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Diversity of Fusarium head blight populations and trichothecene toxin types reveals regional differences in pathogen composition and temporal dynamics

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ABSTRACT

Analyses of genetic diversity, trichothecene genotype composition, and population structure were conducted using 4086 *Fusarium graminearum* isolates collected from wheat in eight Canadian provinces over a three year period between 2005 and 2007. The results revealed substantial regional differences in Fusarium head blight pathogen composition and temporal population dynamics. The 3ADON trichothecene type consistently predominated in Maritime provinces (91%) over the sampled years, and increased significantly ($P < 0.05$) between 2005 and 2007 in western Canada, accounting for 66% of the isolates in Manitoba by the end of the sampling period. In contrast, 3ADON frequency was lower (22%, $P < 0.001$) in the eastern Canadian provinces of Ontario and Québec and did not change significantly between 2005 and 2007, resulting in two distinct longitudinal clines in 3ADON frequency across Canada. Overall, genetic structure was correlated with toxin type, as the endemic population (NA1) was dominated by 15ADON isolates (86%), whereas a second population (NA2) consisted largely of 3ADON isolates (88%). However, the percentage of isolates with trichothecene genotypes that were not predictive of their genetic population assignment (recombinant genotypes) increased from 10% in 2005 to 17% in 2007, indicating that trichothecene type became an increasingly unreliable marker of population identity over time. In addition, there were substantial regional differences in the composition of recombinant genotypes. In western and maritime provinces, NA2 isolates with 15ADON genotypes were significantly more common than NA1 isolates with 3ADON genotypes ($P < 0.001$), and the reverse was true in the eastern provinces of Québec and Ontario. Temporal trends in recombinant genotype composition also varied regionally, as the percentage of 15ADON isolates with NA2 genetic backgrounds increased approximately three fold in western and Maritime provinces, while the opposite trends were observed in Québec and Ontario. The results indicate that *F. graminearum* population dynamics in Canada have been influenced by a complex adaptive landscape comprising different regional selective pressures, and do not reflect a simple model of dispersal and integration following the introduction of a novel pathogen population. In addition, we identified *F. graminearum* strains that produce the recently discovered A-trichothecene mycotoxin (NX-2) for the first time in Canada, representing a significant expansion of the known range of NX-2 producing strains in North America.

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1. Introduction

Fusarium head blight (FHB) is an important disease of wheat, barley and other cereal crops worldwide (Goswami and Kistler, 2004; McMullen et al., 2012), and is responsible for losses to the North American wheat industry exceeding $2 billion (Nganje et al., 2004). Head blight infections can significantly reduce yield and also diminish crop value by contaminating grain with trichothecene mycotoxins that pose a serious threat to food safety and animal health (Desjardins and Proctor, 2007; Goswami and Kistler, 2004). Trichothecenes inhibit eukaryotic protein synthesis

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and can induce anorexia, diarrhea, vomiting, and cell death in addition to altering immune function, phosphokinase-mediated stress pathways, proinflammatory gene expression, gastrointestinal function, and the action of growth hormones (Pestka, 2010; Wu et al., 2014). Trichothecene production is essential for disease spread in hosts such as wheat (Jansen et al., 2005; Proctor et al., 1995) and trichothecene sensitivity is positively correlated with wheat cultivar susceptibility and pathogenesis (Bai et al., 2002; Mesterhazy et al., 1999).

F. graminearum is the primary FHB pathogen in North America and many other parts of the world, and is one of at least 16 species within the *F. graminearum* species complex (FGSC) that are capable of inciting FHB of wheat (O'Donnell et al., 2000; O'Donnell et al., 2004; Sarver et al., 2011). Members of the FGSC typically produce one of three strain-specific profiles (chemotypes) of trichothecene metabolites that are characterized by the presence of a keto group at C-8, which is the defining feature of type B trichothecenes (Alexander et al., 2011; Lee et al., 2002; Yoshio et al., 1973). These B trichothecene chemotypes include nivalenol (NIV), 3-acetyl-deoxynivalenol (3ADON), and 15-acetyl-deoxynivalenol (15ADON) (Miller et al., 1991; Ward et al., 2002). Very recently, several *F. graminearum* strains from the Midwestern U.S. were shown to produce a novel type A trichothecene mycotoxin, 3α-acetoxy, 7α,15-dihydroxy-12,13-epoxytrichothec-9-ene (NX-2), which is structurally similar to 3ADON, but lacks the keto group at C-8 (Liang et al., 2014; Varga et al., 2015). Structural differences among the three B trichothecene chemotypes result from genetic variation in the core trichothecene metabolic gene cluster (Alexander et al., 2011; Kimura et al., 2003; Lee et al., 2002), whereas allelic variation in the unlinked *TRI1* gene is responsible for the NX-2 chemotype (Varga et al., 2015). It is therefore possible to accurately infer chemotype from trichothecene genotype, and the development of high-throughput genotyping methods has facilitated large-scale molecular surveys of trichothecene variation (Boutigny et al., 2014; Liang et al., 2014; Pasquali and Migheli, 2014; Starkey et al., 2007; Umpiérrez-Failache et al., 2013; Ward et al., 2002, 2008; Yli-Mattila et al., 2009; Zhang et al., 2012, 2010).

Analyses of FHB pathogen diversity have demonstrated that there are at least two major genetic populations of FHB pathogens in the Upper Midwest of the U.S. and in western and Maritime provinces of Canada (Gale et al., 2007; Ward et al., 2008; Puri and Zhong, 2010). The dominant genetic population, which we refer to here as NA1, consists largely of isolates with the 15ADON trichothecene type. In contrast, the 3ADON chemotype, which was rare prior to 2004 and has rapidly increased in frequency in the Upper Midwest and western Canada, predominates among isolates from a second genetic population (NA2) that may have been recently introduced into North America (Gale et al., 2007; Ward et al., 2008). Isolates from the emergent NA2 population, on average, produce significantly more trichothecene mycotoxins *in vitro* and *in planta* than isolates from the predominant NA1 population (Gilbert et al., 2010; Puri and Zhong, 2010; von der Ohe et al., 2010; Ward et al., 2008). These findings, and the recent discovery of *F. graminearum* isolates with the NX-2 chemotype, highlight the need to understand the spatial and temporal dynamics of FHB pathogen diversity in order to evaluate changes in pathogen composition and predict consequences for food safety and crop production (Foroud et al., 2012; Ward et al., 2008).

Our previous analyses of *F. graminearum* diversity in Canada were focused largely on western Canada due to small sample sizes in eastern Canada, where temporal analyses were not possible and population genetic analyses were limited to the Maritime province of Prince Edward Island. In the present study, we utilized a greatly expanded, multi-year, collection of FHB isolates from eastern and western Canada to: (1) determine the spatial and temporal dynamics of FHB population and trichothecene diversity across Canada; (2) examine the distribution and prevalence of FHB isolates with the novel NX-2 trichothecene type; (3) characterize the potential for exchange of adaptations between genetic populations; and (4) determine the extent to which trichothecene type can be used as a predictor of genetic population identity.

2. Materials and methods

2.1. Isolates

We conducted genetic analyses on 4117 isolates morphologically identified as members of the FGSC collected from wheat between 2005 and 2007 in eight Canadian provinces. Isolates were accessioned into the U.S. Department of Agriculture, Agricultural Research Service (ARS) Culture Collection (NRRL, http://nrrl.ncaur.usda.gov), Peoria, IL, where they were assigned NRRL numbers. Detailed information about sampled isolates can be found in Supplementary Table 1.

2.2. Species identification and trichothecene genotyping

Isolates were prepared for DNA extraction following methods described in O'Donnell et al. (1998). We used a 39 probe version of the multilocus genotyping (MLGT) assay developed by Ward et al. (2008) to identify each isolate by species and to determine the core trichothecene gene cluster type (NIV, 3ADON, or 15ADON) as a means of predicting trichothecene chemotype (Burlakoti et al., 2011; Gale et al., 2011; Jennings et al., 2004; Ji et al., 2007; Pasquali et al., 2010; Wang et al., 2012; Ward et al., 2002, 2008; Yli-Mattila et al., 2009). MLGT multiplex PCR reactions targeted genetic variation in six genes: four housekeeping genes with probes to differentiate species in the B trichothecene-producing clade, and two genes within the trichothecene biosynthetic cluster (*TRI3* and *TRI12*) with probes to differentiate 15ADON, 3ADON and NIV genotypes (referred to as *TRI*-cluster genotypes throughout).

We also performed *TRI1* PCR-RFLP genotyping to identify *F. graminearum* isolates with the NX-2 genotype following methods described in Liang et al. (2014). *TRI1* Apo I restriction sites that are unique to NX-2-producing *F. graminearum* strains were targeted in the PCR-RFLP assay to distinguish the novel NX-2 genotype from 15ADON, 3ADON and NIV genotypes (Liang et al., 2014). Because the NX-2 type was only recently discovered, it was not feasible to perform *TRI1* PCR-RFLP genotyping on all 4,117 isolates subjected to MLGT. We therefore analyzed a representative subset of 728 isolates that were also selected for population genetic analyses (detailed below in Section 2.4) to investigate the frequency, distribution and population affiliation of isolates with the NX-2 genotype. For all isolates identified as NX-2 by PCR-RFLP, we used gas chromatography-mass spectrometry (GC–MS) based methods described by Liang et al. (2014) to verify NX-2 toxin production *in vitro*.

2.3. Fine-scale spatial analyses of F. graminearum trichothecene types

Given evidence of on-going changes in trichothecene genotype frequencies in western Canada, we conducted a detailed spatial analysis of *F. graminearum* pathogen composition in this region. We analyzed 2,199 isolates collected between 2005 and 2007 from Manitoba, Saskatchewan and Alberta, and to maximize spatial and temporal sampling, we included 411 western Canadian isolates collected from 1998 to 2004, previously described in Ward et al. (2008). The absence of significant sampling prior to 2005 and less specific locality information prevented inclusion of strains from eastern and Maritime provinces. In addition, *TRI1* genotypes were

not available for all 2,610 isolates, thus spatial analyses were based on NIV, 15ADON and 3ADON *TRI*-cluster genotypes. Strain sampling locations corresponded to latitude and longitude coordinates of town or municipality centroids (obtained from ArcGIS, version 10.2, ESRI; Redlands, USA).

We used space–time scan statistics implemented in SaTScan version 9.3, (Jung et al., 2010; Kulldorff, 2009) to examine the time-frame for 3ADON expansion and pinpoint locations where 3ADON frequency was particularly high. For each geographical location and collection year, the number of 3ADON isolates observed was compared to the number expected using maximum likelihood ratios. SaTScan calculates likelihood ratios in scanning windows that are initially centered on each sampling location, and continuously increase in size from zero up to a user-defined maximum. We conducted a series of scans that specified maximum window radii of 50 km, 100 km, 150 km and 200 km to examine heterogeneity of 3ADON prevalence at different spatial scales (Kulldorff, 2014). Likelihood ratios were calculated using a Bernoulli model (Kulldorff, 1997; Kulldorff and Nagarwalla, 1995) that compared the proportion of 3ADON isolates in each window to the proportion in the total sampled population. For each scan, the window yielding the highest likelihood ratio indicated a cluster with unusually high spatio-temporal frequencies of 3ADON isolates. To assess cluster significance, we employed Monte Carlo methods that compared the likelihood ratio statistic to a distribution of ratios calculated from 999 random permutations of the data.

2.4. VNTR genotyping

We selected the following eight variable number tandem repeat (VNTR) loci developed by Suga et al. (2004) for use in population analyses: HK1043, HK913, HK957, HK967, HK1059, HK977, HK630, HK1073. VNTR loci were amplified with fluorescently labeled forward primers in three multiplex reactions using PCR conditions described in Ward et al. (2008). PCR products were visualized and sized relative to a GS500 ROX standard using an ABI3100 Genetic Analyzer and GeneMapper software (version 3.7, Life Technologies 2013). As mentioned previously, 728 isolates were subjected to VNTR genotyping. Isolates were selected based on geographic location and TRI-cluster genotype, with up to 20 3ADON and 20 15ADON isolates selected for VNTR analyses per year from each province where available.

2.5. Population genetic analyses

To characterize genetic structure and examine admixture among populations, we performed Bayesian clustering analyses of VNTR data using STRUCTURE version 2.3.4, (Pritchard et al., 2000). We implemented the admixture model and utilized independent allele frequency models as recommended by Pritchard et al. (2000). The number of simulated clusters (K) ranged from 1 to 5, and we performed 100,000 Monte Carlo Markov Chain (MCMC) iterations following a 25,000 iteration burn-in for each run. Five replicate runs were performed for each K value, and the Evanno et al. (2005) method was used to select the optimal model that maximized ΔK, the rate of change in log likelihood values. Modeling was performed using isolates from all collection years and isolates from each of the three collection years separately. For each isolate, the proportion of membership (q) in each of the K clusters was determined and isolates were assigned to the cluster that had the highest proportion of membership. Results from the most likely model were plotted with the software Distruct (Rosenberg, 2004).

We used GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012) to calculate unbiased gene diversity (H, measured within populations and standardized by sample size), measure genetic distances based on φ_{PT} (a standardized equivalent of F_{ST} for haploid data), and estimate the number of migrants (Nm) exchanged between populations. Significance of φ_{PT} values was assessed from 1000 random permutations of the data.

2.6. Spatial genetic structure of F. graminearum populations in Western Canada

In addition to evaluating the spatial distribution of trichothecene genotypes (described in Section 2.3), we also examined fine-scale spatial genetic structure of *F. graminearum* populations by performing autocorrelation analyses on VNTR genotypes in SPAGeDi version 1.4c (Hardy and Vekemans, 2002). We utilized western Canadian isolates with specific sampling locations and VNTR data, and grouped strains by genetic population (NA1 or NA2), omitting isolates that were potentially admixed (isolates that were weakly ($q < 0.9$) assigned) to a population or that had a *TRI*-cluster genotype that conflicted with the predominant chemotype of the assigned population to eliminate confounding effects of background gene flow. To assess the correlation of alleles between individuals according to distance, we calculated multilocus autocorrelation coefficients (Moran's I for genetic data, Hardy and Vekemans, 1999) at increasing distance classes spanning sampling intervals of nearest neighbors (100–1200 km) (Double et al., 2005; Peakall et al., 2003). Moran's I-values were averaged across loci, and standard errors were calculated using a jackknife procedure to evaluate reliability of multilocus estimates for each distance class. Statistical significance of multilocus I-values was assessed by 1000 random permutations of genotypes among sampling locations, using the 25th and 975th ranked I-values to define 95% confidence intervals around the null hypothesis of a random distribution of genotypes (Hardy et al., 2013; Hardy and Vekemans, 2002). Spatial genetic structure was inferred when I-values did not overlap 95% confidence intervals.

2.7. Statistical analyses

To examine temporal and geographic differences in trichothecene genotype frequency and population assignment rates we performed Fisher's exact tests and Chi-squared tests in R version 3.0.2 (R Core Team, 2013). Individual provinces were analyzed and regional comparisons were made among Maritime (Prince Edward Island, Nova Scotia and New Brunswick), eastern (Québec, Ontario) and western (Manitoba, Saskatchewan, Alberta) provinces. For all statistical analyses $P < 0.05$ was considered significant, and Bonferroni corrections were applied when multiple comparisons among provinces, regions or years were made.

3. Results

3.1. Frequency of TRI genotypes in Canada

Of the 4117 isolates analyzed by MLGT, 99% were identified as *F. graminearum* ($N = 4086$). The remaining 31 isolates were identified as *Fusarium cerealis* ($N = 1$), *Fusarium culmorum* ($N = 6$), *Fusarium pseudograminearum* ($N = 18$), or had MLGT genotypes that were inconclusive for species identity ($N = 6$). These 31 isolates were omitted from further analyses.

Among the 4086 Canadian *F. graminearum* isolates, the majority of isolates had 15ADON (62%) *TRI*-cluster genotypes. The 3ADON type was observed among 37% of the *F. graminearum* isolates, and NIV *TRI*-cluster genotypes were rare ($N = 3$). Analyses of MLGT results by province revealed two longitudinal clines in the

frequency of 3ADON *TRI*-cluster genotypes (Fig. 1). 3ADON frequency was significantly higher in Maritime provinces (91%) compared to eastern (22%) and western (32%) provinces ($P < 0.001$). We observed the highest 3ADON frequencies in Prince Edward Island and New Brunswick (98% and 88% respectively), and the majority of isolates in Nova Scotia (56%) had 3ADON *TRI*-cluster genotypes. 3ADON frequencies decreased significantly ($P < 0.001$) from the Maritime provinces to the eastern Canadian provinces of Québec (30%) and Ontario (11%). However, a second spike in 3ADON genotype frequencies was observed in the western Canadian province of Manitoba (55%), with the 3ADON genotype comprising a significantly smaller ($P < 0.001$) proportion of sampled populations in Saskatchewan (27%) and Alberta (7%).

Temporal trends in *TRI*-cluster genotype frequency varied by region. In Maritime provinces the frequency of 3ADON genotypes remained consistently high, whereas in eastern provinces, 3ADON frequency was relatively low and generally consistent across sampling years (Fig. 1). Although Ontario experienced a modest decrease in the proportion of 3ADON genotypes from 2005 to 2006, followed by a significant increase from 2006 to 2007 (10–21%, $P < 0.001$), the overall change in frequency between 2005 and 2007 was not significant. In contrast, the frequency of 3ADON genotypes increased significantly in western Canada between 2005 and 2007 ($P < 0.05$), specifically in Manitoba (46–66%, $P < 0.05$) and Saskatchewan (17–37%, $P < 0.001$), as compared to Alberta where frequencies of 3ADON remained low across years.

The recent discovery of the novel NX-2 trichothecene toxin type among *F. graminearum* strains from the U.S. prompted us to examine the NX-2 genotype distribution in Canada using a subset of isolates collected during 2005 to 2007. The frequency of NX-2 *TRI1* genotypes was consistently low across the sampled provinces, comprising less than 2% of the subset of isolates examined in any given year. Of the 728 strains tested, 12 NX-2 *TRI1* genotypes were detected, and *in vitro* mycotoxin analyses confirmed that they all produced NX-2 toxin. Four NX-2 isolates were found in Québec, five in Ontario, one in Prince Edward Island and two in Saskatchewan. The NX-2 *TRI1* genotype was only observed in strains with the 3ADON *TRI*-cluster genotype, which was consistent with previous findings (Liang et al., 2014; Varga et al., 2015). For the majority of provinces, isolates with NX-2 *TRI1* genotypes comprised less than 1% of 3ADON *TRI*-cluster genotypes, though in Québec they comprised 2% and in Ontario the proportion was much higher, totaling 11% of strains with 3ADON *TRI*-cluster genotypes.

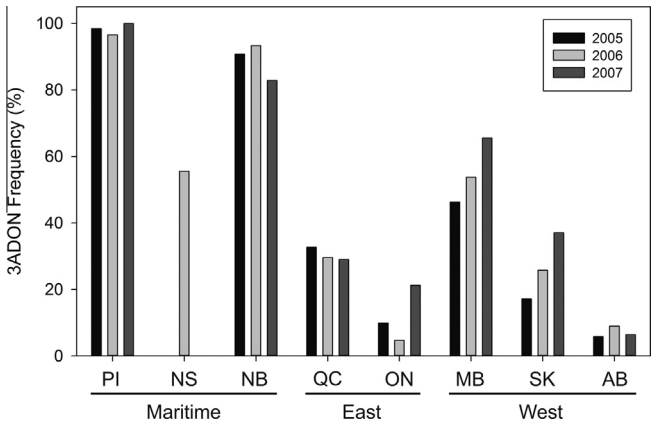

Fig. 1. 3ADON *TRI*-cluster genotype frequency (%) in Canadian provinces (PI = Prince Edward Island, NS = Nova Scotia, NB = New Brunswick, QC = Québec, ON = Ontario, MB = Manitoba, SK = Saskatchewan, AB = Alberta), based on MLGT analyses of 4086 *F. graminearum* isolates collected between 2005 and 2007. Regions are indicated (Maritime, East, West) below province labels.

3.2. Spatio-temporal dynamics of trichothecene variation in Western Canada

Given the evidence of on-going changes in *TRI*-cluster genotype composition in western Canada, we conducted a detailed spatial analysis of 3ADON expansion in this region to examine 3ADON localization at a finer scale (Fig. 2A and B). Pronounced clusters of 3ADON isolates were consistently detected between 2004 and 2007 within a 200-km radius outbreak region in southeastern-Manitoba, indicating that 3ADON isolates were highly concentrated in this area during the last four years of sampling. Within this region and time period, the risk of 3ADON infection was 3.15-fold higher than the population average. The primary focus of this outbreak was located near Winnipeg, Manitoba, wherein the number of 3ADON isolates observed was 2.6-fold higher than expected (Fig. 2B). Analyses conducted across a range of scanning windows (50–200 km radii) showed that the risk of 3ADON infection decreased slightly at broader spatial scales (Fig. 2B), consistent with a pattern of 3ADON diffusion from localized foci. Collectively our findings suggested that habitats near Winnipeg were a primary focal point for 3ADON colonization, and within a few years, the risk of 3ADON infection within the 200 km-radius outbreak region identified in southeast Manitoba increased by at least 3-fold due to localized establishment of 3ADON isolates.

3.3. Genetic population structure and diversity

Bayesian analyses of population structure were conducted on genetic variation at 8 VNTR loci. Analyses with $K = 2$ yielded the highest rate of change in likelihood values (ΔK), with modest increases in likelihood observed for larger K values. Therefore, models indicated at least two coexisting populations in Canada, which we refer to as NA1 and NA2 (Fig. 3), in accordance with Liang et al. (2014). Substantial genetic differentiation was observed between NA1 and NA2 in all regions (average interpopulation $\varphi_{PT} = 0.36$), although genetic divergence between NA1 and NA2 was lower in eastern Canada than in the Maritime or western provinces (Table 1). Within-population differentiation was very low for NA1 and NA2 populations across eastern, Maritime, and western provinces (average within population $\varphi_{PT} = 0.02$), demonstrating continuity of population genetic structure across Canada. Regional differences in gene diversity within a population were also minimal (Table 1). However, gene diversity values were approximately twice as high in NA1 as compared to NA2, which is consistent with the high number of identical genotypes found in NA2 relative to NA1 (80% were identical in NA2 versus 25% in NA1).

Population structure was associated with trichothecene genotype differences in that the NA1 population was dominated by isolates with 15ADON genotypes (86%), with 11% of isolates having 3ADON genotypes and 3% having NX-2 genotypes. In contrast, the vast majority of strains in NA2 had 3ADON genotypes (88%) and a small fraction had 15ADON genotypes (12%). Assignment of the 12 NX-2 isolates to the NA1 population was unexpected because all of these isolates had 3ADON *TRI*-cluster genotypes characteristic of NA2. Analyses specifying $K = 3$ allowed us to explore additional population substructure, and these models placed the NX-2 isolates in a third population, distinct from NA1 and NA2 (data not shown). However, we could not definitively conclude that NX-2 isolates belonged to a separate population because $K = 3$ did not provide a substantial improvement in likelihood scores, and was thus not selected as the optimal model based on the method of Evanno et al. (2005). Given the rarity of NX-2 genotypes, it is difficult to determine if Bayesian assignments of NX-2

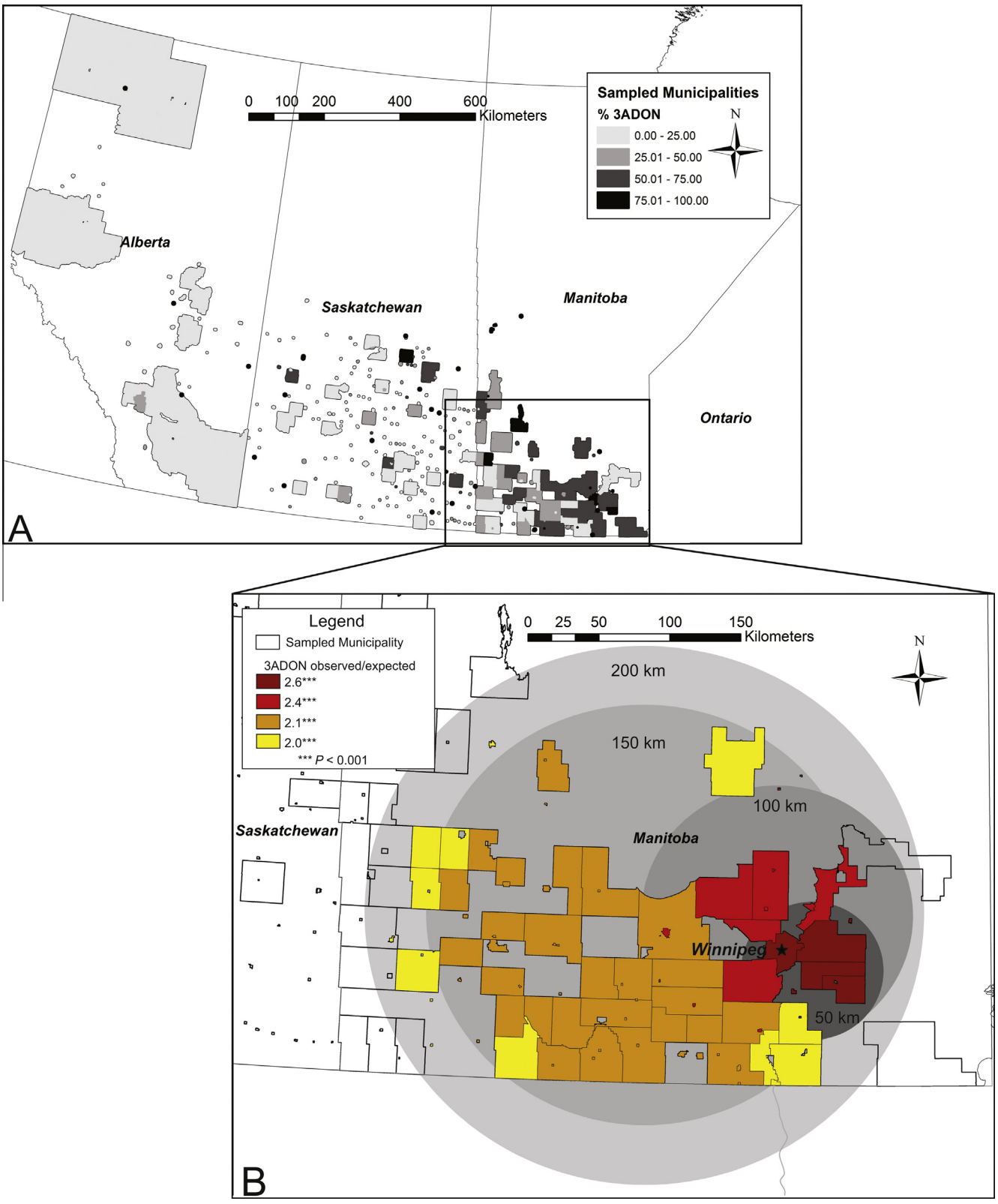

Fig. 2. Spatial dynamics of *F. graminearum* range expansions in western Canada. For spatial analyses, sampling locations corresponded to latitude and longitude coordinates of town/municipality centroids for 2199 *F. graminearum* isolates collected 2005 to 2007 and 411 *F. graminearum* isolates collected 1998 to 2004 (previously published by Ward et al. (2008)). (A) Frequencies (% of total MLGT sampled) of 3ADON observed in sampled municipalities in Alberta, Saskatchewan and Manitoba. (B) Space–time analyses indicated an outbreak of 3ADON *F. graminearum* isolates centered in southeastern Manitoba, 2004–2007. The radii of scanning windows were maximized at 50 km, 100 km, 150 km and 200 km to evaluate cluster intensity at multiple spatial scales. Locations of the highest likelihood clusters are indicated for each window radius tested (grey circles), and observed to expected ratios are reported for municipalities within each cluster. *P*-values indicate cluster significance.

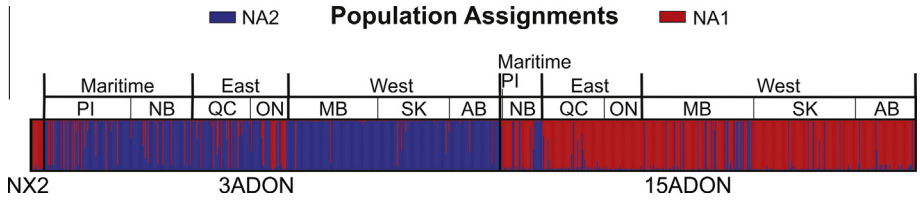

Fig. 3. Population structure analyses of 728 Canadian *F. graminearum* isolates. (A) Bayesian modeling was used to examine genetic clustering and admixture. The number of populations (K) was simulated with five replicate runs for $K = 1$ to 5. Likelihood values were averaged across replicates and the Evanno et al. (2005) method was used to select the K-value having the highest rate of change in likelihood, here $K = 2$. For each strain, the proportion of membership (q) in each of the two populations was determined and strains were assigned to the cluster that had the highest proportion of membership. Vertical bars represent NA2 (blue) and NA1 (red) q-values for each strain. 3ADON strains with majority q in NA1 (red bars) or 15ADON strains with majority q in NA2 (blue bars) indicated recombinant genotypes. Strains were grouped by trichothecene genotype, region (West, East, Maritime) and province (PI = Prince Edward Island, NB = New Brunswick, QC = Québec, ON = Ontario, MB = Manitoba, SK = Saskatchewan, AB = Alberta).

Table 1
Sample size (N), gene diversity (H), genetic differentiation (φ_{PT}), number of migrants (Nm) and the proportion of weakly assigned isolates in genetic populations of *F. graminearum*.

Region	N for Genetic Analysis (%NA1, %NA2)	H[a]		φ_{PT} NA1 vs. NA2[b]	Nm NA1 vs. NA2	% Weak assignments ($q < 0.9$)[c]
		NA1	NA2			
Maritime	**161 (26, 74)**	**0.66**	**0.32**	**0.38**	**0.83**	**6.2**
Prince Edward Island	63 (16, 84)	0.52	0.32	0.46	0.58	9.5
New Brunswick	98 (33, 67)	0.67	0.31	0.37	0.87	4.1
East	**221 (65, 35)**	**0.66**	**0.35**	**0.32**	**1.08**	**8.1**
Québec	119 (55, 45)	0.64	0.35	0.30	1.17	12.6
Ontario	102 (76, 24)	0.67	0.35	0.33	1.01	2.9
West	**346 (46, 54)**	**0.66**	**0.33**	**0.40**	**0.75**	**4.0**
Manitoba	120 (34, 65)	0.60	0.35	0.46	0.59	2.5
Saskatchewan	120 (50, 50)	0.65	0.31	0.41	0.71	5.0
Alberta	106 (56, 44)	0.67	0.31	0.37	0.85	4.7

[a] Gene diversity, standardized by the number of isolates within each group.
[b] Pairwise φ_{PT} values between NA1 and NA2 populations were significant across all provinces and regions.
[c] Bayesian analyses were run for each collection year separately and for all isolates combined. % weak assignments = (Number of weakly assigned isolates ($q < 0.9$) in NA1 and NA2/total number of isolates in each region) * 100.

isolates reflect true population identity or an artifact resulting from underrepresentation of the NX-2 gene pool in our samples.

3.4. Admixture and recombinant genotypes

Most isolates (94%) were assigned to their respective populations with high probability ($q \geqslant 0.9$). However, 42 isolates (24 from NA1 and 18 from NA2) were weakly assigned (q-value 0.5 to 0.9 for assigned population), and when modeling was performed separately for each collection year, the number of weakly assigned isolates increased steadily over time from 6.3% in 2005 to 8.0% in 2007. The number of migrants exchanged between NA1 and NA2 was fairly low (Nm range 0.59–1.17), but Nm was higher in Ontario and Québec than in other provinces (Table 1). Gene flow remained consistent over time in Maritime and eastern provinces. However, the number of migrants exchanged between genetic populations in western provinces steadily increased over time (Nm 2005: 0.69, Nm 2006: 0.74, Nm 2007: 0.83). Interestingly, Nm values were higher in Alberta and Saskatchewan than in Manitoba (Table 1).

To further evaluate the potential for exchange of traits between genetic populations and to assess regional and temporal differences in the utility of trichothecene genotype as a predictor of genetic population identity, we determined the proportion of 15ADON strains with NA2 genetic backgrounds and the proportion of 3ADON strains with NA1 genetic backgrounds by year and by region. Due to the uncertain placement of the NX-2 isolates in relation to population structure, the 12 isolates with NX-2 genotypes were excluded from these analyses. Among the remaining isolates, 13% had trichothecene genotypes that were not predictive of their population assignment, and were defined as having recombinant genotypes. The majority of these recombinant isolates were assigned to one of the two genetic populations with high probability based on the VNTR data ($q > 0.9$ in assigned population).

The frequency of recombinant genotypes increased from 10% in 2005 to 17% in 2007, demonstrating that trichothecene genotype became less predictive of genetic population identity over the course of this study. This finding is consistent with integration of the two genetic populations over time, and indicates that trichothecene type is an increasingly unreliable marker of population identity. However, dramatic regional differences in the composition of recombinant isolates were apparent (Fig. 3). In western provinces, NA2 isolates with 15ADON genotypes were significantly more common than NA1 isolates with 3ADON genotypes (16% versus 4%, $P < 0.001$). This bias in the composition of recombinant isolates was most pronounced in Manitoba where 33% of 15ADON isolates were assigned to NA2 versus 1% of 3ADON isolates assigned to NA1 ($P < 0.001$). We observed a similar pattern in Maritime provinces, where the percentage of 15ADON isolates assigned to NA2 (37%) was nearly 3-fold higher than the percentage of 3ADON isolates assigned to NA1 (13%) ($P < 0.001$). A completely different pattern was observed in the eastern provinces of Québec and Ontario, where 24% of 3ADON isolates were assigned to NA1, only 5% of 15ADON isolates were assigned to NA2, and all of the recombinant isolates in Ontario were NA1 isolates with 3ADON genotypes.

Temporal trends also varied regionally, as the percentage of 15ADON isolates with NA2 genetic backgrounds increased substantially over time in western and Maritime provinces (Fig. 4). By 2007, 50% of the isolates with 15ADON genotypes in Maritime provinces and 30% of the isolates with 15ADON genotypes in western provinces were assigned to the NA2 population. Over this same period of time, the percentage of 3ADON isolates assigned to the NA1 population decreased to less than 5% in western and Maritime provinces. The opposite trends were observed in the eastern Canadian provinces of Québec and Ontario (Fig. 4).

3.5. Spatial scale of genetic structure in Western Canadian F. graminearum populations

To compare the spatial distributions of native and emergent populations we examined fine-scale patterns of spatial genetic structure for NA1 and NA2 isolates in western Canada ($N = 298$, weakly assigned and recombinant isolates were omitted). As expected for established populations with limited dispersal, the

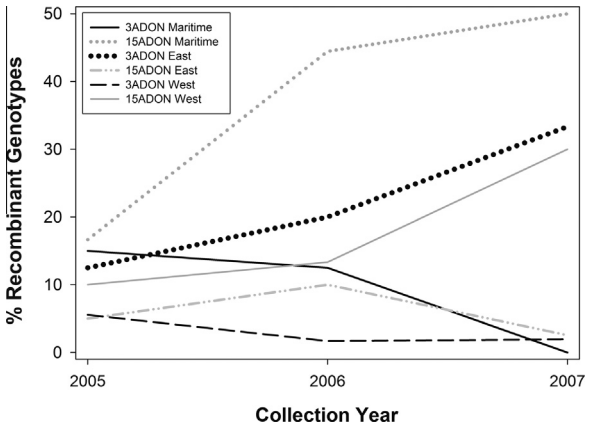

Fig. 4. Regional frequency of recombinant genotypes in 15ADON and 3ADON strains, based on Bayesian assignment tests run separately for each collection year.

NA1 population exhibited significant positive autocorrelation of VNTR genotypes, and the detection threshold for genetic structure was 800 km (Fig. 5). In contrast, VNTR genotypes from NA2 isolates were not autocorrelated, as *I*-values overlapped 95% confidence intervals at all distances analyzed. The lack of genetic structure across vast distances is consistent with geographic expansion and rapid increases in NA2 frequency following a genetic bottleneck.

4. Discussion

We previously identified a rapid adaptive shift in FHB pathogen composition by demonstrating that a novel population of *F. graminearum* characterized by the 3ADON trichothecene type was displacing an endemic population with the 15ADON type in western Canada between 1998 and 2004 (Ward et al., 2008). The same genetic populations (Gale et al., 2007; Ward et al., 2008) and similar temporal trends have been reported in the Upper Midwest of the U.S. (Liang et al., 2014; Puri and Zhong, 2010), and several studies have examined the potential implications of this previously unexpected pathogen diversity for agricultural production and food safety (Foroud et al., 2012; Gilbert et al., 2014, 2010; Puri and Zhong, 2010; Spolti et al., 2014; von der Ohe et al., 2010; Vujanovic et al., 2012; Ward et al., 2008). In the present study, we greatly expanded analyses of FHB pathogen diversity across Canada and demonstrated significant regional differences in pathogen population dynamics. These differences were highlighted by the dual longitudinal clines and distinct temporal trends in trichothecene genotype frequencies (Fig. 1). In the current study, rapid expansion of the 3ADON genotype continued in the western provinces of Manitoba and Saskatchewan, consistent with the previously recognized shift in pathogen populations. The 3ADON type was consistently found at high frequencies in Maritime provinces, suggesting that the shift in pathogen populations that was ongoing in western Canada and the Midwestern U.S. had largely occurred prior to our initial sampling along the Atlantic coast of Canada. In contrast, 3ADON frequencies in the eastern Canadian provinces of Québec and Ontario were significantly lower than the other regions sampled, with no consistent trend in trichothecene genotype frequencies observed between 2005 and 2007. This result suggested that FHB pathogen populations are following a unique evolutionary trajectory in this region. Spolti et al. (2014) cited unpublished data (G.C. Bergstrom) indicating that the frequency of 3ADON genotypes in western New York remained the same in surveys from 2007 and 2011 (15%), which may indicate that the unique FHB population dynamics we observed in Ontario and Québec extend into parts of the northeastern U.S.

The significant differences in trichothecene genotype frequencies and temporal trends observed between regions, led us to determine if 3ADON strains in eastern Canada belonged to the same genetic population that was prevalent in Maritime provinces and was rapidly displacing the endemic population in parts of western Canada and the Midwestern U.S. Indeed, the same two genetically distinct populations co-occurred in all provinces (Fig. 3), and these were equivalent to the endemic NA1 and novel NA2 populations identified in the Midwestern U.S. (data not shown) (Gale et al., 2007; Liang et al., 2014; Ward et al., 2008). We detected minimal substructure within the two populations across the sampled provinces, indicating that regional differences in population dynamics are not due to the presence of cryptic genetic populations that are specific to individual regions. While the genetic populations remain strongly differentiated in all provinces, NA1 and NA2 are integrated to a greater extent in eastern Canada than in Maritime or western provinces (Table 1). Although, this result implies longer periods of sympatry and greater opportunity for the exchange of adaptations between NA1 and NA2 in Ontario and Québec as compared to the other provinces sampled, the regional differences in φ_{PT} are modest.

The linkage between genetic population identity and trichothecene chemotype, reported previously for populations in western Canada, Prince Edward Island, and the Midwestern U.S. (Gale et al., 2007; Liang et al., 2014; Puri and Zhong, 2010; Ward et al., 2008), has resulted in the use of trichothecene type as a proxy for population identity. Although we observed a strong connection between trichothecene type and genetic population overall, our results indicated that trichothecene type is an increasingly poor predictor of population identity and genetic background (Fig. 4), particularly in Manitoba and Ontario. In addition, the extent and nature of the association between trichothecene type and genetic background differs regionally. By 2007, 61% of 3ADON isolates in Ontario and 55% of 15ADON isolates in Manitoba had trichothecene genotypes that conflicted with population assignment. As a result, the NA2 genetic background in western Canada is more prevalent than suggested by 3ADON frequencies and the opposite is true in Ontario and Québec, where a substantial proportion of 3ADON isolates have NA1 genetic backgrounds. Based on our results, we conclude that although the same two genetic populations are present across Canada, the relationship between trichothecene genotype and genetic background or population identity is very different in Ontario and Québec as compared to western Canada or the Maritime provinces. Evidence of biased gene flow favoring the NA2 genetic background was previously reported by Ward et al. (2008), and on that basis, we expected a similar bias among isolates from eastern Canada. However, we observed unique evolutionary dynamics in eastern Canada, as the composition of recombinant genotypes was strongly biased in favor of the NA1 genetic background (Fig. 4). Similar biases in gene flow have been documented in *Fusarium asiaticum* isolates from China following a selective sweep of invasive 3ADON isolates (Zhang et al., 2012, 2010). However, the regional differences in the direction of gene flow bias observed in this study have not previously been reported among FHB pathogen populations.

F. graminearum population dynamics in Canada appear to have been influenced by a complex ecological and adaptive landscape, and do not reflect a simple model of dispersal and integration following the introduction of a novel pathogen population. This is evidenced by the dual longitudinal clines in trichothecene genotype frequencies, the significant regional differences in temporal trends, and region-specific biases in the composition of recombinant genotypes. In comparison to NA1 isolates, NA2 isolates from western Canada, Prince Edward Island, and North Dakota, produced significantly more trichothecene toxin (Foroud et al., 2012; Gilbert et al.,

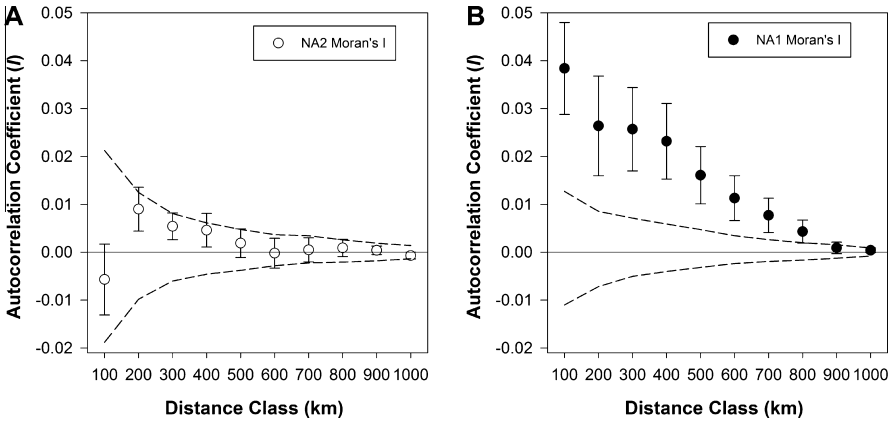

Fig. 5. Genetic spatial autocorrelation of NA2 (A) and NA1 (B) strains in western Canada. Autocorrelation coefficients (Moran's *I*) were calculated within incremental distance classes spanning 100–1000 km to assess the correlation of alleles between individuals according to distance. For each distance class, standard error bars for multilocus *I*-values and 95% confidence intervals (dashed lines) for the null hypothesis (randomly distributed genotypes) are shown. Significant spatial autocorrelation was observed for NA1 at distances ⩽800 km, wherein *I*-values fell outside 95% confidence intervals. In contrast, NA2 isolates did not exhibit genetic spatial autocorrelation, as *I*-values overlapped 95% confidence intervals at all distances analyzed. The lack of genetic structure across vast distances is consistent with geographic expansion and rapid increases in NA2 frequency following a genetic bottleneck.

2010; Puri and Zhong, 2010; von der Ohe et al., 2010; Ward et al., 2008), were more aggressive on some wheat lines (Puri and Zhong, 2010; Foroud et al., 2012), and displayed significantly greater spore production and higher growth rates (Ward et al., 2008), which could impart a direct fitness advantage to NA2 strains. Comparisons of these phenotypes for NA1 and NA2 isolates from eastern Canada have not been reported, but Spolti et al. (2014) assessed 14 different attributes of saprophytic and pathogenic fitness, and found no differences between 15ADON and 3ADON *F. graminearum* from New York. These authors speculated that adaptations introduced with the emergent population may now exist among isolates of both trichothecene types as a result of recombination. Our results suggesting a longer period of sympatry in eastern Canada are consistent with this hypothesis. However, diffusion of adaptive traits from the NA2 population into the NA1 population would not be expected to result in the compositional biases that we observed among recombinant isolates in Ontario and Québec, which strongly favored the NA1 genetic background. In addition, Spolti et al. (2014) did not assess the genetic background or population identity of the New York isolates used in their analyses. Given the percentage of 3ADON isolates in neighboring Ontario and Québec with the NX-2 *TRI1* allele or the NA1 genetic background, direct analyses of phenotype and population identity will be required to determine if the phenotypic differences observed between NA1 and NA2 in western Canada and the Upper Midwest also distinguish the NA1 and NA2 genetic populations in eastern Canada and the northeastern United States.

It is also possible that differences in population dynamics and the composition of recombinant genotypes reflect regional differences in selection pressure favoring NA2 in western and Maritime provinces of Canada as well as the Midwestern U.S., but not in the eastern Canadian provinces of Québec and Ontario. Regional differences in the adaptive landscape could be expected given the marked diversity of agroecosystems that are represented across Canada. Climatic variables, particularly temperature, have been shown to differentially influence the distributions of species within the FGSC (Backhouse, 2014). However, data regarding temperature sensitivity or the influence of temperature on competitive interactions between *F. graminearum* populations are limited and somewhat contradictory (Clear et al., 2013; Gilbert et al., 2014; Spolti et al., 2014; Vujanovic et al., 2012). While climatic factors differ across the regions examined in the current study, western and Maritime provinces have substantially different climates, but similar population dynamics.

Host prevalence could also shape regional pathogen population dynamics. The vast majority (96%) of Canadian wheat was grown in Saskatchewan, Manitoba and Alberta during the course of this study, and corn accounted for less than 3% of the harvested area in western Canada and Prince Edward Island, although it is more common in New Brunswick and Nova Scotia. In contrast, 36% and 50% of the harvested area in Ontario and Québec was devoted to corn production, and wheat accounted for only 15% and 5% of the harvested area in Ontario and Québec, respectively. In addition, spring wheat predominates in western Canada and much of the Maritime provinces, whereas winter wheat is grown primarily in Ontario. Although wheat in Québec is primarily spring wheat, a combined 76% of the harvested wheat acreage in Ontario and Québec is winter wheat due to the small amount of wheat grown in Québec (Government of Canada, 2014). A number of recent studies have suggested that the relative prevalence of different hosts in a region could have a major impact on FHB pathogen species and trichothecene chemotype composition (Boutigny et al., 2011; Gale et al., 2011; Lee et al., 2009; Sampietro et al., 2011; Zhang et al., 2012). Kuhnem et al. (2015) reported no significant structuring of 3ADON and 15ADON types in relation to corn ears, corn stubble, wheat spikes, or the atmosphere in New York. However, the influence of host prevalence on the dynamics of genetic populations within *F. graminearum* has not been characterized, and direct tests of fitness and aggressiveness of different genetic populations on different hosts will be required.

In addition to characterizing regional differences in population dynamics across Canada, we identified *F. graminearum* strains that produce the recently discovered A-trichothecene (NX-2) for the first time in Canada. This represents a significant expansion of the known range of NX-2 producing strains, which were previously found in the Upper Midwest of the U.S. (Liang et al., 2014). Varga et al. (2015) demonstrated that *in planta,* NX-2 was converted to a deacetylated derivative, NX-3, which is equivalent to DON without the keto group at C-8 (Varga et al., 2015). NX-3 and DON have similar abilities to inhibit protein synthesis and additional monitoring will be required to determine the food-safety significance of NX-2 producing isolates of *F. graminearum* across North America (Varga et al., 2015).

Interestingly, NX-2 production and the NX-2 *TRI1* genotype have only been found in strains with the 3ADON *TRI*-cluster genotype (current study; Liang et al., 2014; Varga et al., 2015). This was unexpected given that our VNTR based analyses placed NX-2 isolates with the NA1 genetic population, which is primarily

composed of 15ADON strains. A previous RFLP-based analysis (Liang et al., 2014) grouped NX-2 isolates with the NA2 population, which is consistent with the 3ADON *TRI*-cluster type observed among NX-2 isolates. However, the results of both studies indicated that NX-2 strains may represent a third genetic population, distinct from NA1 and NA2. Additional monitoring and analyses including comparative genomics and assessment of more NX-2 isolates will be required to resolve this issue and to elucidate NX-2 origins and prevalence outside of the regions sampled to date. Regardless of population identity, the complete association of NX-2 *TRI1* genotypes with 3ADON *TRI*-cluster genotypes is somewhat surprising given the percentage of recombinant genotypes observed in this study and the fact that *TRI*-cluster genes and *TRI1* are on different chromosomes (Cuomo et al., 2007). Ward et al. (2002) previously demonstrated that selection has maintained sets of interacting balanced polymorphisms within chemotype determining genes at either end of the *TRI*-cluster. In that context, the association of unlinked and chemotype-determining allelic variation in *TRI1* and the *TRI*-cluster is suggestive of selective pressures similar to those previously described for genes at either end of the *TRI*-cluster.

By characterizing the spatio-temporal dynamics of *F. graminearum* populations across Canada we were able to demonstrate significant regional differences in FHB pathogen composition and temporal trends in trichothecene type. The results indicate that FHB pathogen diversity may be shaped by differences in local selective pressures across Canada, although the ecological factors driving pathogen population dynamics remain unclear. These findings highlight the need to consider population-level variation in disease management and toxin control programs, and also emphasize the need for a regional approach to FHB management. In that context, further studies aimed at elucidating the basis for regional differences in the adaptive landscape could contribute to the development of novel control strategies. The data also indicate that trichothecene type is an increasingly unreliable marker of population identity and genetic background. As such, comparative analyses of pathogen phenotypes need to include a direct analysis of genetic population identity and admixture. Comparative genomic analyses of pathogen populations in relation to agriculturally-relevant phenotypic variation will be of particular utility in identifying specific fitness attributes that could be targeted to reduce disease and toxin contamination of grain. Finally, the detection of strains producing the novel NX-2 trichothecene mycotoxin in Canada reinforces the need for continued monitoring of FHB pathogen communities in North America to further characterize trends in pathogen diversity and promote early detection of invasive populations or novel threats to food safety.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.05.016.

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