Primer Sets Developed To Amplify Conserved Genes from Filamentous Ascomycetes Are Useful in Differentiating *Fusarium* Species Associated with Conifers

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We examined the usefulness of primer sets designed to amplify introns within conserved genes in filamentous ascomycetes to differentiate 35 isolates representing six different species of *Fusarium* commonly found in association with conifer seedlings. We analyzed restriction fragment length polymorphisms (RFLP) in five amplified PCR products from each *Fusarium* isolate. The primers used in this study were constructed on the basis of sequence information from the *H3*, *H4*, and β -tubulin genes in *Neurospora crassa*. Primers previously developed for the intergenic transcribed spacer region of the ribosomal DNA were also used. The degree of interspecific polymorphism observed in the PCR products from the six *Fusarium* species allowed differentiation by a limited number of amplifications and restriction endonuclease digestions. The level of intraspecific RFLP variation in the five PCR products was low in both *Fusarium proliferatum* and *F. avenaceum* but was high in a population sample of *F. oxysporum* isolates. Clustering of the 35 isolates by statistical analyses gave similar dendrograms for *H3*, *H4*, and β -tubulin RFLP analysis, but a dendrogram produced by intergenic transcribed spacer analysis varied in the placement of some *F. oxysporum* isolates.

Fusarium Link: Fr. is a diverse genus consisting of an array of species responsible for damping-off, root rots, cankers, and vascular wilts on a multitude of economically important plant species (37). The confusion regarding the taxonomy of *Fusarium* is perhaps best illustrated by the diversity of proposed taxonomic systems (36, 50). Even the most recent taxonomic systems differ greatly with respect to the number of species recognized. Gerlach and Nirenberg (7) recognize 78 species, while Nelson et al. (38) recognize only 30 well-documented species. Not surprisingly, an expert in fungal taxonomy is required to discern the subtle differences in macro- and microconidial shape and conidiogenous structures to identify *Fusarium* isolates to the species level. Identification to the forma speciales level requires pathogenicity testing.

The advent of DNA-based molecular methods has provided useful tools with which to study the phylogeny of *Fusarium* and to differentiate *Fusarium* species, formae speciales, races, and strains. A variety of methods, including restriction fragment length polymorphism (RFLP) analysis of hybridization data with DNA probes constructed from nuclear (31, 32) or mitochondrial regions (23, 28, 29) or of the ribosomal DNA (17, 29, 30), have been used. The development of the PCR (35) has circumvented the difficulty of obtaining cloned genes to assay polymorphisms in the fungal genome (6). Studies on genetic relatedness and variability among *Fusarium* species have used oligonucleotide primers specific for the internal transcribed spacer (ITS) (48) region of the nuclear ribosomal DNA genes (39, 41). Randomly amplified polymorphic DNA (RAPD) (49) markers were used to examine population variability in *Fusarium graminearum* Schwabe (40) and *F. solani* (Mart.) Sacc. f. sp. *cucurbitae* Snyder and Hans. (5) and to differentiate races of *F. oxysporum* Schlecht. f. sp. *pisi* (J. C. Hall) Snyder and Hans, (15).

In the Pacific Northwest, species of *Fusarium* are commonly

isolated from conifer seed and seedlings and are ubiquitous in bare-root nursery field soils (18, 24). Species of *Fusarium* isolated from conifer seed or seedlings are diverse and include *F. acuminatum* Ellis and Everh., *F. avenaceum* (Fr.) Sacc., *F. moniliforme* Sheldon, *F. oxysporum*, *F. proliferatum* (T. Matsushima) Nirenberg, *F. sambucinum* Fuckel, *F. solani*, *F. sporotrichioides* Sherb., *F. lateritium* Nees: Fr., and *F. poae* (Peck) Wollenw. and Lewis (1a–3, 14, 25, 26, 34). Species of *Fusarium* can also be isolated from diverse sources in conifer container nursery environments, including seedling growing containers, pallets, growing medium, and nursery air (1a).

Fusarium spp. can cause damping-off, hypocotyl rot, and root rot and can contribute to stem canker problems on Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] (3, 19). Pathogenic isolates of *F. oxysporum*, *F. moniliforme*, *F. avenaceum*, and *F. acuminatum* were isolated from diseased and/or healthy Douglas fir seed or seedlings (2, 14, 25). An accurate and efficient technique for identifying *Fusarium* species that associate with conifer seedlings would provide a powerful tool for detecting inoculum sources and aid in the development of disease management strategies.

At present, a method to differentiate a large collection of *Fusarium* species with application to field situations has not been developed, although some preliminary work has been done (30). In this study, we evaluated the effectiveness of primer sets constructed to conserved genes in filamentous ascomycetes (8) to differentiate 35 isolates from six species of *Fusarium* (*F. oxysporum*, *F. proliferatum*, *F. acuminatum*, *F. avenaceum*, *F. moniliforme*, and *F. sporotrichioides*). The primer sets were constructed to protein-coding regions and amplify a segment of DNA that spans one or more intron sequences. We observed that intron sequences diverge more rapidly than protein-encoding DNA sequences among closely related *Neurospora* spp. (7a, 9). These primer sets have been shown to amplify DNA segments that are polymorphic in both size and restriction enzyme sites from a number of filamentous ascomycetes and related deuteromycetes (8).

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TABLE 1. *Fusarium* culture collection

Isolate	Source ^a	Yr isolated	Host	Location
<i>F. acuminatum</i>				
F42	BCRI	1988	Douglas fir seed (seedlot 8706)	Alger Creek, B.C.
9226W	USDA	1992	Sand/soil	Glacier National Park Nursery, West Glacier, Mont.
<i>F. avenaceum</i>				
F34	BCRI	1988	Douglas fir seed (seedlot 9983)	Tsowwin River, B.C.
F62	BCRI	1988	Douglas fir seedling root (seedlot 9983)	Surrey Nursery, Surrey, B.C.
FR8	BCRI	1992	Douglas fir seedling root (pathogenic)	Surrey Nursery, Surrey, B.C.
9142G	USDA	1991	Inner wall, pine cell container	USDA Forest Service Nursery, Coeur d'Alene, Idaho
9217J ^b	USDA	1992	Damped-off Douglas fir seedling root	University of Idaho Research Nursery, Moscow
9221D	USDA	1992	Western white pine root	USDA Forest Service Nursery, Coeur d'Alene, Idaho
<i>F. moniliforme</i>				
FM5 (52-10-2)	BCRI	1992	Douglas fir seed (seedlot 476)	Cowichan Lake, B.C.
FM7 (476-23-13)	BCRI	1992	Douglas fir seedling root (seedlot 476)	Surrey Nursery, Surrey, B.C.
<i>F. oxysporum</i>				
8629G	USDA	1986	Douglas fir seed	University of Idaho Research Nursery, Moscow
8675C	USDA	1986	Diseased Douglas fir seedling root	USDA Forest Service, Coeur d'Alene, Idaho
F30	BCRI	1988	Damped-off Douglas fir germling (seedlot 419) (pathogenic)	Vancouver, B.C.
F41	BCRI	1988	Douglas fir seed (seedlot 8706) (pathogenic)	Alger Creek, B.C.
F44	BCRI	1988	Douglas fir seed (seedlot 476)	Cowichan Lake, B.C.
F71	BCRI	1990	Douglas fir seedling root (seedlot 476)	Surrey Nursery, Surrey, B.C.
F72	BCRI	1990	Douglas fir seedling root (seedlot 9983)	Surrey Nursery, Surrey, B.C.
C2CUP3A	BCRI	1991	5-yr-old Douglas fir root, planted seedling from reforestation site	Chilliwack, B.C.
P4C2P17A	BCRI	1991	5-yr-old Douglas fir root, planted seedling from reforestation site	Pemberton, B.C.
3139	OSU	1993	Douglas fir germling	Brooks, Ore.
9043B	USDA	1990	Douglas fir diseased seedling root	USDA Forest Service Nursery, Coeur d'Alene, Idaho
9065I	USDA	1990	Douglas fir seedling root	University of Idaho Research Nursery, Moscow
9112A	USDA	1991	Diseased blue spruce seedling root	Montana State Nursery, Missoula
<i>F. proliferatum</i>				
8656M ^b	USDA	1986	Douglas fir seedling root	University of Idaho Research Nursery, Moscow
FR9	BCRI	1992	Douglas fir seedling root (pathogenic)	Surrey Nursery, Surrey, B.C.
HC0269	BCRI	1991	Douglas fir root	Fletcher Challenge Nursery, Armstrong, B.C.
HC6701	BCRI	1991	Blue spruce root	Surrey Nursery, Surrey, B.C.
HC25783	BCRI	1991	Douglas fir root	Northwood Nursery, Prince George, B.C.
3183	OSU	Received 1993	Conifer seed	Toledo, Wash.
9112F	USDA	1991	Blue spruce seedling root (pathogenic)	Montana State Nursery, Missoula
9201E	USDA	1992	Bodies of gnats within greenhouse (pathogenic)	University of Idaho Research Nursery, Moscow
9202T	USDA	1992	Inner styroblock container wall (pathogenic)	Potlatch Nursery, Lewiston, Idaho
9207F ^b	USDA	1992	Damped-off Douglas fir seedling	USDA Forest Service Nursery, Coeur d'Alene, Idaho
9219F	USDA	1992	Damped-off Douglas fir seedling	University of Idaho Research Nursery, Moscow
<i>F. sporotrichioides</i>				
3056 ^b	OSU	1992	Soil	Bessy Nursery, Halsey, Neb.

^a BCRI, B.C. Research Inc. culture collection, Vancouver, B.C.; USDA, U.S. Department of Agriculture culture collection, Coeur d'Alene, Idaho; OSU, Oregon State University culture collection, Corvallis.

^b Isolate reidentified on the basis of PCR and RFLP analyses and subsequent microscopic assessment.

Two questions to determine the usefulness of these primer sets as tools for the detection and identification of *Fusarium* species associated with conifers were addressed in this study. First, what is the level of interspecific RFLP variability in the PCR products within the genus *Fusarium*? Second, what is the level of RFLP variability in the PCR products within the population of a single species of *Fusarium*? The studies described here show that these six *Fusarium* species can be differentiated by RFLP analyses of the amplified products by using the five different primer sets. Analysis of RFLPs in the PCR products

within isolates of a single species show that polymorphisms can easily be detected in population samples of *F. oxysporum*, but little RFLP variability is observed in population samples of the remaining species.

MATERIALS AND METHODS

Fungal isolates and species. A number of species of *Fusarium* that are commonly isolated from conifer seed, seedlings, roots, or soil from nursery environments were chosen for this study (Table 1). These initially included 6 isolates of *F. avenaceum* from British Columbia (B.C.) and Idaho, 4 *F. acuminatum* isolates

TABLE 2. RFLP profiles with the H3-1 primer set (H3-1a and H31-b)^a

Isolate	Amplification product ^b digested with:							
	<i>AluI</i>	<i>HhaI</i>	<i>HpaII</i>	<i>MboI</i>	<i>MseI</i>	<i>NlaIII</i>	<i>RsaI</i>	<i>TaqI</i>
<i>N. crassa</i> N2	A	A	A	A	U*	A	A	A
<i>F. acuminatum</i> F42	B	D	B	B	U	U	C	E
9226W	B	D	B	B	U	U	C	E
<i>F. avenaceum</i> F34	C	D	C	B	U	E	B	E
F62	B	D	C	B	B	F	B	E
FR8	B	D	C	B	B	F	B	E
9142G	B	D	C	B	B	F	B	E
9217J	B	D	C	B	B	F	B	E
9221D	B	D	C	B	B	F	B	E
<i>F. moniliforme</i> FM5	C	E	C	B	U	H	B	C
FM7	C	E	C	B	U	H	B	C
<i>F. oxysporum</i> 8629G	B	B	B	B	B	B	B	B
8675C	B	B	B	B	U	B	B	B
F30	B	B	C	B	U	C	B	E
F41	B	C	B	B	U	B	C	B
F44	B	C	C	B	U	D	B	B
F71	B	C	C	B	U	D	B	B
F72	B	B	B	B	U	D	U	B
C2CUP3A	B	C	C	B	U	D	B	B
P4C2P17A	B	B	C	B	U	U	B	D
3139	B	B	C	B	U	U	B	D
9043B	B	B	C	B	U	B	B	B
9065I	B	B	C	B	U	B	B	U
9112A	B	B	C	B	U	B	B	B
<i>F. proliferatum</i> 8656M	B	U	D	B	U	G	B	F
FR9	B	U	D	B	U	G	B	F
HC0269	B	U	D	B	U	G	B	F
HC6701	B	U	D	B	U	G	B	F
HC25783	B	U	D	B	U	G	B	F
3183	B	U	D	B	U	G	B	F
9112F	B	U	D	B	U	G	B	F
9201E	B	U	D	B	U	G	B	F
9202T	B	U	D	B	U	G	B	F
9207F	B	U	D	B	U	G	B	F
9219F	B	U	D	B	U	G	B	U
<i>F. sporotrichioides</i> 3056	B	D	B	B	U	U	C	E

^a Enzyme which did not cut any of the *Fusarium* isolates: *HaeIII*.^b Letters are used to designate unique RFLP patterns within each column. U, uncut; U*, uncut amplification product of different size than U.TABLE 3. RFLP profiles with the H4-1 primer set (H4-1a and H-41b)^a

Isolate	Amplification product ^b digested with:					
	<i>HaeIII</i>	<i>HhaI</i>	<i>MboI</i>	<i>MseI</i>	<i>NlaIII</i>	<i>TaqI</i>
<i>N. crassa</i> N2	U*	A	A	U	A	A
<i>F. acuminatum</i> F42	U	B	B	B	U	B
9226W	U	B	B	B	U	B
<i>F. avenaceum</i> F34	U	B	B	U	U	B
F62	U	B	B	A	U	B
FR8	U	B	B	A	U	B
9142G	U	B	B	A	U	B
9217J	U	B	B	A	U	B
9221D	U	B	B	A	U	B
<i>F. moniliforme</i> FM5	U	B	B	U	C	B
FM7	U	B	B	U	C	B
<i>F. oxysporum</i> 8629G	U	B	B	U	U	B
8675C	U	B	B	U	U	B
F30	U	B	B	U	U	U
F41	U	B	B	U	U	B
F44	U	B	B	U	U	B
F71	U	B	B	U	U	B
F72	U	B	B	U	U	B
C2CUP3A	U	B	B	U	U	B
P4C2P17A	A	B	B	U	U	C
3139	A	B	B	U	U	C
9043B	U	B	B	U	U	B
9065I	U	B	B	U	U	B
9112A	U	B	B	U	U	B
<i>F. proliferatum</i> 8656M	U	B	B	C	B	B
FR9	U	B	B	C	B	B
HC0269	U	B	B	C	B	B
HC6701	U	B	B	C	B	B
HC25783	U	B	B	C	B	B
3183	U	B	B	C	B	B
9112F	U	B	B	C	B	B
9201E	U	B	B	C	B	B
9202T	U	B	B	C	B	B
9207F	U	B	B	C	B	B
9219F	U	B	B	C	B	B
<i>F. sporotrichioides</i> 3056	U	B	B	U	U	B

^a Enzymes that did not cut any of the *Fusarium* amplification products: *AluI*, *HpaII*, and *RsaI*.^b Letters are used to designate unique RFLP patterns within each column. U, uncut; U*, uncut amplification product of different size than U.

from B.C., Idaho, Montana, and Nebraska, 9 *F. proliferatum* isolates from B.C., Washington, Montana, and Idaho, 2 *F. moniliforme* isolates from a single seed lot from B.C., and 14 *F. oxysporum* isolates from B.C., Idaho, Oregon, and Montana. Four of the isolates were subsequently reexamined by Keith Seifert (Agriculture Canada, Ottawa, Ontario) because of anomalous initial results (see Tables 2 to 6). *F. acuminatum* 9217J was reidentified as *F. avenaceum*; *F. avenaceum* 9207F and *F. oxysporum* 8656M were reidentified as *F. proliferatum*; and *F. acuminatum* 3056 differed in PCR and RFLP results from all species tested here and was subsequently reidentified as *F. sporotrichioides*.

DNA isolation. Various strains of *Fusarium* spp. were grown in liquid Vogel's medium (46) at 25°C in stationary cultures for 5 to 10 days. Hyphal mats were recovered by vacuum filtration, and genomic DNA was isolated from the fungal strains according to the method of Stevens and Metzner (44).

Primer selection. The DNA sequence and construction of the primer sets have been described previously (8). Four of the primer sets were derived from the sequence of three *Neurospora crassa* Shear and Dodge genes: *H3*, *H4*, and *Bml*¹ (8). The fifth primer pair was constructed to amplify the ITS region, including the 5.8S ribosomal DNA gene (48). The primer sets selected for this study were based on initial amplification results which showed that discrete bands could be amplified from *F. oxysporum* with the H3-1, H4-1, Bt-1, Bt-2, and ITS primer sets. RFLPs were evident when these DNA amplification products were compared with those from a number of other filamentous ascomycetes.

PCR and RFLP analyses. DNA amplifications were performed as described in Glass and Donaldson (8). Amplifications with genomic DNA from *N. crassa* were used as positive controls. Template-free amplifications containing only primers

TABLE 4. RFLP profiles with the Bt-1 primer set (Bt-1a and Bt1-b)^a

Isolate	Amplification product ^b digested with:						
	<i>AluI</i>	<i>HaeIII</i>	<i>HhaI</i>	<i>MboI</i>	<i>NlaIII</i>	<i>RsaI</i>	<i>TaqI</i>
<i>N. crassa</i> N2	A	U	A	A	A	U	A
<i>F. acuminatum</i> F42	A	U	B	B	E	A	D
9226W	A	U	B	B	E	A	D
<i>F. avenaceum</i> F34	A	U	U	B	E	A	C
F62	A	U	U	B	E	A	C
FR8	A	U	U	B	E	A	C
9142G	A	U	U	B	E	A	C
9221D	A	U	U	B	E	A	C
9217J	A	U	U	B	E	A	C
<i>F. moniliforme</i> FM5	A	F	D	D	C	U	B
FM7	A	F	D	D	C	U	B
<i>F. oxysporum</i> 8629G	A	A	B	U	B	U	B
8675C	A	A	B	U	B	U	B
F30	A	B	B	B	B	A	B
F41	A	A	B	U	B	U	B
F44	A	A	B	U	B	U	B
F71	A	C	B	U	B	U	B
F72	A	A	B	U	B	U	U
C2CUP3A	A	A	B	U	B	U	B
P4C2P17A	A	D	B	U	C	U	B
3139	A	D	B	U	C	U	B
9043B	A	A	B	U	B	U	B
9065I	A	E	C	C	D	A	B
9112A	A	A	B	U	B	U	B
<i>F. proliferatum</i> 8656M	A	B	C	B	C	U	B
FR9	A	B	C	B	C	U	B
HC0269	A	B	C	B	C	U	B
HC6701	A	B	C	B	C	U	B
HC25783	A	B	C	B	C	U	U
3183	A	B	C	B	C	U	B
9112F	A	B	C	B	C	U	B
9201E	A	B	C	B	C	U	B
9202T	A	B	C	B	C	U	B
9207F	A	B	C	B	C	U	B
9219F	A	B	C	B	C	U	B
<i>F. sporotrichioides</i> 3056	A	B	D	B	B	A	D

^a Enzymes that did not cut any of the *Fusarium* amplification products: *HpaII* and *MseI*.

^b Letters are used to designate unique RFLP patterns within each column. These letter designations cannot be compared across columns. U, uncut.

TABLE 5. RFLP profiles with the Bt-2 primer set (Bt-2a and Bt-2b)

Isolate	Amplification product ^a digested with:								
	<i>AluI</i>	<i>HaeIII</i>	<i>HhaI</i>	<i>HpaII</i>	<i>MboI</i>	<i>MseI</i>	<i>NlaIII</i>	<i>RsaI</i>	<i>TaqI</i>
<i>N. crassa</i> N2	A	A	A	A	A	U*	A	A	A
<i>F. acuminatum</i> F42	A	C	C	C	U	A	B	C	B
9226W	A	C	C	C	U	A	B	C	B
<i>F. avenaceum</i> F34	A	C	C	C	U	U	B	C	B
F62	A	C	C	C	U	U	B	C	B
FR8	A	C	C	D	U	U	B	C	B
9142G	A	C	C	C	U	U	B	C	B
9221D	A	C	C	C	U	U	B	C	B
9217J	A	C	C	C	U	U	B	C	B
<i>F. moniliforme</i> FM5	B	B	B	B	U	U	B	B	C
FM7	B	B	B	B	U	U	B	B	C
<i>F. oxysporum</i> 8629G	C	B	B	B	B	A	B	B	C
8675C	C	B	B	B	B	A	B	B	C
F30	C	B	B	B	B	U	B	B	D
F41	C	B	U	B	B	A	B	B	C
F44	C	B	B	B	B	A	B	B	C
F71	C	B	B	B	B	A	B	B	C
F72	C	B	B	B	B	U	B	B	C
C2CUP3A	C	B	B	B	B	A	B	B	C
P4C2P17A	C	B	B	B	B	A	B	B	C
3139	C	B	B	B	B	A	B	B	C
9043B	C	B	B	B	B	A	B	B	C
9065I	C	B	B	B	B	A	B	B	C
9112A	C	B	B	B	B	A	B	B	C
<i>F. proliferatum</i> 8656M	C	B	B	B	B	A	B	B	C
FR9	C	B	B	B	B	A	B	B	C
HC0269	C	B	B	B	B	A	B	B	C
HC6701	C	B	B	B	B	A	B	B	C
HC25783	C	B	B	B	B	A	B	B	C
3183	C	B	B	B	B	A	B	B	C
9112F	C	B	B	B	B	A	B	B	C
9201E	C	B	B	B	B	A	B	B	C
9202T	C	B	B	B	B	A	B	B	C
9207F	C	B	B	B	B	A	B	B	C
9219F	C	B	B	B	B	A	B	B	C
<i>F. sporotrichioides</i> 3056	C	D	B	E	B	U	B	B	B

^a Letters are used to designate unique RFLP patterns within each column. These letter designations cannot be compared across columns. U, uncut; U*, uncut amplification product of different size than U.

were also performed to ensure that reagents were not contaminated with extraneous template DNA. The initial PCR cycling protocol for all of the *Fusarium* isolates was as follows: 94°C for 1 min; 60°C for 1 min; and 72°C for 1 min; this was repeated for 32 cycles and followed by an extension period of an additional 5 s for each cycle. If an amplification product was not observed, the annealing temperature was reduced to 58°C and the number of cycles was increased to a maximum of 45 cycles. If the PCR background was too high, the annealing temperature was increased to 65°C. PCR products from each of the five primer sets were digested individually with nine different 4-bp recognition sequence restriction enzymes. The enzymes used were *AluI*, *HaeIII*, *CfoI*, *HpaII*, *MboI*, *MseI*, *NlaIII*, *RsaI*, and *TaqI* (Boehringer-Mannheim, Laval, Quebec, Canada; and New England BioLabs, Boston, Mass.). Digested amplification products

were subjected to electrophoresis as described in Glass and Donaldson (8). Sizes of restriction fragments were determined by comparison to standard DNA molecular weight markers (Appendix). Fragments smaller than 50 bp and doublets were not included in the analyses.

Statistical analyses of RFLP data. Relationships among *Fusarium* isolates were inferred by using cluster analysis (12) and the SYSTAT program (45). Similar techniques of numerical taxonomy have been used in previous studies with *Fusarium* mitochondrial RFLP (23, 28) and nuclear ribosomal DNA (22) data. Restriction enzyme digestion data were coded as binary tables, with 1 representing the presence of a band and 0 representing the absence of a band. With Jaccard's similarity coefficient (13), a similarity matrix was constructed with the data from the subset of the nine restriction enzyme digests that cut at least one of the *Fusarium* isolates tested. The data include successful restriction

TABLE 6. RFLP profiles with the ITS primer set (ITS1 and ITS4)

Isolate	Amplification product ^a digested with:								
	<i>AluI</i>	<i>HaeIII</i>	<i>HhaI</i>	<i>HpaII</i>	<i>MboI</i>	<i>MseI</i>	<i>NlaIII</i>	<i>RsaI</i>	<i>TaqI</i>
<i>N. crassa</i>									
N2	A	A	A	A	A	A	A	U	A
<i>F. acuminatum</i>									
F42	C	C	D	C	B	B	C	U	C
9226W	C	C	D	C	B	B	C	U	C
<i>F. avenaceum</i>									
F34	C	C	D	C	B	B	C	U	C
F62	C	C	D	C	B	B	C	U	C
FR8	C	C	D	D	B	B	C	U	C
9142G	C	C	D	C	B	B	C	U	C
9221D	C	C	D	C	B	B	C	U	C
9217J	C	C	D	C	B	B	C	U	C
<i>F. moniliforme</i>									
FM5	E	B	B	A	C	B	B	U	B
FM7	E	B	B	A	C	B	B	U	B
<i>F. oxysporum</i>									
8629G	B	B	B	A	C	C	B	U	B
8675C	B	B	B	A	C	C	B	U	B
F30	U	D	C	B	B	B	B	U	C
F41	B	B	B	A	C	C	B	U	B
F44	B	B	B	A	C	C	B	U	B
F71	B	B	B	A	C	C	B	U	B
F72	B	B	B	A	C	C	B	U	B
C2CUP3A	B	B	B	A	C	C	B	U	B
P4C2P17A	U	D	C	B	B	B	B	U	C
3139	U	D	C	B	B	B	B	U	C
9043B	B	B	B	A	C	C	B	U	B
9065I	B	B	B	A	C	C	B	U	B
9112A	B	B	B	A	C	C	B	U	B
<i>F. proliferatum</i>									
8656M	D	D	C	E	B	B	B	A	C
FR9	D	D	C	E	B	B	B	A	C
HC0269	D	D	C	E	B	B	B	A	C
HC6701	D	D	C	E	B	B	B	A	C
HC25783	D	D	C	E	B	B	B	A	C
3183	D	D	C	E	B	B	B	A	C
9112F	D	D	C	C	B	B	B	A	C
9201E	D	D	C	E	B	B	B	A	C
9202T	D	D	C	E	B	B	B	A	C
9207F	D	D	C	E	B	B	B	A	C
9219F	D	D	C	C	B	B	B	A	C
<i>F. sporotrichioides</i>									
3056	B	C	E	C	B	B	D	U	D

^a Letters are used to designate unique RFLP patterns within each column. The letter designations cannot be compared across columns. U, uncut.

digests from all five amplification products. Jaccard's similarity coefficient gives value only to the presence of RFLP bands; the absence of RFLP bands is not interpreted as a similar character between isolates. Undigested amplification products were not counted as similarities. The similarity matrix was subsequently used to construct a dendrogram by means of hierarchical clustering (20), using distance averages between pairs in different clusters (42, 43). This particular method of cluster analysis is more commonly referred to as unweighted paired group analysis using arithmetic means, or UPGMA. Ordering in the dendrogram output of SYSTAT was performed with the algorithm of Gruvaeus and Wainer (16).

RESULTS

DNA amplification. Five primer sets (H3-1, H4-1, Bt-1, Bt-2, and ITS) were selected for use for PCR amplification from

FIG. 1. Level of intraspecific variability in *Fusarium* species in relation to PCR amplification targets. The number of unique profiles was based on information obtained from RFLPs of the PCR products obtained with five different primer sets. The data were collected from 13 *F. oxysporum* isolates, 11 *F. proliferatum* isolates, and 6 *F. avenaceum* isolates.

genomic DNA with the 35 *Fusarium* isolates. DNA amplification products from each of the five primer sets were recovered from each *Fusarium* isolate (175 total amplification products). The H3-1 PCR amplification product was approximately 500 bp in size in all of the 35 isolates of *Fusarium* tested. The approximate sizes for the H4-1, Bt-1, Bt-2, and ITS PCR products were 250, 540, 340, and 550 bp, respectively. Size polymorphisms in amplified PCR products were not observed among the 35 *Fusarium* isolates with any of the five primer pairs.

Restriction enzyme analysis. Each of the 175 PCR amplification products was subjected to restriction enzyme digestion with nine different 4-bp recognition restriction enzymes. The RFLP profiles of the 35 *Fusarium* isolates, using all five primer sets, are summarized in Tables 2 to 6 and are described below. Occasionally, restriction enzyme recognition sequences were not present within specific amplification products, as noted in Tables 2 to 6.

H3-1. RFLPs in the *Fusarium* isolates in the 540-bp H3-1 PCR product were observed in digestion patterns with seven different enzymes (Table 2). Electrophoresis of H3-1 products digested with *MboI* gave an identical pattern for all 35 *Fusarium* isolates. This pattern differed from the *MboI* digestion pattern of the *N. crassa* H3-1 PCR product. By using as few as two restriction enzymes, species tested could be differentiated from each other by unique RFLPs, with the exception of *F. sporotrichioides* and *F. acuminatum*, both of which had identical RFLPs for all nine enzymes (Table 2).

Little intraspecies variability in the H3-1 RFLPs was observed in *F. avenaceum*, *F. acuminatum*, *F. moniliforme*, and *F. proliferatum*. Ten isolates of *F. proliferatum* showed identical H3-1 RFLP patterns with all of the enzymes tested (Table 2); the one exception was 9219F, which differed in the *TaqI* RFLP pattern. Similarly, five of the six isolates of *F. avenaceum* gave identical H3-1 RFLP patterns. The exception was F34, which differed in three RFLP patterns in H3-1.

In contrast to the other *Fusarium* species tested, RFLP variability was observed in 13 isolates of *F. oxysporum* in the H3-1 patterns upon digestion with seven restriction enzymes. Eight distinct classes of RFLPs could be distinguished on the basis of the H3-1 patterns among the 13 *F. oxysporum* isolates (Table 2).

H4-1. As with the H3-1 PCR product, an identical H4-1 RFLP pattern with *MboI* was observed among all of the *Fusarium* isolates (Table 3). Variability in RFLP patterns was not

FIG. 2. Similarity relationships based on cluster analysis with data obtained from restriction endonuclease digestions of PCR products from 35 *Fusarium* isolates, using *N. crassa* as an outgroup. (A) H3-1, H4-1, Bt-1, Bt-2, and ITS PCR products; (B) H3-1, H4-1, Bt-1, and Bt-2 PCR products (data from the ITS PCR product was omitted in constructing the dendrogram); (C) ITS PCR product.

observed among the 11 *F. proliferatum* isolates in any of the nine restriction enzyme digestions performed. The RFLP patterns of the six *F. avenaceum* isolates were also identical, with the exception of F34 (Table 3), which differed only in the *Mse*I digestion pattern.

In contrast to the H3-1 PCR product, H4-1 RFLP variability was much lower among the 13 *F. oxysporum* isolates, although restriction sites were not present for five of the enzymes (*Alu*I, *Hpa*II, *Mse*I, *Nla*III, and *Rsa*I) (Table 3). The 13 *F. oxysporum* isolates separated into only three groups on the basis of RFLP profiles of the H4-1 PCR product. Ten isolates of *F. oxysporum* had identical H4-1 RFLP profiles. Two isolates, P4C2P17A and 3139, formed a second group, while isolate F30 had a unique RFLP profile.

It was difficult to differentiate *Fusarium* species with RFLPs and the H4-1 primer set because of a lack of interspecific polymorphisms. However, *F. acuminatum* and *F. proliferatum* gave distinctive digestion patterns with *Mse*I and *F. moniliforme* was unique in its *Nla*III RFLP pattern (Table 3).

Bt-1. An RFLP pattern common to all of the *Fusarium* isolates was not observed in any of the restriction enzyme digestions of the 540-bp Bt-1 product (Table 4). However, as with RFLP analysis of the H3-1 PCR product, it was possible to differentiate the *Fusarium* species by using different enzymes to digest the Bt-1 PCR products.

Intraspecific RFLPs were not observed in the Bt-1 product in *F. avenaceum*, *F. acuminatum*, or *F. moniliforme* with any of the nine restriction enzymes tested (Table 4). The only pattern variation in the *F. proliferatum* isolates occurred with the *Taq*I digestion in which the Bt-1 PCR product from a single isolate, HC25783, was not digested.

As with the H3-1 PCR products, numerous RFLPs were observed in Bt-1 PCR products among the 13 *F. oxysporum* isolates (Table 4). Restriction enzyme digestion of Bt-1 PCR products with four enzymes identified six distinct groups among the 13 isolates of *F. oxysporum*. *F. oxysporum* isolates F30 and 9065I had unique H3-1 and Bt-1 RFLP patterns that differed from the RFLP patterns of the other 11 isolates of *F. oxysporum* (Tables 2 and 4).

Bt-2. All of the nine restriction enzymes used digested the 340-bp Bt-2 PCR product in at least some of the *Fusarium* isolates (Table 5). However, RFLPs of the Bt-2 PCR products were not sufficiently variable to permit differentiation among the six *Fusarium* species. However, as with *Mbo*I digestions of the H3-1 and H4-1 PCR products, a common RFLP pattern was present in Bt-2 *Nla*III digestions among all 35 *Fusarium* isolates.

The Bt-2 RFLP patterns showed no intraspecific variation in *F. acuminatum*, *F. moniliforme*, or *F. proliferatum*. Ten of the 13 isolates of *F. oxysporum* had identical Bt-2 RFLP patterns with all nine restriction enzymes (Table 5). However, the Bt-2 RFLP patterns of these 10 *F. oxysporum* isolates were indistinguishable from those of the *F. proliferatum* isolates with all nine enzymes and from those of the *F. moniliforme* isolates digested with *Hae*III, *Hpa*II, *Hha*I, *Rsa*I, *Nla*III, and *Taq*I. Only *F. oxysporum* isolates F30, F41, and F72 varied in the Bt-2 RFLP patterns.

ITS. All of the nine restriction enzymes differentially digested the 550-bp ITS PCR product in the *Fusarium* isolates (Table 6). Similar to RFLP analysis of the H3-1, H4-1, Bt-1, and Bt-2 PCR products, the 11 *F. proliferatum* isolates displayed identical ITS RFLPs with eight of nine enzymes, with

FIG. 2—Continued.

the exception of *Hpa*II digestions of the ITS product from 9112F and 9219F. The ITS RFLP patterns were also identical in five of the six *F. avenaceum* isolates, the exception being the *Hpa*II digestion of isolate FR8.

In contrast to the nine and six groups seen among the *F. oxysporum* isolates in RFLPs within the H3-1 and Bt-1 PCR products, respectively, only two groups were defined in RFLP analysis of the ITS PCR product (Table 6). One group consisted of three isolates: F30, P4C2P17A, and 3139. The remaining 10 *F. oxysporum* isolates had identical ITS RFLP profiles.

By using a combination of restriction endonucleases, it was possible to differentiate four of the six *Fusarium* species by RFLP analysis of the ITS PCR product. The exceptions were the *F. acuminatum* and *F. avenaceum* isolates, which could not be differentiated from each other with any of the nine enzymes tested.

Figure 1 shows a bar graph of the RFLP variability observed with each PCR product of *F. avenaceum* (six isolates), *F. proliferatum* (11 isolates), and *F. oxysporum* (13 isolates). Data for the isolates of *F. acuminatum* and *F. moniliforme*, both of which showed no intraspecific RFLP variability, were not included. The *F. oxysporum* isolates exhibited the most RFLP variability for the five PCR products, with the H3-1 and Bt-1 PCR products being the most polymorphic. In contrast, RFLP analyses of the PCR products from the *F. avenaceum* and *F. proliferatum* isolates showed similar levels of polymorphism among the five PCR products. The H4-1 and Bt-2 PCR products showed the least amount of intraspecies RFLP variability, presumably due partly to their small size (260 and 340 bp, respectively).

Statistical analyses of cumulative RFLP data. A dendrogram based on the cumulative RFLP data with all five primer

sets, nine different restriction enzymes, and the 35 *Fusarium* isolates is shown in Fig. 2A. The dendrogram is based on the data acquired from those enzymes which digested the PCR product in at least one of the *Fusarium* isolates examined.

The 11 isolates of *F. proliferatum* surveyed form the least variable group. Only 3 of the 11 isolates, 9219F, 9112F, and HC25783, showed RFLP variability and were aligned closely with the remaining *F. proliferatum* isolates, with only 5% dissimilarity. The *F. proliferatum* isolates were recovered from a variety of sources and locations (Table 1).

Two isolates each of *F. acuminatum* and *F. moniliforme* formed two separate groups, which correlated with the two species. Two isolates within the *F. avenaceum* group, F34 and FR8, could be distinguished from the other four *F. avenaceum* isolates (Fig. 2A); the F34 isolate was the least similar to the rest of the group (88%). The *F. avenaceum* group showed 74% similarity to the *F. acuminatum* group, making these two species relatively difficult to distinguish with this technique.

In sharp contrast to all of the above-mentioned species, *F. oxysporum* exhibited much more diversity in RFLPs in the PCR products (Fig. 2A). Three sets of two isolates each (3139 and P4C2P17A, F44 and C2CUP3A, and 9112A and 9043B) among the 13 *F. oxysporum* isolates were identical in RFLP data (Tables 2 to 6 and Fig. 2A). Isolates F30, P4C2P17A, and 3139 formed a cluster distinct from all of the other *Fusarium* isolates (Fig. 2A). These three isolates group closer to *F. proliferatum* (63% similarity) than to other *F. oxysporum* isolates (48% similarity). The *F. oxysporum* isolates showed 91% similarity among 7 of the remaining isolates and 70% similarity when 10 isolates were included.

Figure 2B shows a dendrogram in which the ITS data have been excluded. The omission of the ITS-derived data resulted in the clustering of isolates 3139 and P4C2P17A with *F. oxysporum* rather than with *F. proliferatum*. The F30 isolate of *F. oxysporum*, however, still groups anomalously. Dendrograms similar to that presented in Fig. 2B were produced when the H3, H4, Bt-1, or Bt-2 RFLP data were omitted from the analysis, in conjunction with the ITS data.

Figure 2C is a dendrogram produced with the ITS data alone. The unusual grouping of *F. oxysporum* isolates F30, 3139, and P4C2P17A is apparent. These three isolates cluster more closely to *F. proliferatum* (74% similarity) and *F. avenaceum* or *F. acuminatum* (45% similarity) than to the other isolates of *F. oxysporum* (30% similarity). Also apparent in Fig. 2C is the inability of the ITS RFLPs to distinguish between *F. avenaceum* and *F. acuminatum* isolates.

DISCUSSION

The objective of this study was to evaluate the effectiveness of primer sets designed to amplify intron sequences in conserved genes for differentiating species of *Fusarium* commonly found in association with conifer seedlings in nursery environments. Prior to the use of these primers as a tool for epidemiological studies, two questions must be addressed. First, what is the level of interspecific RFLPs in the PCR products from isolates of the different species of *Fusarium*? Second, what is the level of intraspecific RFLPs in the PCR products of *Fusarium* isolates of the same species?

The 35 isolates of *Fusarium* spp. described here were assayed with five different sets of primers. For each primer set, the sizes of the amplification products from each of the 35 *Fusarium* isolates were indistinguishable. This is in contrast to size polymorphisms observed in the PCR products from other filamentous ascomycetes (8). This is illustrated best by the Bt-2 PCR product. The Bt-2 PCR product from *N. crassa* is 495 bp

APPENDIX TABLE 1. Restriction fragment sizes^a

Primer set and enzyme	Sizes of fragments (bp) in given RFLP pattern							
	A	B	C	D	E	F	G	H
H3-1								
<i>AluI</i>	120, 70	245, 200	245, 100					
<i>HlaI</i>	345, 70	460, 60	290, 190	375, 105, 75	270, 145, 60			
<i>HpaII</i>	320, 70	365, 175	335, 180	335, 150				
<i>MboI</i>	265, 180	365, 140						
<i>MseI</i>		340, 105						
<i>NlaIII</i>	230	350, 150	220, 130	345, 180	315, 200	315, 220	400, 135	470, 60
<i>RsaI</i>	325, 125	330, 220	330, 140					
<i>TaqI</i>	365, 75	225, 150, 100, 75	260, 180, 75	150, 100, 75	260, 210, 80	150, 100		
H4-1								
<i>HaeIII</i>	190, 55							
<i>HhaI</i>	220, 40	195, 55						
<i>MboI</i>	85, 60	135, 105						
<i>MseI</i>	180, 70	160, 105	200, 50					
<i>NlaIII</i>	170, 90	110, 80	190, 70					
<i>TaqI</i>	100, 80	180, 80	180, 110					
Bt-1								
<i>AluI</i>	420, 120							
<i>HaeIII</i>	295, 125	340, 180	320, 180	295, 245	210, 150	340, 210		
<i>HhaI</i>	220, 190	275, 190	190, 130	275, 130				
<i>MboI</i>	310, 230	360, 190	310, 120	270				
<i>NlaIII</i>	200, 85	320, 85	235, 85	200, 120	140, 120			
<i>RsaI</i>	485, 55							
<i>TaqI</i>	230, 120	150, 120	280, 150, 120	310, 150, 120				
Bt-2								
<i>AluI</i>	190, 100	190, 80	100, 80					
<i>NaeIII</i>	240, 115, 50	135, 115, 50	240, 50	135, 115				
<i>HhaI</i>	240, 95	255, 110	160, 110					
<i>HpaII</i>	145, 110, 45	295, 45	180, 120, 45	200, 45	180, 80, 45			
<i>MboI</i>	280, 180	220, 135						
<i>MseI</i>	210, 135							
<i>NlaIII</i>	240, 150, 110	130, 105						
<i>RsaI</i>	175, 110, 60	150, 115, 75	200, 115					
<i>TaqI</i>	125, 75, 50	125, 120, 105	125, 115, 50	130, 105, 50				
ITS 1-4								
<i>AluI</i>	400, 210	360, 145, 50	450, 120	410, 145	365, 145			
<i>HaeIII</i>	500, 100	350, 120, 90	370, 120, 90	300, 120, 90, 70				
<i>HhaI</i>	260, 90	300, 260	300, 180, 90	220, 180, 90	260, 220, 90			
<i>HpaII</i>	465, 105	315, 180, 105	320, 245	320, 260	250, 155, 105			
<i>MboI</i>	180, 140, 125	190, 160, 120	320, 120					
<i>MseI</i>	365, 210	490, 65	400, 100, 65					
<i>NlaIII</i>	365, 235	310, 245	310, 160, 80	310, 230				
<i>RsaI</i>	460, 100							
<i>TaqI</i>	260, 220	215, 140, 90, 60	240, 210, 60	240, 210, 140				

^a Sizes are given in base pairs. Fragments less than approximately 50 bp are not included. Digestions resulting in doublets are not included.

in length and includes three introns. The Bt-2 PCR product is 475 bp in *Botrytis cinerea* Pers.:Fr and *Sclerotinia sclerotiorum* (Lib.) de Bary, and 360 bp in *Trichoderma reesei* Simmons (8) but only 340 bp in all 35 of the *Fusarium* isolates (this study). Thus, size of the Bt-2 PCR product may be a useful character in differentiating the genus *Fusarium* from other genera of deuteromycetes or filamentous ascomycetes.

Sufficient interspecific RFLP variability was present in the PCR products from the six *Fusarium* species for primer sets to be useful in differentiation and identification of *Fusarium* species. The six *Fusarium* species examined could be differentiated from each other on the basis of a limited number of PCR amplifications, with the H3-1 product being the most informative. The variability observed in the PCR products

among isolates of *F. oxysporum* does not preclude their differentiation from the other *Fusarium* species by this technique. The utility of these primer sets for differentiating *Fusarium* species was illustrated early in this study by anomalous PCR results with four isolates that resulted in their reidentification. This error was possibly due to contamination of two isolates with *F. proliferatum*, misidentification, or mislabeling of isolates.

The second question addressed intraspecific RFLP variability in the PCR products within a single species. RFLPs within the PCR products provided some measure of intraspecific variability in *F. proliferatum*, *F. oxysporum*, and, to a lesser extent, *F. avenaceum*. We can draw no conclusions for *F. acuminatum*, *F. moniliforme*, or *F. sporotrichioides* because of the limited

number of isolates tested. *F. proliferatum* was the least variable species on the basis of studies of 11 isolates from Idaho, Washington, B.C., and Montana. Nine of these isolates clustered at 100% similarity and the remaining two isolates were 94% similar. Although only six *F. avenaceum* isolates were assessed from B.C. and Idaho, these isolates also showed a high level of similarity (88%).

In contrast to the other *Fusarium* species, population analysis of the 13 *F. oxysporum* isolates showed significant RFLP variability in the PCR products. The *F. oxysporum* isolates were from various geographical areas, but no relationship between RFLP pattern and geographic origin was observed. This observation was consistent with other studies (27, 29, 47). Considerable variability among different isolates of *F. oxysporum* has been reported previously (4, 23, 28). Gordon and Okamoto (11) have reported the variable nature of nonpathogenic *F. oxysporum* isolates from a single agricultural field soil in California. Twenty-nine nonpathogenic isolates were classified into six mitochondrial haplotypes, and these isolates are distantly related to *F. oxysporum* f. sp. *melonis*. Genetic variation in *F. oxysporum* has been addressed in a recent review (10).

F. oxysporum recovered from conifer seedlings is rarely given a forma speciales designation. This species can be isolated from visibly healthy seedlings as well as from diseased plants and nursery soil (1a, 18). Since the majority of isolates used in this study were not assessed for pathogenicity, we are uncertain if some of the observed variability is correlated with pathogenicity or is just due to the variable nature of *F. oxysporum* isolates which associate with conifers in nursery environments.

Generally, the clustering of isolates within *Fusarium* species on the basis of total RFLP data was consistent with the taxonomy of *Fusarium*. The only apparent aberration is the clustering of *F. oxysporum* isolates F30, 3139, and P4C2P17A. These three isolates group closer to *F. proliferatum* than to *F. oxysporum*. Microscopic examination of these three isolates has confirmed their identities as *F. oxysporum*. Isolate F30 grouped anomalously even after the removal of the ITS RFLP data. Initial DNA sequence comparison of the ITS regions from isolates F30, P4C2P17A, and 3139 showed a high degree of dissimilarity to ITS regions previously sequenced from *F. oxysporum*, while the DNA sequence from the ITS region of 9065I is quite similar to that of other *F. oxysporum* isolates (39a). This observation may have an impact on studies which use only the ITS region in order to infer phylogenetic relationships between species in various genera (1, 21, 22). It is important to recognize that while these primer sets may be useful for phylogenetic studies, a taxonomic or phylogenetic analysis of the genus *Fusarium* was not the purpose of this study.

A number of approaches to differentiate *Fusarium* species and formae speciales or races of *F. oxysporum* have been reported. Probes have been constructed from mitochondrial DNA to identify RFLPs among *F. oxysporum* formae speciales (29). Species-specific primer sets for *F. graminearum* have been constructed from amplified fragments, using RAPD primers, and RFLP analysis was used to differentiate isolates (40). Manulis (33) used RAPD primers to identify a race-specific RAPD pattern in *F. oxysporum* f. sp. *dianthi*. The advantages of the primer sets described here are that, first, several species of *Fusarium* and possibly other fungi can be identified concurrently from a group of unknown isolates; second, the identification of a species can be confirmed by additional PCR reactions and RFLP analysis, which is useful when the genetic polymorphism within a population sample is unknown; and third, the primer sets are transferable to other filamentous ascomycete species without the extensive screening of suitable primers or the development of specific probes. A blind test to

identify some common *Fusarium* species in a large collection of fungal isolates from conifer seedlings was successful (5a).

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