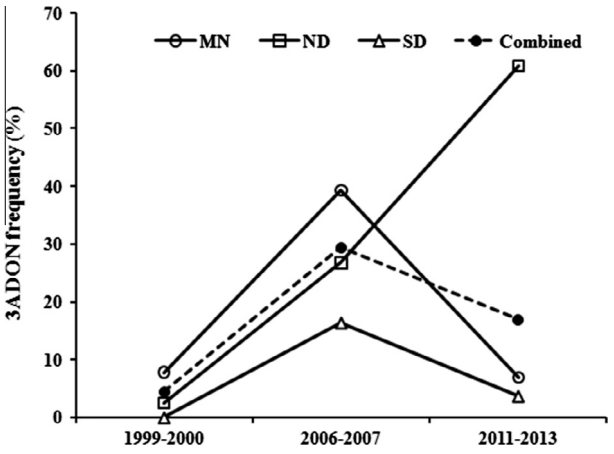

Fig. 3. 3ADON trichothecene type frequencies among *Fusarium graminearum* isolates collected from 1999–2000, 2006–2007 and 2011–2013.

Based on *T*-test comparisons, significant temporal differences in chemotype frequency were observed among *F. graminearum* populations. The frequency of 3ADON producing strains increased between 1999–2000 and 2006–2007 in each state studied (Fig. 3). Overall, the frequency significantly increased more than 6.5-fold from 4.5% (1999–2000) to 29.4% (2006–2007) ($P = 0.016$). However, from 2006–2007 to 2011–2013, the frequency of 3ADON producing strains decreased in both Minnesota and South Dakota, but not in North Dakota. In a combined analyses of all three states, the change in 3ADON frequency between 2006–2007 and 2011–2013 was not significant ($P = 0.434$). We should note that because of difficulty in obtaining samples due to drought and changing agronomic practices, limited numbers of samples were collected in North Dakota from 2011 to 2013. And while 3ADON strains were found in three counties in ND during this time period, nearly 50% of 3ADON strains were isolated from only two fields during 2011–2013 and this may have resulted in oversampling 3ADON genotypes.

The distribution of 3ADON producing strains in each sampling period also showed marked differences. In 1999–2000, an extensive sampling was performed in MN, ND and SD, but only a low rate (4.5%) of 3ADON producing strains was detected in four counties in the upper Midwest area (Fig. 4A) whereas in 2006–2007, 3ADON producing strains expanded southward to the border between MN and SD (Fig. 4B). By 2011–2013, the range of 3ADON producing strains continued to expand toward the western counties sampled, far from the border of these three states (Fig. 4C).

3.2. Population genetic analyses

Ten unlinked PCR-RFLP markers dispersed on all four *F. graminearum* chromosomes were used to genotype strains. (Genes used for diagnosis of trichothecene chemotype were not used to generate haplotypes for population analysis). High genotypic diversity and genetic diversity were found in each temporal and spatial subpopulation (Table 2). The linkage disequilibrium values were very low, with *rD* varying from 0.001 to 0.015. All *rD* values were significantly different from 1 ($P < 0.01$) except for the 2011–2013 ND subpopulation (Table 2).

Genetic variation between any two temporal populations was measured by AMOVA. Much lower genetic variation was detected

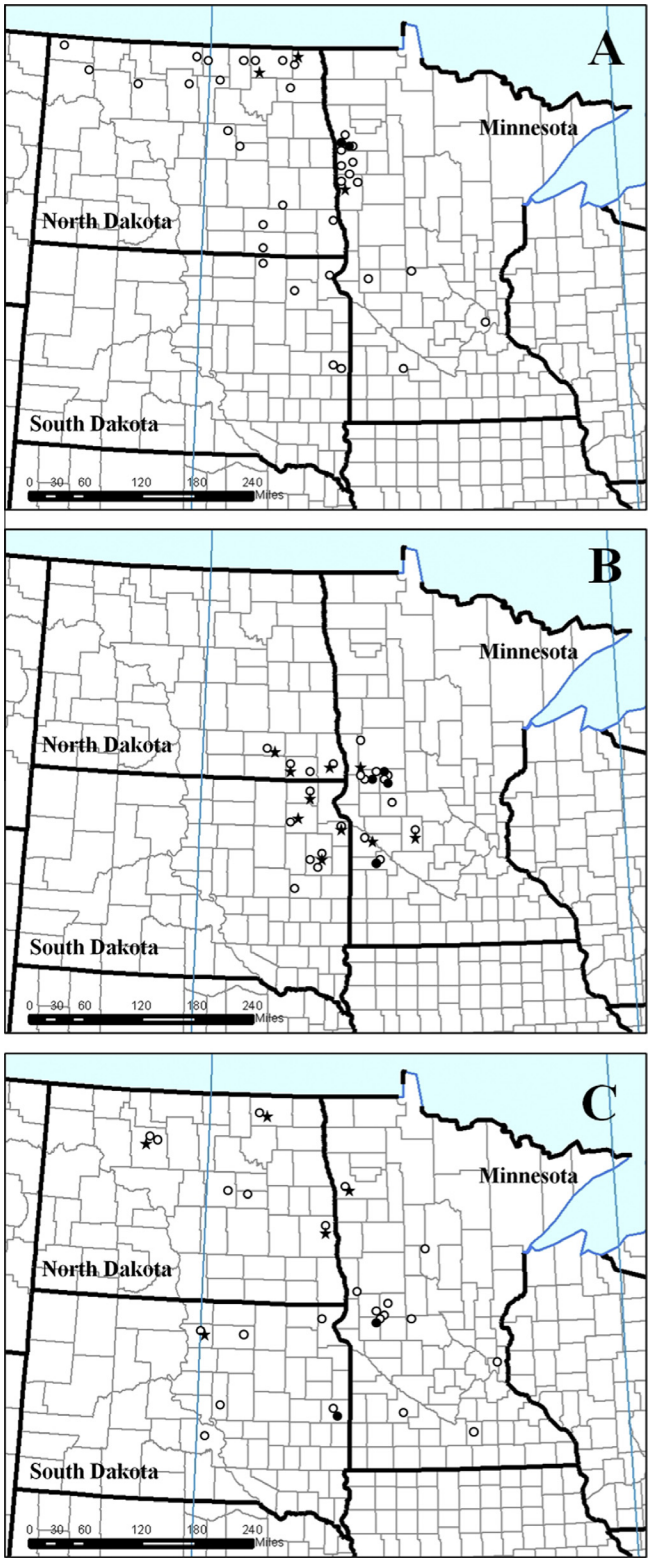

Fig. 4. Approximate county locations for 15ADON producing strains (○), 3ADON producing strains (★) and NX-2 producing strains (●) in three sampling periods. A: 1999–2000; B: 2006–2007 and; C: 2011–2013.

between the 2006–2007 and 2011–2013 populations (3%) than between the other two combinations, 1999–2000 and 2006–2007 (22%) or 1999–2000 and 2011–2013 (20%) (Table 3). When AMOVA was used to estimate genetic differentiation among groups of isolates having different chemotype, significant differentiation was observed among the three chemotype-defined populations ($\Phi pt = 0.202$, $P = 0.001$).

We utilized a Bayesian clustering method to analyze population subdivision of *F. graminearum* in these samples. Based on a previous study (Gale et al., 2007), at least two populations of *F. graminearum* could be detected, the ubiquitous "MW15ADON" population and the upper midwestern "UMW3ADON". (For the purpose of this discussion, these populations will be simply called "NA1" and "NA2" respectively, to avoid conflating population structure defined by neutral PCR-RFLP markers, with chemotype, inferred from polymorphisms at the *Tri1*, *Tri3* and *Tri12* loci, or with geographic origin which may be ephemeral). The optimal value of K was $K = 2$, which resulted in two discrete clusters with most strains being firmly placed in either cluster (Table 5). Consistent with previous analyses, most (71.6%) of the 15ADON genotype strains were assigned to NA1 with a very high average membership value (Q) ($Q = 0.971$[average] ± 0.021[standard deviation]). Additionally, 11.6% of 15ADON genotype strains were also placed into NA1 but at a lower Q value between 0.5 and 0.9 (0.785 ± 0.094). Also, six (9.4% in total) 3ADON genotype strains (06-140, 06-141, 06-248, 06-195, 06-203 and 13-1.2) were assigned to the NA1 population with $Q = 0.748 \pm 0.176$.

Fully 83.3% of the 3ADON genotype strains were assigned to the NA2 population with high Q value ($Q = 0.980 \pm 0.017$) and 7.6% of 3ADON producing strains were also placed into NA2 but with a lower average Q value ($0.5 < Q < 0.9$, $Q = 0.834 \pm 0.083$). In addition, 74 (19.3% in total) 15ADON producing strains were also placed into the NA2 population with $Q = 0.911 \pm 0.137$.

Generally, NX-2 producing strains did not fit NA1 or NA2 populations with very high Q values. Ten of 13 (76.9%) NX-2 producing strains were placed into the NA2 population with $Q = 0.847 \pm 0.077$ whereas the remaining three NX-2 producing strains fit into NA1 but with a lower average Q (0.721 ± 0.161). Therefore it is possible that NX-2 producing isolates belong to a distinct population that is not fully resolved by this method due to the low frequency of NX-2 producers relative to isolates having 3ADON or 15ADON chemotype. To test whether this may be the case, a sampling of isolates balanced for chemotype was assembled. Genotypes for fifteen 3ADON-producing isolates and fifteen 15ADON-producing isolates were chosen at random and pooled with genotypes of all 13 NX-2 producing isolates. Bayesian clustering of this chemotype-balanced sample yielded an optimal K value of $K = 3$ (Table 5) with population structure largely aligning with chemotype (Fig. 5b).

3.3. Reproductive mode within populations

F. graminearum is a homothallic fungus that may reproduce sexually by selfing or outcrossing or reproduce asexually by formation of macroconidia. Therefore mechanisms for both sexual recombination and clonal reproduction are known to exist. Due to its considerable effect on the pathogens' adaptive response, the reproductive mode of NA2 and NA1 populations were tested for the three temporal periods examined. Based on STRUCTURE assignment above, strains which were firmly ($Q > 0.9$) placed into these two clusters were used and grouped into 5 subpopulations based on sampling period and chemotype: 1999–2000 NA1 ($n = 148$), 2006–2007 NA2 ($n = 37$), 2006–2007 NA1 ($n = 66$), 2011–2013 NA2 ($n = 18$) and 2011–2013 NA1 ($n = 67$). The 1999–2000 NA2 population was not tested due to small sample size ($n = 6$). Linkage disequilibrium across 10 PCR-RFLP loci was calculated with the index of rD. Tests of departure from random mating were done with 1000 randomizations of the data. Observed rD values of 1999–2000 NA1 ($rD = 0.008$, $P = 0.059$) and 2011–2013 NA1 ($rD = 0.009$, $P = 0.068$) subpopulations were within the expected rD range from 1000 randomizations with $P > 0.05$ suggesting the

Table 3
Analysis of molecular variance (AMOVA) of *F. graminearum* population among three sampling periods.

	df	Estimated variance	Variation (%)	*Φpt*[a]	*P*[*]
Between 2011–2013 and 2006–2007	1	0.088	3	0.029	0.001
Between 2011–2013 and 1999–2000	1	0.817	20	0.195	0.001
Between 2006–2007 and 1999–2000	1	0.905	22	0.216	0.001

[a] *Φpt*, an analogue of *Fst*, is also the estimate of population genetic differentiation when binary or haploid data are analyzed.
[*] Probability of *Φpt* values was determined by 1000 random permutations.

Table 4
Observed and expected range from 1000 randomizations of linkage disequilibrum in subpopulations of *F. graminearum* based on STRUCTURE assignment.

Subpopulation[a]	*n*	*GD*	*rD*[b]	Range[c]	*P*
1999–2000 NA1	148	0.997	0.008	−0.013 to 0.015	0.059
2006–2007 NA2	37	0.976	0.107	−0.024 to 0.036	<0.001
2006–2007 NA1	66	0.999	0.014	−0.015 to 0.017	0.006
2011–2013 NA2	18	0.952	0.034	−0.074 to 0.252	0.172
2011–2013 NA1	67	0.999	0.023	−0.015 to 0.023	0.068

[a] Subpopulations were grouped by sampling period and cluster detected in STRUCTURE assignment. For analysis, only these isolates with $Q > 0.9$ to NA1 or NA2 cluster were used. The 1999–2000 NA1 subpopulation was not analyzed due to the small sample size ($n = 6$).
[b] *rD*, a measure of linkage disequilibrium corrected for the number of loci based on 10 pairs of PCR-RFLP.
[c] Expected *rD* values range obtained from 1000 randomizations in MULTILOCUS 1.3.

Table 5
Inference of the number of cluster(s) (K) that best explain the genetic structure among isolates of *F. graminearum* from an unbalanced sample size population ($n = 463$) and a balanced sample size population ($n = 43$). For STRUCTURE runs, the mean value of log likelihood was shown directly and ΔK index was computed according to Evanno et al. (2005).

K	Unbalanced sample size[a]		Balanced sample size[b]	
	Log likelihood	Δ*K*	Log likelihood	Δ*K*
1	−4736.3		−449.4	
2	−4149.5	142.5	−339.8	56.0
3	−4116.9	7.9	−293.5	213.5
4	−4206.8	0.9	−307.6	0.4
5	−4045.2	6.2	−323.9	1.2
6	−4133.3	1.4	−320.0	4.7
7	−4002.9	4.9	−326.7	0.6
8	−4143.1	0.4	−328.4	0.5
9	−4347.5	0.7	−338.2	6.5
10	−4463.4	0.3	−394.3	0.7

[a] Unbalanced sample size population includes all the isolates of *F. graminearum* in this study with 13 NX-2 strains, 66 3ADON strains and 384 15ADON strains.
[b] Balanced sample size population includes 43 isolates of *F. graminearum* with 13 NX-2 strains and 15 randomly selected strains each of 3ADON and 15ADON chemotypes.

likelihood of sexual recombination (Table 4). The observed *rD* in 2006–2007 NA1 also was located at the edge of the range but with $P < 0.05$ indicating perhaps a mixture of outcrossing and clonal reproduction. However, reproductive mode varied between the two NA2 subpopulations from the 2006 to 2007 and 2011 to 2013 sampling periods. The 2006–2007 NA2 subpopulation was inferred as a non-randomly mating population with observed *rD* ($rD = 0.107$) located clearly outside of the expected random mating range with $P < 0.001$ (Table 4) while the 2011–2013 NA2 population appears to be randomly mating with observed *rD* of 0.034 ($P = 0.172$) (Table 4).

4. Discussion

The type B trichothecene DON and its acetyl derivatives are harmful mycotoxins found in grain that can have profound impact on human and animal health (Pestka and Smolinski, 2005). The U.S. Food and Drug Administration has proposed guidelines for acceptable levels of these type B trichothecenes in grain and products derived from grain (McMullen et al., 2012). Thus the discovery of the novel type A trichothecene, NX-2, produced by F. graminearum strains infecting wheat, potentially has important consideration for public safety and human health. In this study we sought to develop a quick DNA based assay to identify strains of the fungus capable of producing this novel trichothecene and to understand their geographic distribution and frequency of occurrence over time in the context of population genetic partitions known to exist for *F. graminearum* in the United States.

Specifically, we utilized trichothecene genotype analysis and PCR-RFLP neutral markers to examine the temporal and spatial dynamics of the *Fusarium graminearum* population in wheat from the upper Midwestern United States. The results presented are the first report of the frequency and distribution of strains that produce the novel trichothecene, NX-2. In addition, the results confirmed not only the increasing frequency of 3ADON genotype strains from 1999–2000 to 2006–2007 but also the geographical expansion of these strains from 1999–2000 to 2011–2013. Analysis of DNA polymorphism provides little support for the hypothesis that individuals producing NX-2 form a new population and instead suggest that most NX-2 producing strains may be members of the NA2 population. Nevertheless, this conclusion should be tempered in light of the large discrepancy in the occurrence of strains producing NX-2 ($n = 13$), compared with those producing 3ADON ($n = 66$) or 15ADON ($n = 384$). Because of the small number of NX-2 strains, even divergent genotypes may not be resolved from one of the two more frequently represented populations (i.e. NA1 and NA2).

In order to address this consideration, we created a dataset more balanced for chemotype, by randomly choosing 15 strains each from 3ADON and 15ADON genotypes, and combining these with all NX-2 strains ($n = 13$). This dataset was rerun with the STRUCTURE software using the same settings as before. Interestingly, for the chemotype balanced dataset the best K value obtained was $K = 3$. Forty-three isolates were grouped into three clusters strongly correlated with trichothecene types (Fig. 5b). Therefore it is possible that NX-2 producing strains are members of a population separate from NA1 and NA2. As increasing numbers of NX-2 strains are discovered, we will be eager to determine whether these may indeed form a separate population that may be resolved using a larger dataset.

The assay developed to detect NX-2 producing strains based on polymorphism in the *Tri1* locus was successful; all 13 isolates identified as NX-2 producers by the assay, were shown to actually produce NX-2 in culture. We have recently demonstrated that the *Tri1* allele found in NX-2 producing strains is biochemically responsible for synthesis of the 7-hydroxy, 8-deoxy NX-2 chemotype (Varga et al., 2014). Polymorphisms in the main trichothecene biosynthetic gene cluster are responsible for 3- and 15-position acetylation and 4-position hydroxylation but this cluster does not include the *Tri1* locus. It is interesting to note that all NX-2 producers also have polymorphisms in the main trichothecene gene cluster (i.e. the assayed *Tri3* and *Tri12* genes) normally indicative

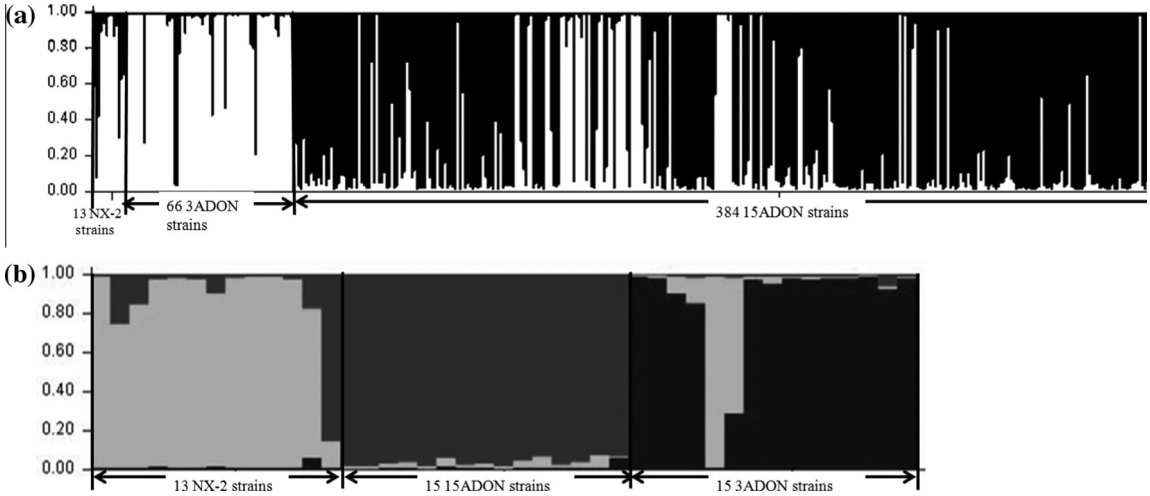

Fig. 5. Population assignment of unbalanced chemotype sample size (Fig. 5a, $n = 463$) and balanced chemotype sample size (Fig. 5b, $n = 43$) of *Fusarium graminearum* isolates collected in the Midwestern United States based on PCR/RFLP defined alleles at 10 loci using STRUCTURE 2.3.1. The horizontal axis represents the isolates each by a vertical bar with the vertical axis denoting estimated membership coefficients for each individual in each cluster. Each shade represents one cluster. In Fig. 5a, all isolates were assigned into two clusters with black corresponding the NA1 cluster and white corresponding the NA2 cluster as in a previous study (Gale et al., 2007). In Fig. 5b, forty-three isolates were assigned into three clusters strongly correlated to trichothecene types with NX-2 strains forming a third cluster.

of a 3ADON chemotype, but in NX-2 producers indicative of acetylation of the 3-position of the novel trichothecene. Because the *Tri1* locus on chromosome 1 and the trichothecene biosynthetic cluster on chromosome 2 are genetically unlinked, it should be formally possible to have recombinants that would have the *Tri1* of NX-2 producers and the trichothecene cluster of a 15ADON producer that presumably would make a trichothecene with a 7-OH, 8-deoxy, 15 acetyl structure. This genotype has yet to be identified.

A considerable increase of 3ADON genotype strains from 1999–2000 to 2006–2007 suggested that these strains were displacing strains with the more prevalent 15ADON chemotype. This increasing trend was also shown in previous studies. The frequency of 3ADON producing strains from Canadian wheat increased 14-fold between 1998 and 2004 (Ward et al., 2008). *F. graminearum* populations from barley also showed a similar increase of 3ADON producing strains over time in North Dakota and Minnesota (Burlakoti et al., 2011). Although the frequency of 3ADON producing strains in this study declined from 2006–2007 to 2011–2013, the frequency in 2011–2013 was still twice the level in 1999–2000. Together these results demonstrate the widespread increasing frequency of the 3ADON chemotype in North America during the time period studied.

Ward et al. (2008) hypothesized that wheat isolates with the 3ADON trichothecene type were rapidly displacing 15ADON strains moving from eastern Canada and into western Canada and the upper Midwestern United States. A previous study (Gale et al., 2007) and the current study both show 3ADON producing strains in a limited area of the Red River Valley defining the border between MN and ND in 1999–2000. However, in 2006–2007, a high percentage of 3ADON genotype strains were found further to the south into SD and in recent years (2011–2013), 3ADON producing strains were detected even in some of western counties far from the Red River Valley. We interpret these results as indicating that in the past decade, 3ADON genotype strains have spread from north to south and west in this three state region.

The very low frequency (2.8%) of NX-2 producing strains found over all time periods suggests that this novel trichothecene type remains somewhat rare in the upper Midwestern United States. Interestingly, the frequency of NX-2 producing strains mirrors the dynamic of 3ADON genotype strains with similar fluctuation over the three sampling periods (1.1% for 1999–2000, 5.0% for 2006–2007 and 1.9% for 2011–2013). This result may indicate a tacit correlation between 3ADON and NX-2 producing stains; NX-2 and 3ADON differ only by the keto group at the 8-C position. In addition, 3ADON producing strains were first reported as occurring in the upper Midwestern United States from collections made in 1999 (Gale et al., 2007). These strains were isolated from wheat mainly grown at the border between MN and ND whereas NX-2 producing strains were gathered at the border of MN, ND and SD (Fig. 4). NX-2 producing strains should be monitored in the future to determine whether they will expand following the route of the 3ADON producing strains described above.

So far, no clear reason has been provided for the rapid increase in the frequency of 3ADON chemotype strains. One possible explanation was that changing wheat cultivars (Burlakoti et al., 2008) or climatic conditions (Yang et al., 2008) could explain this population shift but these explanations were rejected based on the non-significant relationship between these factors and the distribution of 3ADON and 15ADON chemotype isolates. However, Ward et al. (2008) found significantly more trichothecene accumulation, higher growth rates and greater conidia production in the rapidly increasing population typified by the 3ADON chemotype compared to the population typified by the 15ADON chemotype. Meanwhile, non-significant difference between the 3ADON population found in western Canada and NA2 population in the upper Midwestern United States suggested that the NA2 population also had the fitness advantages detected in the Canadian 3ADON population. Irrespective of chemotype, growth related fitness characteristics could potentially explain the rapid increase of the 3ADON enriched NA2 population in the upper Midwestern United States. However, the reason for the numerical (though not statistically significant) decrease in abundance of 3ADON genotype strains from 2006–2007 to 2011–2013 is still unclear. The bottle-neck effect of introduced 3ADON producing strains, changing cropping patterns (deployment of resistant wheat cultivars, widespread fungicide application and increasing corn and soybean acreage) and climatic factors (drought) may be considered. Alternatively, if chemotype is not itself under selection, recombination between populations with different chemotypes should lead to an uncoupling of chemotype from the actual trait(s) under selection. This should lead to a plateau in 3ADON frequency, which would also contribute to the observed changes. Continuous monitoring of *F. graminearum*

populations and related metadata are necessary to fully explain this issue in the future.

In addition to chemotype, the mating system is expected to exert considerable effect on a pathogen's adaptive response: recombining populations likely produce more advantageous gene combinations (McDonald and Linde, 2002) while clonality may allow for the rapid amplification of strongly fit individuals (de Meeus et al., 2007). Linkage disequilibrium tests in the current study revealed potentially different reproductive modes in the NA2 and NA1 populations. Recombination was prevalent in NA1 populations, especially in the 1999–2000 NA1 subpopulation. The lowest *rD* value suggested a frequently outcrossing population as described in Zeller et al. (2004). However, in the 2006–2007 NA2 subpopulation, significant linkage disequilibrium was detected suggesting limited outcrossing. Given the potential fitness advantage of the 3ADON population described by Ward et al. (2008), the founder effect of the introduced NA2 population and clonal expansion of fit individuals within the largely 3ADON population may have driven the increase of 3ADON genotype strains. However, after 5 years, a reduced level of linkage disequilibrium in the 2011–2013 NA2 subpopulation indicates an increased level of recombination. Interestingly, the potential for increased recombination in the 2011–2013 NA2 subpopulation occurs at the same time that the overall representation of the NA2 subpopulation is decreasing (2006–2007 NA2 = 36%; 2011–2013 NA2 = 21%). The possibility then exists that recombination may be resulting in the dilution of particularly fit genotypes in the subpopulation leading to an overall reduction in the prevalence of this population. Alternatively recombination over time may dissociate traits under selection from neutral genetic variation, uncoupling the dynamics of fit genotypes from population identity. Adaptive traits thus may be moving rapidly from one population to another, while genetic structure at neutral loci may take longer to dissipate.

Despite evidence for increased recombination within the NA2 population over time, the contemporary 2011–2013 NA1 and 2011–2013 NA2 subpopulations were still significantly different ($\Phi pt = 0.354$, $P = 0.001$) and the gene flow between them was very low ($Nm = 0.914 < 1$). The factors responsible for this decade-long sympatric population structure remain unknown. But the strong correlation between subpopulations and chemotype lead us to speculate that trichothecene chemotype itself may be one of the contributors to genetic isolation. The barrier to reproduction however does not appear to be absolute so the integration of populations likely will be achieved with time.

Acknowledgments

This work was supported in part by the United States Wheat and Barley Initiative. We are grateful to Franz Berthiller, Gerhard Adam, Gerlinde Wiesenberger and Elisabeth Varga for their cheerful interaction on the project to characterize NX-2. Liane Gale, James Kolmer and Jerry Ochocki are thanked for help with sampling of FHB-infected materials. We dedicate this publication to the late Jerry Ochocki, who greatly enjoyed cereal rust and FHB collection trips and whose joyful manner will be greatly missed.

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