Molecular Phylogenetic Analysis, Trichothecene Chemotype Patterns, and Variation in Aggressiveness of *Fusarium* Isolates Causing Head Blight in Wheat

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Abstract

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Certain *Fusarium* species cause Fusarium head blight (FHB) in wheat and other small grains. Differences in characteristics of the pathogen species/isolates used in breeding programs may affect reaction of host genotypes, leading to erroneous results. To clarify differences among *Fusarium* isolates from different geographical zones, the phylogenetic, chemotypic, and pathogenic abilities of 58 isolates collected from three wheat-producing countries (Canada, Mexico, and Iran) were investigated. Phylogenetic relationships among the isolates were characterized using the *Tri101* gene sequence. All Canadian and Iranian isolates clustered in one group and were identified as *F. graminearum* lineage 7 (= *F. graminearum sensu stricto*) within the *F. graminearum* (*Fg*) clade. The isolates from Mexico were identified as either *F. graminearum* lineage 3 (= *Fusarium boothii*) within the *Fg* clade or *Fusarium*

crookwellense. A polymerase chain reaction assay based on the *Tri12* gene identified three trichothecene chemotypes of 15-ADON, 3-ADON, and NIV, with 15-ADON being the most common. All *F. boothii* isolates from Mexico were of the 15-ADON chemotype, while all *F. crookwellense* isolates were determined to be NIV producers. While we did not find the NIV chemotype among the Canadian isolates, 25.6% of the Iranian isolates were determined to be NIV producers. High level of variation in aggressiveness was also observed among and within the species tested: *F. graminearum sensu stricto* isolates were the most aggressive, followed by those of *F. boothii*, and lastly by *F. crookwellense*. The differences observed among the isolates may explain why wheat lines/cultivars demonstrate different reactions to FHB in different geographical zones.

Fusarium head blight (FHB) is a destructive and economically important disease of wheat, barley, and other small grains throughout the world caused by certain *Fusarium* species. *Fusarium graminearum* Schwabe is the dominant and most widespread pathogen causing the disease (38). In addition to reducing grain yield and quality, FHB may result in grain contaminated with mycotoxins harmful to humans and animals (5,54,67). The most common mycotoxins produced by *F. graminearum* are trichothecenes such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and nivalenol (NIV), as well as an estrogenic mycotoxin, zearalenone (ZEA) (1,5,46,47,54,67).

Historically, two naturally occurring and morphologically distinct populations within *F. graminearum* known as group 1 and group 2 were described based on inability or ability of cultures to form perithecia, respectively (20,56,57). Subsequent analysis based on both morphological characteristics and DNA sequence data indicated that group 1 and group 2 were phylogenetically distinct. Therefore, a new name, *Fusarium pseudograminearum* O'Donnell & T. Aoki, was assigned to group 1, while the name *F. graminearum* was maintained for group 2 (3,4). Later, using the genealogical concordance phylogenetic species recognition (GCPSR) approach and phylogenetic analysis of sequences of nuclear genes such as translation elongation factor (*EF-1 α*), β -tubulin (*TUB*), and trichothecene 3-*O*-acetyltransferase (*Tri101*) from isolates of *F. graminearum* collected from around the world, O'Donnell et al.

(51) detected seven phylogenetically distinct and biogeographically structured lineages. The *F. graminearum* species was characterized as the *F. graminearum* or *Fg* clade, and the lineages were designated as species (51). Using more isolates of *F. graminearum*, six additional lineages (=species) were later discovered (52,53,61,73,75). Therefore, the previously known single species of *F. graminearum* is now considered to be a species complex consisting of at least 13 monophyletic species. These species have different geographic distributions, differ in production of trichothecenes, and may differ in their ability to cause disease on particular crops (15,51,53). The species detected within the *Fg* clade have been formally named, and the use of new species names is recommended by O'Donnell et al. (53). The name *F. graminearum sensu stricto* has been retained for lineage 7, the principal causal agent of FHB in wheat and barley in many parts of the world (53).

Fusarium species produce trichothecenes, which are divided into two broad categories based on the presence (B-trichothecenes) or absence (A-trichothecenes) of a keto group at the C-8 position of the trichothecene ring (68). All members of the *Fg* clade are B-trichothecene producers (73). Miller et al. (45) described two strain-specific chemotypes of DON and NIV within the *Fg* clade and the related species. The DON chemotype produces DON and/or its acetylated derivatives, and NIV chemotype produces NIV and/or its diacetylated derivatives. The DON chemotype is subdivided into two chemotypes of 3-ADON (DON and 3-ADON producers) and 15-ADON (DON and 15-ADON producers). Specific primers have been designed from the sequences of genes such as trichothecene 15-*O*-acetyltransferase (*Tri3*), trichothecene 4-*O*-acetyltransferase (*Tri7*), and trichothecene efflux pump (*Tri12*) to use in chemotype determination (29,34,35,73).

Aggressiveness is an attribute of a pathogen originally defined by Vanderplank (70) as the quantity of disease induced by a pathogenic isolate on a susceptible host. The aggressiveness of *F. graminearum* isolates depends on their DON-producing capacity (40,42). DON-non-producing isolates of *F. graminearum* caused a low level of disease severity in plants (16,17). Bai et al. (7) con-

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 1 appears in color online.

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cluded that the DON-non-producing isolates still could infect wheat spikes but could not spread beyond the initial infection site, suggesting that DON is an aggressiveness factor rather than a pathogenicity factor. There are also several reports indicating that DON-producing isolates are more aggressive than NIV-producing isolates (15,25,42). High variation in aggressiveness has been found among *F. graminearum* isolates from different geographical regions (8,24,39,42,44).

There is evidence that FHB-resistant wheat lines/cultivars tested in one region do not consistently give a resistant reaction in other geographical zones, e.g., Mexico versus Canada (J. Gilbert, *personal communication*). This difference may be attributed to pathogen profile, environmental conditions, and/or the interaction of both. An understanding of genetic profile and diversity of the pathogen may provide insights into its pathological and epidemiological potential, which may lead to an improvement in our strategies for control of the pathogen and management of the disease(s) it causes. The objectives of this study were: (i) to elucidate the phylogenetic relationships among *Fusarium* isolates collected from Canada, Mexico, and Iran based on *Tri101* gene sequencing data, (ii) to determine the trichothecene chemotypes of the isolates based on *Tri12* gene fragments, (iii) to assess the variation in aggressiveness among the isolates, and (iv) to determine if there is an association between phylogenetic properties and chemotype patterns with aggressiveness.

Materials and Methods

***Fusarium* isolates.** A total of 58 *Fusarium* isolates from Canada, Mexico, and Iran were used in this study (Table 1). Isolates 1 to 13 were obtained from the Canadian Collection of Fungal Cultures (DAOM), Ottawa, Ontario, Canada. Isolates 14 to 20 were obtained from Cereal Research Centre (CRC), Winnipeg, Manitoba, Canada (J. Gilbert, *personal communication*). Isolates 21 to 35 were obtained from the International Maize and Wheat Improvement Centre (CIMMYT), Mexico, D.F., Mexico. Isolates 36 to 58 were isolated from FHB-infected wheat spikes collected from wheat fields in Iran. The isolates were chosen to provide a representative sample from *Fusarium*-prone areas in Canada, Mexico, and Iran. Most isolates of the pathogen originated from wheat, but there were also some isolates from barley and maize. Following single-spore on water agar (WA), pure cultures of the isolates were grown on potato dextrose agar (PDA) and carnation leaf agar (CLA) under standard conditions for growth and sporulation, respectively, and the isolates were identified using cultural and morphological characteristics (49).

For phylogenetic analysis, the sequencing data of the *Tri101* gene of the following reference isolates representing 11 species within the *Fg* clade were downloaded from GenBank and used along with the experimental isolates: NRRL 28585 (*Fusarium*

Table 1. Identification code, host, geographic origin, and year of collection of *Fusarium* isolates used to study phylogenetic analysis, trichothecene chemotypes, and variation in aggressiveness

Serial number ^a	Identification code	Host	Geographic origin	Year
1	DAOM 170785	Maize	Ottawa, Ontario, Canada	1998
2-5	DAOM 177406, DAOM 177408, DAOM 177409, DAOM 178148	Wheat	Chatham, Ontario, Canada	1998
6	DAOM 178149	Barley	Petrolia, Ontario, Canada	1998
7-10	DAOM 180376, DAOM 180377, DAOM 180378, DAOM 180379	Maize	Ottawa, Ontario, Canada	1998
11-12	DAOM 192130, DAOM 192131	Wheat	St. Jean, Manitoba, Canada	1998
13	DAOM 213295	Wheat	Burdett, Alberta, Canada	1998
14	EMMB 19/03	Wheat	Plum Coulee, Manitoba, Canada	2003
15	J & R SL 12	Wheat	Swan Lake, Manitoba, Canada	1996
16	MSDS 3/03	Wheat	Beausejour, Manitoba, Canada	2003
17	40/04	Wheat	Somerset, Manitoba, Canada	2004
18	71/04	Wheat	Gretna, Manitoba, Canada	2004
19	98/04	Wheat	Anola, Manitoba, Canada	2004
20	136/04	Wheat	Elkhorn, Manitoba, Canada	2004
21-27	CM-1, CM-2, CM-3, CM-4, CM-5, CM-6, CM-7	Wheat	Toluca, Edo de México, México	1995
28	CM-8	Wheat	El Tigre, Jalisco, México	1997
29	CM-9	Wheat	Jesús María, Jalisco, México	1997
30-33	CM-10, CM-11, CM-12, CM-13	Wheat	Tepatitlan, Jalisco, México	1997
34-35	CM-14, CM-15	Wheat	Patzcuaro, Michoacan, México	1997
36-38	IR-1, IR-2, IR-3	Wheat	Sari, Mazandaran, Iran	2005
39-42	IR-4, IR-5, IR-6A, IR-6B	Wheat	Behshahr, Mazandaran, Iran	2005
43-47	IR-7A, IR-7B, IR-8, IR-9A, IR-9B	Wheat	Aliabad, Golestan, Iran	2005
48-49	IR-10, IR-12	Wheat	Azadshahr, Golestan, Iran	2005
50-58	IR-13, IR-14, IR-16, IR-18A, IR-18B, IR-21, IR-23, IR-24A, IR-24B	Wheat	Moghan, Ardabil, Iran	2005
59	NRRL 28585	Herbaceous vine	Brazil	Unknown
60	NRRL 28436	Sweet potato	New Caledonia	Unknown
61	NRRL 29105	Maize ear	Kaski, Nepal	Unknown
62	NRRL 26754	<i>Acacia mearnsii</i>	South Africa	Unknown
63	NRRL 26156	Wheat	Shanghai, China	Unknown
64	NRRL 28063	Maize stalk	Michigan, USA	Unknown
65	NRRL 29306	Maize	New Zealand	Unknown
66	NRRL 29148	Grape ivy	Pennsylvania, USA	Unknown
67	NRRL 31238	Unknown	Unknown	Unknown
68	NRRL 36905	Wheat	Minnesota, USA	Unknown
69	NRRL 37605	Wheat	Ipolydamásd, Hungary	Unknown

^a Isolates 1 to 13 were obtained from Canadian Collection of Fungal Cultures (DAOM), Ottawa, Ontario, Canada, isolates 14 to 20 from Cereal Research Centre (CRC), Winnipeg, Manitoba, Canada, and isolates 21 to 35 from the International Maize and Wheat Improvement Centre (CIMMYT), Mexico, D.F., Mexico. Isolates 36 to 58 were isolated from *Fusarium* head blight (FHB)-infected wheat spikes collected from Iran. Sequences for isolates 1 to 58 were deposited in the GenBank database under accession numbers JF911426 to JF911483. Isolates 59 to 65 representing seven species within the *Fg* clade were received from USDA/ARS Culture Collection (NRRL, Peoria, IL) to use as reference isolates in chemotype determination and aggressiveness tests. For sequencing purposes, sequences of the *Tri101* gene for isolates 59 to 69 representing 11 species within the *Fg* clade were downloaded from GenBank to use as references. Accession numbers of isolates 59 to 69 were AF212586, AF212582, AF212593, AF212595, AF212599, AF212605, AY225882, AF212589, AY452813, DQ452409, and DQ452412, respectively.

austroamericanum T. Aoki, Kistler, Geiser et O'Donnell), NRRL 28436 (*Fusarium meridionale* T. Aoki, Kistler, Geiser et O'Donnell), NRRL 29105 (*Fusarium boothii* O'Donnell, T. Aoki, Kistler et Geiser), NRRL 26754 (*Fusarium acaciae-mearnsii* O'Donnell, T. Aoki, Kistler et Geiser), NRRL 26156 (*Fusarium asiaticum* O'Donnell, T. Aoki, Kistler et Geiser), NRRL 28063 (*F. graminearum* s.s.), NRRL 29306 (*Fusarium cortaderiae* O'Donnell, T. Aoki, Kistler et Geiser), NRRL 29148 (*Fusarium mesoamericanum* T. Aoki, Kistler, Geiser et O'Donnell), NRRL 31238 (*Fusarium brasiliicum* T. Aoki, Kistler, Geiser et O'Donnell), 36905 (*Fusarium gerlachii* T. Aoki, Starkey, Gale, Kistler et O'Donnell), and 37605 (*Fusarium vorosii* B. Tóth, Varga, Starkey, O'Donnell, Suga et T. Aoki) (Table 1). Sequences of one *F. pseudograminearum* isolate (NRRL 28338) were also used as the outgroup in the phylogenetic analysis.

Mycelium production and DNA extraction. Mycelial disks of *Fusarium* isolates cultured on PDA were transferred to 125-ml flasks containing 60 ml of yeast-malt broth culture medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% dextrose) and grown at 25°C on a rotary shaker (200 rpm) for 3 to 4 days (50). The mycelium was harvested as follows: (i) mycelium suspension was poured into 50-ml centrifuge tubes, centrifuged at 3,500 × g at 25°C for 10 min in an Allegra 6R centrifuge (Beckman Coulter, Brea, CA), and the supernatant discarded, (ii) 10 ml of sterile distilled water was added to the mycelium pellet, (iii) the mixture was centrifuged for another 10 min and the supernatant again discarded, (iv) the mycelium was blotted briefly between sterile paper towels, and (v) the harvested mycelium was lyophilized for 2 days and stored for further use. DNA was extracted using the modified CTAB miniprep method (23). DNA samples were quantified by fluorometry using Hoechst 33258 stain and diluted to 10 ng/μl to use in polymerase chain reaction (PCR), by adding appropriate amounts of 0.1× TE buffer.

DNA amplification and sequencing. The *Tri101* gene was amplified with primers AT1 and AT2 (Table 2). The PCR reaction mixture typically contained 1× PCR buffer (Invitrogen, Carlsbad, CA), 2 mM MgSO₄ (Invitrogen), 0.2 mM of each dNTP (Invitrogen), 0.3 pmol/μl of each primer (Invitrogen), 0.4 ng/μl BSA, 0.02 unit/μl Hi Fi Platinum *Taq* DNA polymerase (Invitrogen), and 10 ng DNA in a reaction volume of 49 μl. PCR products were amplified in a PTC-100 thermal cycler (MJ Research, Waltham, MA) with the following program: (i) an initial denaturing step of 2 min at 94°C, (ii) 35 cycles of 15 s at 94°C for DNA denaturation, 45 s at 60°C for primer annealing, and 1 min at 68°C for primer extension, (iii) a final extension of 10 min at 68°C, and (iv) program held at 15°C. Aliquots of the PCR products were loaded on 1% agarose gel to check the amplification of fragments for *Tri101*.

Following purification of the amplified DNA with a MultiScreen PCR plate (Millipore Corporation, Billerica, MA), cycle sequencing was conducted in a PTC-100 thermal cycler (Applied Biosys-

tems, Foster City, CA) with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the following program: (i) an initial denaturing step of 5 min at 92°C, (ii) 60 cycles of 10 s at 92°C for DNA denaturation, 5 s at 55°C for primer annealing, and 4 min at 60°C for primer extension, (iii) a final extension of 10 min at 60°C, and (iv) program held at 4°C. Amplicons were sequenced with seven additional primers (Table 2) to ensure adequate coverage of all nucleotides in both directions. All sequencing reaction mixtures were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Phylogenetic analysis. DNA sequences were processed using PHRED (18) and assembled with CAP3 (28) as implemented in an internal data pipeline called SOOMOS v0.6 (T. Banks, *personal communication*). Multiple sequence alignments were accomplished using MEGA 4 (64). The sequences obtained in this study were deposited in the GenBank databases under accession numbers JF911426 to JF911483.

Phylogenetic analysis was conducted using PAUP* v. 4.0b10 to estimate the genetic diversity and evolutionary relationships of the isolates from the aligned sequences (63). Maximum parsimony (MP) searches were conducted using the heuristic search option with 1,000 random addition sequences and the tree bisection-reconnection (TBR) method of branch swapping. Bootstrap analysis was performed with 500 pseudoreplicates (19), and values greater than 50 were reported on the phylogenetic tree.

Determination of trichothecene chemotypes. Trichothecene chemotypes were determined by multiplex PCR assays based on the *Tri12* gene. The primers used for the amplification of the *Tri12* gene included 12CON, 12NF, 12-15F, and 12-3F (Table 2). PCR was performed in 10-μl volume with the following reaction mixture: 1× GeneAmp PCR buffer II (Applied Biosystems, Foster City, CA), 2 mM MgCl₂ (Applied Biosystems), 0.16 mM of each dNTP (Invitrogen), 0.2 μmol/μl of each primer (Invitrogen), 0.04 unit/μl AmpliTaq DNA polymerase (Applied Biosystems), and 20 ng DNA. PCR was conducted in a PTC-200 thermal cycler (MJ Research, Waltham, MA) with the following program: (i) an initial step of 2 min at 94°C, (ii) 30 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C, (iii) a final extension of 7 min at 72°C, and (iv) program held at 15°C. PCR products were resolved on 1.2% (wt/vol) agarose gel and scored relative to a 100-bp DNA size ladder (Invitrogen).

Aggressiveness tests and statistical analysis. *Fusarium* isolates were individually inoculated on the susceptible wheat cultivar Roblin to measure disease spread within the spikes (disease severity) as an indication of the isolate's aggressiveness. Plants were grown in plastic pots (16 × 13 × 13 cm³) containing Sunshine Mix #4 Aggregate Plus soil (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada) in the greenhouse, and fertilized with NPK (20:20:20) all-purpose fertilizer (Plant-Prod, Brampton, ON, Canada) weekly. Conditions in the greenhouse were kept at 15 to

Table 2. List of primers used for the *Tri101* gene amplification and sequencing and trichothecene chemotype determination in *Fusarium* isolates

Primer name	Sequence	Forward/reverse	Reference
<i>Tri101</i> gene amplification and sequencing			
PCR primers:			
AT1	AAAATGGCTTTCAAGATACAGC	Forward	51
AT2	C(A/G)TA(C/T)TGCGC(A/G)TA(A/G)TTGGTCCA	Reverse	51
Sequencing primers:			
AT3	TTGATGCTCGACCGGCAATGG	Forward	51
AT4	GTGTGGTAGGTCATGTTTG	Reverse	51
AT6	ATCCATAGCACCCTGTCTGTC	Reverse	51
F140	GACGTACCTGCACAACAAC	Forward	
F158	AGAGTCTTGGTAGCAGCATC	Forward	
F171	CGGAGGTCTTTCACTACAAC	Forward	
R184	GTCAGGGATACGTTGGACT	Reverse	
Trichothecene chemotype determination			
12CON	CATGAGCATGGTGATGTC	Reverse	61
12NF	TCTCCTCGTTGTATCTGG	Forward	61
12-15F	TACAGCGGTGCGAACTTC	Forward	61
12-3F	CTTTGGCAAGCCCGTGCA	Forward	61

isolates when each spike reached 50% anthesis. Using a micropipette, 10 μ l of inoculum (5×10^4 macroconidia/ml) was injected into a single floret in a spikelet positioned 1/3 from the top of the spike, and the inoculated spikes were covered with glassine bags

(Seedburo Equipment Co., Chicago, IL) for 48 h to provide constant high humidity. Three replications (pots) and at least four spikes per replication were used for inoculation by each isolate. Plants were retained in the greenhouse under temperatures of 20 to 28°C and a photoperiod of 16 h to develop disease symptoms. Disease severity was rated as the percentage of diseased spikelets per spike 21 days after inoculation. Average percent diseased spikelet was calculated for each pot, and percentage data were arcsine-transformed prior to analysis. SAS 9.2 (SAS Institute Inc., Raleigh, NC) was used for data analysis and to compare disease severity.

Results

Identification of *Fusarium* isolates. All Canadian and seven out of 15 isolates received from CIMMYT, Mexico were identified as *F. graminearum* based on cultural and morphological characteristics (49). The rest of the isolates from Mexico were determined to be *Fusarium crookwellense* Burgess, Nelson and Toussoun (synonym: *Fusarium cerealis* (Cook) Scc.). A total of 23 isolates were obtained from the infected wheat samples from Iran which also were identified as *F. graminearum*.

Molecular phylogenetic analysis. The length of the *Tri101* gene used to make the sequence data set in this study was 1,350 nucleotides. Results of maximum parsimony analysis showed 1,236 constant nucleotides, 65 parsimony-uninformative variable nucleotides, and 49 parsimony-informative nucleotides in the sequences. The analysis produced 303 most-parsimonious trees at 123 steps long, with consistency index (CI) and retention index (RI) of 0.94 and 0.98, respectively. The tree reported here is a phylogram as shown in Figure 1.

Analyses of the sequence alignment of the isolates including the experimental and the reference isolates detected two distinct clades (Fig. 1). All Canadian, Iranian, and seven Mexican isolates along with the 11 reference isolates of the *Fg* clade clustered together (*Fg* clade), while the remaining eight isolates from Mexico formed a different cluster; both clusters had a bootstrap (BP) value of

100%. Within the *Fg* clade cluster, Canadian and Iranian isolates formed a distinct cluster along with the *F. graminearum* s.s. with 90% BP. The seven isolates from Mexico clustered with the *F. boothii* reference isolate with 100% BP. Sequencing data from the present study support isolates of *F. graminearum* s.s. and *F. boothii* being separate species. The remaining eight Mexican isolates (*F. crookwellense*) formed a cluster different from the *Fg* clade based on phylogenetic analysis of DNA sequences. There was polymorphism among the isolates clustered with *F. graminearum* s.s., and subgroups with BP values of 70 and 59% were detected within the cluster (Fig. 1).

Trichothecene chemotypes. The PCR assay based on *Tri12* gene produced amplicons of approximately 840, 670, and 410 bp corresponding with NIV, 15-ADON, and 3-ADON chemotypes, respectively (61) (Fig. 2). The majority of the isolates tested (46.6%) were of the 15-ADON chemotype, followed by NIV (32.8%) and 3-ADON (20.7%) (Fig. 1 and Table 3). Our results showed that 46.5, 27.9, and 25.6% of the isolates of *F. graminearum* s.s. were 15-ADON, 3-ADON, and NIV producers, respectively. However, all isolates of *F. boothii* and *F. crookwellense* were determined to be 15-ADON and NIV chemotypes, respectively (Fig. 1 and Table 3).

No NIV chemotype was detected among *F. graminearum* s.s. isolates from Canada, while the majority of the isolates received from Iran were of the NIV chemotype (Fig. 1 and Table 1). The majority of the isolates collected across Canada before 1998 were of the 15-ADON chemotype, while more recently collected isolates (after 2004) were identified as 3-ADON producers. Among the Iranian isolates, all three chemotypes were detected in the northern parts of the country (Sari, Behshahr, Aliabad, and Azadshahr), while 15-ADON was the only chemotype detected among the isolates collected from the northwestern region, Moghan (Fig. 1 and Table 1).

Aggressiveness. Three *Fusarium* isolates, namely IR-4, IR-6A, and IR-8, failed to sporulate and were not tested for aggressive-

Fig. 2. Amplification products of *Tri12* gene for *Fusarium* isolates produced by multiplex polymerase chain reaction using the primers 12CON, 12NF, 12-15F, and 12-3F. Amplification fragments of 840, 670, and 410 bp correspond with NIV, 15-ADON, and 3-ADON chemotypes, respectively. Lane M represents a 100-bp ladder (Invitrogen), and lanes 25-48 represent the following *Fusarium* isolates: IR-5, IR-6A, IR-6B, IR-7A, IR-7B, IR-8, IR-9A, IR-9B, IR-10, IR-12, IR-13, IR-14, IR-16, IR-18A, IR-18B, IR-21, IR-23, IR-24A, IR-24B, CM-1, CM-2, CM-3, CM-4, and CM-5.

Table 3. Distribution of trichothecene chemotypes among *Fusarium* isolates collected from Canada, Mexico, and Iran based on *Tri12* gene^a

<i>Fusarium</i> species	Chemotypes		
	NIV	15-ADON	3-ADON
<i>F. graminearum sensu stricto</i> (Canada)	0	11	9
<i>F. graminearum sensu stricto</i> (Iran)	11	9	3
Subtotal (<i>F. graminearum sensu stricto</i> isolates)	11 (25.6%)	20 (46.5%)	12 (27.9%)
<i>F. boothii</i> (Mexico)	0	7	0
<i>F. crookwellense</i> (Mexico)	8	0	0
Total (all isolates)	19 (32.8%)	27 (46.6%)	12 (20.7%)

^a Trichothecene chemotypes were determined by amplification of *Tri12* gene using a multiplex polymerase chain reaction conducted by 12CON, 12NF, 12-15F, and 12-3F primers.

ness. All other isolates infected the susceptible cultivar Roblin and produced disease symptoms in a range of 0.4 to 100% based on percent infected spikelets (disease severity) (Fig. 1). Significant differences in disease severity were observed among the *Fusarium* isolates, evidence of differences in aggressiveness.

The pathogen isolates produced significantly different levels of disease severity according to the geographical origin of the isolates (Fig. 3). As shown in Figure 3, mean disease severities produced by *Fusarium* isolates from Canada and Iran were high and similar as opposed to those of the Mexican isolates. Variation in aggressiveness among the pathogen isolates was also different according to geographical zones. The highest variation in aggressiveness was observed among the Canadian isolates with disease severity ranging from 0.4 to 100%, and the least variation among the Mexican isolates with disease severity in the range of 1.1 to 56.3%. The range of disease severity among Iranian isolates was from 23.7 to 100%. The frequency of isolates with disease severity greater than 50.0% was higher than that of isolates with disease severity lower than 50.0% (Fig. 1).

High variation in aggressiveness was observed among the phylogenetically determined *Fusarium* species in the *Fg* clade (Fig. 4). Mean disease severity among the isolates of *F. graminearum* s.s. was 78.6%, while it was 28.8% among the isolates of *F. boothii*. Therefore, mean disease severity of *F. graminearum* s.s. isolates was more than twice that of *F. boothii* isolates. Isolates of *F. crookwellense*, which is not a species within the *F. graminearum* clade, had a mean disease severity of 12.0%. Therefore, a strong association between *Fusarium* species and aggressiveness was observed, i.e., three species of *F. graminearum*, *F. boothii*, and *F.*

crookwellense showed the highest to lowest aggressiveness, respectively (Fig. 4).

An association between trichothecene chemotype and aggressiveness was also observed. The NIV chemotype had the lowest (33.5%), while the 3-ADON chemotype had the highest mean disease severity (87.5%). The 15-ADON chemotype was intermediate with a mean value of 69.8% (Fig. 5). If the Mexican isolates with significantly lower disease severity values are removed, the pattern of aggressiveness from 3-ADON to 15-ADON to NIV still remains the same.

Discussion

This experiment was designed and conducted to find the reason(s) for different reactions of wheat genotypes to FHB in different regions, i.e., Canada, Mexico, and Iran. To do this, phylogenetic relationships were identified, trichothecene chemotype patterns were determined, and variation in aggressiveness was assessed among 58 *Fusarium* isolates from different origins. In addition, the association between phylogenetic characteristics and chemotype patterns with aggressiveness was determined.

Results showed that all Canadian and Iranian isolates belonged to *F. graminearum* s.s., while the *Fusarium* isolates obtained from Mexico were divided into two clusters: a distinct cluster of *F. boothii* and another cluster which was identified as *F. crookwellense* (Fig. 1). The wide geographic distribution of *F. graminearum* s.s. isolates among the FHB pathogens examined is consistent with previous reports documenting that *F. graminearum* s.s. has a global distribution (53). *F. graminearum* s.s. is reported as the predominant species in Canada (K. O'Donnell, *personal communication*), the United States (14,76,77), Argentina (59), and central Europe

Fig. 3. Comparison of aggressiveness of *Fusarium* isolates from different geographical zones, measured based on percent infected spikelets (disease severity) on the susceptible cultivar Roblin, 21 days after inoculation using single-floret point inoculation. Disease severity values are back-transformed from least squares means of arcsine-transformed data. Bars on the top of each column represent the standard errors.

Fig. 4. Comparison of aggressiveness of different *Fusarium* species, measured based on percent infected spikelets (disease severity) on the susceptible cultivar Roblin, 21 days after inoculation using single-floret point inoculation. Disease severity values are back-transformed from least squares means of arcsine-transformed data. Bars on the top of each column represent the standard errors.

(66). *F. graminearum* s.s. has also been detected in New Zealand (48) and several Asian countries, including China (21), Japan (30,62), and Korea (33). However, *F. boothii* is limited to Africa, Mexico, and Mesoamerica (53). We report for the first time a phylogenetic analysis on *Fusarium* head blight-causing pathogens from western Asia, more specifically Iran, at a relatively extensive level. These isolates covered the predominant areas in Iran in which FHB occurs. Previously, only one isolate of *Fusarium* from maize from Iran was reported to be included in phylogenetic analysis, and was determined to be *F. graminearum* s.s. (51). *F. asiaticum*, which is mainly found in eastern Asia (21,30,33,53), was not detected in our samples from Iran.

F. crookwellense (synonym: *F. cerealis*), a species which had previously been confused with *F. graminearum* and *Fusarium culmorum* (W.G. Smith) Sacc., was described as a distinct species in 1982 by Burgess, Nelson, and Toussoun (13,49). Both species cause FHB in wheat and other small grain cereals. However, in contrast to *F. boothii*, *F. crookwellense* is not a species within the *Fg* clade.

Our results support the previous conclusion that the evolutionary history of NIV, 3-ADON, and 15-ADON chemotypes is different from that predicted by the species phylogeny (51,73). On the other hand, any tricothecene chemotype may arise within each species. Ward et al. (73) showed that each of the tricothecene chemotypes had a single evolutionary origin in the ancestor of extant species within the *Fg* clade, and that polymorphism within these virulence-associated genes has persisted through multiple speciation events in these fungi. They concluded that the polyphyletic distribution of tricothecene chemotypes relative to the *Fg* clade is the result of

trans-species evolution (73), i.e., development based on occurrence of similar alleles in related species.

The 15-ADON chemotype was predominant in this study (Fig. 1 and Table 3), which is consistent with previous reports. Earlier studies report that DON-producing isolates of *F. graminearum* occur more frequently than NIV-producing isolates, and that the 15-ADON chemotype is more prevalent than the 3-ADON chemotype in many countries (11,22,26,58,66). Although *F. graminearum* s.s. segregated for all three chemotypes in the present study, the predominance of a chemotype also depended on the geographic origin of the isolates. All isolates of *F. graminearum* s.s. in this study collected from Canada were determined to be DON producers, and the majority of them were identified as the 15-ADON chemotype. In contrast, the NIV chemotype was predominant among the isolates of Iran, which is in agreement with the results of Haratian et al. (27). Other studies have also revealed some regional differences among mycotoxin chemotypes (35,45,75,78). More specifically, NIV isolates mostly have been reported from the eastern parts of the world, while DON chemotypes are predominant in North America (47). Such ecological differences in chemotype distribution may contribute to establish regional differences in grain contamination (58). There is also evidence of regional difference and variation in chemotype patterns among the isolates of *F. graminearum* s.s. in Iran. While all three chemotypes were detected in northern parts of the country, the isolates collected in northwestern parts were determined to be 15-ADON only. Agricultural practices and wheat cultivation system in northwestern Iran (Moghan, Ardabil) is intensive and uniform in a limited geographical zone. Therefore, there is the possibility of genetic uniformity in pathogen population in northwestern Iran. In contrast, as wheat is grown in a broader geographical area in northern parts of the country, there is the possibility of more variation in pathogen population.

While most Canadian isolates collected earlier than 1998 were determined to be 15-ADON producers, all isolates collected after 2004 were found to be of the 3-ADON chemotype (Fig. 1 and Table 1). This is in agreement with the observations that the dominant 15-ADON FHB pathogen is being replaced by the more toxigenic population of *F. graminearum* s.s. with the 3-ADON chemotype in North America (26,55,74). A significant shift from DON- to NIV-producing *F. graminearum* in northwestern Europe has also been reported (72).

While all isolates of *F. boothii* received from Mexico were identified as the 15-ADON chemotype, all *F. crookwellense* isolates were determined to be the NIV chemotype (Fig. 1 and Table 3). The major secondary metabolites produced by *F. crookwellense*, NIV-related metabolites (45), differ from those produced by *F. graminearum* (32).

Our results showed significant differences in aggressiveness of the pathogen isolates among the geographical zones (Fig. 3). The isolates collected from Canada and Iran produced higher and similar levels of disease severity on cultivar Roblin, while disease produced by the Mexican isolates was significantly lower. Analysis of data based on the *Fusarium* species from the same locations gives a better understanding of the results: disease severity produced by *F. graminearum* s.s. isolates, all collected from Canada and Iran, was significantly higher than that caused by the other species, i.e., *F. boothii* and *F. crookwellense*, both collected from Mexico (Fig. 4). Therefore, the difference in pathogen aggressiveness among geographical zones may be attributed to the presence of different species in different locations. We observed high variation in aggressiveness among species, with isolates of *F. graminearum* s.s. being the most aggressive, followed by *F. boothii* and *F. crookwellense* (Fig. 4). Even though many reports have been published on aggressiveness of *Fusarium* species and *F. graminearum* isolates, only a very few reports have mentioned aggressiveness in *F. graminearum* phylogenetic species or the *Fg* clade. In an investigation on the isolates of *Fusarium* representing eight species of the *Fg* clade and three lineages of *F. culmorum*, Tóth et al. (65) found that *F. boothii* was among the least aggressive species on wheat, while

Fig. 5. Comparison of aggressiveness of *Fusarium* isolates with different tricothecene chemotypes, measured based on percent infected spikelets (disease severity) on the susceptible cultivar Roblin, 21 days after inoculation using single-floret point inoculation. Disease severity values are back-transformed from least squares means of arcsine-transformed data. Bars on the top of each column represent the standard errors.

F. graminearum s.s. isolates were the most aggressive. Alvarez et al. (2) also reported high variation in aggressiveness among *F. graminearum* s.s. isolates from Argentina.

The 3-ADON chemotype had the highest aggressiveness followed by 15-ADON and NIV chemotypes, respectively (Fig. 5). Several earlier reports also confirm that the NIV chemotype has the lowest aggressiveness (15,25,42). The 3-ADON population of *F. graminearum* found in North Dakota was also more aggressive and produced a higher level of DON in grains than the 15-ADON population in spring wheat (55). However, Ward et al. (74) and more recently, von der Ohe et al. (71) did not find significant difference between the two chemotypes in disease development. The contradiction observed in the results in different experiments may be attributed to different methods used for inoculations (55) and different environmental conditions. In spite of lack of significant differences in aggressiveness between 3-ADON and 15-ADON chemotypes under spray inoculation, significant differences in DON content were observed between the two chemotypes, with higher DON produced by 3-ADON (71,74). The possibility of higher production of DON by 3-ADON isolates itself is a concern, especially in susceptible wheat cultivars, particularly if the 3-ADON chemotype continues to increase. Wheat genotype is another factor which may react to trichothecene chemotypes and impact pathogen aggressiveness and DON accumulation (55). While mean disease severity and trichothecene accumulation on wheat cultivars Grandin (susceptible) and ND 2710 (moderately resistant) were significantly greater for 3-ADON isolates than for 15-ADON isolates, no significant differences were observed on cultivar Steele-ND (resistant) between the two chemotypes (55). Similar results were also observed for DON accumulation by Ward et al. (74). We concluded that shifts in *F. graminearum* populations from 15-ADON to 3-ADON chemotypes may result in higher DON accumulation and/or higher FHB level in susceptible to moderately resistant wheat cultivars.

Although our results and other reports indicate an association between chemotype and aggressiveness toward wheat, it does not mean that chemotype itself is the causal factor of aggressiveness. However, trichothecene chemotypes differ in their capacity to produce DON, a factor that contributes to aggressiveness (40,42). As mentioned before, in general, DON production capacity of the 3-ADON chemotype is higher than other chemotypes and they are more aggressive than other chemotypes. Higher aggressiveness of 3-ADON isolates may provide a fitness advantage over 15-ADON isolates, as fungal growth rate, conidial reproduction, and toxin accumulation were mentioned by Ward et al. (74).

Variability in aggressiveness among isolates of a species may cause difficulties in diagnosing the disease in the field and prevent the timely application of control measures (25). The existence of high variability in the pathogen also emphasizes the need for breeders to include a wide range of isolates in their screening for selection of disease resistant varieties (25).

Genetic variation in chemotype patterns and aggressiveness was high among *F. graminearum* s.s. isolates compared to the isolates of *F. boothii* and *F. crookwellense*. Sources of genetic variability in these species are mutation, gene flow, and somatic recombination by heterokaryosis. Sexual recombination is also an important mechanism that changes the patterns of variation in *F. graminearum*. In addition, as the isolates of *F. graminearum* s.s. in this study were originally from diverse locations, hosts, and years (Table 1), they may originally bear diverse genetic variation. Sexual reproduction is not known for either *F. crookwellense* (45) or *F. boothii*, which may explain why these species express low levels of variation. Getting the samples from the more limited geographical area, same host, and same year, and use of smaller sample sizes for both species (Table 1) also may be mentioned.

There is no evidence for stable pathogen races in *Fusarium* species causing FHB (8,41,60). The species of *Fusarium* that cause head blight in wheat can infect many other cereal crops and maize without showing specialization; a host-specific, blight-causing *Fusarium* species has not been documented (69). Recently, how-

ever, Boutigny et al. (11) presented evidence of species-specific differences in host preferences for *Fusarium* species within the *Fg* clade, suggesting a close association of *F. graminearum* s.s. and *F. boothii* with wheat FHB and maize ear rot, respectively. Different species or isolates of *Fusarium* demonstrate high variation in their phylogenetic properties, trichothecene chemotype patterns, and pathogenicity/aggressiveness, which may greatly impact host reaction (Fig. 1). We concluded that differences in reactions of wheat lines/cultivars to FHB in different geographical zones were mainly due to differences in pathogen profile and aggressiveness. Variable environmental conditions, differences in inoculation methods, and evaluation procedures should also be considered.

Use of highly aggressive *Fusarium* isolates as an efficient tool for achieving adequate differentiation among host genotypes and selecting wheat genotypes with high levels of resistance to FHB is recommended in FHB nurseries and wheat breeding programs. In addition, use of a mixture of isolates of the pathogen should be preferred for artificial inoculations reflecting the conditions that occur in natural epidemics.

Resistance to FHB in wheat usually is stable, and resistant cultivars show consistent resistance to different species and isolates of *Fusarium* worldwide. Since the introduction of the wheat line 'Sumai 3' and its derivatives 30 years ago, they are still the major sources of resistance to FHB in wheat breeding programs in China (6,36) and CIMMYT, Mexico (9). These sources of resistance have also been extensively tested for FHB reaction in Japan, the United States, and many European countries using a worldwide collection of *F. graminearum* isolates (5,6,10,31,41). However, to assess the durability of host resistance, study of the structure of pathogen populations is very important (43). If there is a high level of genetic variation in a fungal population of an individual field, then a pathogen can rapidly adapt to the resistance genes of the host (37). Given the large genetic variability that exists in *Fusarium* spp., use of at least a few different resistance genes in a wheat breeding program would be a wise approach (12).

Results of the present study add to our expanding knowledge of the *Fg* complex species and support the premise that there is a relationship between the origin of the pathogen and its phylogenetic features, chemotype patterns, and aggressiveness. The results clearly showed differences among *Fusarium* isolates used in Mexico and the isolates from Canada and Iran. In contrast to Canada and Iran, where FHB pathogen isolates were identified as *F. graminearum* s.s., the Mexican isolates belonged to the less aggressive *F. boothii* within the *Fg* clade or to *F. crookwellense*. These differences in pathogen isolates may explain, at least in part, why advanced wheat lines/cultivars that demonstrate a resistant reaction in Mexico may not express the same reaction in Canada, the United States, or other parts of the world.

In conclusion, we characterized phylogenetic relationships, chemotype patterns, and aggressiveness among *Fusarium* isolates from different regions, and determined the association between phylogenetic features and/or chemotype patterns with aggressiveness. In addition, we argued differences in pathogen profile and aggressiveness as one of the reasons for different reactions of wheat genotypes in different locations. Our conclusions, however, can only be considered preliminary, due to the relatively small size of the pathogen samples from different locations. The approach used in the present study can be extended to cover more geographical zones with larger sample sizes.

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