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Virulence of *Fusarium oxysporum* and *F. commune* to Douglas-fir (*Pseudotsuga menziesii*) seedlings

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Summary

Fusarium species can cause damping-off and root rot of young conifer seedlings, resulting in severe crop and economic losses in forest nurseries. Disease control within tree nurseries is difficult because of the inability to characterize and quantify *Fusarium* spp. populations with regard to disease potential because of high variability in isolate virulence. *Fusarium* isolates were collected from healthy and diseased seedlings of Douglas-fir (*Pseudotsuga menziesii*) and western white pine (*Pinus monticola*) from a nursery in Idaho, USA. Molecular markers such as DNA sequences (mitochondrial small subunit and nuclear translation elongation factor 1-alpha) and amplified fragment length polymorphism were used to identify isolates as either *F. oxysporum* or *F. commune*. In addition, diagnostic primers were developed to detect and distinguish *F. commune* from *F. oxysporum*. *In vitro* and greenhouse virulence tests were completed on Douglas-fir germinants and seedlings. For Douglas-fir germinants and seedlings, *F. oxysporum* isolates generally caused less severe symptoms, whereas most *F. commune* isolates caused mortality through damping-off. This is the first report of direct evidence that *F. commune* can cause damping-off disease on Douglas-fir seedlings under greenhouse conditions.

1 Introduction

Fusaria are important soil-borne pathogens within tree nurseries throughout western North America (James et al. 2000). Damping-off and root rot of young conifer seedlings by Fusaria pathogens cause severe crop and economic losses annually. *Fusarium* spp. are ubiquitous in most container and bareroot nurseries, where they occur on healthy and diseased conifer seedlings, in nursery soils, and on conifer seeds of several species, especially Douglas-fir (*Pseudotsuga menziesii*), western white pine (*Pinus monticola*) and ponderosa pine (*Pinus ponderosa*) (James et al. 1997).

Since the first report of this disease, the major fungal pathogen was previously identified as *F. oxysporum* based on morphology (Bloomberg 1981). *Fusarium oxyporum* pathogenic to Douglas-fir was not differentiated by a formae speciales (Donaldson et al. 1995). However, a recent phylogenetic study (Stewart et al. 2006) of morphologically identified *F. oxysporum* isolates collected from Douglas-fir seedlings and soil suggested that this disease is caused by *F. commune*, a recently named species (Skovgaard et al. 2003). Using amplified fragment length polymorphisms (AFLP) and DNA sequences [mitochondrial small subunit (mtSSU), nuclear translation elongation factor 1-alpha (EF1-α)], Stewart et al. (2006) demonstrated that all highly virulent isolates, which were morphologically indistinguishable from *F. oxysporum*, were phylogenetically separate from low- and non-virulent isolates of *F. oxysporum*. In this manner, these highly virulent isolates were identified as *F. commune*, which represented the first report that *F. commune* is a cause of Fusarium disease (root rot and damping-off) on Douglas-fir germinants under *in vitro* conditions (Stewart et al. 2006).

Management of Fusarium disease in forest nurseries could be greatly enhanced by accurate identification of the *Fusarium* species, especially highly virulent isolates of *F. commune*. Management of Fusarium disease is especially difficult because of the inability to predict disease potential each year and quickly identify populations of highly virulent *F. commune*. Previous genetic studies of *F. oxysporum* have shown that formae speciales are polyphyletic, not monophyletic, which hampers molecular characterization (Baayen et al. 2000).

The primary objectives of this study were to (i) test the roles of *F. commune* and *F. oxysporum* in disease of Douglas-fir using unknown *Fusarium* isolates under *in vitro* and greenhouse conditions; and (ii) design and test species-specific, diagnostic primers for detecting *F. commune.*

2 Materials and methods

2.1 Fungal isolates

During 1992–1998, a total of 53 isolates of *Fusarium* spp. were collected from a forest nursery in Idaho, USA, from (i) seedling roots of healthy (non-symptomatic) and diseased Douglas-fir and western white pine seedlings, and (ii) nursery soil. All 53 isolates were previously classified as *F. oxysporum* based on morphological similarities (Bloomberg 1981), but they had not been previously tested for virulence. Of those 53 isolates, a subset of 14 isolates was selected for virulence studies based on molecular characterization (Table 1).

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Table 1. Isolates of *Fusarium oxysporum* and *F. commune* used in this study.

Taxon[1]	Collection	Isolate[2]	Host/Substrate	GenBank Accession No. (mtSSU; EF-1α)
Fusarium oxysporum	9221G	*Fo*-Q10	Western white pine (diseased)	GQ355919; GQ355932
	9221L	*Fo*-Q12	Western white pine (diseased)	GQ355907; GQ355920
	9225Q	*Fo*-Q17	Western white pine (diseased)	GQ355909; GQ355922
	9456A	*Fo*-Q22	Douglas-fir (healthy)	GQ355918; GQ355931
	9612C	*Fo*-Q33	Douglas-fir (healthy)	GQ355916; GQ355929
	9747M	*Fo*-Q45	Western white pine (healthy)	GQ355908; GQ355921
	9805F	*Fo*-Q54	Western white pine (diseased)	GQ355917; GQ355930
	9955D	*Fo*-Q76	Western white pine (healthy)	GQ355906; GQ355933
	9418D	*Fo*-N16	Douglas-fir (diseased)	DQ016175; DQ016273
F. commune	9319A	*Fc*-Q2	Douglas-fir (diseased)	GQ355913; GQ355926
	9748A	*Fc*-Q4	Douglas-fir (healthy)	GQ355914; GQ355927
	9956E	*Fc*-Q8	Western white pine (healthy)	GQ355915; GQ355928
	9515A	*Fc*-Q29	Douglas-fir (healthy)	GQ355912; GQ355925
	9748C	*Fc*-Q48	Douglas-fir (healthy)	GQ355911; GQ355924
	9950B	*Fc*-Q71	Douglas-fir (diseased)	GQ355910; GQ355923
	9408A	*Fc*-H14	Douglas-fir (diseased)	DQ016153; DQ016251
F. oxysporum f. sp. *lactucae*	0349	*Fo* f. sp. *lact*	Lettuce	DQ016184; DQ016283

[1]All isolates were collected from Idaho, USA, except isolate 0349 that was collected from California, USA; Isolate 0349 was collected by T. Gordon in a pathogenic situation.
[2]Identification code in this article: *Fo*, *Fusarium oxysporum*; Fc, *Fusarium commune.*

2.2 Molecular characterization of *Fusarium* isolates

All 53 isolates were characterized using mtSSU sequences, EF-1α sequences and AFLP data, based on the protocol of Stewart et al. (2006). To select isolates for further study, phylogenetic and AFLP analyses were performed on the 53 isolates, along with previously identified *F. oxysporum* and *F. commune* isolates for which virulence was previously determined (Stewart et al. 2006). Phylogenetic analyses were conducted using PAUP*4.0b10 (Swofford 2003) and MrBayes v.3.0b4 (Huelsenbeck and Ronquist 2001). The mtSSU and EF-1α regions were analysed separately and combined. Isolates of *F. subglutinans* (NRRL 22016: M1431/AF160289) and *F. proliferatum* (NRRL22057: M1431/M1432) were included in the analyses as the outgroup. Sequences of *F. proliferaturm* and *F. subglutinans* were retrieved from matrices (M1431, M1432) in the database TreeBASE (http://www.treebase.org). Sequences of selected *Fusarium* isolates used in virulence tests were deposited into GenBank (Table 1).

Parsimony and Bayesian analyses were implemented on the combined dataset, after an initial partition homogeneity test (Farris et al. 1994; Cunningham 1997; Stewart et al. 2006) to ensure these regions were congruent. Maximum parsimony analysis was conducted using the heuristic search option with 1000 random addition sequences using the tree bisection-reconnection branch swapping. The MULPARS option was off (because of computer constraint) and all characters were weighted equally. DT-ModSel (Minin et al. 2003) determined that TrNef+G was the best-suited, nucleotide-substitution model for the combined dataset. Bayesian analysis was performed using MrBayes with settings suggested by the selected model. For Bayesian analyses, the Markov chain Monte Carlo search was run with four chains for 3 000 000 generations generating 30 001 trees, of which the first 6000 trees were discarded as 'burnin' of the chains.

2.3 Selection of isolates for virulence tests

The resulting AFLP and phylogenetic analyses identified two major clades from which isolates were selected for virulence tests: one clade included all of the low- and non-virulent *F. oxysporum* from Stewart et al. (2006) and 38 *Fusarium* isolates from this study. The second clade contained highly virulent *F. commune* from Stewart et al. (2006) and 15 *Fusarium* isolates from this study (data not shown).

Based on these genetic analyses, a subset of 14 from the 53 isolates was selected to represent the following criteria: (i) genetically distinct (i.e. *F. oxysporum* clade *vs. F. commune* clade) and (ii) derived from diverse sources (i.e. Douglas-fir *vs.* western white pine, healthy *vs.* diseased seedlings). Of these isolates, eight *F. oxysporum* (*Fo*-Q10, *Fo*-Q12, *Fo*-Q17, *Fo*-Q22, *Fo*-Q33, *Fo*-Q45, *Fo*-Q54 and *Fo*-Q76) and six *F. commune* (*Fc*-Q2, *Fc*-Q4, *Fc*-Q8, *Fc*-Q29, *Fc*-Q48 and *Fc*-Q71) were selected for virulence testing on Douglas-fir germinants (*in vitro*) and seedlings (greenhouse; Table 1). Isolates from the previous study (Stewart et al. 2006) were also selected as controls for virulence testing: one non-virulent isolate of *F. oxysporum* (*Fo*-N16), one highly virulent isolate of *F. commune* (*Fc*-H14) and one isolate of *F. oxysporum* f. sp. *lactucae* (0349) (Table 1).

2.4 Development of diagnostic PCR primers for *Fusarium commune*

A species-specific primer pair for *F. commune* was designed to amplify a ca. 295-bp product from EF-1α. The forward and reverse PCR primers are efFc100F (5′-GGGGTATTTCTCAAAGGCAATATGC-3′) and efFc385R (5′-ATGCGCTCATTGAGGTTGTGG-3′), respectively. The PCR programme and conditions were as follows: 95°C for 5 min, followed by 40 cycles of: 94°C for 30 s,

65°C for 30 s and 72°C for 30 s, with a final 72°C step for 10 min, in a 20-μl reaction with 30 ng of template DNA, 1× PCR buffer (New England Biolabs, Ipswich, MA, USA), 0.2 mM dNTP (New England Biolabs), 1 U of *Taq* polymerase (New England Biolabs) and 0.2 μM of each primer (Operon Technologies, Valencia, CA, USA). The primers were tested against *F. oxysporum* and *F. commune* isolates listed in Table 1. In addition, primers were tested against one isolate of *F. oxysporum* (*Fo*47) used in biological control studies, isolates of *F. oxysporum* f. sp. *melonis* (0343), *F. oxysporum* f. sp. *lactucae* (0349), *F. oxysporum* f. sp. *lycopersici* (0350), *F. oxysporum* f. sp. *phaseoli* (9701) and three isolates of *F. proliferatum* (9223F, 9721B, 9816) (Stewart et al. 2006). Isolates *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *lactucae, F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *phaseoli* were collected from melon, lettuce, tomato and bean, respectively. Isolates of *F. proliferatum* were collected from aspen (*Populus tremuloides*), Douglas-fir and whitebark pine (*Pinus albicaulis*), respectively. These isolates were previously tested for pathogenicity on Douglas-Fir (Stewart et al. 2006).

2.5 Inoculum preparation for virulence tests

Inoculum was prepared following the general techniques of Miles and Wilcoxson (1984), which were found effective in previous virulence tests on conifer seedlings (James and Gilligan 1984; James et al. 1989). Inoculum was produced in galvanized metal pans (5 × 25 × 35 cm) lined with a double layer of aluminium foil. Perlite, an inert, inorganic, siliceous rock of volcanic origin commonly used in soil-less planting media in conifer container nurseries, was the matrix for fungal growth. In each metal pan, 150 g of yellow cornmeal was moistened with 300 ml warm 1% potato dextrose agar (PDA) and allowed to stand for 15 min before 75 g of perlite was thoroughly mixed in. The pans were covered with aluminium foil and autoclaved 60 min at 121°C. After cooling, the perlite–cornmeal cake was inoculated with a 1 cm^2 piece of mycelium from each of two 14-day-old cultures for each *Fusarium* isolate grown on PDA. Mycelia were mixed into the perlite–cornmeal cake with a sterile knife, 50 ml of sterile distilled water was added and the pan was sealed with aluminium foil. Sealed pans were incubated in the dark at about 24°C for 24 days. Following incubation, the fungal cake mixtures were air-dried on a tabletop for 3 days and stored in plastic bags at about 8°C until needed. Previous work has shown that this technique produces true-to-type inoculum that can be stored for several months without loss of viability (James et al. 1989). Virulence tests were designed with 24 replicates for the *in vitro* test (total 432 Douglas-fir germinants = 17 *Fusarium* isolates plus one control * 24 replicates) and 25 replicates for the greenhouse test (total 450 Douglas-fir seedlings = 17 *Fusarium* isolates plus one control * 25 replicates). For the completed tests, one replicate of isolate *Fo*-Q33 was missing in the *in vitro* test, and four replicates of isolate *Fc*-Q29 were missing in the greenhouse test.

2.6 *In vitro* virulence test

For the *in vitro* virulence test, we followed the methods of James et al. (1997), which are briefly described here. Seeds of interior Douglas-fir from the Clearwater National Forest in Idaho, USA were placed inside a new mesh bag, soaked in running tap water for 48 h to help remove seed-coat contaminants (James and Genz 1981) and allow imbibition, placed inside a plastic bag and stratified at 2°C for 28 days. After stratification, seeds were germinated on moistened, sterile Whatman No. 3 filter paper within sterile petri dishes and incubated at about 24°C. A single germinant (primary root about 3 mm long) was carefully placed inside a 23-ml glass vial filled to about two-thirds capacity with a 1 : 1 (v/v) *Sphagnum* peat moss/vermiculite growing medium amended with inoculum (described earlier) at a 50 : 1 w/w growing media/inoculum. Each fungal isolate was represented by 24 vials. Sterile water was added as needed and capped vials were incubated under cool, fluorescent, diurnal light (12/12) at about 23°C. Production of disease symptoms was monitored and evaluated over a 14-day period. After 14 days, all germinants were harvested and examined for disease symptoms, and re-isolations onto Komada's medium (Komada 1975) were made from all inoculated germinants. Germinants received one point for each day of survival from 3 to 14 (12 points maximum); another 1, 2 or 4 points depending on type of disease (root rot, damping-off, and no disease, respectively); 1, 2 or 4 points for re-isolation from germinant roots (multiple colonies from root, single colony from root, no colonies produced, respectively); and 3 more points if the root grew to the bottom of the vial. The range of points was 5–25, with higher values reflecting less virulency of the isolate. Points were converted to a reciprocal score of 0–100, with virulence ratings of 0 indicating no fungal infection and 100 indicating germinants were killed within 3 days by the inoculated isolate (Fig. 3a) (James et al. 1990). Based on previous studies (James et al. 1995, 1997, 2000), isolates with moderate-high virulency exhibited virulence ratings above 60, isolates with low virulency exhibited virulence ratings from 40 to 59 and isolates with average virulence ratings below 39 were considered non-pathogenic.

2.7 Greenhouse virulence tests

New individual containers (Ray Leach Cone-tainers™; 2.5-cm diameter, 16-cm length, 66-ml volume, 1076 containers/m^2) were filled with a 1 : 1 (v/v) *Sphagnum* peat moss/vermiculite medium. Assays using the dilution plate method (Nash and Snyder 1962) with Komada's medium were used to ensure that *Fusarium* spp. were not present in the medium. In mid-March, seeds of the same Douglas-fir seed source as the *in vitro* virulence test were stratified as described earlier. After stratification, seeds were rinsed for 24 h in running tap water and three seeds were hand-sown per Cone-tainer™. Seeds were covered with a 1-cm-deep layer of fine-rock mulch and placed on metal tables inside a greenhouse at the USDA Forest Service laboratory in Moscow, Idaho, USA. Target greenhouse temperatures (24–27°C) and photoperiod [natural day light extended to 18 h with a 400-watt, oscillating, high-pressure sodium light (Beamflicker; PARsource, Petaluma, CA, USA) followed the recommendations of Wenny and Dumroese (1992). Most seeds germinated between 7 and 14 days after sowing, and Cone-tainers™ were thinned

to a single seedling. Beginning 3 weeks after sowing and continuing throughout the growth period, seedlings were fertigated (i.e. irrigated with a soluble fertilizer containing 100 mg/L N) when containers weighed 85% of their weight at field capacity (Landis et al. 1989). Peters Professional® Conifer Grower 20-7-19 (The Scotts Company, Marysville, OH, USA) was the nutrient source, and subsequent nutrient ratios were 100 N (58 NO_3^-: 35 NH_4^+: 7 urea): 15 P: 79 K: 4 S: 4 Mg: 2 Fe: 0.3 Cu: 0.3 Mn: 0.3 Zn: 0.12 B: 0.025 Mo.

Three months after sowing (mid-July), seedlings were carefully removed from their containers. For each treatment, 25 seedlings were arbitrarily selected. Control seedlings were transplanted back into their containers with fresh 1 : 1 (v/v) *Sphagnum* peat moss/vermiculite medium. For each isolate, we mixed 1 part inoculum (described earlier) with 50 parts 1 : 1 (v/v) *Sphagnum* peat moss/vermiculite medium (42% moisture content; w/w) and transplanted the seedlings back into their original containers with inoculated/control medium. A subsample of each inoculation medium was collected for assays to verify the presence of *Fusarium* sp. For the duration of the experiment, seedlings were fertigated as described earlier.

Six weeks after transplanting (late August), seedlings were rated for severity of foliar symptoms using the 1–5 classification series of James et al. (1989): 1 – seedling appeared healthy; 2 – <50% of the foliage is chlorotic; 3 – more than 50% of the foliage is chlorotic but <50% is necrotic; 4 – more than 50% of the foliage is necrotic; and 5 – the seedling is dead (Fig. 3b). All seedlings were carefully removed from their containers and gently washed to remove adhering potting medium. From each seedling, 10 lateral roots were randomly selected, and 2- to 3-mm-long tips were aseptically severed. For re-isolation, roots were incubated on Komada's medium. Identification of re-isolated *Fusarium* spp. was determined by DNA sequencing of the mtSSU and EF-1α regions.

2.8 Statistical analyses for virulence tests

Seventeen isolates (plus one control) were used in the *in vitro* virulence test, and virulence was measured for 24 replicates per isolate. Replicates were classified in one of 21 classes corresponding to their virulence rating (0, 5, 10, 15, 20, 25, 30, 35, …, 95, 100), with zero referring to non-pathogenic and 100 being highly virulent. In the greenhouse test, the same isolates were tested for virulence using 25 seedlings (replicates) per isolate. Replicates were classified according to disease-severity rating levels (from 1 to 5), with a disease-severity rating of 1 representing non-pathogenic and a disease-severity rating of 5 representing highly virulent. The resulting data were categorical with an ordinal structure reflecting the order of the scale; these data represented the number of times a certain isolate belonged to a virulence class. Two contingency tables were constructed for the datasets from the *in vitro* and the greenhouse tests (Tables 2 and 3). Virulence scores were used as the response variables and were conditioned both on the species identification that resulted from our phylogenetic analyses and on the isolate used. Accordingly, each row of the contingency table represented a level of prespecified, phylogenetic species and the isolate and was assumed to be independent from all other rows. An extra row was added to the table that represented a control. Each row of the contingency tables was assumed to be a sample from a multinomial distribution independent from the other rows (see Agresti 2002; Zhou et al. 2007; Schütte et al. 2008 for similar examples of such construction).

Using the contingency tables, three models were compared : (i) the null model – assumes each row was sampled from the same multinomial distribution (hence, the isolates are the same in terms of their virulence regardless of their species classification), (ii) the phylogenetic-effects model – assumes a difference exists between the phylogenetic species (*F. commune*, *F. oxysporum* and control) but no difference occurs among the isolates within species group in their virulence and (iii) the full model – states a difference exists between the phylogenetic species and that differences also occur among the isolates within each of the species groups. We used a likelihood ratio test to determine which of these three models best fits the virulence data. Similar to the study of Schütte et al. (2009), significance of the likelihood ratio test was determined using the bootstrap (1000 replicates).

Table 2. In vitro virulence: test pairwise comparison between *Fusarium oxysporum (Fo)* and *F. commune (Fc)* isolates for mean virulence rating per isolate[1].

	Fc-Q2	*Fc*-Q4	*Fc*-Q8	*Fc*-Q29	*Fc*-Q48	*Fc*-Q71	*Fc*-H14
Fc-Q48	0.00140	0.14416	0.00011**	0.00009**			
Fo-Q10	0.00024*	0.03429	0**	0.00001**	0.20391	0.00023*	0.00015**
Fo-Q12	0**	0.00079*	0.00001**	0**	0.01036	0**	0**
Fo-Q17	0.00019*	0.02242	0.00001**	0.00002**	0.14981	0.00010**	0.00004**
Fo-Q22	0**	0.00074*	0**	0**	0.00919	0**	0**
Fo-Q33	0.00130*	0.06316	0.00016**	0.00016**	0.26808	0.00076	0**
Fo-Q45	0**	0.00013**	0**	0**	0.00283	0**	0**
Fo-Q54	0.00210*	0.15267	0.00014**	0.00014**	0.49190	0.00109	0.00055
Fo-Q76	0.00017*	0.01230	0.00002**	0.00003**	0.07882	0.00009**	0.00003**
Fo-N16	0.00001**	0.00199*	0**	0**	0.02125	0.00002**	0.00001**
Fo f. sp. *lact*	0**	0.00001**	0**	0**	0.00020*	0**	0**
Control	0**	0**	0**	0**	0**	0**	0**

[1]Column and rows without significant pairwise comparison are not shown. All isolates were significantly different from the control. Isolates of *F. oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1.
Significant at *0.1 and **0.05 level of significance, after Bonferroni correction.

Table 3. Greenhouse virulence test: pairwise comparison between *Fusarium oxysporum* (*Fo*) and *F. commune* (*Fc*) isolates for mean virulence rating per isolate[1].

	Fc-Q2	*Fc*-Q4	*Fc*-Q8	*Fc*-Q29	*Fc*-Q48	*Fc*-Q71	*Fc*-H14	*Fo*-Q12	*Fo*-Q45	*Fo*-Q54
Fc-Q4	0.31325									
Fc-Q8	0**	0.00005**								
Fc-Q29	0**	0**	0.00807							
Fc-Q48	0.06270	0.11845	0.00094	0**						
Fc-Q71	0**	0**	0.40831	0.00763	0.00019*					
Fc-H14	0**	0**	0.01922	0.41089	0**	0.02206				
Fo-Q10	0.19936	0.34272	0.00005**	0**	0.21162	0.00003**	0**			
Fo-Q12	0.07351	0.01912	0**	0**	0.00237	0**	0**			
Fo-Q17	0.01793	0.03687	0.00205	0**	0.29601	0.00063	0**	0.00025*		
Fo-Q22	0.00960	0.01937	0.01017	0.00001**	0.16499	0.00442	0.00002**	0.00014**		
Fo-Q33	0.02127	0.04311	0.00474	0**	0.27042	0.00161	0.00001**	0.00063		
Fo-Q45	0.43965	0.22497	0.00002**	0**	0.03170	0**	0**	0.00634		
Fo-Q54	0.00015**	0.00036	0.00306	0**	0.05816	0.00050	0**	0**	0**	
Fo-Q76	0.39506	0.21768	0.00003**	0**	0.03698	0**	0**	0.10552	0.43577	0.00004**
Fo-N16	0.40631	0.39196	0.00001**	0**	0.08131	0**	0**	0.01704	0.28609	0.00004**
Fo f. sp. *lact*	0.15800	0.28697	0.00011**	0**	0.26112	0.00002**	0**	0.00404	0.07484	0.00416
Control	0.11409	0.03408	0.00001**	0**	0.00444	0**	0**	0.17800	0.04595	0**

[1]Columns and rows without significant pairwise comparison are not shown. Isolates of *F. oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1.
Significant at *0.1 and **0.05 level of significance, after Bonferroni correction.

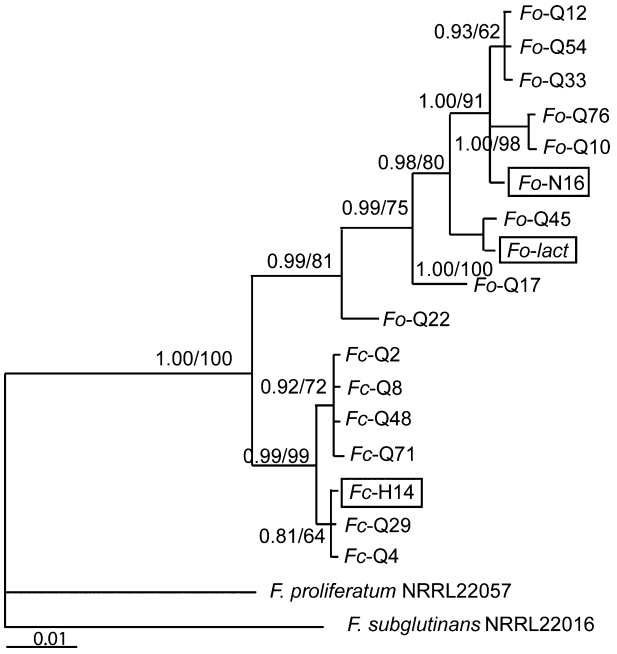

Fig. 1. Bayesian phylogeny generated from combined nuclear translation elongation factor 1-alpha (EF-1α) and mitochondrial small subunit (mtSSU) sequences. Posterior probabilities/parsimony bootstrap values are listed for each clade. Boxed isolates are from the previous study (Stewart et al. 2006). Isolates of *Fusarium oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1.

3 Results

3.1 Molecular characterization of *Fusarium* isolates

Phylogenetic analyses based on DNA sequences (mtSSU and EF-1α regions) showed that the *Fusarium* isolates belonged to two genetically distinct groups, which represented *F. oxysporum* and *F. commune* (Fig. 1). In addition, AFLP marker data also identified the same two groups of isolates; the first group included all *F. oxysporum* isolates, whereas the second group included all *F. commune* isolates (data not shown). Furthermore, each isolate had a unique AFLP phenotype, indicating that our

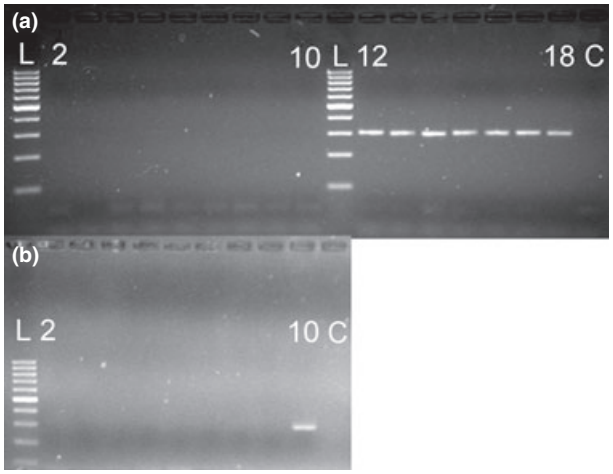

Fig. 2. (a) Comparison of PCR products of *Fusarium oxysporum* (*Fo*) isolates (lanes 2–10) and *F. commune* (*Fc*) isolates (lanes 12–18) using species-specific diagnostic primers. Isolates of *F. oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1. (b) Diagnostic primers were tested against *F. oxysporum* (lane 2) used in biological control studies, isolates of *F. oxysporum* f. sp. *melonis* (lane 3), *F. oxysporum* f. sp. *lactucae* (lane 4), *F. oxysporum* f. sp. *lycopersici* (lane 5), *F. oxysporum* f. sp. *phaseoli* (lane 6), and 3 isolates of *F. proliferatum* (lanes 7–9). *Fusarium commune* isolate *Fc*-H14 (lane 10) was used as a positive control. L: Size markers: 100 base pair ladder C: Negative control.

test set did not contain any clonal isolates. The species-specific set of diagnostic primers, based on sequence differences within the EF-1α region, was effective for identification/detection of *F. commune* (Fig. 2).

Results from the partition homogeneity test indicated that mtSSU and EF-1α were congruent and could be combined (p-value = 0.967), which was further supported by the observed similar topologies previously shown in Stewart et al. (2006). The combined dataset consisted of 1209 characters, and of these, 1087 were constant. Of the 122 variable characters, 57 (4.7% of the total characters) were parsimony informative. The cladograms produced by Bayesian analysis displayed two distinct and well-supported (posterior probability <95%) clades, which separated *F. commune* and *F. oxysporum* on the basis of the mtSSU (not shown), EF-1α (not shown), and combined mtSSU and EF-1α datasets (Fig. 1). Fourteen isolates, not previously characterized, were grouped into one of the two clades, indicating that these isolates were either *F. oxysporum* or *F. commune* based on phylogenetic species recognition. Of the 17 *Fusarium* spp. isolates, which included the three previously characterized isolates, 10 were grouped into the *F. oxysporum* clade and seven grouped into the *F. commune* clade (Fig. 1). Parsimony analysis of the combined dataset showed similar results. In both cases, the node support for each species' clade was over 80%. All isolates grouped in identical locations in both analyses with one exception. In the parsimony phlyogram, *Fo*-N16 grouped in a subclade with *Fo*-Q22, *Fo*-Q33 and *Fo*-Q54, whereas in the Baysesian analyses *Fo*-N16 was placed just outside the *Fo*-Q12-*Fo*-Q33-*Fo*-Q54 and *Fo*-Q10-*Fo*-Q76 subclades (Fig. 1).

After disease scoring under *in vitro* and greenhouse conditions, mtSSU and EF-1α sequence data verified that the re-isolated *Fusarium* spp. from selected diseased and healthy Douglas-fir germinants and seedlings represented the species (*F. oxysporum* or *F. commune*) that was used for the initial inoculation treatments.

3.2 *In vitro* virulence test

Comparing the null model (no difference in virulence between the phylogenetically identified species) against the phylogenetic-effects model resulted in rejection of the null model (p-value = 0). This indicates that the phylogenetic classification explained part of the error, and hence, a model incorporating species identification using the phylogenetic approach did better than one that did not. To determine whether significant variation in virulence exists within the phylogenetic species identification, we compared the full model (which takes into consideration variation within and between the species groups) against the phylogenetic-effects model (accounting for only the variation between species groups). Based on this comparison, the phylogenetic-effects model was rejected (p-value = 0), which indicates there is significant variation in virulence of isolates within each of the species groups.

To evaluate this variation in virulence, pairwise comparisons were performed using the mean virulence of each isolate. We used the underlying multinomial model in a parametric bootstrap strategy to generate the null distribution of the difference between the mean virulence of any two isolates. Comparing the difference obtained from the data against this null distribution allows us to calculate the p-value for such a test. Table 2 presents the p-values for each of these comparisons. Table 2 shows that significant differences exist between *F. commune* (*Fc*) and *F. oxysporum* (*Fo*) in their virulence rating. All *F. commune* isolates, with one exception, were significantly more virulent than most of the *F. oxysporum* isolates (Table 2). Isolate *Fc*-Q48 represented the single exception, as its virulence was only significantly different from the *F. oxysporum* f. sp. *lactucae* and the control (Table 2). Isolates *Fc*-Q48 and *Fc*-Q4 had virulence ratings similar to *Fo*-Q54 and *Fo*-Q33 (Fig. 3a). All isolates had significantly higher virulence ratings than the control (Table 2).

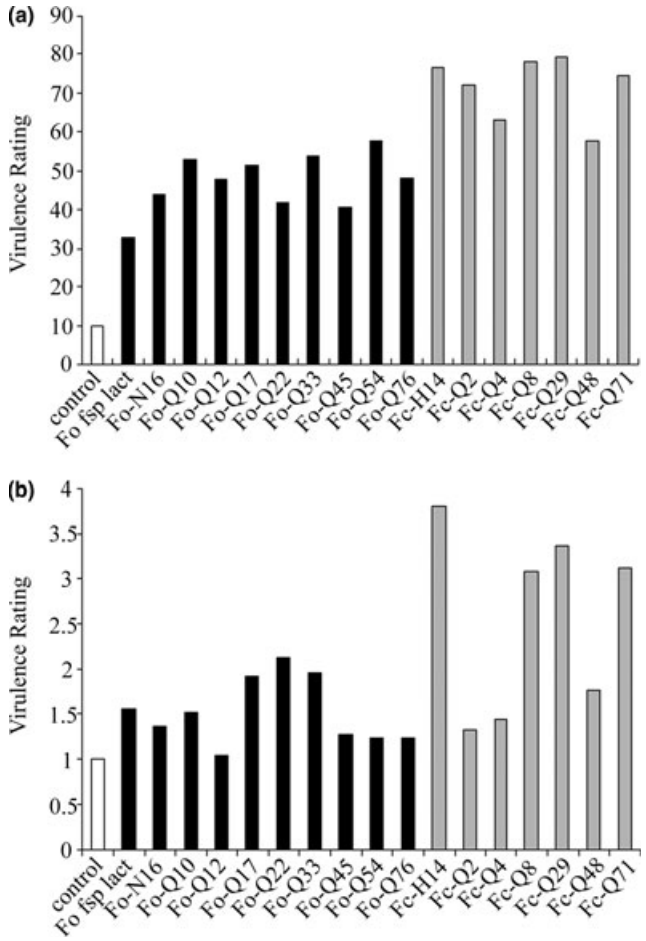

Fig. 3. (a) *In vitro* virulence test, (b) Greenhouse virulence test: Mean virulence ratings for *Fusarium oxysporum* and *F. commune*. Isolates of *F. oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1.

3.3 Greenhouse virulence test

The null model, no difference in virulence of species groups, was also rejected in comparison with the phylogenetic-effects model for the greenhouse test (p-value = 0). This indicates, as in the case mentioned earlier, that the phylogenetic classification explained part of the error, and hence, a model taking into consideration the species groups was more appropriate. Comparing the full model to the phylogenetic-effects model resulted in rejecting the phylogenetic-effects model in favour of the full model (p-value = 0), which demonstrates a significant variation in the virulence of isolates within each of the phylogenetically identified species groups, similar to the *in vitro* test carried out earlier.

Again, to evaluate this variation in virulence, pairwise comparisons were conducted using the mean virulence of each isolate following the bootstrap strategy described earlier. Table 3 presents the p-values for each of these comparisons. Isolates *Fc*-Q29 and *Fc*-H14 are significantly more virulent than all other isolates (Table 3). Isolates *Fc*-Q71 and *Fc*-Q8 had higher virulence ratings than all other isolates, except isolates *Fc*-Q29 and *Fc*-H14, but are not significantly different from isolates *Fo*-Q54, *Fo*-*Q*33, *Fo*-Q22 and *Fo*-Q17 (Table 3). Isolates *Fc*-Q48, *Fc*-Q4 and *Fc*-Q2 had virulence ratings similar to *F. oxysporum* isolates (Fig. 3b).

4 Discussion

To date, little research has been completed on the recently named species *F. commune* (Skovgaard et al. 2003). A recent article, however, showed that highly virulent isolates collected from soil and Douglas-fir seedlings were morphologically similar to *F. oxysporum*, but genetically similar to *F. commune* based on AFLP and DNA sequencing data (Stewart et al. 2006). Using the phylogenetic species concept, which is commonly used for Fusaria (O'Donnell et al. 1998, 2004), these highly virulent isolates were classified as *F. commune* (Stewart et al. 2006). That previous report also presented the first evidence that *F. commune* was associated with disease of Douglas-fir germinants under *in vitro* conditions, representing a potential cause of Fusarium disease in a tree nursery (Stewart et al. 2006). In this study, we demonstrated that *F. commune* is pathogenic to Douglas-fir seedlings under greenhouse conditions, confirming a cause of Fusarium disease. Although isolates used in this study were

collected from a single nursery in Idaho, USA, Skovgaard et al. (2003) showed that *F. commune* is a ubiquitous species found in at least seven countries in the Northern Hemisphere. We have shown that *F. commune* is found on diseased and healthy Douglas-fir and western white pine seedlings; however, our virulence tests were completed only on Douglas-fir. Because of its wide host/substrate range, including western white pine, *F. commune* is potentially pathogenic to more host species, especially other conifers; however, additional studies are needed to determine host range of this pathogen.

Virulence is a difficult phenotype to establish and standardize. Our virulence tests showed that both *F. commune* and *F. oxysporum* can cause disease symptoms (e.g. foliar chlorosis and necrotic tissues) on Douglas-fir seedlings. In all cases, however, *F. oxysporum* isolates did not cause seedling mortality, whereas *F. commune* isolates were deemed highly virulent because of their ability to cause seedling death, within 1 month in some cases. The results of the *in vitro* test showed a clearer distinction of virulence rating between *F. oxysporum* and *F. commune* compared to the greenhouse test. Most likely, this difference was caused by environmental factors, physiological differences, soil microflora and/or other variations. For example, in the *in vitro* virulence test, germinants were grown in a close-to-sterile laboratory environment, whereas in the greenhouse test, extraneous bacterial or fungal interactions were more likely to occur along with more vagaries of temperature, light and moisture conditions. However, the greenhouse test more accurately mimics seedling-production conditions while demonstrating that *F. commune* causes disease leading to death of Douglas-fir seedlings.

Our results confirmed that *F. commune* is highly virulent to Douglas-fir seedlings based on the virulence tests. Results of our likelihood ratio testing showed that the full model, which states that differences exist between the phylogenetic species and among the isolates within each of the species groups, was the more appropriate model for the *in vitro* and greenhouse virulence tests. This outcome, in addition to the pairwise comparison results, shows that not all *F. commune* isolates can be classified as highly virulent under the test conditions.

Previous studies of asexual *Fusarium* spp., especially *F. oxysporum* formae speciales, have suggested that virulence factors may not reflect the evolutionary history of a species because these characters may have been acquired through other mechanisms (Leslie 2001). Mechanisms such as horizontal gene transfer or transposable elements have been suggested as possible modes of non-sexual genetic exchange in putatively asexual fungi (Taylor et al. 1999; Baayen et al. 2000; Rosewich and Kistler 2000). Many *F. oxysporum* formae speciales have been characterized genetically and shown to form polyphyletic species complexes, indicating that pathogenicity determinants may have multiple origins within this species complex (O'Donnell et al. 1998). Similarly, several studies have shown that, in addition to *F. commune* and *F. oxysporum*, other *Fusarium* sp., such as *F. sporotrichioides*, *F. proliferatum* and *F. circinatum*, are capable of causing damping-off in conifers (James et al. 1990, 1995; Juzwik et al. 1995; Donaldson 1999; James and Perez 1999; Steenkamp et al. 1999; Ocamb et al. 2002). The virulence factors may have been developed independently in multiple Fusaria species (polyphyletic character) or by horizontal gene transfer. A study examining the similarities between conifer disease-causing *Fusarium* pathogens may elucidate the relationships among virulence factors among these species.

Fusarium oxysporum and closely related species are difficult to distinguish morphologically. Stewart et al. (2006) showed that although *F. commune* is nearly identical to *F. oxysporum* morphologically, *F. commune* is quite distinct genetically. Furthermore, our virulence tests suggest that *F. commune* is a highly virulent pathogen; whereas *F. oxysporum* is mildly virulent on Douglas-fir seedlings.

In this study, we developed a species-specific diagnostic primer set to detect and identify isolates of *F. commune*. This primer set was tested against *F. oxysporum* found on white pine and Douglas-fir, *F. oxysporum* formae speciales *melonis, lactucae, lycopersici* and *phaseoli* and three isolates of *F. proliferatum* also collected from conifers. For effective disease management, it is critical to easily, quickly, and economically detect highly virulent *F. commune*. Currently, early detection of this pathogen is impractical. This diagnostic primer set can potentially be applied to quantify *F. commune* in nursery soils and seedlings using real-time PCR technology. By associating *F. commune* inoculum density with disease potential, nursery managers could better predict disease severity for the current growing season by quantifying of *F. commune* in the soil (James et al. 2000). With this information, nursery managers could more effectively deploy an appropriate disease-management strategy. Thus, we have shown that *F. commune* isolates caused damping-off leading to death of Douglas-fir seedlings and the species-specific diagnostic tool is effective, with potential applications for managing disease caused by *F. commune* within forest nurseries.

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