

# Molecular Characterization of *Fusarium oxysporum* and *Fusarium commune* Isolates from a Conifer Nursery

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## ABSTRACT

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*Fusarium* species can cause severe root disease and damping-off in conifer nurseries. *Fusarium* inoculum is commonly found in most container and bareroot nurseries on healthy and diseased seedlings, in nursery soils, and on conifer seeds. Isolates of *Fusarium* spp. can differ in virulence; however, virulence and colony morphology are not correlated. Forty-one isolates of *Fusarium* spp., morphologically indistinguishable from *F. oxysporum*, were collected from nursery samples (soils, healthy seedlings, and diseased seedlings). These isolates were characterized by amplified fragment length polymorphism (AFLP) and DNA sequencing of nuclear rDNA (internal transcribed spacer including 5.8S rDNA), mitochondrial rDNA (small subunit [mtSSU]), and nuclear translation elongation factor 1- $\alpha$ . Each isolate had a unique AFLP phenotype. Out of 121 loci, 111 (92%) were polymorphic; 30 alleles were unique to only highly virulent isolates and 33 alleles were unique to only isolates nonpathogenic on conifers. Maximum parsimony and Bayesian analyses of DNA

sequences from all three regions and the combined data set showed that all highly virulent isolates clearly separated into a common clade that contained *F. commune*, which was recently distinguished from its sister taxon, *F. oxysporum*. Interestingly, all but one of the nonpathogenic isolates grouped into a common clade and were genetically similar to *F. oxysporum*. The AFLP cladograms had similar topologies when compared with the DNA-based phylograms. Although all tested isolates were morphologically indistinguishable from *F. oxysporum* based on currently available monographs, some morphological traits can be plastic and unreliable for identification of *Fusarium* spp. We consider the highly virulent isolates to be *F. commune* based on strong genetic evidence. To our knowledge, this is the first reported evidence that shows *F. commune* is a cause of *Fusarium* disease (root rot and damping-off) on Douglas-fir seedlings. Furthermore, several AFLP genetic markers and mtSSU sequences offer potential for development of molecular markers that could be used to detect and distinguish isolates of *F. oxysporum* nonpathogenic to conifers and highly virulent isolates of *F. commune* in forest nurseries.

*Additional keywords:* forest pathology, molecular diagnostics, phylogenetics.

*Fusarium* species can behave as a fungal pathogen causing damping-off and root rot of young conifer seedlings, resulting in severe crop and economic losses in forest nurseries. It is one of the most important soilborne pathogens within nurseries throughout western North America (29). This fungal genus is ubiquitous in most container and bareroot nurseries on healthy and diseased conifer seedlings, in nursery soils, and on conifer seeds of several species, especially Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), western white pine (*Pinus monticola* Dougl.), and ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) (25).

Efforts to improve disease control within tree nurseries have been hampered because of the inability to characterize and quantify *Fusarium* spp. populations with regard to disease potential. Laboratory assays were developed to assess pathogenicity of *Fusarium* spp. isolates on conifer germinants (24). Representative *Fusarium* spp. isolates were collected from conifer seedling nurseries, evaluated for pathogenicity using these techniques, and found to vary greatly in virulence; some isolates were highly virulent, whereas others were nonpathogenic (29). Furthermore, highly virulent isolates could not be differentiated from other isolates based on morphology in culture. In a previous

report, both highly virulent and nonpathogenic isolates were identified as *F. oxysporum*, based on morphology (29,42). Although total *F. oxysporum* Schlechtend:Fr. populations could be estimated quantitatively, population levels were not necessarily correlated with the resulting damage that occurred in the nursery crop (25). Thus, methods are needed to identify and quantify populations of highly virulent *Fusarium* spp.

Molecular genetic techniques can be used to study population structure by examining many traits and revealing information unobtainable by other methods. Molecular markers have been used to compare genetic relationships to pathogenicity in many groups of fungi (3,37). Previous studies used molecular techniques to differentiate pathogenic from nonpathogenic isolates of *F. oxysporum* f. sp. *phaseoli* (55) and other formae speciales of *F. oxysporum* (4–6,35,38,41,52). Genetic differences between pathogenic and nonpathogenic strains of *F. oxysporum* found on conifers were previously examined using the histone-H3 encoding gene and amplified fragment length polymorphisms (AFLPs), and a few genetic differences between high-virulence and low-virulence/nonpathogenic isolates were found (11). These previous results suggested that molecular genetic techniques could perhaps be used to separate these two phenotypes. Furthermore, these techniques can be used to compare the phylogenetic relationships of highly virulent *Fusarium* spp. isolates on conifers from isolates within the *F. oxysporum* species complex. These techniques and tools can also determine if these populations are clonal, which may aid in disease management.

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Development of a genetic marker system to distinguish highly virulent and nonpathogenic isolates would prove useful for managing *Fusarium* root rot in conifer seedlings and a variety of other crops (6,10). Sensitive molecular markers could allow for more cost-effective detection and quantification of the pathogen than conventional methods (33). With such molecular tools, nursery managers could estimate population levels of highly virulent isolates, and thereby better predict disease potential for the current growing season (29,43). Little progress has been made toward developing simple and effective molecular markers for detecting and quantifying highly virulent pathogen populations within conifer nurseries (51).

The aim of this study was to examine the genetic relationships of pathogenic (low, moderate, and high virulence) and nonpathogenic isolates of *Fusarium* spp. that appeared morphologically indistinguishable from *F. oxysporum*, collected from soil and Douglas-fir seedlings. Genetic relationships were analyzed using AFLP and DNA sequencing of the nuclear encoded rDNA (internal transcribed spacer including 5.8S [ITS+5.8S]) and nuclear translation elongation factor 1-alpha (EF-1 $\alpha$ ), and the mitochondrial encoded rDNA (small subunit [mtSSU]). Specific objectives were to (i) determine if highly virulent isolates of *Fusarium* spp. represent a genetically distinct group; and (ii) evaluate potential utility of DNA sequences and AFLP markers to distinguish highly virulent isolates of *Fusarium* spp. The development of molecular markers would facilitate studies on population structure and aid nursery management by identifying isolates that are highly virulent or nonpathogenic on conifers.

## MATERIALS AND METHODS

**Fungal isolates and pathogenicity tests.** Isolates of *Fusarium* spp. were collected from a forest nursery in Idaho during 1992 to 1998. A total of 100 isolates of *Fusarium* spp., collected from soil or roots of either healthy (nonsymptomatic) or diseased conifer seedlings, were evaluated. All 100 isolates were previously classified as *F. oxysporum* based on morphological characteristics (42). Isolates were previously evaluated for pathogenicity on Douglas-fir germinants in laboratory tests (29) using a technique for rapid laboratory assessment for virulence (24).

Briefly, Douglas-fir germinants (recently germinated seeds) were exposed to a cornmeal-perlite inoculum (1:50, wt/wt mixture of inoculum to medium) for each fungal isolate, and disease symptoms were monitored and evaluated over a period of 14 days. Germinants were considered diseased when they displayed standard postemergence damping-off (death) or rotting of the radicle when examined during or at the end of the test. Germinants with damping-off symptoms received higher ratings than those with rotting of the radicle. Three disease categories were used for these assays: damping-off, radicle rotting, and no disease. Numbers were given to germinants in each category, which were then added to other criteria (length of time germinants survived, primary root growth, and re-isolation of inoculated isolates) for a final virulence rating (24). The possible numerical rating ranged from 0 to 100, with higher values reflecting greater virulence. Each isolate had an average rating of numerical virulence that was used to compare isolates. Based on previous studies (26–28), highly virulent isolates exhibited scores of 80 to 100, moderately virulent isolates from 60 to 79, isolates with low virulence from 40 to 59, and isolates with average scores below 39 were considered nonpathogenic. Detailed methods are described in James et al. (29).

A total of 41 of the 100 isolates were selected to represent the following criteria: (i) diversity of sources (i.e., soil, healthy seedlings, and diseased seedlings) and (ii) availability of virulence ratings (high virulence versus medium-low virulence versus nonpathogenic). Of these selected isolates, 16 exhibited high virulence, three isolates were moderately virulent, five isolates had

low virulence, and 17 were deemed nonpathogenic to Douglas-fir seedlings (Table 1). As possible outgroups for phylogenetic and genetic analyses, we included single isolates of *F. oxysporum* f. sp. *melonis* (0348), *F. oxysporum* f. sp. *lactucae* (0349), *F. oxysporum* f. sp. *lycopersici* (0350), a nonpathogenic isolate of *F. oxysporum* (F047) currently used as a biological control for pathogenic isolates of *F. oxysporum* (7,16), and three isolates of *F. proliferatum* (9223F, 9721B, and 9816A) (Table 1).

**DNA extraction.** In preparation for DNA extraction, isolates were placed on 0.2- $\mu$ m-pore nylon filters (Millipore Corp., Billerica, MA) to avoid direct contact with potato dextrose agar medium in a petri dish and incubated at 22°C for 1 week. Approximately 100 mg of fresh mycelia was scraped from the nylon membrane for DNA extraction and purification with DNeasy Plant Mini DNA extraction kits following the manufacturer's protocol (Qiagen Inc., Valencia, CA). DNA was quantified using a TD-360 fluorometer (Turner Designs, Sunnyvale, CA).

**AFLP Analyses.** AFLP analyses were performed following the protocol of Vos et al. (53) with some modifications. For restriction digests, 350 ng of genomic DNA was digested with *EcoRI* and *MspI* (New England BioLabs, Inc., Beverly, MA) to serve as the template. Resulting DNA fragments were ligated to adapters and diluted 1:10 with sterile distilled water prior to pre-amplification. Pre-amplifications were performed following protocols developed for *Fusarium* (3) and *Armillaria* species (34) with some modifications. Pre-amplification reaction mixtures (total 30  $\mu$ l) contained 6  $\mu$ l of diluted restriction/ligation mixture as template, 1 $\times$  polymerase chain reaction (PCR) buffer, 3 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 300 nM of each preselective primers (*EcoRI*-no extension and *MspI*-no extension), and 1.5 units of *AmpliTag* DNA polymerase (Applied Biosystems, Inc., Foster City, CA). The PCR conditions for pre-amplification were 72°C for 2 min, 20 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min 30 s followed by a final step at 72°C for 10 min.

For selective amplification, we chose two primers (*EcoRI*-GC/*MspI*-CC and *EcoRI*-GC/*MspI*-CA) used by Baayen et al. (3). Selective amplification mixtures (total 25  $\mu$ l) contained 5  $\mu$ l of diluted pre-amplification products (1:20 with low Tris-EDTA buffer [10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0]) as a template, 1 $\times$  PCR buffer, 2.4 mM MgCl<sub>2</sub>, 300  $\mu$ M dNTPs, 100 nM of *EcoRI*-GC primer, 300 nM of *MspI*-CC or *MspI*-CA primers, and 1.25 units of *AmpliTag* Gold polymerase (Applied Biosystems). Amplifications were performed using the following PCR conditions: 94°C for 2 min, 14 cycles at 94°C for 30 s (1°C per s to 65°C), 65°C for 30 s (–0.7°C per cycle), and 72°C for 1 min, 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min (+1 s per cycle), and finally 72°C for 10 min. All AFLP reactions were conducted using an MJ PTC-200 thermocycler (Bio-Rad Laboratories, Waltham, MA).

Selective amplification products were separated in an ABI 3700 DNA automated sequencer (Applied Biosystems) at the University of Wisconsin–Biotechnology Center (Madison, WI). Genotyper 3.7 NT (Applied Biosystems) was used to identify peaks with a fluorescent intensity greater than the threshold value (ca. 150 units) in at least one sample. Categories were made from these identified peaks for scoring samples.

AFLP bands were scored as present (1) or absent (0) using Genotyper 3.7 NT and a binary matrix was developed with molecular sizes ranging from 70 to 325 bp. Each band was checked visually using GeneScan 3.7 NT (Applied BioSystems). The resulting binary matrix was used to calculate Dice's similarity coefficients using NTSYS-pc version 2.1 (Exeter Software, Setauket, NY). Distances were graphically displayed by unweighted paired-group method with arithmetic means (UPGMA). Maximum parsimony analyses were also implemented with PAUP\* 4.0b10 (Sinauer Publishing, Sunderland, MA) using the heuristic search option with 1,000 random addition sequences using the tree

bisection-reconnection (TBR) branch swapping option. The MULPARS option was off (because of computer constraint) and all characters were weighted equally. Robustness and support for parsimony groupings were obtained using 1,000 bootstraps with random addition (10 replicates) for each heuristic search. The gel fragment size and binary data sets have been deposited into TreeBASE, as well as the UPGMA and parsimony trees (study accession no. S1494).

**DNA sequencing.** The ITS+5.8S, mtSSU, and EF-1 $\alpha$  regions were amplified using PCR. Primers ITS-1F/ITS-4 (17,54), MS1/MS2 (54), and EF1/EF2 (46) were used to amplify the ITS+5.8S, mtSSU, and EF-1 $\alpha$ , respectively. Each 50- $\mu$ l reaction mixture

contained 10 ng of template DNA (or no DNA template for a negative control), 2.5 units of AmpliTaq DNA polymerase, 1x PCR buffer, 4 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, and 0.5  $\mu$ M of each primer. The PCR conditions were as follows: (i) for ITS+5.8S, 95°C for 2 min, 30 cycles at 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min followed by a final step at 72°C for 10 min; (ii) for mtSSU, 94°C for 2 min, 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min 30 s followed by a final step at 72°C for 10 min; and (iii) for EF-1 $\alpha$ , 94°C for 2 min, 30 cycles at 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min followed by a final step at 72°C for 10 min. All reactions were conducted with an MJ PTC-200 thermocycler. PCR products were electrophoresed in

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

Taxon	Collection <sup>a</sup>	Isolate <sup>b</sup>	Host/substrate	Virulence rating <sup>c</sup>	GenBank accession no. (ITS+5.8, mtSSU, EF-1 $\alpha$ )
<i>F. commune</i>	9245H	<i>Fc</i> -H1	Soil	High	DQ016195, DQ016145, DQ016242
<i>F. commune</i>	9302G	<i>Fc</i> -H2	Soil	High	DQ016197, DQ016143, DQ016252
<i>F. commune</i>	9313Q	<i>Fc</i> -H3	Soil	High	DQ016201, DQ016143, DQ016255
<i>F. commune</i>	9312F	<i>Fc</i> -H4	Soil	High	DQ016200, DQ016148, DQ016245
<i>F. commune</i>	9302A	<i>Fc</i> -H5	Soil	High	DQ016196, DQ016144, DQ016254
<i>F. commune</i>	9312D	<i>Fc</i> -H6	Soil	High	DQ016191, DQ016146, DQ016253
<i>F. commune</i>	9447B	<i>Fc</i> -H7	Soil	High	DQ016203, DQ016151, DQ016256
<i>F. commune</i>	9447G	<i>Fc</i> -H8	Soil	High	DQ016204, DQ016152, DQ016247
<i>F. commune</i>	9447J	<i>Fc</i> -H9	Soil	High	DQ016199, DQ016147, DQ016244
<i>F. commune</i>	9448M	<i>Fc</i> -H10	Soil	High	DQ016205, DQ016153, DQ016251
<i>F. commune</i>	9449A	<i>Fc</i> -H11	Soil	High	DQ016206, DQ016154, DQ016250
<i>F. commune</i>	9526E	<i>Fc</i> -H12	Soil	High	DQ016208, DQ016156, DQ016248
<i>F. commune</i>	9665K	<i>Fc</i> -H13	Soil	High	DQ016210, DQ016158, DQ016258
<i>F. commune</i>	9408A	<i>Fc</i> -H14	Douglas-fir (diseased)	High	DQ016202, DQ016150, DQ016246
<i>F. commune</i>	9458B	<i>Fc</i> -H15	Douglas-fir (healthy)	High	DQ016207, DQ016155, DQ016257
<i>F. commune</i>	9613A	<i>Fc</i> -H16	Douglas-fir (healthy)	High	DQ016209, DQ016157, DQ016249
<i>F. oxysporum</i>	9315G	<i>Fo</i> -M1	Soil	Medium	DQ016212, DQ016161, DQ016259
<i>F. oxysporum</i>	9312H	<i>Fc</i> -M2	Soil	Medium	DQ016211, DQ016159, DQ016243
<i>F. oxysporum</i>	9348A	<i>Fo</i> -M3	Soil	Medium	DQ016213, DQ016160, DQ016260
<i>F. oxysporum</i>	9411C	<i>Fo</i> -L1	Soil	Low	DQ016216, DQ016164, DQ016263
<i>F. oxysporum</i>	9302E	<i>Fo</i> -L2	Soil	Low	DQ016214, DQ016165, DQ016261
<i>F. oxysporum</i>	9447E	<i>Fo</i> -L3	Soil	Low	DQ016217, DQ016163, DQ016264
<i>F. oxysporum</i>	9665J	<i>Fo</i> -L4	Soil	Low	DQ016218, DQ016166, DQ016265
<i>F. oxysporum</i>	9312L	<i>Fo</i> -L5	Soil	Low	DQ016215, DQ016162, DQ016262
<i>F. oxysporum</i>	9343A	<i>Fo</i> -N1	Soil	Non	DQ016219, DQ016170, DQ016267
<i>F. oxysporum</i>	9343E	<i>Fo</i> -N2	Soil	Non	DQ016192, DQ016169, DQ016266
<i>F. oxysporum</i>	9343G	<i>Fo</i> -N3	Soil	Non	DQ016220, DQ016171, DQ016268
<i>F. oxysporum</i>	9348F	<i>Fo</i> -N4	Soil	Non	DQ016221, DQ016172, DQ016269
<i>F. oxysporum</i>	9348J	<i>Fo</i> -N5	Soil	Non	DQ016222, DQ016173, DQ016270
<i>F. oxysporum</i>	9411B	<i>Fo</i> -N6	Soil	Non	DQ016223, DQ016174, DQ016271
<i>F. oxysporum</i>	9448J	<i>Fo</i> -N7	Soil	Non	DQ016194, DQ016167, DQ016274
<i>F. oxysporum</i>	9449C	<i>Fo</i> -N8	Soil	Non	DQ016226, DQ016176, DQ016275
<i>F. oxysporum</i>	9449D	<i>Fo</i> -N9	Soil	Non	DQ016227, DQ016177, DQ016276
<i>F. oxysporum</i>	9449E	<i>Fo</i> -N10	Soil	Non	DQ016228, DQ016178, DQ016277
<i>F. oxysporum</i>	9449F	<i>Fo</i> -N11	Soil	Non	DQ016229, DQ016179, DQ016278
<i>F. oxysporum</i>	9449H	<i>Fo</i> -N12	Soil	Non	DQ016230, DQ016180, DQ016279
<i>F. oxysporum</i>	9449K	<i>Fo</i> -N13	Soil	Non	DQ016231, DQ016181, DQ016280
<i>F. oxysporum</i>	9450A	<i>Fo</i> -N14	Soil	Non	DQ016232, DQ016182, DQ016281
<i>F. oxysporum</i>	9418C	<i>Fo</i> -N15	Douglas-fir (diseased)	Non	DQ016193, DQ016168, DQ016272
<i>F. oxysporum</i>	9418D	<i>Fo</i> -N16	Douglas-fir (diseased)	Non	DQ016224, DQ016175, DQ016273
<i>F. commune</i>	9418E	<i>Fc</i> -N17	Douglas-fir (diseased)	Non	DQ016198, DQ016142, DQ016241
<i>F. oxysporum</i> f. sp. <i>melonis</i> <sup>d</sup>	0348	...	Melon	N/A	DQ016233, DQ016183, DQ016282
<i>F. oxysporum</i> f. sp. <i>lactuca</i> <sup>d</sup>	0349	...	Lettuce	N/A	DQ016234, DQ016184, DQ016283
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> <sup>d</sup>	0350	...	Tomato	N/A	DQ016235, DQ016185, DQ016284
<i>F. oxysporum</i> <sup>e</sup>	Fo47	<i>Fo</i> -Fo47	Soil	Non	DQ016236, DQ016186, DQ016285
<i>F. proliferatum</i>	9223F	...	Aspen	High	DQ016238, DQ016188, DQ016287
<i>F. proliferatum</i>	9721B	...	Douglas-fir	High	DQ016239, DQ016189, DQ016288
<i>F. proliferatum</i>	9816	...	Whitebark pine	High	DQ016240, DQ016190, DQ016289

<sup>a</sup> All isolates were collected in Idaho, except isolates 0348, 0349, and 0350, which were collected in California, and isolate Fo47, which was collected in France.

<sup>b</sup> Identification codes: *Fo*-M, *Fo*-L, and *Fo*-N indicate *F. oxysporum* medium virulence, low virulence, and nonpathogenic, respectively, and *Fc*-H, *Fc*-M, and *Fc*-N indicate *F. commune* high virulence, medium virulence, and nonpathogenic, respectively.

<sup>c</sup> Three disease categories were used for these assays: damping-off, radicle rotting, and no disease. Numbers were given to germinants in each category, which were then added to other criteria (length of time germinants survived, primary root growth, and re-isolation of inoculated isolates) for a final virulence rating (24). The possible numerical rating ranged from 0 to 100, with higher values reflecting greater virulence. Each isolate had an average rating of numerical virulence that was used to compare isolates. Based on previous studies (26–28), highly virulent isolates exhibited scores of 80 to 100, moderately virulent isolates from 60 to 79, isolates with low virulence from 40 to 59, and isolates with average scores below 39 were considered nonpathogenic.

<sup>d</sup> Isolates 0348, 0349, and 0350 were collected by T. Gordon in pathogenic situations.

<sup>e</sup> Isolate Fo47 was collected by C. Alabouvette and has been used as a biological control against pathogenic isolates of *F. oxysporum*.

1.5% agarose gels with 0.5× Tris-borate-EDTA (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) buffer. Gels were stained with ethidium bromide (0.5 µg/ml) and bands were visualized using UV light. PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced with an ABI 3700 DNA sequencer at the Davis Sequencing Facility (Davis, CA) and the University of Wisconsin–Biotechnology Center. All regions were sequenced with the same primers used for initial amplification.

**Phylogenetic analyses.** Raw sequences from both strands were aligned and edited visually using BioEdit (19). Alignments of sequences were also examined using Clustal X Multiple Sequence Alignment Program version 1.81 (30). Any duplicate sequence types were removed from the data set prior to phylogenetic analyses. All sequences used for phylogenetic analyses were deposited into GenBank (Table 1).

Phylogenetic analyses were conducted using PAUP and MrBayes version 3.0b4 (23). The ITS+5.8S, mtSSU, and EF-1 $\alpha$  regions were analyzed separately and combined (excluding the ITS+5.8S region). For the combined analyses, sequences (mtSSU and EF-1 $\alpha$ ) of four isolates of *F. oxysporum* (NRRL 31073, AF362290 for mtSSU/AF362274 for EF-1 $\alpha$ ; NRRL 31074, AF362289/AF362273; NRRL 26442, AF250562/AF246834; and NRRL 25603, AF008453/AY527534), one isolate of *F. oxysporum* f. sp. *batatas* (NRRL 26409, AF008450/AF008484) (47), one isolate of *F. oxysporum* f. sp. *lycopersici* (NRRL 26383, AF008468/AF008502) (47), one isolate of *F. oxysporum* f. sp. *melonis* (NRRL 26406, AF008470/AF008504) (47), six isolates of *F. commune* Skovgaard, O'Donnell et Nirenberg (NRRL 22900, AF362288/AF362272; NRRL 22903, U34509/AF008513; NRRL 25043, AF362277/AF362261; NRRL 26897, AF362286/AF362270; NRRL 28058, AF324293/AF324333; and NRRL 31081, AF362285/AF362269) (49), and one isolate of *F. subglutinans* (NRRL 22016, M1431/AF160289) were obtained from GenBank. Sequences of *F. subglutinans* were found in a matrix (M1431) from TreeBASE (49) and included in the analyses for comparison. Care was taken to ensure sequences of the mtSSU and EF-1 $\alpha$  regions were from the same isolate. An isolate of *F. redolens* (NRRL 31255, M1431/M1432 from TreeBASE) served as the outgroup. All indels within the sequence alignment were coded using a simple gap-coding method (48). Maximum parsimony and Bayesian analyses were performed on all regions to search for optimal trees. Maximum parsimony analyses were performed using the heuristic search option with 1,000 random addition sequences using TBR branch swapping with the MULPARS option on, and all characters were weighted equally. Robustness and support for parsimony groupings were obtained using 1,000 bootstraps with random addition (10 replicates) for each heuristic search. DT-ModSel (39) was used to determine nucleotide substitution models best suited for the data sets. Model selections for ITS+5.8S, mtSSU, and EF-1 $\alpha$  were TrNef, HKY+I, and K80+I, respectively. These models were used for Bayesian analysis, which was performed with settings suggested by the selected models. For Bayesian analyses, the Markov chain Monte Carlo search was run with four chains for 3,000,000 generations generating 30,001 trees, the first 6,000 trees were discarded as “burnin” of the chains. The remaining 24,001 trees were used to make 90% majority-rule consensus trees using PAUP. Preliminary analyses for the ITS+5.8S showed, however, that the highly virulent isolates of *Fusarium* sp. clustered with *F. proliferatum*. For this reason, we obtained a sequence for *F. beomiforme* from GenBank (U61674) to serve as the outgroup for analyses of the ITS+5.8S region. Partition-homogeneity test implemented using PAUP was conducted to evaluate concordance and combinability of the mtSSU and EF-1 $\alpha$  data sets (15,22). Partition-homogeneity test, which corresponds to the incongruence length difference (ILD) test (9,14), was conducted using 1,000 heuristic searches with random addition sequence and TBR branch swapping. The

MULPARS option was not in effect because of computer memory constraints. Parsimony and Bayesian analyses were completed on the combined data. Maximum parsimony analysis was completed on the combined data set with identical options in effect for the separate regions. Again, DT-ModSel was used to determine that SYM+I+G was the best-suited nucleotide-substitution model for the combined data set. Bayesian analyses were 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, the first 6,000 trees were discarded as “burnin” of the chains. The remaining 24,001 trees were used to make a 90% majority-rule consensus tree using PAUP. All phylogenetic trees generated from rDNA sequence data have been deposited into TreeBASE (study accession no. S1494).

## RESULTS

**AFLP analyses.** Analysis of two selective primer combinations yielded a total of 146 presumptive loci for all 48 isolates included in the analyses, 41 *Fusarium* spp. isolates, one isolate of *F. oxysporum* f. sp. *melonis* (0348), one isolate of *F. oxysporum* f. sp. *lactucae* (0349), one isolate of *F. oxysporum* f. sp. *lycopersici* (0350), one nonpathogenic isolate of *F. oxysporum* (*Fo*-Fo47), and three isolates of *F. proliferatum* (9223F, 9721B, and 9816A). Of the 146 AFLP loci, 142 (97.3%) were polymorphic. Each of the 48 isolates had a unique AFLP phenotype. Examinations of the 41 isolates from the conifer nursery (Table 1) yielded a total of 121 presumptive loci, of which 111 (92%) loci were polymorphic. Of the 111 polymorphic AFLP loci evaluated, 30 alleles were unique to only highly virulent isolates (*Fc*-H1 to *Fc*-H16) and 33 alleles were unique to only nonpathogenic isolates (*Fo*-N1 to *Fo*-N16). The AFLP phenotype for one isolate (*Fc*-N17; nonpathogenic), however, showed similarity to AFLP phenotypes for highly virulent isolates; this was the only nonpathogenic isolate that clustered with highly virulent isolates (Table 1; Figs. 1 to 5). A cluster analysis using UPGMA identified two groups of isolates (Fig. 1). The first group included all nonpathogenic isolates (except *Fc*-N17), all isolates of low virulence, and most isolates of moderate virulence, whereas the second group contained all highly virulent isolates (*Fc*-H1 to *Fc*-H16), one moderately virulent isolate (*Fc*-M2), and one nonpathogenic isolate (*Fc*-N17) (Fig. 1).

**DNA sequencing.** Each sequenced region (ITS+5.8S, mtSSU, and EF-1 $\alpha$ ) had a different number of unique haplotypes used for the analyses (Table 2). The ITS+5.8S region had six unique haplotypes and analyzed sequences were 94 to 99% similar. Of this region's 534 characters, 35 (6% of the total) of 50 variable characters were parsimony informative. The mtSSU region had seven haplotypes, with sequences that were 94 to 99% similar. Of the mtSSU region's 652 characters, 46 (7% of the total) of 47 variable characters were parsimony informative. The EF-1 $\alpha$  region had 17 unique haplotypes and 669 characters; all 71 (11% of the total) variable characters were parsimony informative. Results from the partition homogeneity test indicated that mtSSU and EF-1 $\alpha$  were congruent and could be combined ( $P = 0.104$ ). The combined data set yielded 35 unique haplotypes with 117 (9%) informative and 84 uninformative characters (Table 2).

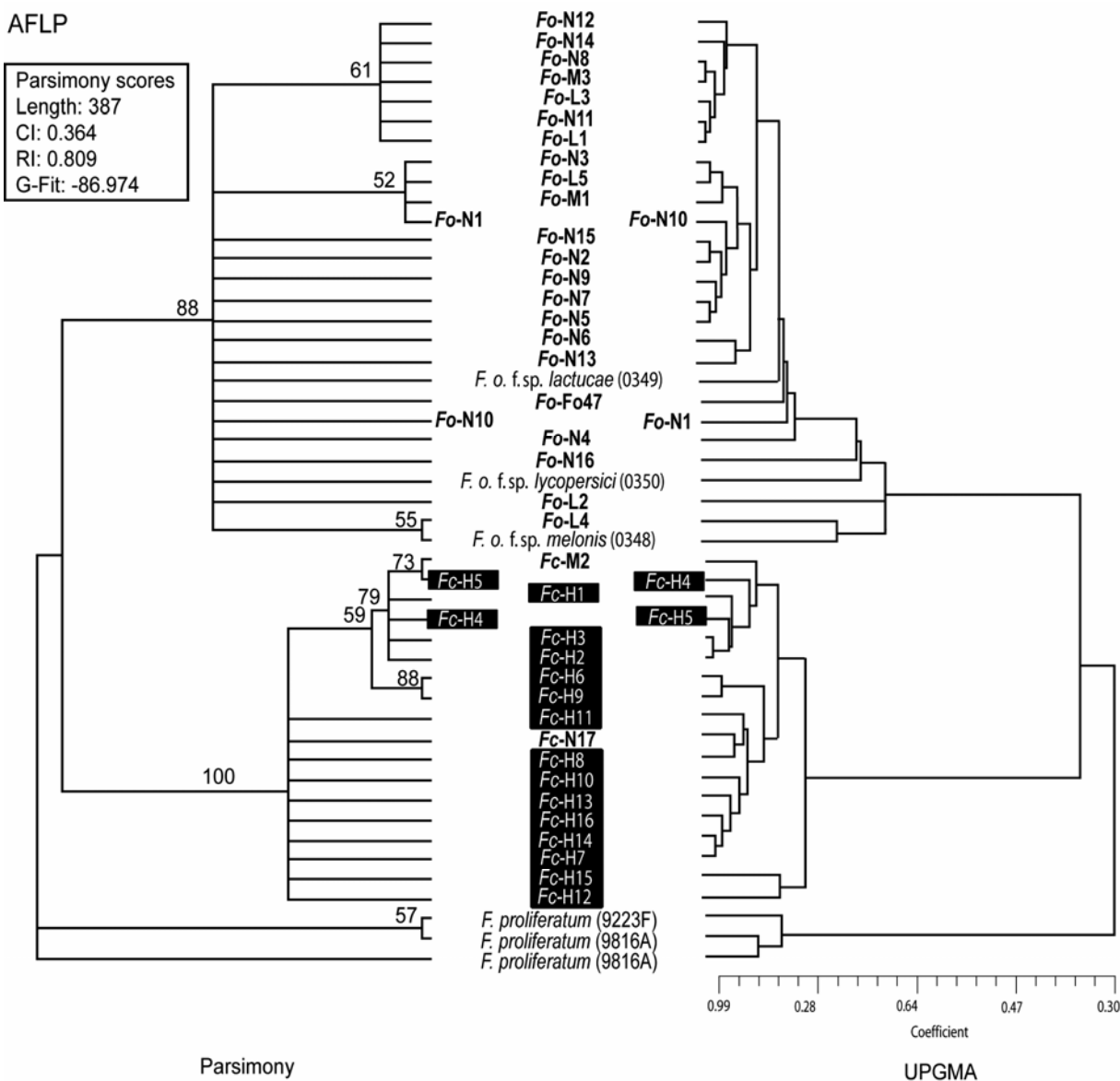
**Phylogenetic analyses.** Maximum parsimony and Bayesian analyses on ITS+5.8S, mtSSU, and EF-1 $\alpha$  yielded similar results (Figs. 2 to 4). Bayesian analyses tended to generate a more conservative phylogram dependent on the majority-rule posterior probability cutoffs at 90% compared with 50% bootstrap cutoffs used for parsimony analyses. For all regions, however, separation of the highly virulent isolates into a single clade was consistently supported with bootstrapping of over 80% (Figs. 2 to 4). All nonpathogenic isolates, with the exception of *Fc*-N17, also clustered within a common clade. In addition, all low to moderately

virulent isolates also grouped with the nonpathogenic isolates, with the exception of *Fc*-M2, which consistently clustered within the highly virulent clade (Figs. 2 to 4). A heuristic search, with the optimality criterion set to parsimony in PAUP, found 10 equally parsimonious trees for the EF-1 $\alpha$  region; one parsimonious tree was found when the ITS+5.8S and mtSSU regions were analyzed.

The number of clusters within a clade, and the cluster each isolate nested into, varied depending on the DNA region analyzed. For example, the clade comprising nonpathogenic isolates with some low to moderately virulent isolates, contained eight clusters based on the EF-1 $\alpha$  region, but only four and five clusters based on the ITS+5.8S and mtSSU regions, respectively (Figs. 2 to 4). With the EF-1 $\alpha$  and mtSSU regions, the three *F. proliferatum* isolates formed a separate cluster. Isolates from other *F. oxysporum* formae speciales, *F. oxysporum* f. sp. *lactucae*, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *lycopersici*, and nonpathogenic isolate (*Fo*-Fo47) clustered within the same clade for all regions. In the ITS+5.8S region, few polymorphisms were observed between sequences of highly virulent and nonpathogenic isolates.

Sequences of highly virulent *F. oxysporum* and *F. proliferatum* differed by only three base pairs, and *F. oxysporum* and *F. proliferatum* were clustered together in the phylogenetic analysis of the ITS+5.8S region (Fig. 2).

The combined data set of mtSSU and EF-1 $\alpha$  and the AFLP cladograms had similar topologies when compared with the other regions analyzed (Figs. 1 and 5). Cladograms produced using maximum parsimony displayed two distinct clades: one contained all the highly virulent isolates with one nonpathogenic isolate and one moderately virulent isolate. The second clade contained nonpathogenic and low to moderately virulent isolates, which were again supported with bootstrapping of over 88% (Figs. 1 and 5). Sequences for isolates within the *F. oxysporum* species complex (obtained from GenBank) clustered within the nonpathogenic clade, whereas sequences of *F. commune* isolates (obtained from GenBank and TreeBASE) clustered within the highly virulent clade. In fact, two highly virulent isolates (*Fc*-H2 and *Fc*-H6) shared an identical sequence type with *F. commune* isolate NRRL 22903 (Fig. 5). A heuristic search, with the optimality criterion set to parsimony in PAUP, found eight equally parsimonious trees.



**Fig. 1.** Parsimony and unweighted pair-group method with arithmetic average (UPGMA, right) trees generated from amplified fragment length polymorphism (AFLP) data. Isolates of *Fusarium oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1. Bold numbers represent *Fo* and *Fc* isolates and boxed numbers represent highly virulent *Fc* isolates used in our study. The image shows a 50% majority-rule bootstrap support for maximum parsimony analysis based on 1,000 bootstraps (left).

monious trees when using the combined data set and 1,560 equally parsimonious trees when using the AFLP binary data set.

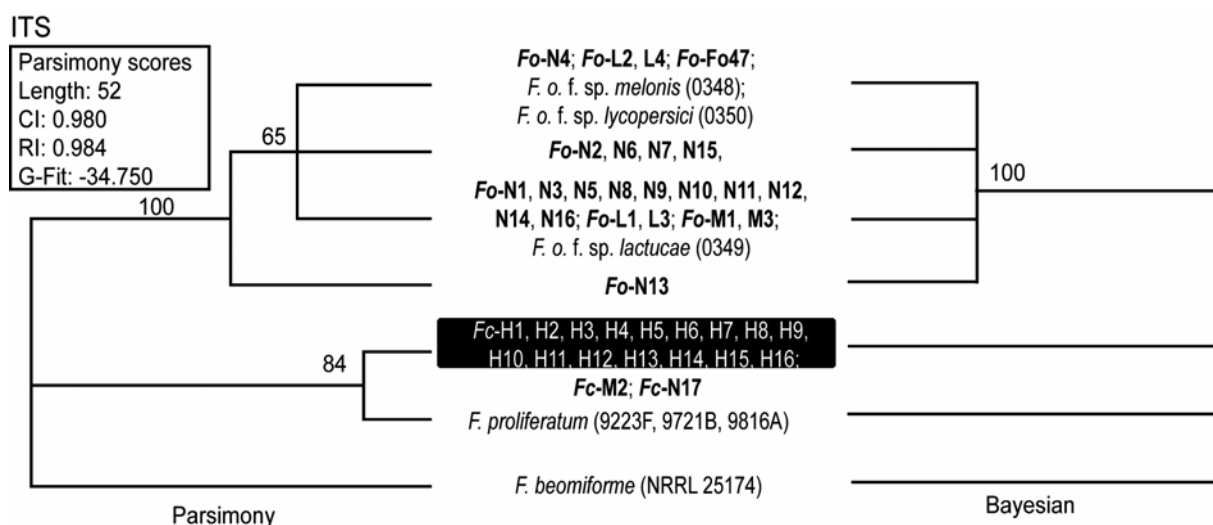
**Phylogenetic species recognition.** DNA sequence data from mtSSU and EF-1 $\alpha$  indicated that the highly virulent isolates (*Fc*-H1-H16), one nonpathogenic (*Fc*-M2), and one moderately virulent isolate (*Fc*-M1) appeared to be *F. commune* (Fig. 5) that was recently distinguished from its putative sister taxon, *F. oxysporum* (49). Most of our nonpathogenic (*Fo*-N1-N16), low (*Fo*-L1-L5) and moderately virulent (*Fo*-M1, *Fo*-M3) isolates appeared to be *F. oxysporum* (Fig. 5).

## DISCUSSION

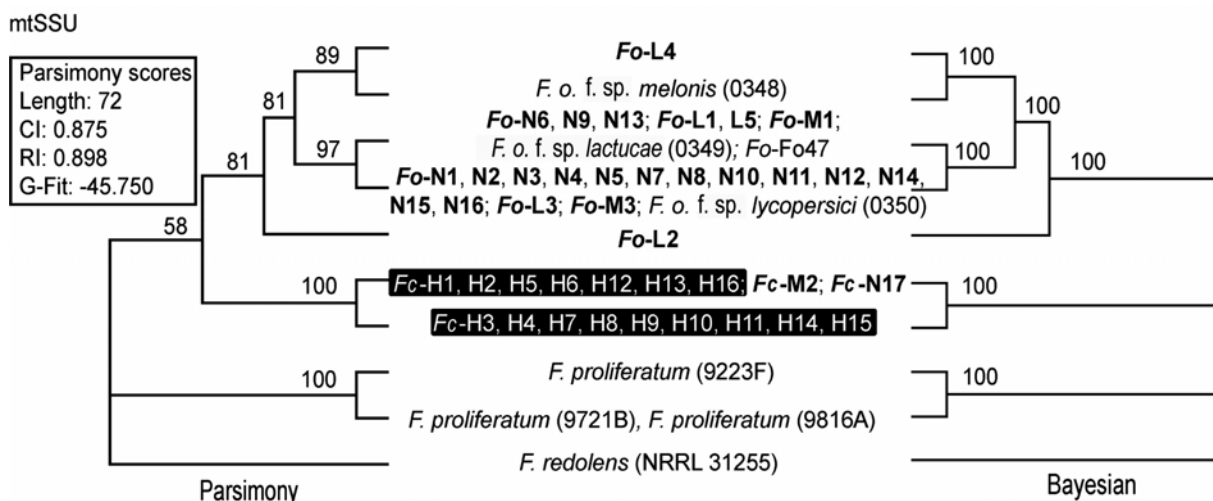
Based on AFLP markers and DNA sequences of ITS+5.8S, mtSSU, and EF-1 $\alpha$  regions, we determined that highly virulent isolates that caused damping-off and root rot on Douglas-fir seedlings were *F. commune*, although they were originally classified as *F. oxysporum* based on morphology. Those isolates were genetically distinct from nonpathogenic isolates of *F. oxysporum*

collected from the same forest nursery. Furthermore, mtSSU or EF-1 $\alpha$  sequence data or AFLP markers showed potential utility for identifying highly virulent *F. commune* isolates among populations of *Fusarium* spp. collected from conifer seedlings or nursery soil. Nearly 30 and 33 AFLP loci were unique to only highly virulent and nonpathogenic isolates, respectively. Unique loci associated with highly virulent isolates could be sequenced and used to develop diagnostic primers for identifying highly virulent isolates of *F. commune* within the nursery setting.

Sequences of the ITS+5.8S region allowed us to distinguish highly virulent isolates from most nonpathogenic isolates (collected from soil and Douglas-fir roots), but this region was not useful for differentiating highly virulent isolates from *F. proliferatum*. O'Donnell and Cigelnik (45) showed that species within the *Gibberella fujikuroi* complex, which includes *F. proliferatum*, contain two nonorthologous copies of ITS, type 1 and type 2. Our study showed that *F. proliferatum* and our isolates of *F. commune* contain ITS type 2, while nonpathogenic isolates of *F. oxysporum* contain ITS type 1. Low and moderately virulent



**Fig. 2.** Parsimony and Bayesian trees generated from internal transcribed spacer including 5.8S (ITS+5.8S) rDNA sequences. Isolates of *Fusarium oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1. Bold numbers represent *Fo* and *Fc* isolates and boxed numbers represent highly virulent *Fc* isolates used in our study. The image shows a 50% majority-rule bootstrap consensus tree for maximum parsimony based on 1,000 bootstrap analyses (left) and a 90% majority-rule posterior probability consensus tree based on 24,001 trees (right).



**Fig. 3.** Parsimony and Bayesian trees generated from the mitochondrial small subunit (mtSSU) rDNA sequences. Isolates of *Fusarium oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1. Bold numbers represent *Fo* and *Fc* isolates and boxed numbers represent highly virulent *Fc* isolates used in our study. The image shows a 50% majority-rule bootstrap consensus tree for maximum parsimony based on 1,000 bootstrap analyses (left) and a 90% majority-rule posterior probability consensus tree based on 24,001 trees (right).

isolates contained ITS type 1 or ITS type 2, depending on their similarity to either *F. commune* or *F. oxysporum*. The heterogeneity of intergenic spacer and ITS regions within single genomes or among strains has been reported in several groups of fungi (1,32,34,36,44). Sequence heterogeneity can confound phylogenetic analysis, so meticulous care is needed to recognize and interpret such heterogeneity (34). Bao et al. (5) showed that the ITS+5.8S region could not distinguish between the isolates previously used as a biocontrol agent (7) and other nonpathogenic isolates of *F. oxysporum*. Similar results were found in our study; isolate Fo-Fo47, previously used as a biocontrol agent against virulent *F. oxysporum* strains (7,16), grouped with our isolates that were nonpathogenic on conifers for all regions analyzed (Figs. 2 to 4). Although all of these nonpathogenic isolates appeared closely related genetically, the nonpathogenic isolates in this study have not been tested for potential biocontrol effects. Such evaluations could prove useful because nursery-derived isolates may be better adapted to nursery environments than commercially available biocontrol agents that were developed for other agricultural systems.

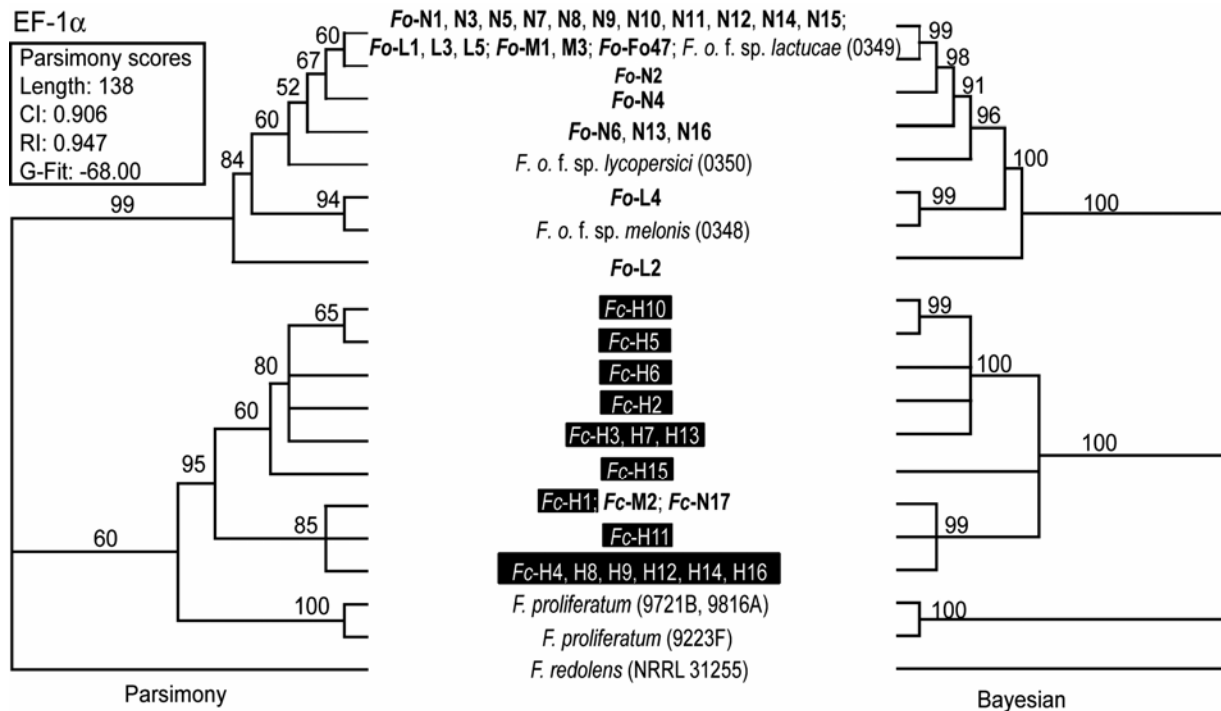
Several isolates of *F. oxysporum*, such as f. sp. *lactucae*, f. sp. *melonis*, and f. sp. *lycopersici*, which are pathogenic to lettuce, melon, and tomato, respectively, clustered with isolates that were nonpathogenic to Douglas-fir. Thus, it cannot be assumed that isolates nonpathogenic on Douglas-fir are also nonpathogenic on other plant species. Similarly, *F. oxysporum* f. sp. *lilii* isolates were shown to be highly pathogenic to lily, but were not pathogenic to gladiolus or tulip (2). Many additional studies have shown similar host specificity for *F. oxysporum* formae speciales (18).

Our highly virulent isolates clustered with isolates described as *F. commune* (Fig. 5), a recently characterized taxon closely related to *F. oxysporum* and *G. fujikuroi* species complexes, although independent of these species complexes (49). Because our highly virulent isolates appeared to be *F. commune* based on DNA sequences, morphological traits were examined to determine if these highly virulent isolates were similar to the morphological species description of *F. commune*. Morphological charac-

teristics of *F. commune* include the formation of polyphialides, long, slender monophialides, and chlamydoconidia (49). Extensive microscopic examination revealed that only one of our highly virulent isolates produced a structure resembling a polyphialide while being grown on synthetic low-nutrient agar media under dark conditions (49). All *Fusarium* spp. isolates used in this study appeared morphologically similar regardless of pathogenicity. However, we conclude that our highly virulent isolates, previously classified as *F. oxysporum*, are *F. commune* based on definitive DNA sequence data.

To our knowledge, this is the first report that shows *F. commune* is a cause of Fusarium disease (root rot and damping-off) on Douglas-fir seedlings. Because isolates of *F. commune* in our study were morphologically indistinguishable from *F. oxysporum*, previous reports of disease on Douglas-fir caused by *F. oxysporum* may be attributable to what is now recognized as *F. commune*. Two isolates of *F. commune* used in this paper for comparison purposes, NRRL 22900 and NRRL 22903, were collected from Douglas-fir seedlings and previously classified as *F. oxysporum* based on morphological features (10,49). In fact, the majority of newly recognized isolates of *F. commune* were previously classified as *F. oxysporum* (49). The morphological differences between these two species are few, and include plastic characteristics such as polyphialide production, which can be variable. It seems highly likely that *F. commune* taxon includes many isolates that are morphologically indistinguishable from *F. oxysporum*. At present, it appears that genetic characterization is the only reliable method to identify *F. commune*.

Pathogenic isolates from Douglas-fir have not been classified into a forma specialis because of the lack of evidence for host specialization within *F. oxysporum* associated with forest nurseries (8). The information presented here supports this conclusion. Moreover, it remains unclear whether *F. oxysporum* can be pathogenic to Douglas-fir or other conifers. Perhaps species that are morphologically similar to *F. oxysporum*, like *F. commune*, have been misidentified because genetic-based identification techniques were lacking. Several studies have shown that numerous



**Fig. 4.** Parsimony and Bayesian trees generated from the nuclear translation elongation factor 1-alpha (EF-1 $\alpha$ ) sequences. Isolates of *Fusarium oxysporum* (Fo) and *F. commune* (Fc) are described in Table 1. Bold numbers represent Fo and Fc isolates and boxed numbers represent highly virulent Fc isolates used in our study. The image shows a 50% majority-rule bootstrap consensus tree for maximum parsimony based on 1,000 bootstrap analyses (left) and a 90% majority-rule posterior probability consensus tree based on 24,001 trees (right).

*Fusarium* spp., such as *F. sporotrichioides*, *F. proliferatum*, and *F. circinatum*, are capable of causing damping-off in conifers (11,26,27,28,31,43,50). Studies examining the genetic association of these species would prove useful in understanding the complexity and relatedness of *Fusarium* spp. causing diseases of conifers.

Similarities between phylogram topologies of DNA sequences and AFLP data sets strengthen the conclusions of this study and indicate that distinct genetic differences exist between *F. commune* and *F. oxysporum*. In all analyses, separation of the highly virulent clade had over 80% bootstrap and posterior probability support. None of the AFLP profiles for isolates in our study were identical; however, isolates within a particular clade were 70 to 99% similar. The fact that none of the AFLP profiles for the isolates examined were identical suggests that our test set did not contain clonal isolates. Thus, considerable genetic diversity among *F. commune* and *F. oxysporum* isolates was present within a single nursery. The high number of haplotypes and variation of AFLP profiles, within each clade, may indicate that the nursery has had numerous introductions of both *F. oxysporum* and *F. commune* over time. Pre-plant soil fumigation may not completely manage *Fusarium* spp. effectively (13,20,21,40). In some situations, *Fusarium* propagules can infest soil and plant materials and may be introduced on seeds or other organic matter brought

into nurseries (31,43). The complexity of managing *Fusarium* spp. is greatly increased because of numerous pathogen introductions into nurseries and high genetic diversity among isolates. For this reason, an integrated approach for managing *Fusarium* spp. is prudent (12).

Isolates used in this study were collected from one particular nursery in Idaho; isolates from a wider range of forest nurseries would help determine if our results are applicable to other areas. Our long-term goal is to provide a basis for developing molecular

TABLE 2. Comparison of DNA sequence and amplified fragment length polymorphism (AFLP) tree statistics

Analysis	No. of unique haplotypes <sup>a</sup>	No. of characters	Synapomorphies/ autapomorphies
ITS+5.8S <sup>b</sup>	7	534	35/15
mtSSU <sup>c</sup>	10	664	48/9
EF-1 $\alpha$ <sup>d</sup>	20	661	71/45
mtSSU+EF-1 $\alpha$	35	1,287	117/84
AFLP	48	146	124/17

<sup>a</sup> Number of unique haplotypes includes outgroups.

<sup>b</sup> ITS+5.8S = internal transcribed spacers 1 and 2 including 5.8S rDNA.

<sup>c</sup> mtSSU = mitochondrial small subunit rDNA.

<sup>d</sup> EF-1 $\alpha$  = nuclear translation elongation factor 1-alpha.

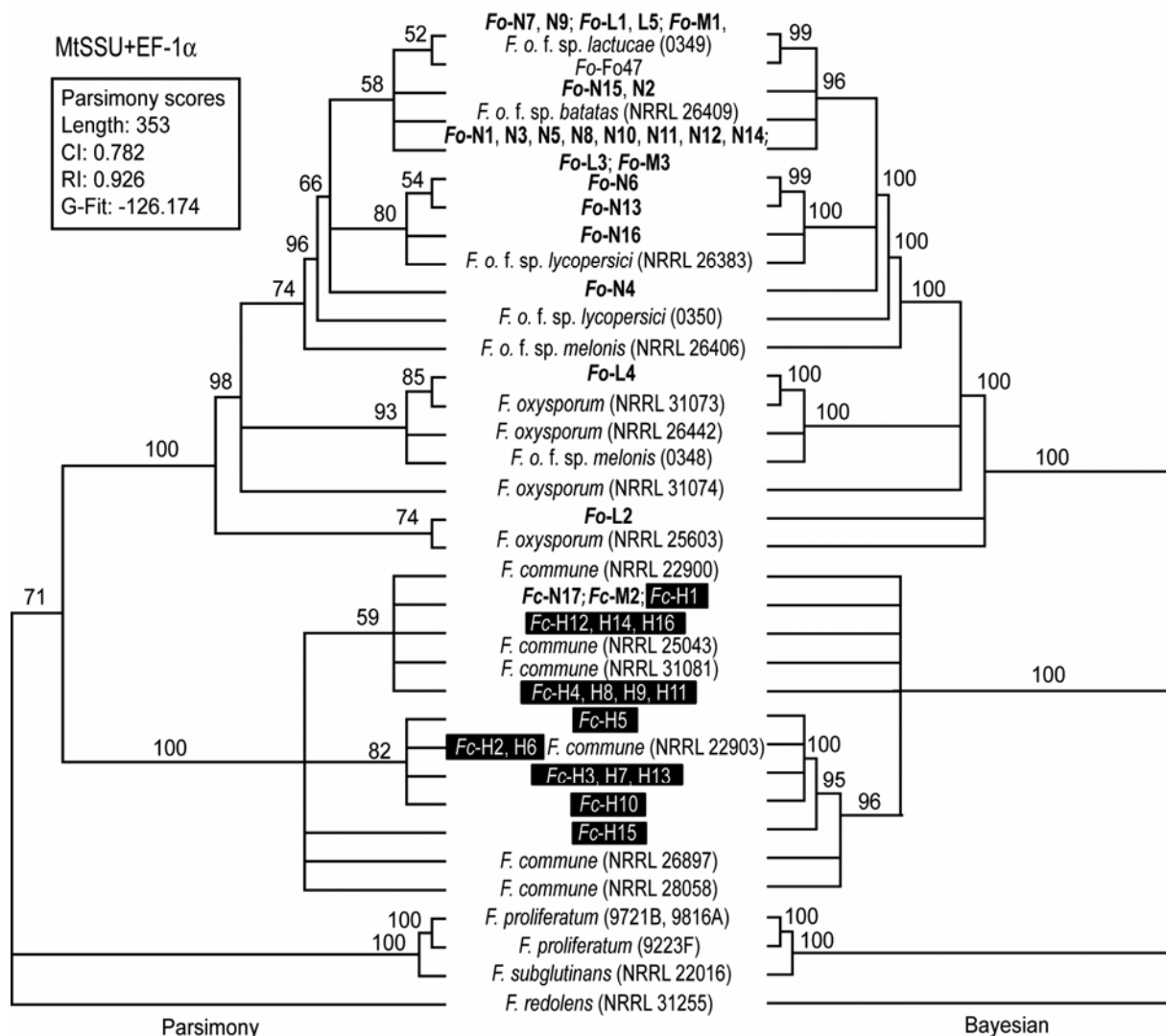


Fig. 5. Parsimony and Bayesian analyses generated from combined mitochondrial small subunit (mtSSU) rDNA and the nuclear translation elongation factor 1-alpha (EF-1 $\alpha$ ) sequences. Isolates of *Fusarium oxysporum* (*Fo*) and *F. commune* (*Fc*) are described in Table 1. Bold numbers represent *Fo* and *Fc* isolates and boxed numbers represent highly virulent *Fc* isolates used in our study. The image shows a 50% majority-rule bootstrap consensus for maximum parsimony analysis based on 1,000 bootstraps (left) and a 90% majority-rule posterior probability consensus for Bayesian analysis based on 24,001 trees (right).



markers for easy identification of highly virulent *Fusarium* isolates within nurseries that represent high risk to conifer seedlings. We have shown that several AFLP genetic markers and mtSSU sequences offer potential for development of such markers. After such markers are developed, further field testing will be required to validate their utility for characterizing pathogenic potential of *Fusarium* populations within forest nurseries.

### ACKNOWLEDGMENTS

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