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Disease Control and Integrated Management

e-Xtra*

Diversity of *Fusarium* spp. Associated with Wheat Node and Grain in Representative Sites Across the Western Canadian Prairies

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ABSTRACT

Fusarium head blight (FHB) and Fusarium crown and root rot (FCRR) are major wheat diseases. Populations of FHB and FCRR pathogens are highly dynamic, and shifts in these populations in different regions is reported. Analyzing fungal populations associated with wheat node and grain tissues collected from different regions can provide useful information and predict diseases that might affect subsequent crops and effective disease management practices. In this study, wheat node and grain samples were collected from four representative sites across the western Canadian prairies in the 2018 growing season to characterize the major *Fusarium* spp. and other mycobiota associated with wheat in these regions. In total, 994 fungal isolates were recovered, and based on culture and molecular diagnostic methods, three genera constituted over 90% of all fungal isolates, namely *Alternaria* (39.6%), *Fusarium* (27.8%), and *Parastagonospora* (23.9%). A quantitative PCR (qPCR) diagnostic toolkit was developed to quantify the most frequently isolated *Fusarium* spp. in infected wheat tissues: *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae*. This qPCR specificity was validated in silico, in vitro, and in planta and proved specific to the target species. The qPCR results showed that *F. graminearum* was not detected frequently from wheat node and grain samples collected from four locations in this study. *F. poae* was the most abundant *Fusarium* species in grain samples in all tested locations. However, in node samples, *F. culmorum* (Beaverlodge and Scott) and *F. avenaceum* (Lacombe and Lethbridge) were the most abundant species. Trichothecene genotyping showed that the 3ADON is the most dominant trichothecene genotype (68%), followed by type-A trichothecenes (29.5%), whereas the 15ADON trichothecene genotype was least dominant (2.5%) and the NIV genotype was not detected. Moreover, a total of 129 translation elongation factor 1-alpha (*TEF1α*) sequences from nine *Fusarium* spp. were compared at the haplotype level to evaluate genetic variability and distribution. *F. avenaceum* and *F. poae* exhibited higher diversity as reflected by higher number of haplotypes present in these two species compared with the rest.

Keywords: *Alternaria*, disease control and pest management, fungal pathogens, Fusarium head blight, *Parastagonospora*, root and crown rot, wheat

Wheat-associated mycobiomes consist of diverse fungal communities, among which *Alternaria* and *Fusarium* species are the most abundant (Fernandez and Jefferson 2004; Gilbert and Tekauz 2000; Orina et al. 2017; Tralamazza et al. 2016). A number of *Fusarium* spp. are pathogens that pose a threat to wheat production and cause diseases like Fusarium head blight (FHB) and Fusarium crown and root rot (FCRR). In Canada, FHB is one of the most damaging diseases of wheat and can also affect barley, oats, rice, corn, triticale, and rye (Aboukhaddour et al. 2020; Osborne and Stein 2007). FHB is a disease complex in which several species may or may not be involved. Most FHB pathogens are members of the *Fusarium graminearum* species complex (FGSC; also known as *F. graminearum* sensu lato), which includes *F. graminearum* sensu stricto and at least 15 other phylogenetically distinct *Fusarium* spp. (O'Donnell et al. 2008) that show different geographic distributions (O'Donnell et al. 2008) and some degree of host preferences (Sampietro et al. 2011). In addition to members of FGSC, other species complexes are also associated with FHB in Canada and elsewhere: *F. tricinctum* species complex (FTSC), *F. sambucinum* species complex (FSSC), and *F. incarnatum–equiseti* species complex (FIESC) (Aoki et al. 2012; van der Fels-Klerx et al. 2012). FCRR is another major disease of wheat worldwide, with *F. culmorum* and *F. pseudograminearum* as the most common causal agents (Knight and Sutherland 2017; Moya-Elizondo et al. 2011; Scherm et al. 2013). FCRR has been reported in all areas where wheat is grown, including western Canada (Fernandez and Conner 2011), where *F. avenaceum*, *F. culmorum,* and *F. pseudograminearum* were most commonly isolated (Fernandez and Jefferson 2004; Fernandez and Zentner 2005).

Molecular methods have previously been applied to detect and identify many important *Fusarium* spp. (reviewed in Chandra et al. 2011; Nicholson et al. 2003). Several genes/DNA regions have been used to define species boundaries within the genus *Fusarium*, with the *TEF1α* gene being the most frequently used sequence (Geiser et al. 2004; O'Donnell et al. 2008). Several molecular markers have been designed to detect and quantify FHB pathogens, but the specificity of these markers is questionable (Boutigny et al. 2019; Hafez et al. 2020a; Scherm et al. 2013).

Many *Fusarium* spp. are toxigenic and produce mycotoxins. Trichothecenes are a major family of chemically related mycotoxins produced by several fungal genera, including *Fusarium*, and cause severe toxicosis in humans and animals (Rocha et al. 2005).

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*The *e-Xtra* logo stands for "electronic extra" and indicates there are supplementary tables and supplementary figures published online.

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Fusarium trichothecenes are grouped into two classes: type-A (such as diacetoxyscirpenol [DAS], NX-2, T-2, and HT-2 toxins) and type-B (such as deoxynivalenol [DON], nivalenol [NIV], and fusarenon X [FUS-X]). The two types differ at the functional group attached to the carbon 8 position (McCormick et al. 2011). Among the type-B trichothecenes, the following are considered to have a significant impact on food and feed safety: DON, NIV, and their acetylated derivatives, 3ADON, 15ADON, and 4ANIV (Alexander et al. 2011). *Fusarium* mycotoxins were found to be frequently associated with wheat and maize (Ahmed et al. 2020; Luo et al. 1990; Tanaka et al. 1988). Taxonomy of the genus *Fusarium* has been revised numerous times since the genus was first described (Link 1809). Distinguishing *Fusarium* spp. was initially entirely based on morphological character, and, subsequently, biological and phylogenetic species concepts were introduced (Leslie and Summerell 2008; Summerell 2019). Following the phylogenetic species concept, many new *Fusarium* spp. were described based on DNA sequence analysis (Geiser et al. 2013). The number of toxigenic *Fusarium* spp. is still uncertain because of the aforementioned taxonomic revisions; as a result, the toxigenic capabilities of many *Fusarium* spp. are still unknown.

Populations of FHB pathogens are highly dynamic, and shifts in these populations in different countries is reported (Valverde-Bogantes et al. 2019). Some examples include the displacement of *F. graminearum* by *F. poae* in Italy (Shah et al. 2005), displacement of *F. culmorum* by *F. graminearum* in The Netherlands (Waalwijk et al. 2003), displacement of *F. asiaticum* NIV-producing population with a more aggressive 3ADON population in China (Yang et al. 2008), and a highly toxigenic 3ADON-producing *F. graminearum* population displacing the existing 15ADON population in North America (Kelly et al. 2015; Schmale et al. 2011; Ward et al. 2008). The 3ADON chemotype is rapidly invading and replacing the 15ADON populations in western Canadian provinces, especially Manitoba and Saskatchewan, and, in recent years, a novel NX-2 type-A trichothecene chemotype was also reported in *F. graminearum* populations recovered from southern Canada and the northern U.S.A. (Kelly et al. 2016). In addition to FHB population shifts, introduction of foreign FHB species or populations into new areas were also reported, such as the detection of *F. asiaticum* outside of Asia (Del Ponte et al. 2013; Umpiérrez-Failache et al. 2013). Population shifts are usually associated with higher yield losses, changes in the mycotoxin(s) accumulated in the grain, climate change, regional environmental conditions, and crop rotation regimes (Valverde-Bogantes et al. 2019). Due to the complexity (where several *Fusarium* spp. are involved) and the dynamic nature (shifts in population and mycotoxin genotypes) of FHB, accurate detection and quantification of FHB-associated pathogens in a given area is essential. For these reasons, the present work aims to (i) characterize the mycobiota, especially *Fusarium* spp., associated with wheat node and grain samples from distanced locations across the western Canadian Prairies; (ii) determine the trichothecene genotypes associated with *Fusarium* isolates recovered from node and grain samples; and (iii) design specific quantitative PCR (qPCR) assays for rapid detection and quantification of the most abundant FHB pathogens.

MATERIALS AND METHODS

Sampling, fungal isolations and statistical analysis. Wheat node and grain samples were collected during the 2018 growing season from four experimental sites: Scott (western Saskatchewan) and three locations representing northern, central, and southern Alberta, at Beaverlodge, Lacombe, and Lethbridge, respectively. Samples were collected randomly from each location at physiological maturity, with a total of 48 node and 48 grain sample sets from each of the four sites (with a grand total of 192 node samples and 192 grain samples from all four sites). Five lower stem pieces (node with ~0.5-cm segments on both sides) and five wheat grains were randomly selected from each sample set and surface-sterilized with 2% sodium hypochlorite for 3 min, then rinsed twice in sterile distilled water. Samples were plated on 9-cm-diameter plates with potato dextrose agar (PDA; Difco Laboratories, Franklin Lakes, NJ, U.S.A.) amended with neomycin sulfate (0.12 g/liter) and streptomycin sulfate (1 g/liter), and the plates were incubated at room temperature for 7 to 10 days. Growing cultures were exposed to fluorescent white light placed 25 cm above the plates. Suspected *Fusarium* isolates were transferred to Spezieller Nährstoffarmer agar (Nirenberg 1981) for 5 to 10 days to enhance sporulation. *Fusarium* isolates were initially identified using microscopy by the presence of macroconidia and identified to species level according to Leslie and Summerell (2008). *Parastagonospora nodorum* and *Parastagonospora avenaria tritici* 1 isolates were identified as described in Hafez et al. (2020b). All other fungal isolates (including leaf spot pathogens and saprophytes) were purified on PDA via subcultures and were initially identified using microscopy. Relative abundance (RA) and frequency of isolation (FI) for each species were calculated as follows:

$$\text{RA}\ (\%) = [\text{total number of isolates from particular species} / \text{total number of all isolates}] \times 100$$

$$\text{FI}\ (\%) = [\text{number of samples in which a species occurred} / \text{total number of all samples}] \times 100$$

χ^2 tests were conducted to determine if differences in the observed number of each fungal species were significantly different. The four sampling sites were compared in a pairwise fashion with regard to the number and kind of fungal species isolated from node and grain samples. The count data were first separated into grain and node groups, and the tests described below were run on these groups separately. Initial χ^2 tests were done using all locations (Beaverlodge, Lacombe, Lethbridge, and Scott) and genera (*Alternaria*, *Fusarium*, and *Parastagonospora*) to determine if there were significant differences between any of the locations. A series of pairwise χ^2 tests were then done with all genera (e.g., Beaverlodge versus Lacombe, Beaverlodge versus Lethbridge) to determine which specific locations exhibited significant differences in counted isolates. To account for the multiple comparisons, *P* values were adjusted using the Bonferroni method. The adjusted *P* values were then compared with bar charts to infer relationships.

DNA extraction, PCR, and phylogenetic analysis. To confirm species identities, fungal genomic DNA (gDNA) was extracted from single spore cultures using the DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) based on the manufacturer's recommendations. PCR reactions were performed in a final volume of 50 µl using the Taq PCR Core Kit (Qiagen) with the following reagent concentrations: CoralLoad PCR buffer (1×), dNTP mixture (200 µM each), forward and reverse primers (0.2 µM each), Taq DNA polymerase (1.25 U/50 µl), ~20 to 50 ng of gDNA template, and the total volume of the PCR reaction adjusted to 50 µl with nuclease-free H_2O. The primer pair ef1/ef2 (O'Donnell et al. 1998) was used to amplify the *TEF1α* gene from *Fusarium* isolates with an initial denaturation step at 94°C for 3 min, followed by 35 cycles: 94°C for 1 min, 50°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The internal transcribed spacer (ITS) region was amplified from other fungal isolates using the primers BMBC-R and ITS-4b (White et al. 1990). ITS PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. All PCR amplicons were analyzed by gel electrophoresis through 1 to 2% agarose gels in 1× TBE buffer (89 mM Tris-borate, 10 mM EDTA, pH 8.0). Sizes of the PCR amplicons were estimated against a 1-kb plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and visualized under UV light after staining with RedSafe

(iNtRON Biotechnology, Seongnam, South Korea). PCR amplicons were purified and sequenced in two directions by Psomagen (Rockville, MD, U.S.A.).

Online BLAST (Basic Local Alignment Search Tool; Altschul et al. 1990) was used to confirm species identity for fungal isolates. Initial nucleotide sequence alignments were done with Clustal-X v2.0.7 (Thompson et al. 1997) and then refined with GeneDoc v2.5.010 (Nicholas 1997). PHYLIP (v3.6) was used for phylogenetic analysis (Felsenstein 1993). DNADIST was used to generate distance matrices using *TEF1α* or *Tri5* alignments to create neighbor-joining trees. SEQBOOT was used to generate 1,000 bootstrap replicates, and a majority-rule consensus tree was constructed with the CONSENSE program and visualized using iTOL v3 (Letunic and Bork 2016). GenBank accession numbers for *TEF1α*, ITS, and *Tri5* sequences generated during this study and sequences used in phylogenetic analysis and haplotype network construction are listed in Supplementary Table S1. Reference *Fusarium* isolates were obtained from the culture collection of the Agricultural Research Service, U.S. Department of Agriculture (USDA-ARS, Peoria, IL, U.S.A.), and gDNA was extracted from *F. acuminatum* (NRRL13559), *F. avenaceum* (NRRL40579), *F. cerealis* (NRRL43802), *F. culmorum* (NRRL3288), *F. graminearum* (NRRL31729), *F. poae* (NRRL13714), *F. pseudograminearum* (NRRL40886), and *F. sporotrichioides* (NRRL13441). The *TEF1α* gene was amplified with ef1/ef2 as described previously, sequenced, and compared with the published sequence at GenBank for each species with 100% homology.

Trichothecene genotyping. The *Tri5* gene (trichodiene synthase) was amplified using the primer pair Tox5-1/Tox5-2 (Niessen and Vogel 1998). Thermal cycler conditions were: initial denaturation at 95°C for 4 min with five cycles of 60 s at 95°C, 2 min at 60°C, and 3 min at 72°C followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C, with a final extension for 5 min at 72°C. PCR amplicons were purified and sequenced in two directions by Psomagen (Supplementary Table S1 includes *Tri5* GenBank accession numbers). Trichothecene genotype determination was performed according to Ward et al. (2002) using two multiplex PCRs based on sequence polymorphisms in *Tri3* (trichothecene 15-O-acetyltransferase) and *Tri12* (trichothecene efflux pump) genes. A PCR/restriction fragment length polymorphism protocol based on restriction digestion of *Tri1* gene with *Apo*I enzyme was used to screen for the presence of NX-2 genotype in the *F. graminearum* isolates recovered during the present study from wheat according to Kelly et al. (2016). All PCR amplicons were analyzed by gel electrophoresis as described above.

Haplotype network construction. DNA polymorphisms among 129 *TEF1α* and 59 *Tri5* sequences from *Fusarium* isolates recovered during the present study were determined using DNA Sequence Polymorphism software (DnaSP v.5.10; Librado and Rozas 2009). Haplotype data files prepared by DnaSP were used in PopART v.1.7 (Leigh and Bryant 2015), generating haplotype networks to visualize the genetic differences among the recovered *Fusarium* spp. The method from Templeton et al. (1992) (TCS) within PopART was used to build the haplotype network. The haplotype networks were then edited with CorelDraw x4 Graphic to improve readability.

qPCR primer design and specificity. Specific qPCR assays for quantifying the most abundant *Fusarium* spp. in the tested sites—*F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae*—were developed. Several sets of candidate primers and probes were designed for the four targeted *Fusarium* spp. using different software: primer 3 (Untergasser et al. 2012) and PrimerQuest Tool (Owczarzy et al. 2008). Their specificity was first assessed in silico by BLAST with *Fusarium*-ID (Geiser et al. 2004), *Fusarium* MLST (O'Donnell et al. 2010), and NCBI databases (Altschul et al. 1990). A total of 105 *TEF1α* sequences from 25 *Fusarium* species (Supplementary Table S2) were retrieved from GenBank, aligned using Clustal X, and examined by GeneDoc to validate specificity of the designed primers and probes, and multiple primer pairs were chosen for each species when possible, targeting a 75- to 150-bp amplicon from different regions within the *TEF1α* gene to prevent cross-hybridization and nonspecific binding. The primer pair that showed 100% specificity to the targeted species in silico (and low homology to nontarget *Fusarium* spp.) was chosen and validated in conventional PCR with nine different *Fusarium* spp. A final set of four primer pairs was chosen (Table 1): qFavF/R (for *F. avenaceum*), qFcuF/R (for *F. culmorum*), qFgrF/R (for *F. graminearum* sensu stricto), and qFpoF/R (for *F. poae*). The qFgrF primer is modified from FgssF primer (Hafez et al. 2020a) but with a higher melting temperature for optimum pairing with the qFgrR primer. The PCR reaction mix was prepared as previously described for *TEF1α*. PCR amplification included an initial denaturation step at 94°C for 3 min, followed by 35 cycles: 94°C for 1 min, 60°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min.

Specific qPCR assays optimization and validation. For singleplex qPCR assays, each 20 µl reaction contained 10 µl of PrimeTime Gene Expression Master Mix (Integrated DNA Technologies [IDT], Coralville, Iowa, U.S.A.), 1 µl of PrimeTime std qPCR assay (IDT; 5 nmol each primer and 2.5 nmol probe), and 4 µl of template DNA, and the total reaction volume was adjusted to 20 µl with ultrapure nuclease-free H_2O. For multiplex qPCR assays, 1 µl of each PrimeTime std qPCR assay was used and the total reaction volume was adjusted to 20 µl with ultrapure nuclease-free H_2O.

TABLE 1. Primers and probes used to detect and quantify *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae*

Assay	Primer/ probe	Sequence (5′ to 3′)	Tm (°C)	Product size (bp)	Ct	LOD (pg)[a]	LOQ (pg)
qFav	qFavF	ACCACTGTAAGTACAACCATCAGCGAGTC	61.3	102	21	>0.4	4
	qFavR	CGGTCTGTCAAGAGTTAGCAAGATGTCG	60.5				
	qFavP	**FAM**/TCTGCACTC/**ZEN**/GGAACCCGCCAAACCTG/**3IABkFQ**	66.6				
qFcu	qFcuF	ATTTTGCGGCTTTGTCGTAATTTTTCTG	58.0	111	23	<4	4
	qFcuR	TGACACGTGATGGTGCGCGCCT	66.0				
	qFcuP	**HEX**/CAGGCGCTT/**ZEN**/GCCCTCTTCCCACAAACCA/**3IABkFQ**	67.4				
qFgr	qFgrF	TGCGGCTTTGTCGTAATTTTTTYCCC[b]	61.2	123	19	>0.4	4
	qFgrR	AGTGACTGGTTGACACGTGATGATGA	59.9				
	qFgrP	**FAM**/CAGGCGTCT/**ZEN**/GCCCTCTTCCCACAAACCA/**3IABkFQ**	67.4				
qFpo	qFpoF	GCGGGGTAGACTCAACATGCACT	61.3	159	22	<4	4
	qFpoR	ATTCGAGTGATGGATCGAGGGAAAGT	59.5				
	qFpoP	**HEX**/ATGCTTGAC/**ZEN**/AGACCGGTCACTTGTACATCCAGTG/**3IABkFQ**	65.1				

[a] Failure rates at 0.4 pg were 25, 55, 10, and 65%, respectively (descending), and no assays failed at 4 pg. Tm, melting temperature; Ct, quantification threshold cycle; LOD, limit of detection; LOQ, limit of quantification; qFav, primer/probe for *F. avenaceum*; qFcu, primer/probe for *F. culmorum*; qFgr, primer/probe for *F. graminearum*; qFpo, primer/probe for *F. poae*. The assays were designed to target species-specific regions within the *TEF1α* gene. TaqMan probes were labeled with the reporter dye FAM or HEX on the 5′ end. All probes contained 3IABkFQ (Iowa Black FQ) as a quencher at the 3′ end with additional internal quencher ZEN in the middle of the probe. Quenchers and reporter dyes are indicated by bold letters.

[b] Y = C or T.

Cycling conditions were set at 95°C for 3 min, 40 cycles of 95°C for 5 s, and annealing/extension at 60°C for 30 s. Reactions were run in MicroAmp Fast Optical 96-well plates (Applied Biosystems) using the QuantStudio 6 Flex real-time PCR system (Applied Biosystems). Standard curves were generated using 10-fold serial dilutions of DNA from each *Fusarium* spp. that ranged from 40 to 40^{-5} ng. All probes used were TaqMan-labeled (IDT) with the reporter dye FAM or HEX on the 5′ end and contained 3IABkFQ (Iowa Black FQ) as a quencher at the 3′ end with additional internal quencher ZEN in the middle of the probe. Limit of detection (LOD) and limit of quantification (LOQ) for each assay were determined by running seven-point standard curves (40 to 40^{-5} ng), with 20 "unknown" replicates for the lowest three concentrations. The concentration at which a single detection failure among 20 replicates was recorded (95% confidence) was deemed the LOD, and, when the standard deviation of the threshold cycle (Ct) was <0.5, it was deemed the LOQ.

To further validate the efficiency and accuracy of this test, an additional qPCR experiment was run in replicated duplex reactions with a mix of different ratios of known quantities of *F. avenaceum* and *F. culmorum* gDNA. Several DNA mixtures (mixtures 1 to 8) were prepared using different DNA concentrations from both *F. avenaceum* and *F. culmorum* ranging from 20 to 0.002 ng (Supplementary Table S3 provides detailed information).

Quantification of *Fusarium* spp. in field samples. Wheat node and grain samples were ground into powder with liquid nitrogen (LN_2) with a mortar and pestle. Node samples were very dry/fibrous and difficult to homogenize, so sterile fine sand 40/100 mesh (Acros Organics, Geel, Belgium) was added. A second round of homogenization was done using Precellys-24 bead beating homogenizer (Bertin Technologies, Rockville, MD, U.S.A.), and total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) based on the manufacturer's recommendations using 80 mg of node or grain LN_2 powder. DNA was also extracted from healthy leaf tissue of 'Glenlea' wheat genotype (used to generate the wheat DNA standard curve) using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA quantity and quality was determined by a Nanodrop 1000 spectrophotometer (Thermo Scientific). All node and grain samples were normalized to 2 ng/µl and 20 ng/µl, respectively, and 4 µl of normalized DNA was used from each sample as a template in the qPCR analysis to quantify *F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae* DNA in both node and grain samples. The *Waxy-D1* gene was used as the wheat internal reference gene (Yang et al. 2017). *Waxy-D1*, *F. graminearum*, and *F. poae* were quantified in singleplex qPCR assays, whereas *F. culmorum* and *F. avenaceum* were quantified together in a multiplex qPCR. The reporter dye FAM was used to label the probe of *Waxy-D1*, *F. graminearum*, and *F. avenaceum* assays, while HEX reporter dye was used to label the probes of *F. culmorum* and *F. poae*. Detailed information about primers and probes is listed in Table 1.

Ancestral state reconstruction for *Fusarium*-associated trichothecenes. To trace the evolutionary history of trichothecene mycotoxin production potentiality by *Fusarium* spp., the ancestral states of the internal nodes were reconstructed using MESQUITE v3.61 (Maddison 2008). Twenty-eight *TEF1α* sequences representing 27 *TEF1α* haplotypes (*F. graminearum TEF1α* haplotype number 26 was used twice to represent 3ADON and 15ADON genotypes) were aligned with MUSCLE (included in MESQUITE), and the alignment was used to reconstruct ancestral nucleotide states using a 50% majority rule consensus tree. The evolutionary history of type-A and type-B (3ADON and 15ADON) trichothecene genotypes was traced over the tree using the parsimony reconstruction method. Each taxon was scored for presence (score of 1) or absence (score of 0) of each trichothecene mycotoxin based on the *Tri5*, *Tri3*, and *Tri12* PCR results.

The genus *Fusarium* includes numerous toxigenic species that can produce a wide variety of mycotoxins. The literature was examined and information collected about trichothecene mycotoxins reported in species within the genus *Fusarium*. A total of six trichothecene mycotoxins were reported in 28 *Fusarium* species (Altomare et al. 1995; Desjardins 2006; Desjardins et al. 1987; Desjardins and Proctor 2007; Goswami and Kistler 2005; Jestoi 2008; Kelly et al. 2016; Leslie and Summerell 2008; Logrieco et al. 1990; Marasas et al. 1984; Marín et al. 2012; Munkvold 2003, 2017; Neish et al. 1982; Pettersson 1991; Sugiura et al. 1993; Tóth et al. 2008). A species was considered a producer for a particular mycotoxin when two or more isolates were reported to produce it in at least two publications. GenBank accession numbers for all *TEF1α* sequences from 28 *Fusarium* species and the associated mycotoxins used in this analysis, in addition to the matrix used to reconstruct the mycotoxins' ancestral state, are indicated in Supplementary Table S4. The absence and presence of particular mycotoxins was recorded as 0 and 1, respectively. Estimation of the ancestral character states for the presence and absence of trichothecene mycotoxins within the 28 *Fusarium* species was done with MESQUITE. Ancestral states were reconstructed for six *Fusarium*-associated trichothecene mycotoxins: DAS, HT-2, T-2, NX-2 (type-A), DON, and NIV (type-B). The *TEF1α* consensus tree created previously used as the base for the reconstruction of the ancestral states. The phylogenetic tree included 28 different *Fusarium* species and was rooted with *F. solani*. The evolutionary history of each character (i.e., mycotoxin) was traced over the tree using parsimony reconstruction.

RESULTS

Diversity of mycobiota associated with wheat node and grain. A total of 994 fungal isolates were recovered, with 480 isolates from nodes and 514 from grains. These samples were collected from four distant locations in Alberta (Beaverlodge, Lacombe, and Lethbridge) and Saskatchewan (Scott). Twenty-seven species belonging to 15 genera were identified based on colony characteristics, spore structure, and *TEF1α* and ITS sequences. *Alternaria*, *Fusarium*, and *Parastagonospora* represented the most prevalent genera at 91.3% of the collected isolates (Fig. 1 and Supplementary Table S5). *Alternaria* was the most abundant genus (RA, 39.6%). Three species were identified in the genus *Alternaria*: *Alternaria infectoria*, recovered from both node and grain samples (RA, 30%); *A. triticina*, recovered from only grain samples (RA, 8.5%); and *A. alternata*, recovered from only node samples (RA, 0.9%). Two species were identified within the genus *Parastagonospora*: *P. nodorum* and *P. avenaria tritici* 1, with the former mainly from node samples and the latter exclusively from grain. Certain species were isolated only from grain samples (e.g., *A. triticina*, *Epicoccum nigrum*, and *Bipolaris sorokiniana*), and some were isolated from only node samples (e.g., *F. culmorum*, *F. torulosum*, and *F. equiseti*). A third group was recovered from both node and grain samples (e.g., *A. infectoria*, *P. nodorum*, and *F. poae*). The diversity of fungal species was much higher in the node samples (22 species) than in the grain (14 species; Fig. 1 and Supplementary Table S5).

The results of the initial χ^2 tests with all locations showed that significant differences were present between locations for grain samples and node samples (Supplementary Table S6). Most of the pairwise tests showed significant differences ($P \leq 0.05$) between individual locations. The locations that showed no significant differences for grain samples were Lethbridge and Scott ($P = 0.68$); similarly, in the node samples, Lethbridge and Scott were not significantly different ($P = 1.21$). Based on the χ^2 tests and the bar chart (Fig. 2A), we can determine that a relationship exists between *Parastagonospora* and *Fusarium* species in node samples whereby, when one genus count is high, the other is correspondingly lowered. Similarly but perhaps less conclusive is the relationship between *Alternaria* spp. and all other genera in grain samples (Fig. 2B), whereby *Alternaria* spp. dominates and other

species counts are reduced, but the relationship with specific genus is less clear.

Nine *Fusarium* spp. were recovered from node samples and are listed here in order from the most to least abundant: *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. torulosum*, *F. pseudograminearum*, *F. sporotrichioides*, *F. acuminatum*, *F. graminearum*, and *F. poae* (Fig. 2C). Only three species were recovered from grain samples: *F. poae*, *F. avenaceum*, and *F. graminearum*, from most to least abundant (Fig. 2D). *F. avenaceum* was the most abundant species in node samples (RA, 9.8%; FI, 32.8%), whereas *F. poae* was the most abundant in grain samples (RA, 4.5%; FI, 12%; Figs. 1 and 2C and D; Supplementary Table S5). *F. avenaceum* was the only *Fusarium* species recovered from node and grain samples in all sampled locations (Beaverlodge, Lacombe, Lethbridge, and Scott). *F. poae* was recovered from grain samples (collected from Beaverlodge, Lacombe, and Lethbridge) and node samples (collected from Lethbridge), but was not recovered from Scott. *F. graminearum* was recovered from node and grain samples collected from Lethbridge and Scott and was not detected in Beaverlodge or Lacombe. *F. culmorum* was exclusively recovered from node samples in all four locations and was the most abundant species in Beaverlodge (Fig. 2C and Supplementary Table S5).

Genetic variability and haplotype distribution. Sequence analysis of *TEF1α* gene from 129 *Fusarium* isolates indicate that this gene consists of four exons and three introns, with no insertions or deletions (indels) observed in the exons. However, 141 indel sites were observed in the introns, which contributed to length variability between different species. Intron 2 showed the highest length variability (from 223 bp in *F. poae* to 251 bp in *F. graminearum*). Several nucleotide polymorphisms were observed in intron 2, which were used to design species-specific primers in the qPCR assays. The numbers of polymorphic (segregating) sites were found to be 14 and 113 in exons and introns, respectively. Among the 14 polymorphic sites within the coding region (exon sequences), 13 sites were parsimonious informative sites (sites that have a minimum of two nucleotides that are present at least twice) and one site was singleton (mutations appearing only once among the sequences). Interestingly, all these 14 polymorphic sites were third base position substitutions that do not change the amino acid sequence (silent/synonymous mutation; Fig. 3A).

Three *Fusarium* species complexes were identified from wheat node and grain samples: (i) FTSC, including *F. acuminatum*, *F. avenaceum*, and *F. torulosum*; (ii) FIESC, including *F. equiseti*; and (iii) FSSC, including *F. culmorum*, *F. graminearum*, *F. poae*, and *F. sporotrichioides* (Supplementary Table S1). A total of 129 *TEF1α* sequences were analyzed, and there were 27 haplotypes reported: 15 haplotypes in FTSC (Fig. 3B), 9 haplotypes in FSSC (Fig. 3C), and 3 haplotypes in FIESC (Supplementary Fig. S1). The number of haplotypes also varied among *Fusarium* species, in which *F. avenaceum* showed the highest number of haplotypes (n = 10), followed by *F. torulosum* (n = 4), *F. poae* (n = 4), *F. equiseti* (n = 3), and *F. sporotrichioides* (n = 2), and only one haplotype was observed in each of the remaining *Fusarium* spp. Haplotype H10 (*F. avenaceum*) and haplotype H27 (*F. culmorum*) occurred at the highest frequencies (35 and 33 sequences, respectively), whereas haplotypes H2, H3, H7, H12, H23, and H24 showed the lowest frequencies and were each present once (Fig. 3B and C). Several *TEF1α* haplotypes were found to be restricted to certain locations like H22 (Lacombe) and H4 (Beaverlodge). The two most abundant haplotypes, H10 (*F. avenaceum*) and H27 (*F. culmorum*), were observed in all investigated locations (Fig. 3B and C, respectively). Among the 27 *TEF1α* haplotypes, only 6 haplotypes from three *Fusarium* spp. were associated with wheat grains: H10 (*F. avenaceum*), H19-H22 (*F. poae*), and H26 (*F. graminearum*).

Species-specific *TEF1α*-based TaqMan qPCR assays. Species-specific primers and probes were designed based on *TEF1α* sequence polymorphism to detect four *Fusarium* species: *F. avenaceum* (qFav), *F. culmorum* (qFcu), *F. graminearum* (qFgr), and *F. poae* (qFpo; Table 1). Primer specificity was tested initially using conventional PCR with nine *Fusarium* spp., and results showed that the four primer pairs were specific and produce a single band with expected lengths for the targeted species; however,

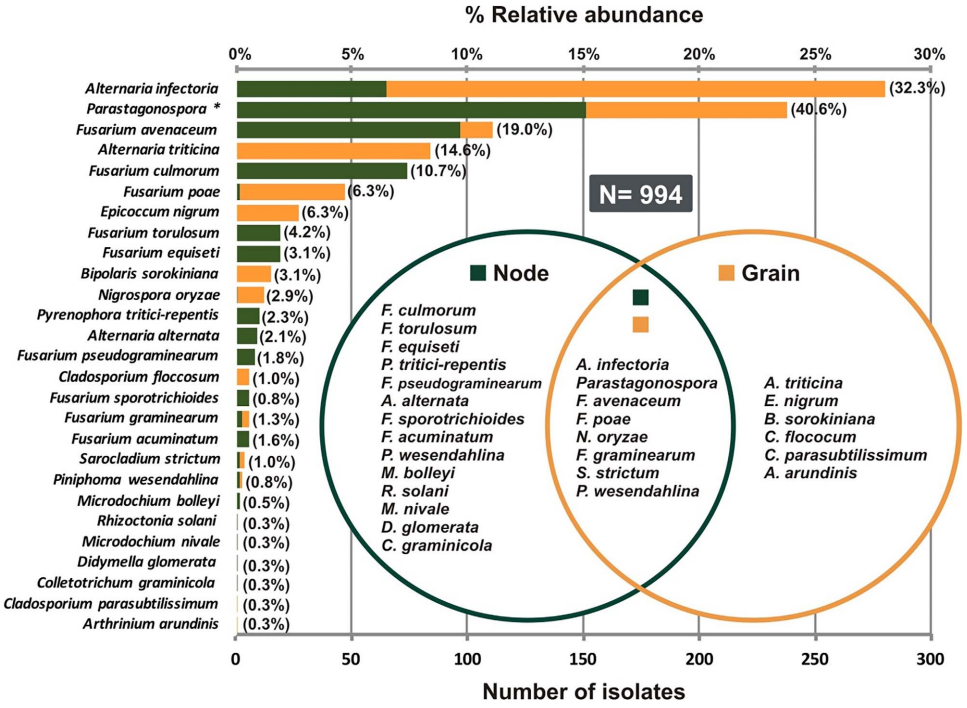

Fig. 1. The relative abundance of fungal species associated with wheat node and grain samples collected from four sites in Alberta (Beaverlodge, Lacombe, and Lethbridge) and Saskatchewan (Scott) during the 2018 growing season. Numbers in parentheses next to each bar represent frequency of isolation for each species from all node and grain samples in the four locations. The total number of fungal isolates (N) recovered from both node and grain samples in all sites is indicated. Asterisk: the genus *Parastagonospora* includes two species: *P. nodorum* and *P. avenaria tritici*.

no bands were obtained with nontarget species (Supplementary Fig. S2A). The qPCR specificity testing results showed high specificity to the corresponding targeted species; fluorescent signals were detected with the targeted species only when the corresponding qPCR assay was used, and no signals were detected with nontarget species (Supplementary Fig. S2B). Assay efficiencies ranged from 102 to 107, and quantification threshold cycle (Ct) values ranged from 19 to 23 (Table 1 includes detailed information). The LOD for qFgr is slightly >0.4 pg, with that concentration having 10% detection failure (2 of 20 replicates) and no failures at 4 pg. The LOQ for qFgr is 4 pg with a standard deviation <0.5. The LOD for qFav is >0.4 pg (25% failure; 5 of 20 replicates), with no failures at 4 pg. The LOD for qFcu and qFpo are closer to, but still less than, 4 pg, with failure rates of 55 and 65%, respectively (11 and 13 of 20 replicates, respectively). The LOQ of all four assays is 4 pg; at that concentration, the standard deviation of the Ct was <0.5 for each. The qWaxy assay, used to quantify plant DNA, had an LOD between 0.5 (no failures) and 0.05 pg (20% failure rate; 4 of 20 replicates) and an LOQ of 0.5 pg. In multiplex qPCR, we tested how well this assay would function when different ratios of known quantities of *F. avenaceum* and *F. culmorum* were mixed in the same sample. The results showed that there was no competition for the PCR reagents to favor the amplification of the more abundant DNA template in the qPCR mixture, and the measured DNA quantities for higher and lower DNA concentrations in the mixture were tightly close to the known DNA concentrations (Supplementary Table S3).

***Fusarium* quantification in wheat field samples.** The developed qPCR assays were used to quantify four *Fusarium* spp. in wheat field samples collected from distant sites in western Canadian prairies. *F. graminearum* and *F. poae* DNA concentration in wheat node (n = 192) and grain (n = 192) samples were assessed using qFgr or qFpo single-plex qPCR assays, respectively. *F. avenaceum* and *F. culmorum* DNA concentration in node and grain samples were quantified together using a qFav/qFcu multiplex qPCR assay. Total DNA concentration in node samples ranged from 2 to 29 ng/µl, and, in grain samples, it ranged from 35 to 209 ng/µl (Supplementary Table S7 includes detailed information on DNA and qPCR results for each sample). *Fusarium* DNA was normalized to wheat DNA using the *Waxy-D1* qPCR assay and expressed as picograms of fungal DNA per microgram of wheat DNA (pg/µg). Standard curves for the four assays were produced by plotting the DNA concentration (log [fg DNA]) and Ct values using pure fungal 10-fold DNA dilutions (Supplementary Fig. S3). A standard curve of *Waxy-D1* assay using pure wheat DNA was generated to quantify plant DNA. *F. poae* showed very low RA (0.2%) and FI

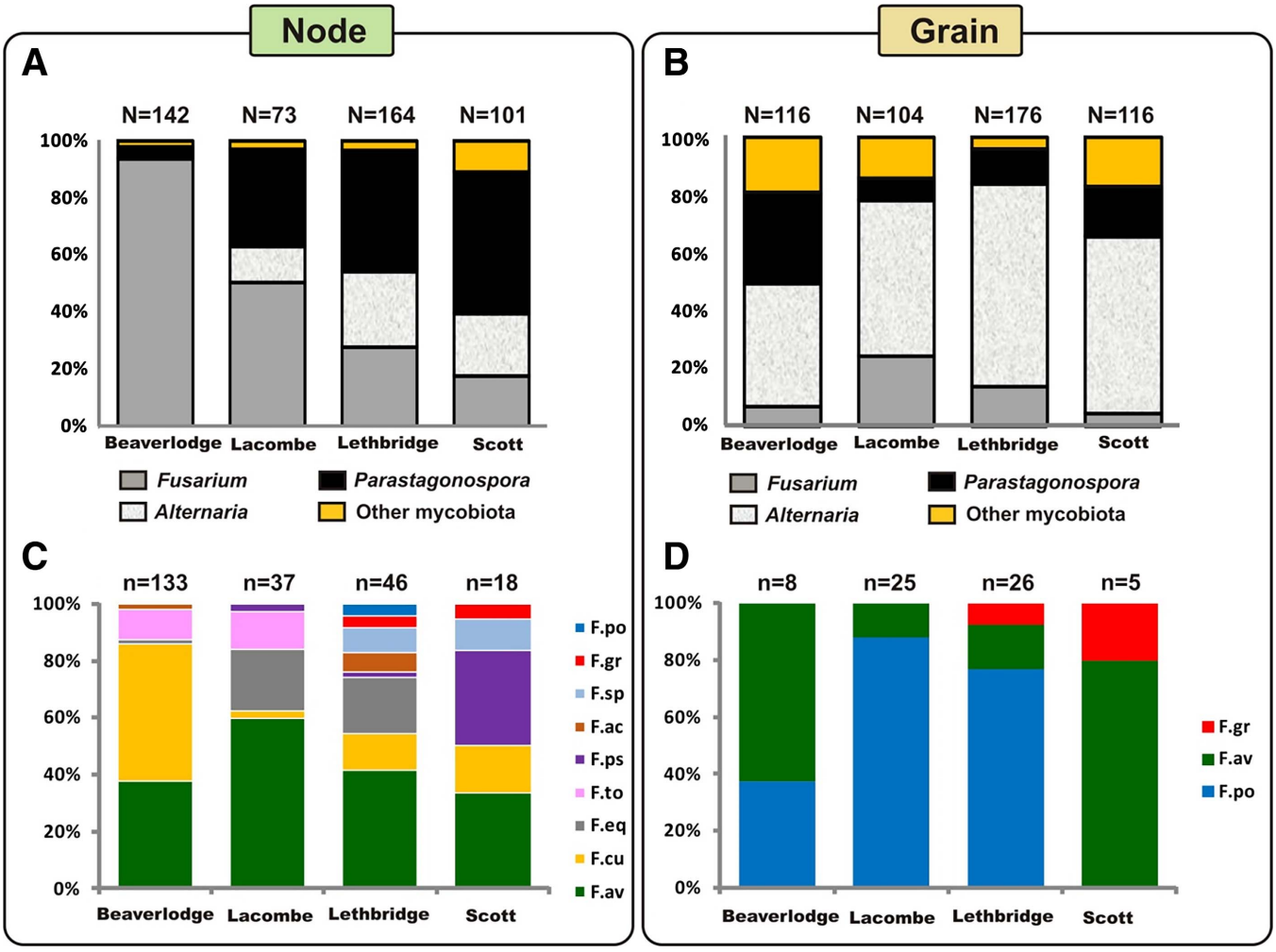

Fig. 2. A, Diversity of the most abundant fungal genera (*Alternaria*, *Fusarium*, and *Parastagonospora*) recovered from node samples in four locations: Beaverlodge, Lacombe, Lethbridge, and Scott. **B,** Diversity of the most abundant fungal genera (*Alternaria*, *Fusarium*, and *Parastagonospora*) recovered from grain samples in four locations: Beaverlodge, Lacombe, Lethbridge, and Scott. *Fusarium* was the most abundant genus in node samples (relative abundance, 48.75%), and *Alternaria* was the most abundant genus in grain samples (relative abundance, 62.06%). The total number of all isolates (N) recovered from each location is indicated. **C,** *Fusarium* species diversity in wheat node samples. **D,** *Fusarium* species diversity in wheat grain samples. *Fusarium* diversity in node samples (nine species) is much higher than in grain samples (three species). *Fusarium avenaceum* was the only *Fusarium* species recovered from both node and grain samples in all locations. Total number of *Fusarium* isolates (n) recovered from each location is indicated.

(0.5%) values in node samples, but relatively higher RA (2.5%) and FI (6.8%) values in grain samples based on the culture-based experiments. For this reason, *F. poae* was quantified only in grain samples.

In node samples, *F. culmorum* showed the highest biomass in Beaverlodge (212,300 pg/µg) and Scott (31,100 pg/µg), whereas *F. avenaceum* was found to be the most abundant *Fusarium* species in Lacombe (15,200 pg/µg) and Lethbridge (3,900 pg/µg). Interestingly, *F. graminearum* was not detected in Beaverlodge and Lacombe and was rarely detected in Scott (2,620 pg/µg) and Lethbridge (<100 pg/µg; Fig. 4A). In grain samples, *F. poae* gave the highest biomass in all locations, with the largest in Lethbridge (216.3 pg/µg), followed by Lacombe (146.8 pg/µg) and Beaverlodge (27.7 pg/µg). *F. avenaceum* was the second most abundant species in grain samples from Lacombe (49.1 pg/µg) and Beaverlodge (17.8 pg/µg), with very low DNA amounts in grain samples from Lethbridge and Scott. *F. graminearum* was not detected in grain samples from Beaverlodge or Lacombe but was detected in very small amounts in grain samples from Scott (2.4 pg/µg) and Lethbridge (1.2 pg/µg). *F. culmorum* was not detected in grain samples from any location (Fig. 4B).

***Fusarium*-associated trichothecene genotypes.** A total of 276 *Fusarium* isolates belonging to 9 *Fusarium* spp. were tested for trichothecene genotypes (Supplementary Table S8). Results from the *Tri5* PCR assay showed that 149 isolates (53.9%) belonging to the three species—*F. avenaceum*, *F. equiseti*, and *F. torulosum*—did not amplify *Tri5* (*Tri*$^{-}$), whereas 127 isolates (46.1%) amplified the *Tri5* gene (*Tri*$^{+}$). Type-B trichothecene genotyping showed that three different genotypes can be recognized based on the *Tri3* and *Tri12* multiplex PCR, respectively: NIV (840/840 bp), 15ADON (610/670 bp), and 3ADON (243/410 bp). *Fusarium* isolates that amplified *Tri5* and gave no bands for *Tri3* and *Tri12* were considered type-A trichothecene producers. The most dominant genotype was 3ADON (68%; *F. culmorum*, *F. pseudograminearum*, and *F. graminearum*), followed

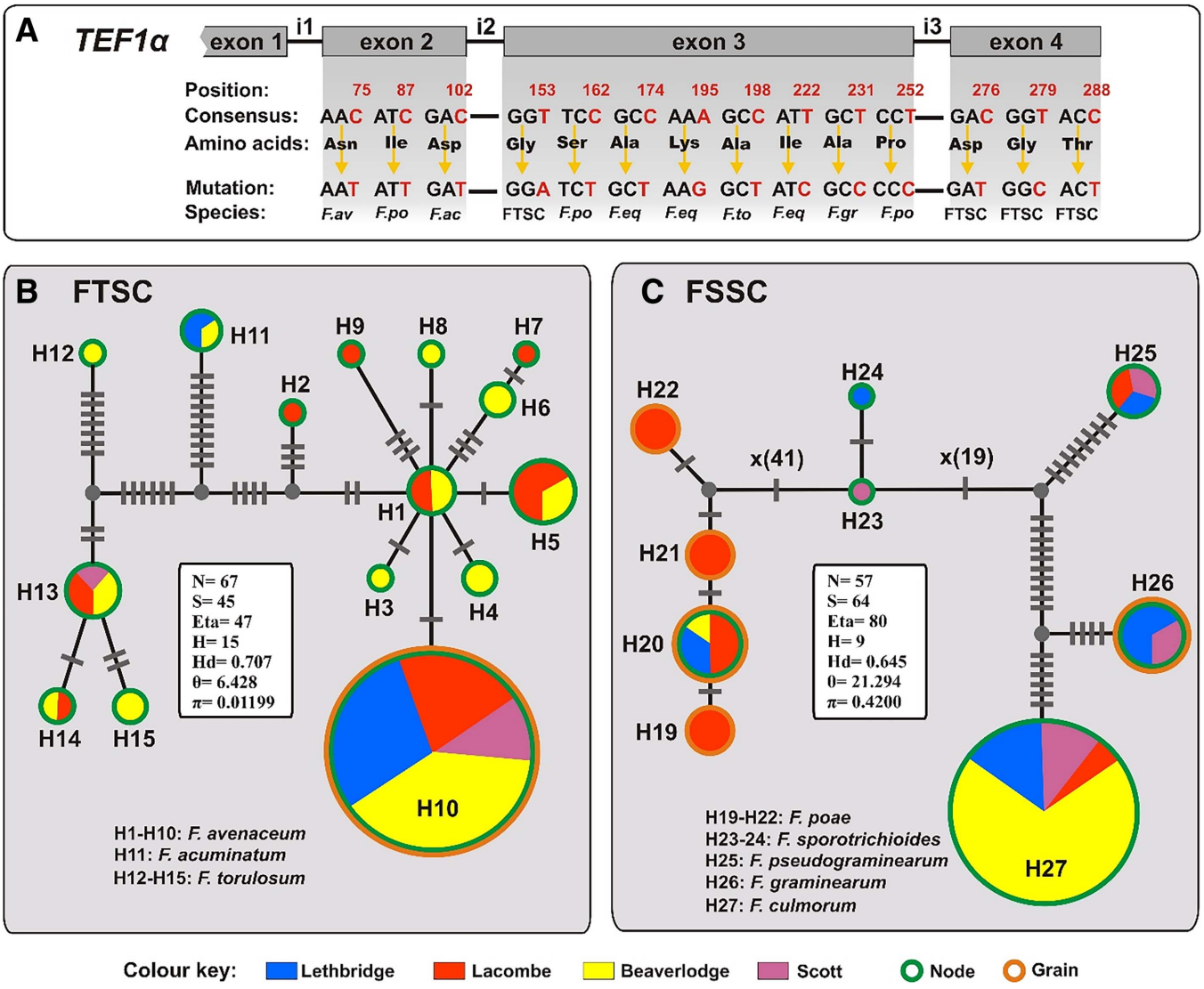

Fig. 3. *TEF1α* sequence polymorphism. **A,** Among 127 polymorphic sites in the *TEF1α* gene, only 14 sites were found in coding region (exons 2 to 4). All polymorphic sites in the coding region were third-position synonymous mutations resulting in no amino acid change. Polymorphic sites were numbered in relation to the start codon in *Fusarium pseudograminearum TEF1a* mRNA (XM_009265097). **B,** *TEF1α*-based Templeton et al. (1992) (TCS) haplotype networks for *F. tricinctum* species complex (FTSC). **C,** *TEF1α*-based TCS haplotype networks for *F. sambucinum* species complex (FSSC). The size of the circle indicates the relative frequency belonging to a particular haplotype (smallest circle, 1 sequence; largest circle, 35 sequences). Hatch marks along the network branches indicates the number of mutations. Each color represents a different location, and haplotype(s) within each species is indicated. Haplotypes found in species recovered from nodes and grains were marked with green and orange circle rims, respectively. The haplotype networks were generated by PopART v. 1.7 (Leigh and Bryant 2015), and statistics for each network are indicated: number of polymorphic/segregating sites (S), total number of mutations (Eta), number of haplotypes (h), haplotype diversity (Hd), nucleotide polymorphisms (θ), and nucleotide diversity (π), and N represents the number of sequences analyzed within each network. Networks were edited by CorelDRAW X4 Graphic.

by type-A (29.5%; *F. poae*, *F. acuminatum*, and *F. sporotrichioides*), and 15ADON was found to be the least observed trichothecene genotype (2.5%; *F. graminearum*) in all tested trichothecene-producing *Fusarium* spp. Notably, none of the *Fusarium* isolates were of the NIV genotype (Supplementary Fig. S4). PCR amplification of *Tri1* (1,700 bp) from six *F. graminearum* isolates followed by restriction digestion with *Apo*I produced a pattern with two bands of equal size, 850 bp each. This pattern indicates that NX-2 genotype is absent in the screened *F. graminearum* isolates, as the pattern for NX-2 consists of three bands (851, 482, and 407 bp). A phylogenetic analysis of select isolates based on the *TEF1α* and *Tri5* sequences showed that trichothecene genotype differences are not well correlated with the species' evolutionary relationships (Supplementary Fig. S5). The results presented here might indicate an adaptive evolution within trichothecene genes.

Trichothecene production profiling and ancestral state reconstruction. Results of mycotoxin ancestral state reconstruction showed that all trichothecene-producing species are located in a monophyletic group (FSSC) except for *F. acuminatum*, which was the only species in the FTSC to amplify the *Tri5* gene (Fig. 5A). The trichothecene producers were further divided into two clades: strict type-A producers (*F. poae* and *F. sporotrichioides*) and strict type-B producers (*F. pseudograminearum*, *F. culmorum*, and *F. graminearum*). Trichothecene mycotoxins were absent in all species within the FIESC and FTSC except for *F. acuminatum*. Three *F. acuminatum* isolates recovered from Beaverlodge (B203-1) and Lethbridge (G108-1 and G311-1) were found to amplify *Tri5* with 100% similarity to the *F. poae Tri5* (Supplementary Table S1).

Ancestral state reconstruction was done to understand the evolutionary history of trichothecenes produced by different species within the genus *Fusarium*. We have used the results of trichothecene genotyping generated during the present study (Supplementary Fig. S4) and the previously published trichothecene genotyping/chemotyping reports (Supplementary Table S4) to predict the evolutionary history of trichothecene biosynthetic genes and to reconstruct the ancestral state of each gene. Information from our results and previous reports on the mycotoxin production potentiality of 27 *Fusarium* spp. within three species complexes (FTSC, FIESC, and FSSC) were used to reconstruct the ancestral state of mycotoxin production potentiality and to predict a "model" for potential gain and loss of trichothecene biosynthetic genes (Fig. 5B). The results indicate that the ability to produce trichothecenes (+A and +B) was found in a common ancestor to FIESC and FSSC. The trichothecene-producing species were further divided into three clades: a clade with both type-A and type-B producers (Fig. 5B, clade 1), a clade of strict type-A trichothecene producers that lost the ability to produce type-B trichothecenes (Fig. 5B, clade 2), and a clade with strict type-B trichothecene producers that lost the ability to produce type-A trichothecenes (Fig. 5B, clade 3). *F. graminearum* was the only species in clade 3 with the capability to produce type-A trichothecenes (NX-2). *F. acuminatum* was the only species in the FTSC with the capability of producing trichothecenes (type-A: DAS, HT-2, and T-2).

DISCUSSION

The development of specific primers and probes to quantify four FHB species. In this study, we developed specific qPCR assays to identify/quantify the four species *F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae* based on the *TEF1a* gene. The qPCR developed was validated in silico, in vitro, and in planta and showed unique specificity to the target species and no detection of other *Fusarium* spp. The newly developed qPCR assays we presented also showed very high sensitivity, which allows for detection and quantification of the pathogen of interest even if found in very low biomass (for example, during the early stages of infection). *TEF1α* is the most frequently used sequence to detect and identify *Fusarium* spp., and the gene is highly conserved, highly informative, and is present as a single copy in *Fusarium*, and, for these reasons, it was selected as the barcode of choice to identify *Fusarium* spp. in the two currently available *Fusarium* databases: *Fusarium*-ID (Geiser et al. 2004) and *Fusarium* MLST (O'Donnell et al. 2010). Previously, several *TEF1α*-based primers have been developed to differentiate between different *Fusarium* spp., but the specificity of these primers is questionable. For example, two primer pairs were often used to detect *F. graminearum*: UBC85F/UBC85R (Schilling et al. 1996) and Fg16F/Fg16R (Nicholson et al. 1998). However, the division of *F. graminearum* sensu lato (i.e., FGSC) into different species (O'Donnell et al. 2004) renders these primers invalid because they are no longer specific for *F. graminearum* sensu stricto. These primers are unable to distinguish between members of the FGSC as they were designed before *F. graminearum* was split into multiple species. In addition, false negatives have been reported for these two primer pairs when tested

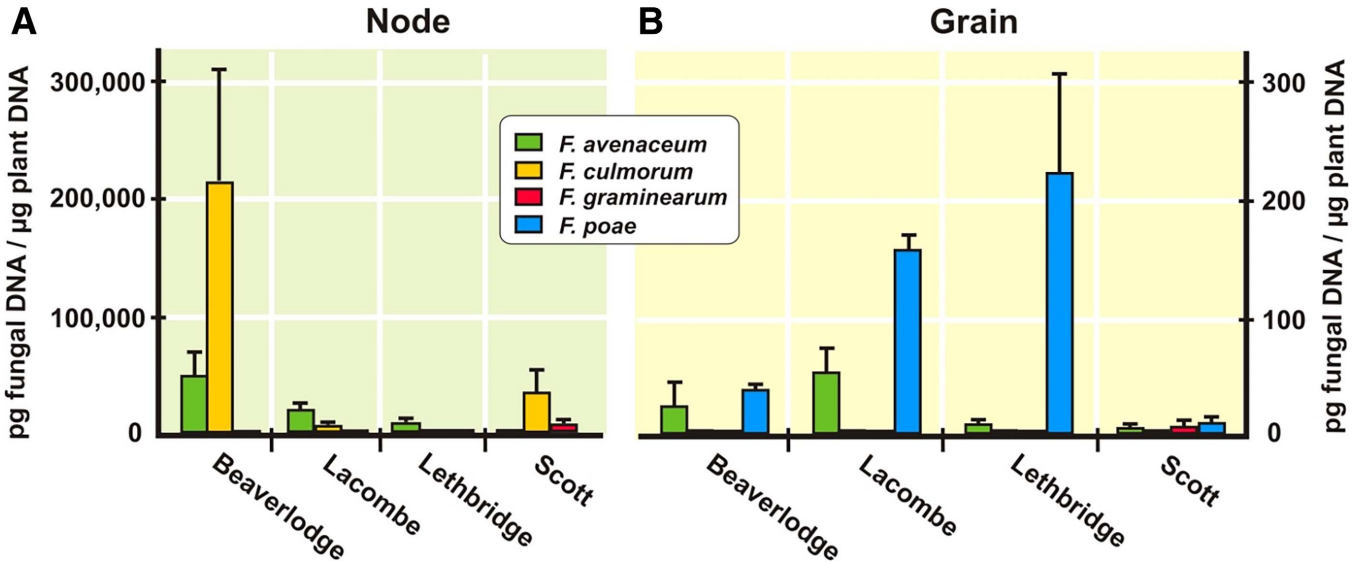

Fig. 4. A, *Fusarium* DNA amount in wheat node samples as measured by singleplex quantitative PCR (qPCR; *Fusarium graminearum* and *F. poae*) and multiplex qPCR (*F. avenaceum* and *F. culmorum*) assays. **B,** *Fusarium* DNA amount in grain samples as measured by singleplex qPCR (*F. graminearum* and *F. poae*) and multiplex qPCR (*F. avenaceum* and *F. culmorum*) assays. *Fusarium* DNA was normalized using wheat *Waxy-D1* gene and expressed as picograms of fungal DNA per microgram of wheat DNA.

against different species within the FGSC (Hafez et al. 2020a). In the present study, we checked the specificity of many published PCR primers in silico, and the vast majority did not distinguish *F. graminearum* sensu stricto from other species in the FGSC (FgramB379fwd and FgramB411rev primers [Nicolaisen et al. 2009]; EF1-FCFG-F/EF1-FG-R primers [Boutigny et al. 2019]). For these reasons, the specific qPCR assay for *F. graminearum* sensu stricto developed here will add significant value for the detection and quantification of this important plant pathogen.

Previously released primers that were considered specific to *F. culmorum* based on several genes/DNA regions were found nonspecific and could not distinguish *F. culmorum* from *F. cerealis* or *F. graminearum* (reviewed in Scherm et al. 2013). There are also many *TEF1α*-based real-time qPCR assays for quantification of different FHB pathogens in infected host tissues, but their specificity appears weak and they produce nonspecific results, especially for the more closely related species like members of the FSSC (Nicolaisen et al. 2009; Sonia et al. 2018; Zitnick-Anderson et al. 2018). Recently, a primer set based on sequence polymorphism in the *TEF1α* gene was released (Boutigny et al. 2019), but the authors stated that this protocol was unable to discriminate between *F. graminearum* sensu stricto and other closely related species in the FGSC, and also failed to differentiate between *F. sporotrichioides* and *F. sibiricum*. The same nonspecificity issue was also observed in another SYBR Green protocol to quantify 11 *Fusarium* species (Nicolaisen et al. 2009). The authors stated that the *F. culmorum* qPCR assay (FculC561 fwd/FculC614 rev) also detected *F. cerealis*, and the *F. graminearum* assay (FgramB379 fwd/FgramB411 rev) could not differentiate between *F. graminearum* sensu stricto and species in the FGSC except for *F. cortaderiae* and *F. brasilicum*.

Fungal species diversity in node and grain samples. Several members of *Alternaria* complex were reported to colonize wheat at high levels in various geographic regions, causing disease symptoms on both leaf and grains (Perelló and Larran 2013; Ramires et al. 2018). In Canada, little is known about the diversity of wheat-associated *Alternaria* species. *A. alternata* (the causal agent of black point disease) was the main reason for the soft white spring wheat crop downgrade between 1975 and 1981 (Conner and Thomas 1985). Recently, 128 Canadian strains of *Alternaria* were investigated to determine their toxigenic potentiality and food contamination risk (Kelman et al. 2020). In this study, three species in the genus *Alternaria*—*A. triticina*, *A. alternata*, and *A. infectoria*—

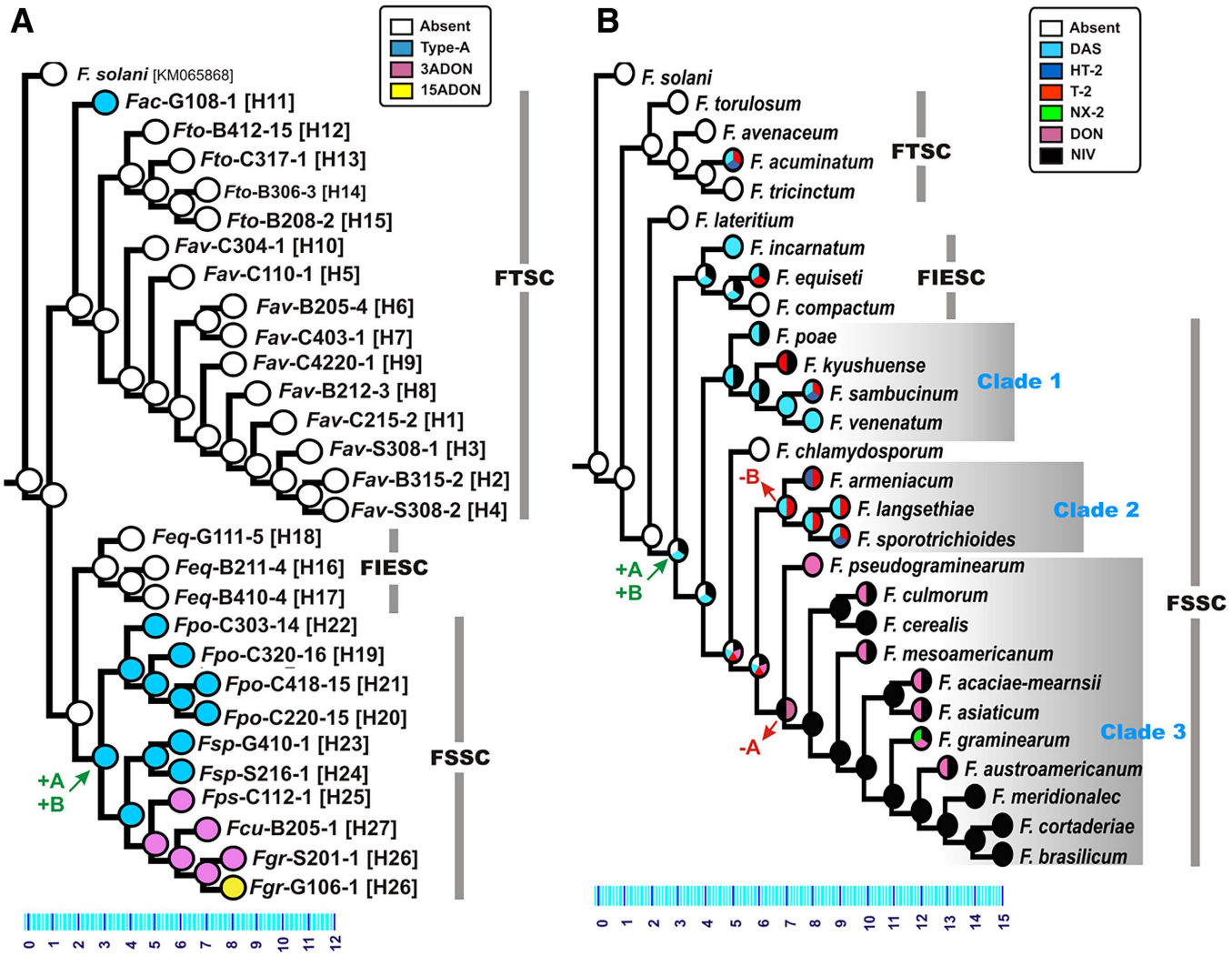

Fig. 5. A, *TEF1α*-based tree to show the distribution and ancestral state reconstructions for trichothecene mycotoxins in *Fusarium* isolates recovered from tested sites based on parsimony model within MESQUITE v 3.61 software. Colored pies in the terminal and internal nodes represent the most parsimonious distribution (presence/absence) for type-A and type-B (3ADON and 15ADON) trichothecene genotypes. Each node is labeled with the species name (first three letters) followed by the isolate number, and the *TEF1α* haplotype number is indicated between brackets. **B,** Ancestral state reconstruction for trichothecene mycotoxins in *Fusarium tricinctum* species complex (FTSC), *F. incarnatum–equiseti* species complex (FIESC), and *F. sambucinum* species complex (FSSC) based on parsimony model within MESQUITE v 3.61 software. Colored pies in the terminal and internal nodes represent the most parsimonious distribution (presence/absence) for type-A (DAS, HT-2, T-2, and NX-2) and type-B (DON and NIV) trichothecenes. The predicted ability to produce (+A and +B) and lost ability to produce (–A and –B) type-A and type-B trichothecenes, respectively, is indicated.

were recovered from grain, node, and grain/node samples, respectively. In the present study, species of *Alternaria* (saprophytic), *Fusarium* (hemibiotrophic), and *Parastagonospora* (necrotrophic) constitute >90% of all fungal isolates inhabiting wheat node and grain samples. The interaction between members of these three important genera in host tissues should be considered in future studies to understand the competitive interactions and mycotoxins produced during host colonization.

***Fusarium* species diversity in relation to tissue type.** In this study, we observed variations in the abundance and diversity of *Fusarium* spp. recovered from grain samples in the four locations. Spatial variation in *Fusarium* spp. was also noticed in node samples. This variation can be explained based on the tissue type or on spatial conditions; such a link was observed in fungal diversity in different wheat tissues like roots (Bateman and Kwaśna 1999; Dawson and Bateman 2001), stems/nodes (Łukanowski 2009), leaves (Robinson et al. 1994), and spikes (Rojas et al. 2020; Xu and Nicholson 2009). All of these reports found that plant tissues are densely colonized by large numbers of microorganisms, including pathogenic and nonpathogenic species (growing asymptomatically), which compete in the same niche and thereby influence plant performance (Rojas et al. 2020).

Analyzing the *Fusarium* populations associated with wheat node/stem tissues can provide useful information and help to predict diseases that might affect wheat stem and root as well as spikes in subsequently grown cereal crops. FCRR is an important disease that is caused by three main fungal species: *F. culmorum*, *F. pseudograminearum*, and *F. graminearum*. FCRR is devastating disease in many regions and can cause as much as 35% yield loses in bread wheat (Hollaway et al. 2013; Smiley et al. 2005; Zhou et al. 2019). The predominant pathogens can also differ depending on which geographical region and climate is being discussed. For example, in Saskatchewan, wheat root and crown rot is generally caused by *B. sorokiniana* and *Fusarium* spp. (Fernandez and Jefferson 2004; Fernandez et al. 2009). Among *Fusarium* spp., *F. avenaceum* was reported as one of the most abundant pathogens associated with wheat (Fernandez et al. 2002, 2007; Fernandez and Zentner 2005). This is consistent with our results, as we have analyzed one location in Saskatchewan (Scott), and *F. avenaceum* was found to be the most frequently isolated species from both node and grain samples. Moreover, unlike spike infection, crown and root infections by pathogenic *Fusarium* spp. are likely less affected by environmental conditions, and this would allow the maintenance of high levels of inoculum in years with less suitable conditions for developing FHB, which then may contribute to further spread of this disease when environmental conditions become optimal (Fernandez et al. 2009).

***Fusarium* species diversity in relation to soil type.** Classifying soils in Canada began with the first soil survey in Ontario in 1914 and has been refined and modified numerous times since then (Anderson and Scott Smith 2011). There are 10 orders of soil recognized in Canada based on the nature of the overall soil environment and the effects of the dominant soil-forming process. Each order is further divided into "great groups" and "subgroups" (Canadian Society of Soil Science 2020). In the grassland regions of Canada (including western Canadian prairies), chernozemic soils are the dominant soil order (Pennock et al. 2011). Chernozemic soils are classified into four great groups based on the color of the A horizon, which reflects the amount of organic matter present in the horizon. The four great groups of chernozemic soils are black (5 to 8.5%), dark gray (3.5 to 5.5%), dark brown (3.5 to 5%), and brown (2.5 to 3.4%), with numbers in parentheses representing the percentage of typical organic matter in each soil type (Canadian Society of Soil Science 2020). In this study, four locations were investigated: Beaverlodge (dark gray soil), Lacombe (black soil), and Lethbridge and Scott (dark brown soil). *F. graminearum* was detected only in node and grain samples from Lethbridge and Scott (dark brown soil), which is consistent with previous work (Turkington et al. 2002, 2011). In Alberta, *F. avenaceum*, *F. culmorum*, and *F. pseudograminearum* were found to be the most frequently isolated *Fusarium* spp., whereas *F. graminearum* showed infrequent occurrence from cereal, grass, and corn residue collected in 2001 to 2003 (Turkington et al. 2011). Recently, *F. graminearum* populations associated with corn stalk rot and wheat FHB in Alberta were investigated from samples collected in 2017, with the majority of *F. graminearum* isolates recovered from corn (82%) and only 14 isolates (12%) obtained from wheat samples (Ahmed et al. 2020). Our results are consistent with previous studies, and likely indicate the low frequency of *F. graminearum* associated with wheat in Alberta. The low levels of *F. graminearum* detected in Alberta reported here and in previous studies justify giving more concern to the other highly detected *Fusarium* spp. associated with wheat like *F. avenaceum*, *F. culmorum*, and *F. poae*.

In Canada, *F. graminearum* is highly detected in black soil zones, a zone that usually receives more moisture than other soil zones (Clear and Patrick 2000; Zentner et al. 2002). Fernandez and Hooligan (2009) indicated that *Fusarium* spp. associated with crown rot in cereal crops grown in Saskatchewan was linked to the soil type, with the occurrence of *F. culmorum* higher in brown soils than in the black/dark-gray soil zone and the occurrence of *F. avenaceum* highest in the black/dark-gray soil zone. It is worth noting that the prevalence of each *Fusarium* species involved in FHB can be dependent on climatic conditions in each geographical area (Clear et al. 1996; Parry et al. 1995), which is further influenced by agronomic factors like crop rotation regimes (Dill-Macky and Jones 2000; Lamprecht et al. 2006) and tillage (David Miller et al. 1998; Knight and Sutherland 2017; Lori et al. 2009). In Canada, *F. graminearum* was the main FHB-causing pathogen and the most frequently isolated species from *Fusarium*-damaged kernels in Manitoba (Wong et al. 1992). Since the 1980s, *F. graminearum* has been moving west from southern Manitoba toward Saskatchewan and Alberta to replace the less pathogenic FHB species (Clear and Patrick 2000).

Genetic variability in *Fusarium* populations. In this study, we generated a *TEF1α* haplotype network for each species complex separately to study the genetic variability in tested species/isolates in the four locations. Four and ten haplotypes were found in *F. poae* and *F. avenaceum*, respectively. *F. poae* was the most abundant *Fusarium* species in grain samples, whereas *F. avenaceum* was the only *Fusarium* species recovered from both node and grain samples in the four locations. The high number of haplotypes in these two species indicates a higher level of genetic variability in *F. poae* and *F. avenaceum* populations associated with wheat in this study. However, all *F. culmorum* isolates had an identical *TEF1a* haplotype, and these isolates were all recovered from the node samples but were absent from the grain samples. Moreover, the *Tri5* gene sequence in all *F. culmorum* isolates was identical. These results indicate that the *F. culmorum* populations in tested locations may be a monophyletic group and are specialized to infect wheat node/stem and cause FCRR disease.

In the FTSC and FSSC networks, a mixture of ancestral (central) and surviving descendant (terminal) haplotypes was observed, a pattern that is usually found in naturally occurring field populations (Ramdial et al. 2017). The most frequent haplotype in *F. avenaceum* populations (H10) and in *F. poae* populations (H20) were the only haplotypes recorded in isolates recovered from both grain and node samples in all locations, and these two haplotypes, H10 and H20, were located terminally and centrally, respectively (Fig. 3B and C).

Trichothecene genetic profiles and evolution. Distribution of certain trichothecene biosynthesis key genes in relation to phylogeny was explored in the isolates recovered during the present study and in several other species of FTSC, FIFSC, and FSSC (published in Fig. 5B and Supplementary Table S4) to have more comprehensive results. The presence of key genes involved in trichothecene biosynthesis was demonstrated in all three species complexes

(Fig. 5). *F. acuminatum* was the only species within the FTSC that was reported to produce type-A (DAS, HT-2, and T-2) trichothecene mycotoxins (Logrieco et al. 1992; Munkvold 2017; Tan et al. 2012). All *F. equiseti* isolates recovered in the present study failed to amplify *Tri5* gene; however, this species and others within the FIESC (like *F. incarnatum*) produce type-A trichothecenes (DAS and T-2) and type B (NIV) in different regions (Barros et al. 2012; Munkvold 2017). Tox5-1 and Tox5-2 primers used in the present study to amplify *Tri5* gene were designed based on *F. graminearum*, *F. sporotrichioides*, and *F. poae Tri5* sequence (Niessen and Vogel 1998). These primers were able to amplify *Tri5* gene from three of four *F. equiseti* strains screened by PCR (Niessen and Vogel 1998); however, *F. equiseti Tri5*-specific primers is needed to avoid reporting false-negative results as a result of primer nonspecificity. The FSSC contains many important plant pathogens, and three monophyletic clades were recognized within this species complex (Fig. 5B). The capability to produce trichothecenes may have been gained in a common ancestor of these three species complexes and then got lost in FTSC (except *F. acuminatum*), or may have been independently gained in *F. acuminatum* and other species in the FIESC and FSSC. Mapping mycotoxin production capabilities on a phylogenetic tree could help us to predict the mycotoxin production capability of certain species within any clade; however, *F. graminearum* (in the strict type-B producers clade) was recently reported to produce a novel type-A trichothecene mycotoxin called NX-2 (Kelly et al. 2016; Varga et al. 2015). This finding reflects the importance of continuous screening and identification of the trichothecene genotypes/chemotypes associated to *Fusarium* populations. A phylogenetic analysis of selected *Fusarium* isolates based on the *TEF1α* and the *Tri5* gene sequences showed that trichothecene genotype differences are not well correlated with the species evolutionary relationships of FHB-associated *Fusarium* spp. (Supplementary Fig. S5). These results might suggest an ancestral polymorphism predate speciation event. The results presented here extend the previous knowledge about the adaptive evolution within trichothecene genes to different environments, different hosts, and different geographic regions (Kelly et al. 2016; Proctor et al. 2018; Ward et al. 2002, 2008).

Conclusion. As a result of the complex and dynamic nature of *Fusarium* populations associated with wheat, detection and quantification of these pathogens in a given area is essential for taking the right management approach. Many of these *Fusarium* spp. are highly similar and very difficult to differentiate even at the molecular level. The specificity of several molecular markers not sensitive enough to the differentiate between targeted *Fusarium* species (Boutigny et al. 2019; Nicolaisen et al. 2009; Scherm et al. 2013), especially for the closely related species of *F. graminearum*, *F. cerealis*, *F. culmorum*, and members of the FGSC. Here we developed a qPCR test for specific detection and quantification of the most abundant wheat-associated *Fusarium* spp.: *F. avenaceum, F. culmorum, F. graminearum*, and *F. poae*. This test was validated in silico, in vitro, and in planta and showed unique specificity to the target species and no detection of other *Fusarium* spp. Our results showed that *F. graminearum* was not detected frequently from the 2018 wheat node and grain samples collected from distant locations in western Canada. More concern should be given to the other *Fusarium* spp. that were recovered frequently and detected with high amounts using culture- and molecular-based methods, respectively (e.g., *F. avenaceum*, *F. culmorum*, and *F. poae*).

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