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Identification of Root Rot Fungi in Nursery Seedlings by Nested Multiplex PCR

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The internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) subunit repeat was sequenced in 12 isolates of *Cylindrocladium floridanum* and 11 isolates of *Cylindrocarpon destructans*. Sequences were aligned and compared with ITS sequences of other fungi in GenBank. Some intraspecific variability was present within our collections of *C. destructans* but not in *C. floridanum*. Three ITS variants were identified within *C. destructans*, but there was no apparent association between ITS variants and host or geographic origin. Two internal primers were synthesized for the specific amplification of portions of the ITS for *C. floridanum*, and two primers were designed to amplify all three variants of *C. destructans*. The species-specific primers amplified PCR products of the expected length when tested with cultures of *C. destructans* and *C. floridanum* from white spruce, black spruce, Norway spruce, red spruce, jack pine, red pine, and black walnut from eight nurseries and three plantations in Quebec. No amplification resulted from PCR reactions on fungal DNA from 26 common contaminants of conifer roots. For amplifications directly from infected tissues, a nested primer PCR using two rounds of amplification was combined with a multiplex PCR approach resulting in the amplification of two different species-specific PCR fragments in the same reaction. First, the entire ITS was amplified with one universal primer and a second primer specific to fungi; a second round of amplification was carried out with species-specific primers that amplified a 400-bp PCR product from *C. destructans* and a 328-bp product from *C. floridanum*. The species-specific fragments were amplified directly from infected roots from which one or the two fungi had been isolated.

Root rots are among the most serious conifer nursery diseases in Canada (25, 26). *Cylindrocarpon destructans* Zins. Scholt. and *Cylindrocladium floridanum* Sobers and Seymour are two important causal agents of root rots in nurseries (2). While the former is a ubiquitous soil organism (16), the latter is most often found in nurseries and may have been introduced into northern North America. It was first reported in nurseries in Minnesota in 1962 (1) and in Ontario and Quebec in the late 1960s (20, 26).

The impact of this disease on nursery seedling production is twofold. First, these fungi can cause seedling mortality in nurseries. Up to 50% mortality was reported to be caused by root rots in affected nursery beds (1, 2). Second, infected seedlings have a lower survival rate after outplanting to reforestation sites. Mortality rates of 40% the first year after outplanting for *Picea mariana* and 60% two years after outplanting for *Pinus resinosa* were reported (25, 27). An important factor compounding this problem is that in some cases the symptoms are not visible on infected seedlings but disease can develop after transplantation. Seedlings that were apparently healthy had up to 25% root rot-induced mortality after transplantation (25).

It is not known whether these pathogens can become established in plantations on sites where they were not previously present and, if so, what damage they can cause. *C. destructans* was recently reported to cause root rots on English walnut in Italy (18). *C. floridanum* was isolated from the soil in plantations that had been reforested with infected seedlings (2). Since both fungi have broad host ranges and can infect deciduous trees as well as other annual and perennial plants (9), it is important to develop the proper tools to monitor these pathogens following outplanting.

Because of the severe losses reported and the potential for spreading these pathogens, certification or grading programs have been put in place in Quebec and Ontario to cull out seedlings showing symptoms. In the province of Quebec, 262 million seedlings were inspected in 1993 for certification of health and 4 million seedlings were destroyed because they showed symptoms of root rot (2). The certification is done by visual inspection followed by random sampling in nursery beds and isolation and identification of organisms present in roots and root collars. In Ontario, 400,000 seedlings were culled out in 1986 and 1987 and incidence levels were evaluated between 1 and 40% within individual fields (26). However, the certification process is time-consuming and can lack sensitivity, in particular when aggressive contaminants are present in the roots and prevent isolation of the causal agent (25).

Detection of plant pathogens directly from infected tissues has been reported for several agricultural plant pathogens (5, 14, 15, 17, 19, 22, 23, 28). Also, PCR has been used to monitor mycorrhizal symbionts associated with nursery seedlings (11) and to detect soil pathogens directly from infested soil (13). These molecular detection approaches are ideally suited for the study of root rot organisms because of the difficulty in isolating and identifying some of these fungi.

The objective of this study was therefore to develop an efficient and reliable detection method based on PCR for the identification and detection of these two organisms directly from infected seedlings.

MATERIALS AND METHODS

Infected material. Fungal cultures were made from seedlings collected from several nurseries in Quebec. Between 4 and 16 wood chips approximately 5 by 10 mm were aseptically excised from roots and root collars of seedlings showing symptoms of the disease in order to isolate the two organisms, placed on potato dextrose agar (PDA), and incubated for 10 days at 20°C. Meanwhile, the seed-

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TABLE 1. Isolates used in this study with their host and geographic origin[a]

Fungal species and isolate	Host of origin	Geographic origin (administrative region)[b]	Species-specific primers[c]	
			Dest1-4 (ITS variant)[d]	Flor 2-5
C. destructans				
1005	*P. mariana*	Normandin (02)	+ (b)	−
89-0090 (1147)	*P. glauca*	Duchesnay (03)	+ (c)	−
89-0283 (1254)	*P. mariana*	St-Modeste (01)	+ (b)	−
94-1365	*P. abies*	Martinville (05)	+ (c)	−
94-0893 (1330)	*P. rubens*	Berthierville (14)	+ (a)	−
94-0001 (1331)	*P. banksiana*	Albanel (02)	+ (a)	−
94-1628	*P. glauca*	St-Prosper (12)	+ (a)	−
St-Amable	*P. glauca*	St-Amable (06)	+ (a)	−
94-0003	*P. resinosa*	St-Jean-Chrysostome (06)	+ (a)	−
94-1685	*P. glauca*	Normandin (02)	+ (c)	−
Normandin	*P. mariana*	Girardville (02)	+ (a)	−
C. floridanum				
92-1382 (1073)	*P. mariana*	Duchesnay (03)	−	+
94-1259	*P. mariana*	Martinville (05)	−	+
92-1482 (1070)	*P. mariana*	Normandin (02)	−	+
92-1826 (1071)	*P. mariana*	Berthierville (14)	−	+
92-1486 (1072)	*P. glauca*	Normandin (02)	−	+
Berthier	*P. glauca*	Berthierville (14)	−	+
92-1743 (1309)	*P. mariana*	St-Modeste (01)	−	+
92-1708 (1258)	*P. mariana*	Ste-Luce (01)	−	+
94-0893 (1329)	*P. rubens*	Berthierville (14)	−	+
94-1913	*P. mariana*	Ste-Luce (01)	−	+
94-1583	*P. glauca*	Grandes-Piles (04)	−	+
93-2023	*Juglans nigra*	Berthierville (14)	−	+

[a] Samples were isolated from conifer roots and identified by cultural and morphological means.
[b] Administrative regions (2).
[c] Presence or absence of species-specific amplicon is indicated by a + or − for each set of primers. Primer sequences are given in Table 2.
[d] The three ITS variants within *C. destructans* are described in the legend to Fig. 2.

lings were stored at 2°C. All seedlings from which either *C. floridanum* or *C. destructans* were isolated were subsequently transferred into a freezer at −20°C until DNA was extracted from the infected root systems.

Fungal isolates. Isolates were obtained from infected nursery seedlings belonging to six conifer and one broad-leaved species from eight nurseries and three plantations situated in eight administrative regions in the province of Quebec (Table 1).

Additional isolates were obtained from the fungal collection of the Quebec Ministry of Natural Resources. Cultures belonging to the genus *Cylindrocladium* were transferred to PDA dishes and exposed to light until the formation of vesicles to confirm the species (6, 7). Cultures identified as *Cylindrocarpon* spp. were transferred to synthetic nutrient agar (SNA) petri dishes, cultured for a month, and monitored for the formation of chlamydospores. Isolates were classified to species according to their morphological, cultural, and sporulation characteristics (3, 8, 24).

Eleven isolates of *C. destructans* and 12 of *C. floridanum* that clearly satisfied the species criteria were included for sequencing and as positive controls for the species-specific primers. These isolates were grown in a liquid broth at 20°C for 10 days. The mycelium was then filtered through filter paper (Whatman no. 1) and rinsed with sterile distilled water, lyophilized, and stored at −20°C until DNA extraction. Isolates from 26 contaminants commonly encountered when isolating fungi from roots were cultured and processed for DNA extraction, as described for root rot organisms. These included *Botrytis cinerea*, *Sirococcus conigenus*, *Pestalotiopsis funerea*, *Gliocladium roseum*, *Mycelium radicis atrovirens*, and nine isolates belonging to *Fusarium* spp., one from *Phomopsis* spp., and one from *Coniothyrium* spp., in addition to three unidentified hyphomycetes, three coelomycetes, one deuteromycete, and three nonsporulating basidiomycetes. In addition, isolates identified as *Cylindrocarpon* spp. other than *C. destructans* were tested.

DNA extraction. DNA was extracted according to a modification of a protocol described elsewhere (31). Approximately 10 mg of lyophilized mycelium was mixed with diatomaceous earth (10 mg) and ground in 700 μl of extraction buffer (700 mM NaCl, 50 mM Tris-HCl [pH 8], 10 mM EDTA, 1% β-2-mercaptoethanol, 1% cetyl-trimethyl-ammonium bromide). The mixture was incubated at 65°C for 1 h and extracted by adding an equal volume of chloroform-isoamyl alcohol (24:1), vortexing, and centrifuging for 5 min at 10,000 × *g*. The aqueous phase was precipitated with cold isopropanol and centrifuged for 5 min at 10,000 × *g*. The pellets were washed with 70% ethanol, air dried, resuspended in 50 μl of TE8 (10 mM Tris-HCl [pH 8], 1 mM EDTA), and stored at −20°C until needed.

For DNA extractions directly from root collars, the outer bark of the collar was removed with a scalpel and two to four strips of wood (approximately 3 by 10 mm) were excised and ground in 400 μl of Qiagen extraction buffer (100 mM Tris-HCl, pH 9.5, 2% CTAB, 1.4 M NaCl, 1% polyethylene glycol 8000, 20 mM EDTA, 1% β-mercaptoethanol) mixed with approximately 10 mg of diatomaceous earth. Extracts were vortexed, incubated at 65°C for approximately 1 h, extracted once with 400 μl of phenol:chloroform:isoamyl alcohol (25:24:1), and centrifuged at 10,000 × *g* for 5 min. The supernatant was transferred to a new microtube, precipitated with one volume of isopropanol, and centrifuged at 10,000 × *g* for 5 min. Pellets were washed with 70% ethanol, air dried overnight, and resuspended in 20 μl of TE buffer, pH 8. For DNA extractions from roots, entire rootlets were cut into small pieces and processed as above.

DNA amplifications, purification, and sequencing. The entire region containing portions of the small subunit (18S), both internal transcribed spacers (ITS), and the 5.8S of the rDNA repeat unit was amplified with oligonucleotides ITS1F (fungus specific: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ [10]) and ITS4 (universal: 5′-TCCTCCGCTTATTGATATGC-3′ [29]). PCR reactions were carried out in volumes of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.0001% gelatin, 400 μM deoxynucleoside triphosphates, 1 μM of each of ITS1F and ITS4 primers, 1 unit of *Taq* DNA polymerase (Boehringer Mannheim Biochemica, Mannheim, Germany), and 1 μl of template DNA. The reactions were carried out on a MJ-Research PTC-60 thermal cycler (Watertown, Mass.) and consisted of an initial denaturation at 95°C for 3 min followed by 30 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The reaction was completed by a 10-min extension at 72°C.

PCR products were purified on a minispin column (Wizard Kit, Promega, Wis.) and sequenced with a DNA Direct Sequencing kit (Gibco-BRL, Gaithersburg, Md.) according to the manufacturer's recommendations with [γ-^{33}P] ATP (Amersham) labelling of the primers.

Primer synthesis and two-step nested PCR. Following sequence alignment (GCG software package) with related fungi in GenBank, primers were synthesized on a Beckman synthesizer according to the manufacturer's recommendations. In order to conduct multiplex PCR, primers were designed to amplify PCR products of different lengths for the two target species. Specificity of the primers Dest1 and Dest4 for *C. destructans* and Flor2 and Flor5 for *C. floridanum* was tested on all *C. floridanum* and *C. destructans* DNA templates, as well as with DNA templates of the 26 common contaminants.

A nested primer PCR combined with a multiplex PCR approach (Fig. 1) was used for detection of infected tissues (12, 13). Fungi present in roots and root collars of 34 infected seedlings and 2 healthy seedlings were identified by isolation as described above. The DNA was extracted from the root collars and

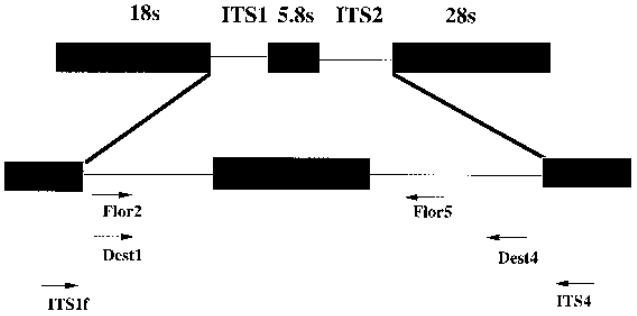


FIG. 1. Diagram showing the nested multiplex PCR approach. Primers ITS1f and ITS4 are used in a first-round fungus-specific amplification directly from infected seedling extracts. PCR products of the first round are used in a second-round amplification using all four specific primers, Flor2, Flor5, Dest1, and Dest4. PCR products of 328 and 400 bp can be visualized, respectively, for *C. floridanum* and *C. destructans.*

diluted 1:100; 1 μl was pipetted in a PCR using ITS1F and ITS4 primers to amplify the entire ITS region by using the amplification profile described above. A second amplification with all four species-specific internal primers was conducted by using as a template 1 μl of PCR product from the previous fungus-specific amplification. Only 15 cycles were run with denaturation at 94°C for 20 s and an annealing temperature of 60°C to increase specificity. Positive (genomic DNA from the target fungus) and negative (no template DNA) controls were included in all tests.

PCR products were separated by gel electrophoresis on 1.5% agarose in TAE buffer or on 1% agarose and 1% Synergel (Certified Biotech) in TPE buffer and visualized by UV fluorescence.

RESULTS

All isolates of *C. floridanum* had the same ITS sequence. However, some intraspecific variability was found in the ITS sequences within our collections of *C. destructans* (Fig. 2). Three groups were identified within *C. destructans* based on their ITS sequences. One group, Dest C, was clearly more divergent from Dest A and Dest B. There were 4 insertions or deletions (indels), 16 transitions, and 12 transversions between Dest A and B and Dest C (Fig. 2). There were no indels and only one transition and one transversion between Dest A and Dest B. There was also some variability within the groups; for example, there was a transversion within Dest A at position 433 and two insertions within Dest C at positions 435 (T) and 537 (A) (results not shown).

Genetic variability seemed to be evenly distributed between the ITS1 and ITS2. There were 13 mutations and three indels in the ITS1 and 14 mutations and 1 indel in the ITS2. Only a single mutation in the 5.8S coding region was present within *C. destructans* (Fig. 2).

All *C. destructans* isolates were formally identified as belonging to this species according to the following morphological and cultural characteristics: (i) chlamydospores were produced in the mycelium and sometimes in the macroconidia; (ii) microconidia (6 to 11 by 2 to 4 μm) with zero to one septum were present; (iii) macroconidia had one to three or four septa and were straight and cylindrical with a round apical cell, and (iv) the cultures were pigmented brown to dark brown.

No clear association was found between ITS variants within *C. destructans* and host or geographic origin. For example, isolates belonging to Dest A originated from a plantation of *Pinus banksiana* in Lac-Saint-Jean (northern Quebec) and from container nursery seedlings on *Picea rubra* in Berthierville (along the St. Lawrence River; Table 1). Also, both Dest B and Dest C were found on *Picea abies* and *Picea glauca* in a nursery in Normandin (Table 1).

There were size differences between the species and groups

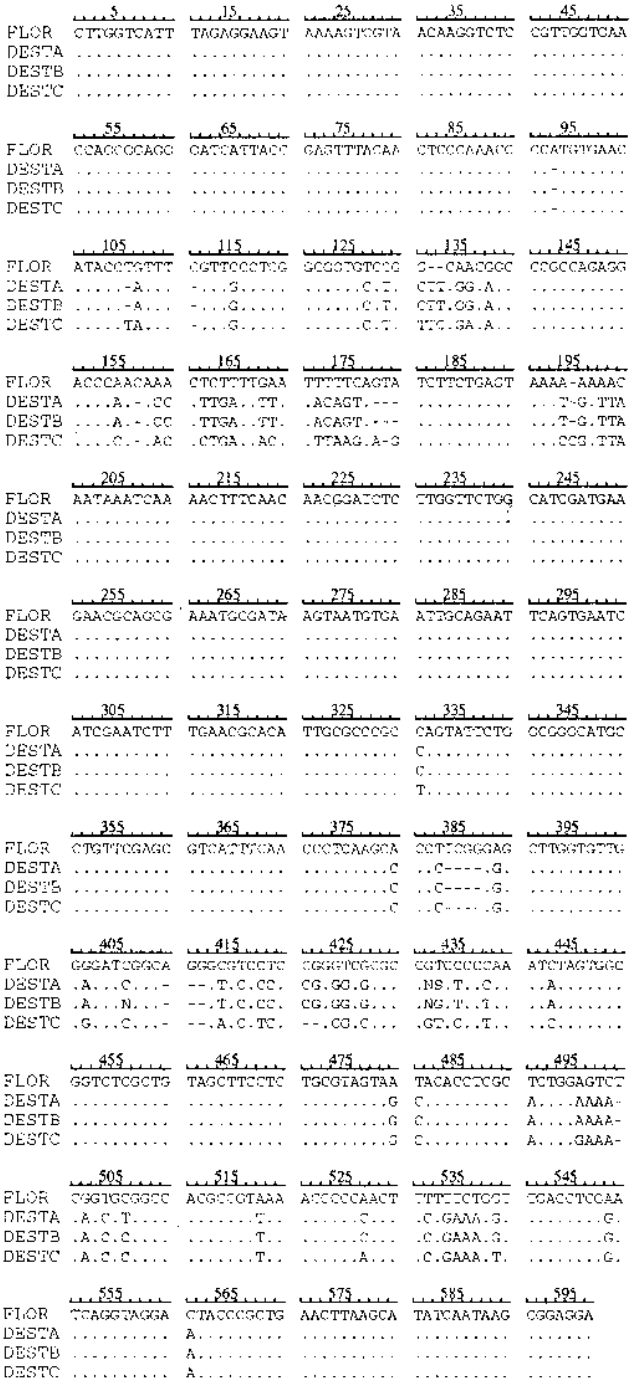


FIG. 2. Sequence of the ITS region, including the 5.8S coding region, of *C. floridanum* and *C. destructans*. A dot indicates the same nucleotide is present as in the top sequence. A dash indicates an insertion or deletion.

sequenced. *Cylindrocladium floridanum* had an ITS of 592 bp, while *C. destructans* belonging to groups Dest A and Dest B had an ITS of 582 bp and those belonging to *C. destructans* group Dest C had an ITS of 584 bp (Fig. 2).

Species-specific primers were selected in regions that were variable between, but fairly conserved within, species. Primers Flor2 and Flor5 were designed to amplify a 328-bp PCR product starting at position 109 in *C. floridanum* (Fig. 2; Table 2).

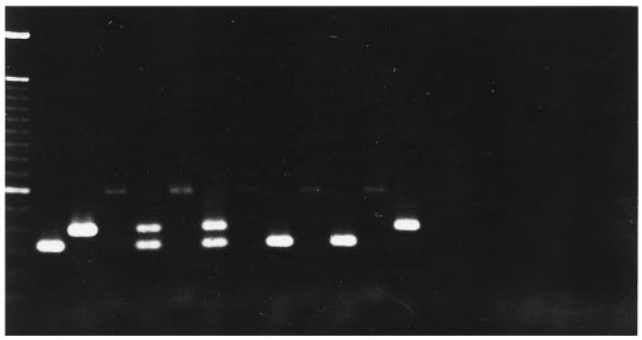
TABLE 2. List of primers used for specific amplification of *C. destructans* and *C. floridanum*

Primer name	Primer sequence	Size of PCR product (bp)
Flor2	5′-TTCGTTCCCTCGGCGGTGTCCGGCAACG-3′	
Flor5	5′-CAGGGCGTCCTCCGGGTCGCGCCGT-3′	328
Dest1	5′-TTGTTGCCTCGGCGGTGCCTG-3′	
Dest4	5′-GGTTTAACGGCGTGGCCGCGCTGTT-3′	399–400

In *C. destructans*, it was difficult to target regions that were conserved within and variable between species and that also produced a PCR fragment of a size different from that of *C. floridanum* so that it could be amplified in a multiplex reaction and resolved by electrophoresis. Two primers were designed to amplify a 400-bp amplicon in variants Dest A and Dest B and a 399-bp amplicon in Dest C of *C. destructans* (Fig. 2; Table 2). The mismatch at the priming site in Dest C was situated in the center of the primer Dest4; at the annealing temperature used in our study, amplification of a product in Dest C was not prevented by this mismatch (Table 2). The primers were therefore successfully designed to overcome the genetic variability present within *C. destructans.*

Specificity of the primers was confirmed on genomic DNA from all cultures of *C. floridanum* and *C. destructans*. No amplification resulted from PCRs using either set of primers with contaminant genomic DNA as template, including closely related species of *Cylindrocarpon* (Fig. 3).

The first round of amplification from infected tissues by using primers ITS1F and ITS4 resulted in PCR products of variable sizes, as would be expected if several fungal species were associated with decaying roots (Fig. 3). However, the second round of amplification with all four specific primers in a multiplex PCR yielded either one or two amplicons of the expected size (Fig. 3). *C. floridanum* was detected in 100% infected seedlings from which it was isolated. In one case, the fungus was detected in the seedlings by PCR but was not isolated. In the two healthy seedlings, the fungus was not isolated and the diagnostic PCR was negative.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 3. Amplification of the ITS in root rot organisms and associated fungi. Lane 1, Pharmacia's 100-bp ladder; lane 2, *C. floridanum* positive control (1070); lane 3, *C. destructans* positive control (1005); lanes 4 to 11, root collar DNA extracts and lanes 12 and 13 rootlet extracts from infected seedlings; lanes 14 to 17, root collar extracts from healthy seedlings; lanes 4, 6, 8, 10, 12, 14, and 15 were amplified with ITS1f and ITS4; lanes 5, 7, 9, 11, 13, 16, and 17, second round of PCR amplification with primers Flor2, Flor5, Dest1, and Dest4 using amplification products from ITS1f-ITS4 (lanes 4, 6, 8, 10, 12, 14, and 15) as a template; lanes 18, 19, and 20, *Fusarium* spp., *Gliocladium roseum*, and *Botrytis cinerea*, respectively. *C. floridanum* and *C. destructans* were both isolated from seedlings in lanes 4 to 7. Only *C. floridanum* was isolated from seedlings in lanes 8 to 11, and only *C. destructans* was isolated from seedlings in lanes 12 and 13.

C. destructans was detected in 20 of 27 infected seedlings from which the fungus was isolated and two seedlings from which only *C. floridanum* was isolated. On five infected seedlings, the diagnostic PCR on DNA extracts from root collars was negative for *C. destructans* but the fungus was isolated, but only from rootlets. The multiplex PCR was effective, as the two expected products were amplified simultaneously in 20 reactions (Fig. 3).

DISCUSSION

The work presented here illustrates the potential for detecting root rot fungi directly from infected seedlings with a minimum of manipulation. This approach is particularly well suited to soil organisms that are difficult to identify or isolate because of the presence of other aggressive species.

The presence of variability within the ITS of *C. destructans* was not totally unexpected. This fungus is a ubiquitous soil pathogen (16). In another ubiquitous soil pathogen, *Fusarium sambucinum*, three ITS variants were found in 86 isolates of *F. sambucinum* (21). However, the rate of intraspecific divergence reported for *F. sambucinum* was apparently greater than that reported here for *C. destructans*. Only four indels were found between the two most divergent groups of *C. destructans*, but 14 such mutational events were reported for *F. sambucinum* (21). It must be pointed out, however, that isolates of *F. sambucinum* from a broader range of geographic origins were investigated in that study (21). Comparable numbers of transversions and transitions were found in the two species (32 for *F. sambucinum*, 28 for *C. destructans*). In contrast to our results, the ITS2 of *F. sambucinum* was twice as variable as that of the ITS1 (21). In both *C. destructans* and *F. sambucinum*, there was no apparent correlation between ITS variants and geographic origin or host of origin.

We were able to extract large quantities of DNA from the root collars of both infected and healthy seedlings. However, DNA extraction and amplification from rootlets was not very efficient. Such rootlets may have been dead for some time before the seedlings were harvested or they might have been more susceptible than root collars to desiccation in storage. We have compared several different extraction protocols and found that the one used here was the most reliable. Some of the extraction buffers based on sodium dodecyl sulfate or hexadecyltrimethyl ammonium bromide yielded DNA extracts that contained high levels of inhibitors; diluting these extracts 1:1,000 and adding 1 μl to a PCR reaction already containing genomic DNA of the target fungus completely prevented amplification of the expected PCR product (results not shown).

Some of the DNA samples were extracted from seedlings that had been in storage at −20°C for 1 year; this has important implications for detection on an operational basis since large numbers of samples could be frozen during the fall inspection and processed during the winter before outplanting in the spring.

Amplification of species-specific PCR products directly from DNA extracts from the roots without a first round of fungus-specific amplification was not very consistent, probably because of the variability in the ratio of target DNA to non-target DNA. Multiplex PCR with all four primers directly from infected roots without a first round of amplifications was extremely unreliable.

Amplification of the species-specific PCR fragments with individual primer combinations (Flor2 and Flor5, or Dest1 and Dest4) from genomic DNA was generally more successful than

multiplex PCR with all four primers. When a mixture of genomic DNA from the two fungi was used as a template with all four primers, competition between the two amplicons appeared to cause inconsistent results, i.e., often only one PCR product was amplified even though genomic DNA from the two fungi was used as a template. Similar results were reported for multiplex PCR in other organisms (4).

By contrast, the nested multiplex PCR approach presented here yielded highly consistent and reproducible results. The first amplification with fungus-specific primers preferentially increased the population of fungal ITS molecules, thereby increasing the ratio of fungal:host ITS molecules. Competition between the two PCR products was apparently reduced during the second round of amplification since large numbers of ITS molecules served as a template to the species-specific primers.

Detection with the PCR assay was mostly concordant with results from the isolation procedure. All seedlings infected by *C. floridanum* tested positive with the PCR assay. This is important because this fungus is believed to be the primary root rot pathogen in conifer nurseries in northeastern North America (2). In addition, this fungus was detected by PCR in seedlings from which it had not been isolated. This can be explained by the fact that isolations are not always successful, in particular if aggressive contaminants are also present on the roots. Alternatively, infected seedlings with dead mycelium might test positive even though the fungus cannot be isolated, since DNA can be amplified from dead mycelium (13).

The fungus was frequently detected by PCR in seedlings where *C. destructans* was not isolated in the root collars but was isolated in rootlets. This could be an indication that the pathogen was present in the root collars at some point during the etiology but receded to the roots subsequently. This fungus was found to survive on infected roots of cultivated strawberry for 15 weeks after which it was replaced by other saprophytes (16). Since *C. destructans* is believed to be either a weak pathogen or a secondary parasite (16), it may be less important to detect than *C. floridanum*. DNA extractions from fresh rootlets in addition to root collars might improve our detection ability for *C. destructans.*

Depending on the proposed application of this PCR-based diagnostic assay, it could be desirable to amplify DNA from nonviable fungal material, such as for a survey of fungus incidence. However, to assess in a certification program the potential for introducing a pathogen into a new site, it would be preferable to discriminate between viable and nonviable pathogens.

Two obvious practical problems might limit the use of this technique in an operational setting. The first one is the danger of carry-over contamination. In spite of careful laboratory manipulations by trained personnel, there were occurrences of carry-over contaminations in some of our experiments and these had to be repeated with new reagents. Different means of circumventing these problems, including enzymatic digestions and UV irradiation, have been proposed and could be implemented (30). The second practical problem concerns the cost of such analyses. Although it would be impossible to carry out routine diagnostics with PCR on nursery seedlings, it could be possible to use the technique presented here as an aid in identification or to solve difficult cases. Another prospect would be the use of bulk samples followed by a quantification assay to determine the proportion of infected seedlings. We are currently developing colorimetric assays to achieve this goal.

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