Genetic Diversity and Aggressiveness of *Fusarium* spp. Isolated from Canola in Alberta, Canada

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Abstract

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Canola (*Brassica napus*) is one of the most economically important oilseed crops in Canada. Fusarium seedling blight is a root disease with the potential to cause severe yield reductions in canola. *Fusarium* spp. are commonly isolated root pathogens from fields in Alberta. *Fusarium* infection can also cause root rot in adult plants. In this study, 128 isolates identified as *Fusarium* spp. were recovered from field soils in central Alberta and from the roots of diseased canola plants with typical Fusarium seedling blight symptoms. Six species of *Fusarium* were identified, with *Fusarium acuminatum* as the predomi-

nant species (57 of 128 isolates, 44.5%). Phylogenetic analyses based on the translation elongation factor 1- α and the internal transcribed spacer sequence data were used for evaluation of genetic variations, and also used for *Fusarium* spp. identification in combination with morphological characteristics and polymerase chain reaction-based analyses. Based on disease ratings in pathogenicity tests, six isolates of *F. avenaceum* showed high aggressiveness on canola. Also, the aggressiveness varied within all *Fusarium* spp. No correlation was observed between aggressiveness and the geographic origin of the isolates.

Canola (*Brassica napus* L.) is one of the most economically important oilseed crops in Canada, and its production has significantly increased in recent years (22). In Canada, most canola is grown mostly on the prairies, consisting of the provinces of Alberta, Saskatchewan, and Manitoba, where a cool climate and fertile soils provide ideal conditions for growing the crop. Diseases caused by *Fusarium* spp. on canola include Fusarium seedling blight, Fusarium root rot, and Fusarium wilt. Fusarium seedling blight, caused by several species, is one of several seedling diseases in canola (3). Fusarium seedling blight may cause failure of seed to germinate, death of newly emerged seedlings (pre- and post-emergence damping-off), root rot in adult plants, and yield reductions (3). Fusarium wilt of canola, caused by *Fusarium oxysporum* and *F. avenaceum*, can also result in yield loss in susceptible cultivars (4,25). Based on assays of soil samples from Saskatchewan, *Fusarium* spp. were the most common root pathogen in canola fields, followed by *Pythium* and *Rhizoctonia* spp. (22).

Because more than one species of *Fusarium* was isolated from canola (3,22), the identification of the species and the evaluation of their virulence are important. However, the identification and evolutionary history of *Fusarium* spp. is quite complex. Species identification of *Fusarium* is based on morphological characteristics described in the taxonomic systems of Wollenweber and Reinking (44). However, the concept of a species within the genus has varied greatly between broader and narrower concepts (26). In addition, isolates within a particular species may vary considerably, both morphologically (26,36) and genetically (2,10,12,19), which has caused difficulties in species identification (26). However, molecular approaches have become more and more valuable for species identification and evaluation of the genetic variation among

Fusarium isolates. Some *Fusarium* spp. can be identified by using polymerase chain reaction (PCR) analysis with specific primer sets (8,9,41). Studies of the population structure of *Fusarium* spp. by using this molecular approach are also helpful for clarifying the disease etiology and devising effective management strategies.

The translation elongation factor 1- α (EF-1 α) gene sequence has proven to be an excellent phylogenetic marker for resolving inter- and intraspecies relationships within the *Fusarium* species complex. This has led to the creation of a single locus database for species identification, FUSARIUM-ID v. 1.0 (15), which has been updated to a multiple sequence database FUSARIUM-ID (<http://isolate.fusariumdb.org>) at Pennsylvania State University's Department of Plant Pathology, and to the Fusarium MLST (<http://www.cbs.knaw.nl/fusarium>) at the CBS-KNAW Fungal Biodiversity Center (31). At the species level, the internal transcribed spacer (ITS) region of ribosomal (r)DNA is commonly used to identify and detect fungi based on the microbiological molecular phylogeny (6,16,40). The ITS rDNA region has been formally proposed to the Consortium for the Barcode of Life for adoption as the primary fungal barcode marker (37), and has been added as one of the core plant barcodes for land plants (5,7,27). Phylogenetic approaches are commonly used to identify taxonomic units in fungi and are often more effective than morphological methods (37).

In the present study, 128 isolates of *Fusarium* spp. were isolated from canola fields in Alberta. Species identification, genetic diversity, and phylogenetic relationships of these isolates were examined. Pathogenicity tests were also conducted to assess the aggressiveness of the *Fusarium* isolates.

Materials and Methods

Fungal isolation and purification. Soil samples and diseased canola seedlings were collected from a total of 120 canola fields in 10 counties in central Alberta during the summers of 2009, 2010, and 2011 (Table 1). For each soil sample, an aliquot of about 500 ml was placed in a 700-ml plastic cup which was sown with 10 seeds of 'Westar' canola, a cultivar that is susceptible to Fusarium root rot. The plants were grown in a greenhouse at about 25°C, with a 12-h photoperiod. Root tips and stem crowns of the canola seedlings were harvested 2 to 3 weeks after seeding and gently washed under running tap water. The greenhouse-grown canola

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Table 1. Source of *Fusarium* isolates, their species identification, and median disease rating (MDR), mean rank (Ri), and estimated relative effects (pi) of root rot severity of *Fusarium* isolates on canola

Species, isolate	Location	Species identification ^a		MDR	Ri	pi	Confidence interval (95%) for relative treatment effect	
		FaF/FaR	J1AF/J1AR				Lower	Upper
<i>Fusarium avenaceum</i>								
F185	Yellowhead	+	+	3.7	75.8	0.84 (0.009)	0.68	0.91
F168	Wetaskiwin	+	+	3.7	74.8	0.83 (0.009)	0.72	0.89
F058a	Camrose	+	+	3.6	73.8	0.81 (0.009)	0.72	0.88
F087	Ponoka	+	+	3.7	73.8	0.81 (0.009)	0.69	0.89
F169	Wetaskiwin	–	+	3.5	69.8	0.77 (0.009)	0.69	0.83
F085	Parkland	–	+	3.3	66.6	0.73 (0.008)	0.63	0.82
F066	Leduc	–	+	2.0	48.6	0.53 (0.006)	0.50	0.57
F119	Strathcona	+	+	2.0	42.6	0.47 (0.005)	0.39	0.55
F195	Yellowhead	+	+	1.3	36.9	0.40 (0.004)	0.32	0.50
F124	Strathcona	+	+	1.1	31.2	0.34 (0.004)	0.28	0.41
F108	Strathcona	+	+	0.8	26.2	0.29 (0.003)	0.20	0.40
F023	Camrose	+	+	0.9	25.6	0.28 (0.003)	0.21	0.37
F123	Strathcona	+	+	0.6	13.3	0.14 (0.002)	0.09	0.23
F058b	Camrose	+	+	0.4	12.3	0.13 (0.001)	0.07	0.25
F122	Strathcona	+	+	0.4	11.4	0.12 (0.001)	0.06	0.28
<i>F. acuminatum</i>								
F094	Strathcona	+	–	2.6	301.8	0.96 (0.006)	0.91	0.98
F174	Yellowhead	+	–	3.1	327.7	0.95 (0.006)	0.77	0.98
F081	Parkland	+	–	2.4	281.3	0.93 (0.005)	0.88	0.96
F070	Leduc	+	–	2.4	313.1	0.89 (0.005)	0.73	0.96
F140	Thorhild	+	–	2.1	263.1	0.87 (0.005)	0.74	0.94
F116	Strathcona	+	–	2.3	294.8	0.82 (0.005)	0.54	0.95
F193	Yellowhead	+	–	2.1	257.5	0.81 (0.005)	0.65	0.91
F089	Ponoka	+	–	2	263.4	0.79 (0.005)	0.72	0.85
F101	Strathcona	+	–	1.6	224	0.79 (0.005)	0.58	0.91
F030	Camrose	+	–	1.8	220.1	0.77 (0.004)	0.42	0.93
F090	Ponoka	+	–	1.9	242.8	0.75 (0.004)	0.6	0.86
F172	Wetaskiwin	+	–	1.5	215.9	0.74 (0.004)	0.51	0.88
F115	Strathcona	+	–	2	270.6	0.72 (0.004)	0.56	0.84
F134	Thorhild	+	–	1.8	219.2	0.69 (0.004)	0.61	0.77
F083	Parkland	–	–	2	238.9	0.68 (0.004)	0.3	0.91
F061	Leduc	+	–	1.7	214.7	0.67 (0.004)	0.56	0.76
F086	Parkland	+	–	2	252.1	0.67 (0.004)	0.37	0.87
F121	Strathcona	+	–	1.7	229.3	0.67 (0.004)	0.49	0.8
F163	Wetaskiwin	–	–	1.5	184.9	0.66 (0.004)	0.57	0.73
F194	Yellowhead	+	–	1.8	214.8	0.62 (0.004)	0.38	0.8
F192	Yellowhead	+	–	1.8	231.6	0.59 (0.003)	0.4	0.75
F011	Barrhead	+	–	1.3	159.2	0.57 (0.003)	0.29	0.8
F068	Leduc	+	–	1.7	217.2	0.57 (0.003)	0.23	0.85
F095	Strathcona	+	–	1.4	186.8	0.57 (0.003)	0.23	0.85
F002	Barrhead	+	–	1.6	207.7	0.56 (0.003)	0.44	0.68
F150	Sturgeon	+	–	1.6	221.3	0.56 (0.003)	0.42	0.69
F082	Parkland	+	–	1.7	222.6	0.54 (0.003)	0.45	0.63
F141	Sturgeon	+	–	1.2	133.9	0.50 (0.003)	0.26	0.73
F196	Yellowhead	+	–	1.5	190.7	0.50 (0.003)	0.33	0.67
F161	Wetaskiwin	–	–	1.2	141.8	0.49 (0.003)	0.3	0.67
F147	Sturgeon	–	–	1.4	148.9	0.48 (0.003)	0.42	0.54
F151	Sturgeon	+	–	1.5	177.8	0.47 (0.003)	0.23	0.73
F133	Thorhild	–	–	1	115.1	0.46 (0.003)	0.24	0.69
F099	Strathcona	+	–	0.8	133.8	0.44 (0.003)	0.11	0.84
F142	Sturgeon	–	–	1.8	201.6	0.44 (0.003)	0.09	0.87
F084	Parkland	+	–	1.5	180.2	0.41 (0.002)	0.17	0.7
F143	Sturgeon	+	–	1.3	160.6	0.41 (0.002)	0.27	0.57
F114	Strathcona	+	–	0.9	104.8	0.40 (0.002)	0.19	0.65
F049	Camrose	+	–	0.6	79.2	0.36 (0.002)	0.13	0.68
F109	Strathcona	–	–	1.3	155.2	0.36 (0.002)	0.19	0.58
F102	Strathcona	+	–	1.1	113.6	0.34 (0.002)	0.24	0.46
F037	Camrose	+	–	0.9	84.8	0.32 (0.002)	0.24	0.41
F078	Parkland	–	–	1.2	136.8	0.31 (0.002)	0.16	0.52
F034	Camrose	+	–	0.9	90.3	0.26 (0.002)	0.15	0.42
F092	Strathcona	+	–	1	99.1	0.26 (0.002)	0.14	0.45
F026	Camrose	+	–	1	97	0.24 (0.001)	0.16	0.35
F053	Camrose	+	–	1.2	123.5	0.23 (0.001)	0.1	0.45
F031	Camrose	+	–	0.8	64.6	0.22 (0.001)	0.13	0.35
F048	Camrose	+	–	0.7	83.2	0.21 (0.001)	0.07	0.51
F050	Camrose	+	–	0.5	45.1	0.20 (0.001)	0.08	0.43
F051	Camrose	+	–	0.9	91.8	0.19 (0.001)	0.07	0.46
F036	Camrose	+	–	1	97.4	0.14 (0.001)	0.09	0.22

(continued on next page)

^a Results of polymerase chain reaction-based assays with both primer sets; + = target band observed and – = target band absent.

tissues and the diseased samples from the canola fields were surface sterilized with 1% bleach for 30 s and washed under running tap water. All the tissues were placed on fresh Difco potato-dextrose agar (PDA) plates (Becton, Dickinson and Company) for 2 days at room temperature with 12-h lighting,

then transferred to peptone-pentachloronitrobenzene medium (29) and incubated for 10 to 14 days with 12 h lighting at room temperature. Single-conidium isolates were established and cultured on PDA for 13 to 15 days at room temperature and stored at 4°C for later use (26).

Table 1. (continued from preceding page)

Species, isolate	Location	Species identification ^a		MDR	Ri	pi	Confidence interval (95%) for relative treatment effect	
		FaF/FaR	J1AF/J1AR				Lower	Upper
F056	Camrose	+	–	0.4	29.4	0.14 (0.001)	0.08	0.23
F097	Strathcona	+	–	0.5	64.3	0.11 (0.001)	0.03	0.34
F047	Camrose	+	–	0.6	48.3	0.07 (0.000)	0.04	0.13
F052	Camrose	+	–	0.4	45.8	0.07 (0.000)	0.03	0.19
F027	Camrose	+	–	0.5	24.5	0.05 (0.000)	0.02	0.15
<i>F. culmorum</i>								
F001	Barrhead	–	–	1.5	25.8	0.70 (0.020)	0.50	0.83
F167	Wetaskiwin	–	–	1.3	25.2	0.69 (0.019)	0.49	0.81
F175	Yellowhead	–	–	1.2	24.8	0.67 (0.019)	0.49	0.80
F135	Thorhild	–	–	0.7	16.1	0.43 (0.012)	0.30	0.58
F014	Barrhead	–	–	0.4	10.8	0.28 (0.008)	0.17	0.47
F054	Camrose	–	–	0.2	8.4	0.22 (0.006)	0.12	0.47
<i>F. equiseti</i>								
F004	Barrhead	–	–	1.9	118.6	0.94 (0.007)	0.86	0.96
F139	Thorhild	–	–	1.4	99.1	0.78 (0.006)	0.63	0.88
F179	Yellowhead	–	–	1.1	90.8	0.72 (0.006)	0.60	0.81
F155	Wetaskiwin	–	–	1.2	87.1	0.69 (0.005)	0.45	0.85
F006	Barrhead	–	–	0.9	81.9	0.65 (0.005)	0.44	0.80
F069	Leduc	–	–	1.3	79.9	0.63 (0.005)	0.34	0.84
F103	Strathcona	–	–	0.9	77.3	0.61 (0.005)	0.52	0.69
F003	Barrhead	–	–	0.9	71.3	0.56 (0.004)	0.34	0.76
F106	Strathcona	–	–	0.9	71.2	0.56 (0.004)	0.34	0.76
F100	Strathcona	–	–	0.8	68.6	0.54 (0.004)	0.39	0.68
F020	Barrhead	–	–	0.7	67.4	0.53 (0.004)	0.36	0.70
F044	Camrose	–	–	0.6	63.8	0.50 (0.004)	0.31	0.69
F045	Camrose	–	–	0.7	58.3	0.46 (0.004)	0.29	0.64
F007	Barrhead	–	–	0.5	49.5	0.39 (0.003)	0.22	0.60
F041	Camrose	–	–	0.6	45.6	0.36 (0.003)	0.22	0.53
F018	Barrhead	–	–	0.6	44.9	0.35 (0.003)	0.21	0.53
F075	Parkland	–	–	0.5	42.5	0.33 (0.003)	0.16	0.57
F118	Strathcona	–	–	0.4	39.9	0.31 (0.002)	0.16	0.54
F015	Barrhead	–	–	0.3	31.3	0.24 (0.002)	0.13	0.42
F012	Barrhead	–	–	0.0	24.2	0.19 (0.001)	0.06	0.54
F019	Barrhead	–	–	0.2	20.3	0.16 (0.001)	0.11	0.23
<i>F. torulosum</i>								
F186	Yellowhead	–	–	1.7	156.9	0.97 (0.012)	0.94	0.98
F137	Thorhild	–	–	1.0	106.7	0.86 (0.010)	0.75	0.92
F064	Leduc	–	–	1.1	112.3	0.85 (0.010)	0.65	0.94
F184	Yellowhead	–	–	1.2	123.9	0.85 (0.010)	0.65	0.94
F191	Yellowhead	–	–	1.2	115.6	0.84 (0.010)	0.76	0.90
F190	Yellowhead	–	–	1.5	144.8	0.82 (0.010)	0.69	0.90
F180	Yellowhead	–	–	1.3	117.8	0.77 (0.009)	0.62	0.86
F131	Thorhild	–	–	0.3	53.8	0.75 (0.009)	0.57	0.87
F166	Wetaskiwin	–	–	1.3	120.2	0.71 (0.008)	0.63	0.78
F110	Strathcona	–	–	1.1	114.3	0.69 (0.008)	0.59	0.78
F197	Yellowhead	–	–	1.2	120.9	0.69 (0.008)	0.60	0.77
F162	Wetaskiwin	–	–	0.9	94.6	0.56 (0.007)	0.47	0.64
F164	Wetaskiwin	–	–	1.0	104.1	0.56 (0.007)	0.47	0.64
F024	Camrose	–	–	0.4	44.0	0.53 (0.006)	0.47	0.58
F145	Sturgeon	–	–	0.8	71.6	0.47 (0.006)	0.38	0.56
F149	Sturgeon	–	–	0.6	44.2	0.44 (0.005)	0.38	0.50
F153	Sturgeon	–	–	0.8	69.4	0.41 (0.005)	0.35	0.47
F021	Camrose	–	–	0.6	56.5	0.39 (0.005)	0.32	0.47
F176	Yellowhead	–	–	0.7	71.7	0.37 (0.004)	0.28	0.48
F105	Strathcona	–	–	0.7	67.4	0.30 (0.004)	0.24	0.37
F157	Wetaskiwin	–	–	0.6	52.0	0.23 (0.003)	0.17	0.31
F148	Sturgeon	–	–	0.5	48.3	0.19 (0.002)	0.13	0.27
F187	Yellowhead	–	–	0.6	46.1	0.19 (0.002)	0.13	0.27
F076	Parkland	–	–	0.6	49.8	0.16 (0.002)	0.09	0.30
F146	Sturgeon	–	–	0.6	52.0	0.16 (0.002)	0.09	0.30
F171	Wetaskiwin	–	–	0.6	57.0	0.13 (0.002)	0.08	0.19
F065	Leduc	–	–	1.2	95.5	0.08 (0.001)	0.04	0.18
F107	Strathcona	–	–	0.4	33.8	0.02 (0.000)	0.02	0.06
<i>F. redolens</i>								
F125	Strathcona	–	–	–	–	–	–	–

DNA extraction, PCR amplification, sequencing, and identification of species. Prior to DNA extraction, all isolates were subcultured on PDA for 10 to 14 days at room temperature. Approximately 25 mg of mycelium was collected from a PDA plate of each isolate with a sterilized toothpick for fungal genomic DNA extraction using the modified rapid mini-preparation method of Feng et al. (13). The DNA concentration and quality was estimated using a Thermo Scientific NanoDrop 1000 Spectrophotometer (Fisher Scientific) with the default set for DNA assays. In total, 128 isolates were tested by PCR using two *F. avenaceum*-specific primer pairs, J1AF/J1AR (41) and FaF/FaR (9) (Table 2). The PCR reactions were amplified in a final volume of 15 µl, which contained 1× PCR buffer (1.5 mM MgCl₂ and 125 µM each dNTP), 250 nM each primer, 0.5 unit of GoTaq polymerase (Promega Corp.), and 25 to 60 ng of genomic DNA. Thermocycler conditions consisted of an initial heat denaturation step at 95°C for 3 min; 38 cycles of 30 s at 95°C, 30 s at 58°C (60°C for FaF/FaR), and 40 s at 72°C; followed by a final elongation step of 5 min at 72°C. The amplicons obtained were mixed with a safegreen loading dye (Applied Biological Materials) and separated on a 1.2% agarose gel in 1× Tris-acetate-EDTA buffer at 100 V for 1.2 h. The gels were visualized under UV light with a Gel DocTM XR System (Bio-Rad).

The translation EF-1 α and the ITS region of 128 isolates were amplified with the primer sets EF-1/EF-2 (32) and ITS4/ITS5 (43) (Table 2), respectively, in a final reaction volume of 30 µl. Each amplification reaction mixture contained 1× PCR buffer (1.5 mM MgCl₂ and 125 µM each dNTP), 250 nM each forward and reverse primer, 0.5 unit of GoTaq polymerase (Promega Corp.), and 25 to 60 ng of genomic DNA. The PCR reactions were performed with an initial denaturation at 95°C for 5 min; followed by 38 cycles of 30 s at 94°C, 30 s at 52°C, and 45 s (60 s for ef1/ef2) at 72°C; and a final elongation step of 5 min at 72°C (Table 2). PCR products were purified with a Wizard SV Gel and PCR clean-up system (Promega Corp.) for DNA sequencing with BigDye terminator reagent (Applied Biosystems) following the manufacturer's instructions. All purified PCR products were sequenced at the University of Calgary. The DNA sequences obtained were manually adjusted and aligned using ClustalW embedded in the BioEdit v7.1.3 (17).

Based on the phylogenetic analysis described below, 1 *F. redolens* isolate from clade I, 3 isolates from clades II to V (Fig. 1), and 16 isolates from clade VI were characterized morphologically after 7 to 10 days of growth on 60-by-15-mm PDA and 14 to 21 days on carnation leaf-piece agar (CLA) with 12 h of lighting at room temperature (16). A volume of 20 µl of 30% glycerine was pipetted onto the CLA surface, and spores were then harvested to make slides for examination under a Zeiss AxioImager M1 fluorescent microscope (Carl Zeiss). Digital images of conidia were taken under the ×40 objective lens, and the length and width of 20 conidia of selected isolates of each species were estimated with the aid of image analysis software AxioVision v4.8.2.

The ITS and EF-1 α DNA sequences of each isolate from each phylogenetic clade were aligned by means of the BLASTn algorithm at the National Center for Biotechnology Information web-

site and the *Fusarium*-specific database (FUSARIUM-ID) to identify species using 97 to 100% sequence identity as the threshold to identify isolates to their corresponding species. If an isolate had 97 to 100% sequence identity to more than one known species, further species identification was performed by employing the PCR-based assays, phylogenetic analyses, and conidial characterization. All species identification was based on a combination of molecular phylogenetic results and morphological characterizations.

Phylogenetic analysis. All of the obtained sequences used for phylogenetic analyses were deposited in GenBank (accession number JX534245-500). The sequence characters were weighted equally in all phylogenetic analyses.

Bayesian inference (BI) was applied to estimate phylogenetic relationships among isolates using MrBayes v3.1.2 (20,35), with *F. oxysporum* as an outgroup. Prior to BI analysis, the best-fit nucleotide substitution model was selected using the program ModelTest 3.7 (34). BI was performed with four chains (one cold and three heated under default heating values) in each of two parallel runs, with each chain starting with a random tree, and subsequently run for 2,000,000 generations. Trees were sampled every 100 generations. Based on the 40,002 trees sampled, a consensus tree was calculated after omitting the first 25% of the trees as burn in.

Maximum parsimony (MP) analysis was performed with PAUP v 4.0b10 (39). Gaps were treated as a "fifth base" because the sequence gaps generally provide significant phylogenetic signals. The heuristic searches were conducted using tree-bisection-reconnection branch-swapping with 200 random additional replicates, with unordered character option (100 random replications, starting seed 678756052 for ITS and 290116450 for EF-1 α sequences). Bootstrap support values (11) were calculated from the 200 replicate analyses, using a single random addition replication per bootstrap resampling. All phylogenetic trees were graphically edited with FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>) to produce figures.

Pathogenicity tests. The 128 isolates of *Fusarium* were tested for their pathogenicity on canola '45H29' (Pioneer Hi-Bred), a widely grown cultivar in Alberta and susceptible to *Fusarium* spp. For each single-conidium-derived isolate, two 8-mm-diameter agar plugs with live mycelium were cut and transferred to two separate PDA plates and incubated at room temperature under 12 h of ambient room lighting for 13 to 15 days. The two PDA plates fully covered with mycelium (30 ml of propagulae, in total, for each isolate) were cut into pieces of approximately 1 to 2 mm² and suspended in about 90 ml of distilled water. A 20-ml aliquot of the suspension was evenly added to the surface of a 13-cm-diameter plastic cup filled with 500 ml of sterilized Promix PGX soil (Evergro Canada Inc.). Ten seeds of 45H29 canola were sown on the top of each plastic cup, covered with 40 ml of the same sterilized Promix PGX soil, and grown at about 25°C under a 12-h photoperiod in a greenhouse. Two PDA plates without any fungal colonization were suspended in distilled water and inoculated onto the plants as previously described to serve as the negative control. For each isolate, six replications (cups) were inoculated. Cups were placed randomly following a randomized complete block design.

When sunken brown lesions were observed on the basal stem, canola plants were collected and washed under tap water 20 days after inoculation. The washed roots were assessed for presence and severity of root rot symptoms on a 0-to-4 scale according to Hwang et al. (22), where 0 = no symptoms, 1 = light symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions on the stem and diminished plant vigor, and 4 = stem rotten and plant dead. The pathogenicity test of all isolates on canola 45H29 was repeated once under the same greenhouse conditions. A nonparametric marginal effects analysis was performed to determine the root rot severity within each species using an SAS macro (available for download at http://www.ams.med.uni-goettingen.de/makros/run_npar.html). The datasets consisted of the following variables: isolate, severity rating, and subject (a unique identifier that is needed for calculating confidence intervals; 38). Within each dataset, severity was ranked within each isolate, and

Table 2. Primers used for polymerase chain reaction and DNA sequencing in this study

Primer	Sequence (5'–3')	Temp (°C) ^a	Time (s) ^b
ITS 5	GGAAGTAAAAGTCGTAACAAGG	52	45
ITS 4	TCCTCCGCTTATGATATGC	...	...
EF-1	ATGGGTAAAGGA(A/G)GACAAGAC	52	60
EF-2	GGA(G/A)GTACCACT(G/C)ATCATGTT	...	...
J1AF	GCTAATTCTTAACCTACTAGGGGCC	60	45
J1AR	CTGTAATAGGTTATTACATGGGCG	...	...
FaF	CAAGCATTGTCGCCACTCTC	58	45
FaR	GTTTGGCTCTACCGGACTG	...	...

^a Annealing temperature.

^b Extension time.

analysis of variance of the ranks was conducted using the Proc Mixed option. The disease severity of each isolate on canola was represented by the estimated relative effects. Median and mean rank values for each isolate within the species were calculated and isolate with π values outside the confidence interval for an isolate were deemed significantly different from that isolate.

Results

Phylogenetic analysis. In total, 128 *Fusarium* isolates were obtained from the 120 commercial fields in central Alberta in the summers of 2009, 2010, and 2011 (Table 1). The EF-1 α fragments from the 128 isolates ranged from 595 to 653 bp and the number of

Fig. 1. Bayesian inference tree based on elongation factor 1- α sequence data. Numbers above the branches indicate the posterior probabilities for each cluster. Phylogenetic clades are given based on the species identification. Clade I: *Fusarium redolens*, clade II: *F. culmorum*, clade III: *F. equiseti*, clade IVa and clade IVb: *F. acuminatum*, clade V: *F. avenaceum*, and clade VI: *F. torulosum*.

sequence characters in the aligned sequence matrix was 680. Nucleotide frequencies were A = 0.2354, C = 0.3034, G = 0.2003, and T = 0.2609. After alignment with the BioEdit program, the data optimal nucleotide substitution model was applied to the data matrix. The best-fit model (HKY+G) was selected using the program ModelTest.

The ITS fragments from the 128 isolates ranged from 469 to 527 bp and the number of the sequence characters in the aligned sequence matrix was 531. The variation in length largely resulted from indels among the different species. Nucleotide frequencies were A = 0.2697, C = 0.2645, G = 0.2305, and T = 0.2353. After alignment with the BioEdit program, the data optimal nucleotide

Fig. 2. Maximum parsimony consensus tree based on elongation factor 1-α sequence data. Numbers above the branches indicate bootstrap support (percentage) for each cluster.

substitution model was applied to the data matrix. The best-fit model (TrNef+G) was selected using the program ModelTest.

For MP analyses of the EF-1 α sequence data, 270 characters were parsimony-informative, 329 characters were constant, and 81 variable characters were parsimony-uninformative. The outgroup-rooted major consensus tree is shown in Figure 2. For MP analyses

of the ITS sequence data, 130 characters were parsimony-informative, 382 characters were constant, and 19 variable characters were parsimony-uninformative. The outgroup-rooted major consensus tree is shown in Figure 3.

Both the MP and BI analyses of the EF-1 α and ITS sequences yielded the same results for phylogenetic clades I, II, and III (Figs.

Fig. 3. Maximum parsimony consensus tree based on internal transcribed spacer sequence data. Numbers above the branches indicate bootstrap support (percentage) for each cluster.

1–4). Isolate F125 was the only member of clade I (phylogenetic branch highlighted in green); isolates F001, F167, F054, F135, F014, and F175 were clustered together in clade II (highlighted in red); and 21 isolates were clustered in clade III (highlighted in light blue) in all of the BI and MP trees. However, different tree topologies were observed among the BI and MP trees of the remaining 100 isolates of clades IV, V, and VI (Figs. 1–4).

Among the remaining 100 isolates, based on the EF-1 α sequence data, 54 isolates were clustered in clade IV-a of the BI tree, and 53 of these isolates were clustered together (branches highlighted as dark blue) in the MP tree (Figs. 1 and 2). Based on the ITS sequence data, 56 isolates were clustered together (55 dark blue branches and 1 pink branch) in the BI tree but 12 more isolates (11 black branches and 1 dark blue branch) were clustered

Fig. 4. Bayesian inference tree based on internal transcribed spacer sequence data. Numbers above the branches indicate posterior probabilities for each cluster.

together in the MP tree (Figs. 3 and 4). In the BI tree generated with the EF-1 α data, isolates F089 and F150 were included in clade IV-a but isolates F124, F142, F174, and F196 were not.

For the clade V, based on the EF-1 α sequence, 15 isolates (highlighted in pink) were clustered together in the BI tree but isolate F122 was not clustered together with the other 14 isolates in the MP tree (Figs. 1 and 2).

The phylogenetic relationships of the remaining isolates were complex because their topological positions were different in the phylogenetic trees (Figs. 1–4). Based on the EF-1 α sequence data, the isolates formed four subclades on the BI tree (Fig. 1) but only two subclades on the MP tree (Fig. 2). Based on the ITS sequence data, two subclades were formed on both the BI and MP trees.

Species identification. Two sets of *F. avenaceum*-specific primers, FaF/FaR and J1AF/J1AR, were used for PCR-based identification of all 128 isolates. Amplicons of the target fragments were obtained from 15 isolates (11.7% of the total number) with the primer pair J1AF/J1AR and from 61 isolates (47.7% of the total number) with primer pair FaF/FaR (Table 2). Those 15 isolates, which produced the target fragments with the primers J1AF/J1AR, were clustered to a clade with 0.99 posterior probabilities in the BI tree based on the EF-1 α sequence data. Isolate F122 was excluded from this clade in the MP tree, because its EF-1 α sequence information was not readable in the MP tree construction. However, F122 clustered into the same clade with other *F. avenaceum* isolates in both the BI and MP trees based on the ITS data, with strong support of its phylogenetic position in the *F. avenaceum* clade (clade V; the posterior probability was 0.84 in the BI tree and the bootstrap value was 53% in the MP tree).

There were three more isolates (F142, F174, and F196) which had a close relationship to the 15 *F. avenaceum* isolates, with a high posterior probability value, which could be clustered into one clade. However, no target bands were obtained from these three isolates with the primers J1AF/J1AR. They clustered together with other isolates on the BI tree based on the ITS data. And, because macroconidia of these three isolates were wider and much shorter than expected for *F. avenaceum*, F142, F174, and F196 did not match the description for this species. The macroconidia of the 15 isolates described above were very long and slender, with a thin wall and slightly curved appearance. The average size of the mac-

roconidia was 65 by 3.5 μ m. The apical cell was long and tapering to a point and the basal cell was notched (Fig. 5d). Three to five septa were observed. No microconidia were seen in these isolates (Fig. 5d). Based on the phylogenetic analyses and the conidial characteristics, those 15 isolates (11.7% of the total number of isolates) that produced the target fragment with primer set J1AF/J1AR were identified as *F. avenaceum*. Based on a comparison of the phylogenetic trees and megablast results of the EF-1 α and ITS sequence data with the nucleotide collection database of GenBank, only one isolate, F125, was identified as *F. redolens* (Fig. 5c), with 100% sequence identity to the published EF-1 α sequences of *F. redolens* (GenBank accession number HQ731063; 0.8% of the total number of isolates) (25); six isolates (F001, F167, F054, F135, F014, and F175) were identified as *F. culmorum* (Fig. 5a), with 99 to 100% sequence identity to the published EF-1 α sequences of *F. culmorum* (GenBank accession numbers JF278585-90 and GU370469-70; 4.7%) (30); and 21 isolates (F003, F015, F139, F179, F004, F103, F012, F019, F006, F075, F020, F045, F044, F118, F007, F106, F018, F041, F100, F069, and F155) were identified as *F. equiseti* species complex (Fig. 5b), with 96 to 99% sequence identity (16.4%; GenBank accession numbers HQ702572, FJ939679-80, and GQ505599). The species identification results based on phylogenetic relationship were confirmed for these three species by the shapes and sizes of their macroconidia, together with the characteristics of the microconidia on CLA plates (26; Fig. 5b).

Isolate F125 (*F. redolens*) produced robust and thick-walled macroconidia (Fig. 5c), with average dimensions of 37 by 5.4 μ m, and microconidia with dimensions of 9.2 by 4.2 μ m. The widest part was at the upper third of the macroconidia and each had a hooked apical cell and slightly foot-shaped basal cell. The macroconidia of *F. culmorum* isolates were robust, relatively short, quite wide relative to their length and thick walled, with an average size of 37 by 6.8 μ m for macroconidia (Fig. 5a). The widest part was at the midpoint of the macroconidium, and it had a rounded and blunt apical cell. The basal cell was notched and without a distinct foot shape. No microconidia were observed for this species.

The isolates of *F. equiseti* had long and slender macroconidia with a similar shape and size, and usually had five septa. The average dimensions of the macroconidia were 55 by 4.4 μ m. The apical

Fig. 5. Microscope images of conidia from representative isolates of identified species. The scale bar corresponds to 10 μ m. **a**, Macroconidia of *Fusarium culmorum* (F001); **b**, macroconidia of *F. equiseti* (F106); **c**, macroconidia and microconidia of *F. redolens* (F125); **d**, macroconidia of *F. avenaceum* (F087); **e**, macroconidia and microconidia of *F. acuminatum* (F194); and **f**, macroconidia of *F. torulosum* (F021).

cell was tapered and elongated and the basal cell had an obvious foot-shape (Fig. 5b).

The 54 isolates of clade IV-a (Fig. 1) were clustered together in all phylogenetic trees. Three more isolates of clade IV-b were clustered together with the above 54 isolates based on the ITS sequences (Figs. 3 and 4) and were not identified as *F. avenaceum* based on PCR assay results or conidial characteristics; therefore, these 57 isolates (44.5% of the total) were identified as *F. acuminatum*, a designation supported by the blast results obtained with the EF-1 α and ITS sequence data. Moreover, this designation was confirmed by the conidial characteristics of these isolates. The macroconidia were thick walled and the curvature of the conidia was moderately equal. The average size of the macroconidia was 52 by 3.9 μ m. Their basal cells had a distinct foot shape. The apical cell tapered and was slightly elongated (Fig. 5e). Sparse microconidia were observed in cultures of a few of isolates.

For the remaining 28 isolates, species identification was more difficult and complex. Based on blast results of the EF-1 α sequence of isolate F076 in the Fusarium-ID and Fusarium MLST databases, the most closely matched species were *F. negundis* and *F. tricinctum*, with 96.1 and 96.8% identity, respectively. Based on the ITS sequence of F076, the most closely matched species were *F. flocciferum* and *F. tricinctum*, with 99.8% identity in the Fusarium-ID database, and *F. tricinctum*, with 100% identity in the Fusarium MLST database. Based on the blast results of the EF-1 α and ITS sequence of isolate F076 against the nucleotide collection database of GenBank (blastn suite), the most closely matched species were *F. flocciferum* (KC999486 and AJ543574), *F. torulosum* (KC999484-5), *F. tricinctum* (HQ702589, KF381076, and GQ922561), and *F. avenaceum* (EU255800-04), all with 96 to 100% identity. For isolate F171, the most closely matched species were *F. flocciferum* (JX397824-25) and *F. reticulatum* (DQ854864), with 99 and 97% identity, respectively, based on the EF-1 α sequence; and *F. flocciferum* (GQ505465) and *F. tricinctum* (JF273496,) with 100% identity based on the ITS sequence blast result against the GenBank database. These 28 isolates was assigned to four species—*F. reticulatum*, *F. flocciferum*, *F. tricinctum*, or *F. torulosum*—based on the blast results for each isolate (above 98% identity to the reference sequence; data not shown). Morphological examination revealed that the characteristics of the macroconidia were similar for all of the 28 isolates. Macroconidia with three to five septa were common for most of the isolates. The average size of the macroconidia was 36 by 4.6 μ m. The basal cells had a slight foot shape and microconidia were absent or very rare (Fig. 5f). Therefore, they could not be *F. tricinctum* because *F. tricinctum* produces abundant microconidia on its aerial mycelia (26). *F. reticulatum* is a common synonym of *F. heterosporum* Nees & T. Nees but the shape of the macroconidia of *F. heterosporum* is slender and straight or gently curved, which was different from that of the 28 isolates. These 28 isolates were identified as belonging to the *F. torulosum* species complex, because the conidial characteristics closely matched the species description. The macroconidia of *F. torulosum* are comparatively short and falcate with pointed apical cell and foot-shaped basal cell and three to five septa, and microconidia are absent or very rare (26).

Pathogenicity tests. Sunken brown lesions were observed on the stems of diseased canola plants 20 days after inoculation. Based on the results from the nonparametric marginal effects analysis of the severity values in the pathogenicity tests, aggressiveness of *Fusarium* isolates varied in all species (Table 1). Median disease rating of isolates were 0.4 to 3.7 in *F. avenaceum*, 0.4 to 3.1 in *F. acuminatum*, 0.2 to 1.5 in *F. culmorum*, 0.0 to 1.9 in *F. equiseti*, 0.3 to 1.7 in *F. torulosum*, and 0.7 in *F. redolens*. In *F. avenaceum*, three pathogenicity groups could be distinguished, based on aggressiveness on canola. F058a, F085, F087, F168, F169, and F185 were highly aggressive on canola (median disease rating of 3.3 to 3.7, estimated relative effect of 66.6 to 75.8), while F023, F058b, F108, F122, and F123 were less aggressive on canola (median disease rating of 0.4 to 0.9, estimated relative effect of 11.4 to 26.2). The other four isolates showed moderate aggressiveness on

canola. In *F. culmorum*, three isolates (F001, F167, and F175) showed higher aggressiveness, while two isolates (F014 and F054) were less aggressive on canola. However, for the other species, the aggressiveness on canola varied among the isolates. In *F. acuminatum*, one isolate (F174) showed significantly higher aggressiveness on canola. Within *F. torulosum*, F186 showed significantly higher aggressiveness on canola. In *F. equiseti*, F004 was the most highly aggressive on canola compared with other isolates in this species. There was no correlation between disease severity and locations in any species.

Discussion

Fusarium seedling blight, caused by *Fusarium* spp., is one of several seedling diseases in canola (3). The pathogens can cause damping off and root rot (21). Fusarium wilt, caused by *F. oxysporum* and *F. avenaceum*, has emerged as a disease of canola in recent years (25). The symptoms of Fusarium wilt include yellowing, wilting, and dark-brown or black discoloration of the vascular tissue on one side of the stem (4,25). These symptoms are distinct from those associated with Fusarium seedling blight and root rot of canola. In the current study, 15 isolates of *F. avenaceum* were identified from root tissues showing typical symptoms of root rot but no isolates of *F. oxysporum* were collected. In all, 57 (44.5%) of 128 *Fusarium* isolates were classified as *F. acuminatum*, which was the predominant *Fusarium* sp. isolated from the sampled canola fields. These results suggest that Fusarium seedling blight and root rot may now be more important diseases of canola in Alberta than Fusarium wilt, which could reflect the widespread cropping of canola cultivars with effective resistance against the latter.

The composition of *Fusarium* spp. collected from canola in the current study differs from that found in other crops in recent reports; *F. avenaceum* was identified as the primary causal pathogen of root rot on pea (*Pisum sativum*) and lupin (*Lupinus angustifolius*) in Alberta (12,19). High levels of genotypic diversity were detected among the *F. avenaceum* isolates from lupin fields in central Alberta (19). For the 15 isolates of *F. avenaceum* in the current study, genetic diversity was relatively low and this species was not the predominant *Fusarium* pathogen of canola in central Alberta, although eight of the *F. avenaceum* isolates were among the most highly aggressiveness on canola. Target bands were obtained only from these 15 isolates with the primer set J1AF/J1AR. However, obvious target bands were also obtained from 12 isolates of *F. avenaceum* and 26 isolates of *F. acuminatum* with the primer set FaF/FaR. The results of PCR-based assays indicated that the species concept of *F. avenaceum* for primer set J1AF/J1AR was much stricter and narrower than that of primer set FaF/FaR.

For species identification, a clear phylogenetic relationship was shown to exist for *F. redolens*, *F. culmorum*, and *F. equiseti*, and similar but distinct topologic structures were constructed for *F. avenaceum*, *F. acuminatum*, and *F. torulosum* in the phylogenetic trees based on the EF-1 α and ITS sequences. Phylogenetic analysis of multilocus sequence data using BI and MP analysis showed that *F. culmorum* from wheat is a single phylogenetic species, with no significant linkage disequilibrium and little or no lineage development related to geographic origin (30). The EF-1 α sequence of *F. redolens* isolate F125 had a 100% sequence identity to the *F. redolens* isolate 34.92 (HQ731063), which was isolated from chickpea (*Cicer arietinum*) in Spain (25) and an isolate of *F. redolens* from Italy (accession number EU281660), indicating that genetic diversity might be low for this species. The isolates of *F. equiseti* were clustered to a distinct clade in all of the phylogenetic trees, which coincides with results of research on human *Fusarium* pathogens (33). The phylogenetic relationships for species of *F. avenaceum*, *F. acuminatum*, and *F. torulosum* were very close, especially in the phylogenetic trees based on the ITS sequences, but they could be differentiated from each other by the phylogenetic trees based on the EF-1 α sequences as well as by their conidial characteristics. However, the reverse situation existed between *F. redolens* and *F. oxysporum* f. sp. *ciceris*. *F. redolens* is not easily differentiated from *F. oxysporum* f. sp. *ciceris* using morphology-based diagnosis

but molecular protocols helped to avoid misdiagnoses in chickpea (25). Both molecular phylogenetic analyses and morphological characterization were used for all of the isolate identifications in this study to avoid any misdiagnoses.

The ITS region is a primary fungal barcode marker (37) and EF-1 α is an excellent phylogenetic marker for resolving phylogenetic relationships within the *Fusarium* genus (15). Both DNA regions are good DNA barcode markers for the genus *Fusarium*, and their universality is suggested by the observation that DNA fragments from both regions could be successfully amplified and sequenced from all of the *Fusarium* isolates in this study. Within the most recently updated database FUSARIUM-ID, portions of the EF-1 α , RPB2, ITS + LSU rDNA, and RPB1 genes were used for species identification (31). However, it is not necessary for all of these genes to be used for species identification or the analysis of phylogenetic relationships. The core barcode system has been separately standardized for animals, land plants, and fungi, and generally consists of less than three DNA regions (7,18,37). In MP analyses of this study, 270 parsimony-informative characters were found for the EF-1 α sequence, which were higher than the 130 parsimony-informative characters for the ITS sequence. Additionally, *F. avenaceum*, *F. acuminatum*, and *F. torulosum* could be differentiated from each other based on phylogenetic trees of EF-1 α sequences but not based on the ITS sequences (Figs. 1–4). This shows that the EF-1 α sequence could provide more phylogenetic information than the ITS region within the *Fusarium* genus, which agreed with the results of Watanabe et al. (42).

For *F. avenaceum* identification, two specific primer sets, FaF/FaR and J1AF/J1AR, were used and combined with the phylogenetic approach from ITS and EF-1 α sequences and morphological characteristics. The results of PCR assays using two primer sets were distinct from each other. Only 15 isolates produced target bands with J1AF/J1AR but 61 isolates had positive amplification with FaF/FaR. The primer set J1AF/J1AR was developed to detect *F. avenaceum*, with *F. tricinctum*, *F. culmorum*, *F. graminearum*, and *F. poae* as some of the reference species (41). *F. avenaceum* and *F. tricinctum* have similar colony morphologies on PDA but can be distinguished by the shape of their macroconidia, which are falcate in the former and almost lunate in the latter (26). ITS restriction fragment length polymorphism marker data also revealed a close relationship between *F. avenaceum* and *F. tricinctum* (41). The primer set FaF/FaR was developed to detect *F. avenaceum* using *F. culmorum*, *F. poae*, *F. graminearum*, and some other species as reference; however, no closely related species such as *F. tricinctum* or *F. acuminatum* were used (9). Based on the results of this study, the primer set FaF/FaR could not distinguish *F. avenaceum* from *F. acuminatum*; therefore, it was not reliable for identification of *F. avenaceum* in the present study. Additionally, with the same primer set, PCR conditions such as the composition of the PCR reaction mixture and the amplification parameters also affected the results. Using the DNA sequence of a specific gene region could avoid this problem. This is the reason for DNA barcoding, an approach to identify species based on sequences from a short, standardized DNA region, which was developed for the identification of organisms (5,7,18,37). In this study, 128 isolates belonging to six species of *Fusarium* were tested for aggressiveness on canola. Among them, only one isolate belonged to *F. redolens*. Light disease symptoms were shown and could be rated as 0.68 (average data from different replicates). In total, 57 isolates were identified as *F. acuminatum*, which was the predominant species in the sampled canola fields in central Alberta. However, their aggressiveness on canola varied. The second most predominant species was *F. torulosum*, with 28 isolates. One isolate was more highly aggressive on canola compared with the other isolates. *F. avenaceum* was one of the pathogens causing seedling blight and root rot (4,21). However, 15 isolates were identified in the present study. Six isolates (40% of the species) showed an extremely high aggressiveness on canola, with disease ratings of 3.3 to 3.7. However, five of the isolates (F023, F058b, F108, F122, and F123) were much less aggressive on canola (disease ratings of 0.4 to 0.9).

This indicated that there were variations of aggressiveness within the species.

Most of the previous evaluations of genetic diversity and aggressiveness of *Fusarium* spp. were focused on only a single species (1,12,19,24,28). The current study attempted an examination of composition of the *Fusarium* pathogen complex on canola in central Alberta, and revealed the presence of at least six *Fusarium* spp. associated with seedling blight or root rot of canola. Although different species showed different levels of aggressiveness on canola, some isolates belonging to *F. avenaceum* were highly aggressive. However, among these species, the number of *F. acuminatum* isolates indicated that it was the predominant.

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