Detection of Latent *Sphaeropsis sapinea* Infections in Austrian Pine Tissues Using Nested-Polymerase Chain Reaction

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ABSTRACT

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Sphaeropsis sapinea is the causal agent of Sphaeropsis tip blight disease of pines. Past surveys of diseased and symptomless Austrian and Scots pines revealed that latent infections of symptomless shoots by *S. sapinea* are common. The role of these latent infections in the tip blight disease is unknown. A sampling technique and nested-polymerase chain reaction (PCR) protocol were developed to detect latent *S. sapinea* in symptomless pine shoots. The sampling protocol was designed to be minimally destructive to the shoot so it could be preserved for further studies. The primers that were developed were specific for *S. sapinea* DNA and did not amplify DNA from any of 13 other endophytic fungal

species that were commonly isolated from symptomless pine shoots. The PCR primers also amplified DNA of *Botryosphaeria obtusa*, which was, however, rare in symptomless Austrian pine tissues. The protocol detected as little as 0.93 pg of *S. sapinea* DNA in terminal bud samples and 10.4 pg of DNA in bark samples. Correlation (chi-square) analyses indicated that the nested-PCR protocol detected latent *S. sapinea* infections in both bud and bark samples with an efficiency that was statistically equivalent to isolating the fungus from the tissue. The nested-PCR protocol will make it possible to more quickly identify latent *S. sapinea* infections in symptomless pine shoots and should be useful in future studies of the latency phenomenon.

Additional keywords: *Diplodia pinea*, Diplodia tip blight, endophyte.

During the last decade, Sphaeropsis tip blight (previously known as Diplodia tip blight) has caused significant mortality in Kentucky of landscape Austrian pines (*Pinus nigra*) and Scots pines (*P. sylvestris*), and of Scots pine Christmas trees. Sphaeropsis tip blight is caused by the imperfect fungus *Sphaeropsis sapinea* (Fr.:Fr) Dyko & Sutton in Sutton (syn. *Diplodia pinea* (Desmaz.) J. Kickx fil.). The most obvious symptoms of the disease are necrotic, stunted shoot tips and needles. Worldwide, *S. sapinea* infects more than 30 conifer species, which can result in serious economic losses of exotic and native trees in managed plantations (26,30,36). In addition to tip blight, *S. sapinea* can cause seedling damping off and collar rot, root disease, stem cankers, top dieback, and blue stain of cut timber (16-19,27, 28,33).

S. sapinea can be readily isolated from symptomless tissues of both diseased and apparently healthy pine trees (3,10,25,27). Latent *S. sapinea* infections have been reported on various pine hosts and in several different regions of the world (3,10,25,27). Other tree pathogens, including *Botryosphaeria* spp. causing cankers of woody plants, *Cytospora kunzei* on conifers, and *Hypoxylon atropunctatum* on oak, also have latent phases as part of their life cycles (1,22,24). For all of these pathogens, it has been widely assumed that latent infections can become pathogenic if the host tree becomes stressed (24). Although it was addressed in one study (29), to our knowledge this hypothesis has never been tested directly. If such a transition from latency to pathogenicity actually occurs, it raises some very intriguing questions about the molecular controls of that transition. Our long-term goal is to study the molecular mechanisms of latency, and of the transition from latency to pathogenicity if it occurs, in *S. sapinea*.

The goal of this study was to develop a rapid, reliable, and minimally destructive identification technique for latent *S. sapinea* infections in pine shoots. A polymerase chain reaction (PCR) detection protocol would potentially allow quick, sensitive, and specific screening of symptomless tissues for the detection of latent *S. sapinea* infections. The internal transcribed spacer (ITS) regions of the ribosomal RNA (rDNA) genes have been used to develop species-specific PCR primers for other organisms (11, 12,14,21). The ITS regions are good candidates for development of species-specific primers because they are relatively variable between species (32). Furthermore, the ITS sequences can be amplified from many different fungi by using universal primers that are complementary to the highly conserved flanking regions of the rDNA gene sequences (14,32). Amplification is very efficient because the rDNA gene sequence occurs as multiple copies in the genome (32), and this tends to increase the sensitivity of the PCR (14).

Species-specific primers were developed based on the ITS rDNA region and tested in a nested-PCR protocol for their ability to detect latent *S. sapinea*. The primers also were analyzed for their ability to distinguish *S. sapinea* from other common endophytic and saprophytic fungi found in Austrian pine tissues or as laboratory contaminants. A sampling regime was developed that was minimally destructive to the pine shoots, thus preserving them for further studies. This protocol should be useful for future investigations of the role of latent infections in the Sphaeropsis tip blight disease cycle.

MATERIALS AND METHODS

Fungal cultures. *S. sapinea* strains used in this study (Table 1) were obtained from other researchers or were isolated from Austrian or Scots pine tissues according to the protocol of Flowers et al. (10). Thirteen species representing the most commonly isolated fungal endophytes from symptomless Austrian pine shoots in

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our area were chosen for this study (Table 1). *Penicillium brevicompactum*, found growing as a contaminant on a culture plate, also was included. Each fungal isolate was grown according to the protocol of Flowers et al. (10) and single-spored with a micro-manipulator device (Microscore LCC, Georgetown, KY). Stock cultures of all single-spored isolates were stored on potato dextrose agar (PDA) slants (Difco Laboratories, Sparks, MD) at 4°C.

DNA extraction. Fungal stationary cultures were grown in potato dextrose broth (Difco Laboratories) for up to 1 week under ambient laboratory conditions. DNA extraction and purification were performed according to the protocol of Thon et al. (31). DNA pellets were resuspended in 50 µl of sterile deionized water. Some DNA samples were further purified by partitioning with phenol and chloroform. All DNA samples were quantified, diluted to 1 ng/µl, and stored at 4°C.

The protocol used to extract DNA from fresh pine tissue was modified from Zhang et al. (34) and Hamelin et al. (12). Fresh Austrian pine tissue samples (described below) were first surface-disinfested by agitating in a 10% bleach solution containing a few drops of Tween 20 for 2 min, rinsed twice in sterile deionized

water, and then dried by blotting with sterile paper towels. The pine tissue samples were crushed with a mortar and pestle and incubated for 1 h at 65°C in 800 µl of DNA extraction buffer with periodic vortexing. After cooling for a few minutes at room temperature, 600 µl of chloroform/isoamyl alcohol (24:1) was added to each sample. The samples were vortexed vigorously and then centrifuged. The top aqueous phase of each sample was transferred to a new tube and an equal volume of ice-cold isopropanol was added. The samples were incubated for 10 min on ice followed by centrifugation. The DNA pellet was washed with 70% ethanol, air dried, and redissolved in 20 µl of sterile deionized water.

ITS amplification, sequencing, and primer design. PCR was performed using 1 µl of fungal DNA as a template. Each 50-µl PCR reaction contained 0.5 µM ITS5 primer (32), 0.5 µM ITS4 primer (32), 2 µM dNTPs, 1× PCR buffer, 5 µM MgCl₂, and 0.5 µl of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The reactions were incubated for 30 cycles, each consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Ten microliters of each PCR reaction product was electrophoresed through a 1% agarose

TABLE 1. Fungal isolates used in this study

Isolate number	Species	Host	Origin	Collector	GenBank Accession No.
11B	<i>Sphaeropsis sapinea</i>	<i>Pinus nigra</i>	Urbana, IL	J. Hartman	AY 156718
103-1	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Hartman	AY 156719
113-1	<i>S. sapinea</i>	<i>P. sylvestris</i>	Lexington, KY	J. Hartman	AY 156720
12 S	<i>S. sapinea</i>	<i>P. nigra</i>	Urbana, IL	J. Hartman	AY 156721
125-1	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Hartman	AY 156722
15 SB	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Hartman	AY 159245
150-2	<i>S. sapinea</i>	<i>P. sylvestris</i>	Lexington, KY	J. Hartman	AY 159246
157 D-1	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 159247
157 D-2	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 159248
158 S	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 159249
158 N	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 159250
159 S	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 159251
159 N	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 159252
144-3	<i>S. sapinea</i>	<i>P. sylvestris</i>	Lexington, KY	J. Hartman	AY 159253
61 SB	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Hartman	AY 159254
70 D	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Hartman	AY 159255
21 S	<i>S. sapinea</i>	<i>P. nigra</i>	Lexington, KY	J. Hartman	AY 160196
25 B	<i>S. sapinea</i>	<i>P. nigra</i>	Bloomington, IN	J. Hartman	AY 160197
28 A	<i>S. sapinea</i>	<i>P. sylvestris</i>	Ohio	J. Hartman	AY 160198
230	<i>S. sapinea</i>	<i>P. nigra</i>	Dijon, France	J. Hartman	Not sequenced
232	<i>S. sapinea</i>	<i>P. nigra</i>	Angers, France	J. Hartman	Not sequenced
233	<i>S. sapinea</i>	<i>P. nigra</i>	Paris, France	J. Hartman	Not sequenced
128 WT ^a	<i>S. sapinea</i>	<i>P. resinosa</i>	Wisconsin	M. Palmer	AF 027758
411 WT ^a	<i>S. sapinea</i>	<i>P. resinosa</i>	Minnesota	M. Palmer	AF 243409
CMW1184 SA ^a	<i>S. sapinea</i>	<i>P. radiata</i>	South Africa	W. J. Swart	AY 260545
CMW1185 SA ^a	<i>S. sapinea</i>	<i>P. radiata</i>	South Africa	W. J. Swart	AY 260544
CMW1187 SA ^a	<i>S. sapinea</i>	<i>P. radiata</i>	South Africa	W. J. Swart	AY 260543
CMW4240 SA ^a	<i>S. sapinea</i>	<i>P. radiata</i>	South Africa	W. J. Swart	AY 260542
113 WT ^{ab}	<i>S. sapinea</i>	<i>P. banksiana</i>	Wisconsin	G. Adams	AY 160199
124 WT ^{ab}	<i>S. sapinea</i>	<i>P. banksiana</i>	Wisconsin	M. Palmer	AF 027757
215 WT ^{ab}	<i>S. sapinea</i>	<i>P. resinosa</i>	Wisconsin	M. Palmer	AF 243410
457 WT ^{ab}	<i>S. sapinea</i>	<i>P. resinosa</i>	Wisconsin	M. Palmer	AY 160200
1LEX	<i>Botryosphaeria obtusa</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	Not sequenced
96-2 WI	<i>B. obtusa</i>	Woody shrub	New York	G. Samuels	AF 027759
96-115 WI	<i>B. obtusa</i>	<i>Prunus persica</i>	Georgia	K. Britton	AF 243408
B.doth	<i>B. dothidea</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160208
L.theo1	<i>Lasiodiplodia theobromae</i>	<i>P. nigra</i>	Lexington, KY	J. B. Magnin	AY 160201
L.theo2	<i>L. theobromae</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160214
E.nigr	<i>Epicoccum nigrum</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160213
Phoma	<i>Phoma</i> sp.	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160204
P.brev	<i>Penicillium brevicompactum</i>	N/A	Lexington, KY	J. Flowers	AY 160203
Fusa1	<i>Fusarium</i> sp.	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160205
Fusa2	<i>Fusarium</i> sp.	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160209
Fusa3	<i>Fusarium</i> sp.	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160207
Pest	<i>Pestalotiopsis</i> sp.	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160206
H.dem	<i>Hormonema dematiodes</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160202
A.alt	<i>Alternaria alternata</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160211
Sord	<i>Sordaria</i> sp.	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160210
B.tris	<i>Blakeslea trispora</i>	<i>P. nigra</i>	Lexington, KY	J. Flowers	AY 160212

^a WI or SA have been added to the original numbers to distinguish these isolates from those in our own collection.

^b B morphotype *S. sapinea* isolates. All other *S. sapinea* isolates are A morphotype.

gel and stained with ethidium bromide. Individual PCR products were recovered from the gels for sequencing with a PCR purification kit (QIAquick; Qiagen, Valencia, CA) according to the manufacturer's instructions.

PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), following the manufacturer's protocols, on an automated sequencer (ABI Prism 310; Applied Biosystems).

Sequences were analyzed and a multiple sequence alignment was produced using the Double Twist Gene Tool lite version 1.0 software package (BioTools Inc., Edmonton, Canada). Parameters for the multiple sequence alignment included a gap penalty of 5 and a gap size penalty of 5. All alignments were visually inspected prior to further analysis. Homology searches (GenBank blastn) were performed using the ITS1 and ITS2 regions and the 5.8S rDNA gene of all the fungal isolates. Each of the endophytic or contaminant fungal strains was initially identified based on morphology. Their identities were later confirmed by the results of the homology searches.

Areas of the ITS1 and ITS2 regions that were completely conserved for *S. sapinea* isolates, but divergent in the other fungi, were targeted for *S. sapinea*-specific primer development. Primer pairs were developed using either the PRIME program in the Wisconsin Package (GCG, 1996), or manually, based on visual inspection of the multiple alignment. Potential primer pairs were analyzed for melting temperature, G/C content, and primer dimer or hairpin formation using the Integrated DNA Technologies (IDT, Coralville, IA) software package Oligo Analyzer 2.5 and the Cprimers package (G. Bristol and R. Anderson, University of California, Los Angeles).

Nested-PCR. In the first round of the nested-PCR protocol, the universal ITS1 and ITS4 primers (32) were used. Each 50- μ l reaction contained either 1 ng of purified fungal DNA or 2 μ l of the DNA extracts from Austrian pine tissue as a template and 0.4 μ M each ITS primer, 2 μ M dNTPs, 1 $\times$ PCR buffer, 5 μ M MgCl₂, and 0.5 μ l of *Taq* DNA polymerase. Sterile water or 1 ng of purified *S. sapinea* DNA was used as a negative or positive control, respectively, in each nested-PCR experiment. The reactions were incubated at 94°C for 3 min followed by 30 cycles each consisting of 94°C for 30 s, 65°C for 15 s, and 72°C for 30 s. The resulting PCR product was diluted 1:50 and used as a template for the second round of PCR in the nested-PCR protocol.

The nested-PCR reaction was performed with 2 μ l of the diluted primary PCR product as a template, and the same PCR conditions as described previously, except 0.375 μ M each of the *S. sapinea*-specific primers was used in place of the universal ITS primers. Ten microliters of each PCR reaction product was electrophoresed through a 1% agarose gel and stained with ethidium bromide.

The nested-PCR protocol using the universal ITS primers, ITS1 and ITS4, and the *S. sapinea*-specific nested primers, S.sapFOR3 and S.sapREV3, was evaluated for sensitivity and specificity. Sensitivity was determined by amplifying known concentrations of purified *S. sapinea* genomic DNA in either sterile deionized water; a DNA extract from uninfected 4-year-old greenhouse-grown Austrian pine terminal buds; or a DNA extract from uninfected 4-year-old greenhouse-grown Austrian pine bark samples (described below). For each experiment, control pine samples comparable to those used for DNA extraction were incubated on culture media according to Flowers et al. (10) to confirm that the control pines were free of *S. sapinea* infection. PCR control reactions containing only the extracts from the buds or bark, with no added *S. sapinea* DNA, also were included for each experiment.

Purified genomic DNAs from each of the endophytic and contaminant fungi, along with several *S. sapinea* isolates representing different geographic locations, hosts, and morphotypes (A and B), were used to test the specificity of the nested-PCR protocol. Primer specificity was tested further by conducting a GenBank blastn search, using the nested primer sequences as queries, in

order to identify sequences from other organisms included in the databases that had the potential to be amplified by the primers.

Sampling of diseased and symptomless Austrian pine tissues. A previous study showed that *S. sapinea* is located primarily within the buds and outer tissues of latently infected Austrian pine shoots (including the bark and phloem tissues down to the cambium) (10). To develop a sampling technique that would be minimally destructive to the shoot, terminal buds and bark samples were chosen as the tissues that would be least likely to cause death of the shoot upon removal.

The distribution of the fungus within the terminal buds and bark of symptomless shoots was evaluated. The entire current year's lignified growth was collected from five symptomless shoots from each of 13 tip blight-diseased trees (a total of 65 shoots) during the late summer of 2001. The samples were placed in individual plastic bags and stored at 4°C for no more than 2 days until pathogen isolation. Leafless shoots with their terminal buds still attached were surface-disinfested according to the protocol of Flowers et al. (10). The terminal buds were aseptically removed and then cut longitudinally. A cork borer (0.6-cm-diameter) was used to remove bark disks (including the bark and phloem tissues down to the cambium) from the top (1 cm below terminal bud), middle, and base (1 cm above sampling cut) of each shoot. *S. sapinea* was isolated from the terminal bud halves and bark disks on acidified PDA and subcultured onto water agar plates for verification according to the protocol of Flowers et al. (10).

The distribution of *S. sapinea* within individual bark disk samples was further characterized. Bark disks were removed approximately 1 cm below the terminal bud from 57 symptomless shoots on three tip blight-diseased Austrian pines. The 57 bark disk samples were surface-disinfested by agitating in a 10% bleach solution containing a few drops of Tween 20 for 2 min followed by two rinses in sterile deionized water, and then each was cut in half aseptically. Each half was incubated separately to identify *S. sapinea* according to the protocol of Flowers et al. (10).

Analysis of the correlation between culture isolation and nested-PCR. To evaluate the degree of correlation between culture isolation and the nested-PCR protocol, terminal buds and bark samples (from the top of the shoot only) were recovered, as described previously, from 114 symptomless shoots collected from 10 tip blight-diseased Austrian pine trees on the University of Kentucky campus between December 2001 and March 2002. Sixteen terminal bud and 18 bark disk samples were collected from infection-free control Austrian pines that had been grown in the greenhouse for use as negative controls, and three terminal buds and 20 bark disk samples were collected from shoots with symptoms of tip blight in the field for use as positive controls. Each bud or bark sample was cut in half and surface-disinfested as described previously. One half of each sample was incubated on PDA acidified with 1 ml of 85% lactic acid per liter followed by subculturing of putative *S. sapinea* colonies onto 2% water agar as described previously (10). The other half of each sample was placed in a 1.5-ml Eppendorf tube and stored at 4°C for up to 6 days until the DNA was extracted and used as a template in the nested-PCR protocol described previously. A data set that included only latently infected samples (determined by a positive result for the presence of *S. sapinea* by at least one of the two detection protocols) was used for statistical analysis. A chi-square test was used to compare detection frequency of *S. sapinea* by the culture isolation or the nested-PCR protocol for each sample.

RESULTS

ITS amplification, sequencing, and primer design. The 5.8S rDNA gene and flanking ITS regions of the endophytic and saprophytic fungi were sequenced and compared with the sequences of representative *S. sapinea* isolates (Fig. 1). As expected, most of the variation between species was observed within the ITS1 and

ITS2 regions. In agreement with previous studies, the ITS sequences of 29 different isolates of *S. sapinea* were virtually identical to one another (data not shown) (7,15). The *S. sapinea*-specific primers S.sapFOR3 (5'-GCTTTGGCGGCTCTTTG-3') and S.sapREV3 (5'-CTACTACGCTTGAGGGCTGAA-3') were chosen from the different primer pairs that were tested as the most sensitive and specific for *S. sapinea* DNA (Fig. 1).

Nested-PCR. The universal ITS primer pair and the *S. sapinea*-specific primer pair SsapFOR3 and SsapREV3, used in the nested-PCR protocol, amplified a product of approximately 350 bp from the genomic DNA of all 29 *S. sapinea* isolates tested, including both the A and B morphotypes (data not shown). No product was amplified from any of the endophytic or contaminant fungi with these primers (Fig. 2A).

GenBank blastn search results using each of the *S. sapinea*-specific primers as queries indicated that both sequences were identical to sequences only of *S. sapinea* and the closely related fungus *B. obtusa*. We tested the ability of the primers to amplify DNA from isolates of *B. obtusa* in this study. The nested-PCR protocol amplified a product from the DNA of all three isolates of

B. obtusa tested (Table 1). This product was the same size as that produced by amplification of *S. sapinea* DNA (Fig. 2B). The GenBank blastn searches also indicated that the SsapFOR3 and SsapREV3 primer sequences differ from *S. sapinea* f. sp. *cupressi* and *B. stevensii* by only 1 bp (close to the 3' end of primer S.sapFOR3). Thus, it is likely that these primers could also amplify a product from the genomic DNA of these fungi, which are, however, not known to infect *Pinus* spp. We did not test this possibility experimentally.

The sensitivity of the nested-PCR protocol depended on the source of the sample. A PCR product of the expected size was amplified from as little as 0.5 pg of purified *S. sapinea* genomic DNA per µl of diluted in sterile deionized water, but sensitivity dropped slightly, to 0.93 pg/µl, for purified genomic DNA diluted in bud extract, and was reduced about 20-fold, to 10.4 pg/µl, for purified genomic DNA diluted in extract from bark samples (Fig. 3).

Pathogen distribution of *S. sapinea* within symptomless terminal buds and bark samples. Of the 65 symptomless Austrian pine shoots that were tested, 50 (77%) were latently infected by *S. sapinea*, as determined by isolation in culture. *S. sapinea* was isolated from 26 (52%) of the terminal buds taken from the latently infected shoots and from at least one of the three bark samples in 39 (78%) of the shoots. *S. sapinea* was most frequently recovered from bark samples taken from the top of the shoots (data not shown). There was a highly significant chi-square probability of 0.0094 that the position of the sample at the top of the shoot and the presence of *S. sapinea* were interdependent. Therefore, for subsequent experiments bark disks were taken only from the tops of the shoots.

Pathogen distribution of *S. sapinea* in terminal buds and bark samples. The distribution of *S. sapinea* within terminal bud and bark samples appeared to be discontinuous. The fungus was isolated from both halves of only 13 of 26 bisected, latently infected terminal buds. *S. sapinea* was isolated from only one of the two halves in the remainder of the samples. Of the 57 bark samples that were bisected and tested in culture, 41 (72%) were latently infected with *S. sapinea*. The fungus was isolated from both halves of these latently infected bark samples two-thirds of the time. In the remaining third of the samples, *S. sapinea* could be isolated from only one of the two halves.

Correlation between culture isolation and nested-PCR. The ability of the nested-PCR protocol to detect latent *S. sapinea* shoot infections was compared statistically with the culture isolation technique by a chi-square analysis. The terminal bud and/or the bark were colonized by *S. sapinea* in 101 shoots of 114 sampled (89%). *S. sapinea* was isolated from 96% of the bark samples taken from these latently infected shoots and from 38% of the terminal buds. The correlation between results obtained by culture isolation and by the nested-PCR protocol was highest for the bark tissue (Fig. 4). This correlation was statistically highly significant, with a chi-squared probability of 0.003 (<0.05 is statistically significant). All control samples removed from infection-free greenhouse-grown Austrian pines tested negative for the presence of *S. sapinea* with both the culture isolation technique and the nested-PCR protocol. All control Austrian pine tissues with symptoms of tip blight taken from the field tested positive for the pathogen by both detection techniques.

DISCUSSION

In this study, a sampling technique and a nested-PCR protocol were developed that can rapidly and accurately detect *S. sapinea* in symptomless pine shoots while preserving the latently infected shoots for further study. This is an improvement over previously published methods that are more time-consuming and destructive.

Members of the species *Pinus* subgenus *Diploxylon*, which includes Austrian and Scots pines, are reportedly colonized by many fungal endophytes (13). In developing a PCR detection protocol, it

	S.sap FOR3
S.sapin	GCTTTGGC-G--GC-----TCT-T---T-G
S.sap B	GCTTTGGC-G--GC-----TCT-T---T-G
B.obtus	GCTTTGGC-G--GC-----TCT-T---T-G
B.dothei	GCTTTGGC-G--GCCCGCGGTCC-T---C-C
L.theob	GCTTTGGC-G--GC-----TC-----
E.nigr	TCCTCGGC-G--GG-----TC-----
Phoma s	TCCTCGGC-G--GG-----TC-----
P.brevi	GCTTCGGC-GA-GCCTGCCTTTTGG---CTG
Fusariu	TCCTTGGT-G--GG-----TT-----
Fusariu	GCCTCGGC-G--GA-----TCAG-----C
Fusariu	TCCTTGGT-G--GG-----TT-----
Pestalo	GCCTCGGCAGAACG-TGC--TCGGTGCACT
H.demat	GCTTTGGC-G--GGACCGTTTCGGT---C-T
Alternat	TCCTTGGT-G--GG-----TT-----
Sordari	GCCTCGGC-GCTGGCGG--TCCGGAAGGCCC
B.trisp	GCAGCGTTTGAGGGATG--TTCT-TGTGCTAT

	S.sap REV3
S.sapin	CTGTTTCAGCC-CTCA-AGCGTA-GTAGAATAC
S.sap B	CTGTTTCAGCC-CTCA-AGCGTA-GTAGAATAC
B.obtus	CTGTTTCAGCC-CTCA-AGCGTA-GTAGAATAC
B.dothei	C-GTCTTGCC--TCA-AGCGTA-GTAGAACAT
L.theob	CTCTTCAGCC-CTCA-AGCGTA-GTAGAATAC
E.nigr	C-GTATTGAT-TTCGGAGCGCA-GTACA-T--
Phoma s	C-GTATTGAT-TTCGGAGCGCA-GTACA-T--
P.brevi	C-GTCCGGTC-CTCA-AGCGTATGGGGCTTTG
Fusariu	C-CTACTGGT-TTCGGAGCGCA-GCACAAGTC
Fusariu	CTG--CAGCC-TCCATTGCGTA-GTAGC-TA-
Fusariu	C-CTACTGGT-TTCGGAGCGCA-GCACAAGTC
Pestalo	CGATAT--CC--TCTGAGCGTA-GTA-ATTTT
H.demat	TTCAACCAAC-TTCGG-GCGTA-GTAGAGTTA
Alternat	C-CTACTGGT-TTCGGAGCGCA-GCACAAGTC
Sordari	CTCGCTAGTCACACCGAGCGTA-GTAC--TCT
B.trisp	-----

Fig. 1. Multiple sequence alignment of representative *Sphaeropsis sapinea* isolates, endophytic fungi isolated from Austrian pine, and contaminant fungi. Only the portions encompassing the S.sapFOR3 and S.sapREV3 primers are shown. S.sapFOR3 is located in internal transcribed spacer 1 (ITS1) region and S.sapREV3 is located in ITS2 region. The fungi included in the alignment below, in order of their appearance in the list, are *S. sapinea* (28 A, Kentucky isolate, A morphology); *S. sapinea* (457, Wisconsin isolate, B morphology); *Botryosphaeria obtusa*; *Botryosphaeria dothidea*; *Lasiodiplodia theobromae* (*L.theo1*); *Epicoccum nigrum*; *Phoma* sp.; *Penicillium brevicompactum*; *Fusarium* sp. 1; *Fusarium* sp. 2; *Fusarium* sp. 3; (none of these three *Fusarium* spp. were further identified); *Pestalotiopsis* sp.; *Homonema dematioides*; *Alternaria alternata*; *Sordaria* sp.; and *Blakeslea trispora*. More information about each of these isolates can be found in Table 1.

was important to ensure that the PCR primers did not amplify the DNA of common fungal endophytes. The endophytic fungal isolates chosen for this analysis were those most commonly cultured in no more than 1 week from symptomless, surface-disinfested Austrian pine tissue in our area. With the exception of *Blakeslea trispora*, all of these fungi have been reported previously as endophytes on pine tissue (5,6,9,20,22–25). *Penicillium brevicompactum* is a common laboratory contaminant and was included for that reason. The pair of PCR primers developed for this study, S.sapFOR3 and S.sapREV3, was specific for *S. sapinea* and did not amplify a product from any of the other cultured endophytic or saprophytic fungi tested.

The primers developed for this study amplified a product of equal size from strains of both the A and B morphotypes of *S. sapinea*. Although representative strains were not tested, the C morphotype of *S. sapinea*, which has an ITS sequence that is identical to that of the A morphotype (8), also would be expected to be amplified by these primers. Type B isolates are morpho-

logically distinct from type A and are less aggressive on red and jack pines (2). Type C isolates are more similar to type A isolates but they have been found only in Indonesia (4,8). A type B isolate was reportedly isolated in Kentucky from a Virginia pine (28), but the thousands of isolates of *S. sapinea* that we have recovered over the past several years from diseased and symptomless Austrian pine tissues have all had the typical type A morphology. It is important to note that we have not characterized these isolates further by using molecular markers, which would be a more accurate method for assigning morphotype. However, it seems likely that B and C isolates are rare or nonexistent in Austrian pines in Kentucky, and so their ability to react with the *S. sapinea* primers should not seriously impact the future use of this assay to detect latent infections in our regional population of Austrian pines.

Results of a GenBank blastn homology search indicated that the *S. sapinea*-specific primers would also probably amplify a product from the genomic DNA of the fungus *B. obtusa*. This prediction

Fig. 2. Nested-polymerase chain reaction products amplified from purified genomic DNAs of *Sphaeropsis sapinea*, *Botryosphaeria obtusa*, and various pine endophytic fungi. **A**, Lane 1, 1-kb Plus Ladder (Invitrogen, Carlsbad, CA); lane 2, negative control (no DNA template); lane 3, *S. sapinea* (0.45 ng); lane 4, *B. dothidea* (1 ng); lane 5, *Lasiodiplodia theobromae* 1 (1 ng); lane 6, *L. theobromae* 2 (1 ng); lane 7, *Fusarium* sp. 1 (1 ng); lane 8, *Fusarium* sp. 2 (1 ng); lane 9, *Fusarium* sp. 3 (1 ng); lane 10, *Epicoccum nigrum* (1 ng); lane 11, *Phoma* sp. (1 ng); lane 12, *Penicillium brevicompactum* (1 ng); lane 13, *Pestalotiopsis* sp. (1 ng); lane 14, *Hormonema dematioides* (1 ng); lane 15, *Alternaria alternata* (1 ng); lane 16, *Sordaria* sp. (1 ng); and lane 17, *Blakeslea trispora* (1 ng). **B**, Lane 1, 1-kb Plus Ladder (Invitrogen); lane 2, negative control (no DNA template); lane 3, *S. sapinea* (0.45 ng); lane 4, *B. obtusa* 96-2 WI (1 ng); and lane 5, *B. obtusa* 96-115 WI (1 ng).

Fig. 3. Nested-polymerase chain reaction detection of **A**, purified *Sphaeropsis sapinea* DNA diluted in sterile deionized water (lanes 3 to 5) or DNA extracts from *S. sapinea* infection-free Austrian pine terminal buds (lanes 6 and 7), or **B**, DNA extracts from *S. sapinea* infection-free Austrian pine bark plugs (lanes 2 to 6). **A and B**, Lane 1 contains 1-kb Plus Ladder (Invitrogen, Carlsbad, CA). **A**, Lane 2, negative control (no DNA template); lane 3, 0.05 ng of DNA; lane 4, 0.5 pg of DNA; lane 5, 0.05 pg of DNA; lane 6, 0.9 pg of DNA; and lane 7, 2 μ l of DNA extract from infection-free control Austrian pine terminal buds. **B**, Lane 2, negative control (no DNA template); lane 3, 64 pg of DNA; lane 4, 26 pg of DNA; lane 5, 10.4 pg of DNA; and lane 6, 2 μ l of DNA extract from infection-free control Austrian pine bark plugs.

was subsequently tested and confirmed. *B. obtusa* is common in temperate regions on many hosts, including pine (4,9). It produces a serious canker disease of apples (24) but it is not known to cause tip blight symptoms on pine. *B. obtusa* and *S. sapinea* are closely related (15). The sexual stage of *S. sapinea* is unknown, but the teleomorphs of *Sphaeropsis* spp. are often *Botryosphaeria* spp. (35). *B. obtusa* and *S. sapinea* have similar, though distinguishable, colony and spore morphology and can easily be confused (15). The two fungi have virtually identical rDNA sequences, with only 2 bp differences within the 5.8S rDNA gene. Unfortunately, neither of these changes is located in a region that would be suitable for primer development, so it is unlikely that a primer pair could be designed based on the rDNA sequences that would distinguish among these two fungi. It is possible that some pine shoots could be falsely identified by the nested-PCR method as containing latent *S. sapinea* infections when they actually contain *B. obtusa*. However, our data suggest that the percentage of such false positives is likely to be very small. More than 1,300 symptomless shoots from both Austrian and Scots pine have been tested for the presence of *S. sapinea* by culture isolation in our laboratory since 1998. The anamorphic state of *B. obtusa* has been isolated only twice from among those 1,300 shoots, once from a nondisinfested Austrian pine shoot and once from a surface-disinfested Scots pine shoot. The identification of *B. obtusa* was initially based on spore morphology which was then confirmed by sequencing of the ITS regions. Other results of the Blastn search suggest that the *S. sapinea* primers might also amplify a product from the DNA of *S. sapinea* f. sp. *cupressi* or *B. stevensii*, both of which differ from the primer sequences by only a single base pair. Since these species have not been reported to occur on pine, we did not test this prediction experimentally.

The nested-PCR protocol described here amplified a detectable product from as little as 0.5 pg of purified *S. sapinea* DNA per μ l. However, sensitivity was substantially reduced (by about 20-fold) when purified *S. sapinea* DNA was diluted in DNA extracts of bark tissue from infection-free Austrian pine trees that had been grown in the greenhouse. This suggests that pine bark tissues contain inhibitors of the PCR reaction. Nevertheless, the high degree of correlation between culture isolation and the PCR technique using bark disk samples demonstrates that most latently infected tissue samples contain enough *S. sapinea* DNA for successful amplification. Thus, the presence of inhibitors does not appear to be a significant impediment to the use of this protocol for detection of latent *S. sapinea* infections.

An important goal of this study was to develop a less destructive sampling protocol that would preserve latently infected shoots

for further study. A previous study found that *S. sapinea* was located primarily within the outer tissues (including terminal and lateral buds, bark and phloem tissue down to the cambium, and needle bundles) of latently infected shoots (10). Initially, needles were considered an ideal tissue for sampling, but we found that the needles were rarely latently infected (J. Flowers, unpublished data). A large majority (96%) of the bark disks that were removed from symptomless, latently infected shoots on diseased trees in our correlation study were colonized by *S. sapinea*. This is in contrast to the lower colonization rate (38%) for terminal buds. The intact shoots from which a bark plug had been removed for the present study were observed in the field 10 months later, and all of the shoots appeared healthy, with the cork-borer wounds completely calloused over. Thus, the most suitable sample for nondestructive detection of latent *S. sapinea* shoot infections seems to be a bark disk that is removed from the top of the shoot with a cork borer.

The nested-PCR technique was statistically equivalent to the culture isolation technique for detection of latent *S. sapinea* infections. Uneven distribution of the pathogen is one likely explanation for cases in which results of the two methods were not correlated. The pathogen appears to be discontinuous in both bud and bark tissues, as suggested by the finding that *S. sapinea* could be isolated from both halves of a terminal bud only 50% of the time and from both halves of a bark disk only 66% of the time. Another possibility is overgrowth of *S. sapinea* in culture by other endophytic fungi or bacteria, preventing it from being identified in the culture dish. The nested-PCR technique can theoretically amplify DNA from dead *S. sapinea* cells, which could explain why some samples were positive with the nested-PCR technique but not with the culture isolation. Spurious amplification in the PCR cannot be ruled out as another possible explanation for cases in which the two methods did not agree, although negative controls produced from uninfected samples were included and always failed to detect any contamination.

As more laboratories begin to use molecular techniques for plant disease diagnosis, the nested-PCR protocol could replace culture isolation for the quick diagnosis of *Sphaeropsis* tip blight. However, symptoms and signs of this disease are easily recognized in landscape Austrian and Scots pines. When pathogen identification is needed, the primary advantage of the nested-PCR technique is that it can positively identify the pathogen within 1 day. It can, therefore, provide a confirmation of the initial diagnosis much more quickly than culturing, which takes about 3 weeks. Disadvantages include the increased cost of PCR over culturing and the requirement for more specialized technical expertise.

A combination of the nested-PCR technique described here and culture isolation could enhance the efficiency of certification programs designed to identify disease-free nursery stock. However, it is very important to emphasize that both techniques can produce false negative results. Due to sampling issues and the apparently discontinuous nature of *S. sapinea* in symptomless shoots, we feel it would be inappropriate to use either or both of these procedures to guarantee that nursery stock was completely free of *S. sapinea* colonization.

The hypothesis that latent *S. sapinea* infections become pathogenic when the host becomes stressed has yet to be tested directly. Barriers to addressing this question in the past have included the relatively long periods of time required to identify latently infected shoots by culturing, limiting the number of samples that can be tested, and the destruction of the whole shoot in the process. With the sampling and nested-PCR protocol developed here, latently infected shoots can now be identified less destructively and far more quickly. These latently infected shoots could be manipulated to simulate various stresses and then observed for tip blight symptom development. Future experiments of this type should provide important insights into the possible role of latent infections in the *Sphaeropsis* tip blight disease cycle.

Fig. 4. Comparison of the culture isolation and the nested-polymerase chain reaction techniques for detection of *Sphaeropsis sapinea* in symptomless, latently infected Austrian pine terminal buds or bark samples. Of the 114 symptomless shoots sampled, 101 were latently infected with *S. sapinea*, indicated by a positive result using one or both techniques. Only these 101 latently infected shoots are represented in the figure.

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LITERATURE CITED

- Bassett, E. N., and Fenn, P. 1984. Latent colonization and pathogenicity of *Hypoxyylon atropunctatum* on oaks. *Plant Dis.* 68:317-319.
- Blodgett, J. T., and Stanosz, G. R. 1997. A and B morphotypes of *Sphaeropsis sapinea* differ in aggressiveness, but both infect non-wounded red or jack pines. *Plant Dis.* 81:143-147.
- Botes, W. M., Swart, W. J., and Crous, P. W. 1996. Frequencies of fungal endophytes isolated from pine needles in South Africa. (Abstr.) *Phytopathology* 86(suppl.):S66.
- Burgess, T., Wingfield, M. J., and Wingfield, B. W. 2001. Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Appl. Environ. Microbiol.* 67:345-362.
- Carroll, G. C. 1995. Forest endophytes: Patterns and process. *Can. J. Bot.* 73:S1318-S1324.
- Carroll, G. C., and Carroll, F. E. 1978. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. *Can. J. Bot.* 56:3034-3043.
- de Wet, J., Wingfield, M. J., Coutinho, T., and Wingfield, B. 2000. Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico, and Indonesia. *Plant Dis.* 84:151-156.
- de Wet, J., Wingfield, M. J., Coutinho, T., and Wingfield, B. 2002. Characterization of the "C" morphotype of the pine pathogen *Sphaeropsis sapinea*. *For. Ecol. Manag.* 161:181-188.
- Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. *Fungi on Plants and Plant Products in the United States*. The American Phytopathological Society, St. Paul, MN.
- Flowers, J., Nuckles, E., Hartman, J., and Vaillancourt, L. 2001. Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. *Plant Dis.* 85:1107-1112.
- Hamelin, R., Berube, P., Gignac, M., and Bourassa, M. 1996. Identification of root rot fungi in nursery seedlings by nested multiplex PCR. *Appl. Environ. Microbiol.* 62:4026-4031.
- Hamelin, R., Bourassa, M., Rail, J., Dusabenyagasani, M., Jacobi, V., and Laflamme, G. 2000. PCR detection of *Gremmeniella abietina*, the causal agent of Scleroderis canker of pine. *Mycol. Res.* 104:527-532.
- Hata, K., and Futai, K. 1996. Variation in fungal endophyte populations in needles of the genus *Pinus*. *Can. J. Bot.* 74:103-114.
- Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* 31:81-109.
- Jacobs, K. A., and Rehner, S. A. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* 90:601-610.
- Nicholls, T. H., and Ostry, M. E. 1990. *Sphaeropsis sapinea* cankers on stressed red and jack pines in Minnesota and Wisconsin. *Plant Dis.* 74:54-56.
- Palmer, M. A. 1991. Isolate type of *Sphaeropsis sapinea* associated with main stem cankers and top-kill of *Pinus resinosa* in Minnesota and Wisconsin. *Plant Dis.* 75:507-510.
- Palmer, M. A., and Nicholls, T. H. 1985. Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. *Plant Dis.* 69:739-740.
- Peterson, G. W. 1977. Infection, epidemiology, and control of *Diplodia* tip blight of Austrian, ponderosa, and Scots pines. *Phytopathology* 67:511-514.
- Petrini, O., and Fisher, P. J. 1988. A comparative study of fungal endophytes in xylem and whole stem of *Pinus sylvestris* and *Fagus sylvatica*. *T. Brit. Mycol. Soc.* 91:233-238.
- Schmidt, O., and Moreth, U. 2000. Species-specific PCR primers in the rDNA-ITS region as a diagnostic tool for *Serpula lacrymans*. *Mycol. Res.* 14:69-72.
- Schoeneweiss, D. F. 1983. Drought predisposition to *Cytospora* canker in blue spruce. *Plant Dis.* 67:383-385.
- Sieber, T. N., Rys, J., and Holdenreider, O. 1999. Mycobiota in symp-tomless needles of *Pinus mugo* ssp. *uncinata*. *Mycol. Res.* 103:306-310.
- Sinclair, W. A., Lyon, H. H., and Johnson, W. T. 1987. *Diseases of Trees and Shrubs*. Cornell University Press, Ithaca, NY.
- Smith, H., Wingfield, M. J., Crous, P. W., and Coutinho, T. A. 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. in South Africa. *S. Afr. J. Bot.* 62:86-88.
- Stanosz, G. R., and Cummings Carlson, J. 1996. Association of mortality of recently planted seedlings and established saplings in red pine plantations with *Sphaeropsis* collar rot. *Plant Dis.* 80:750-753.
- Stanosz, G. R., Smith, D. R., Guthmiller, M. A., and Stanosz, J. C. 1997. Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. *Mycologia* 89:525-530.
- Stanosz, G. R., Swart, W. J., and Smith, D. R. 1999. RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. *Mycol. Res.* 103:1193-1202.
- Stanosz, G. R., Blodgett, J. T., Smith, D. R., and Kruger, E. L. 2001. Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phytol.* 149:531-538.
- Swart, W. J., and Wingfield, M. J. 1991. Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant Dis.* 75:761-765.
- Thon, M., Nuckles, E., and Vaillancourt, L. 2000. Restriction enzyme-mediated integration used to produce pathogenicity mutants of *Colletotrichum graminicola*. *Mol. Plant-Microbe Interact.* 13:1356-1365.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York.
- Wingfield, M. J., and Knox-Davies, P. S. 1980. Association of *Diplodia pinea* with a root disease of pines in South Africa. *Plant Dis.* 64:221-223.
- Zhang, Y., Uyemoto, J. K., and Kirkpatrick, B. C. 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *J. Virol. Methods* 71:45-50.
- Zhou, S., Smith, D., and Stanosz, G. 2001. Differentiation of *Botryosphaeria* species and related anamorphic fungi using inter simple or short sequence repeat (ISSR) fingerprinting. *Mycol. Res.* 105:919-926.
- Zwolinski, J. B., Swart, W. J., and Wingfield, M. J. 1995. Association of *Sphaeropsis sapinea* with insect infestation following hail damage of *Pinus radiata*. *For. Ecol. Manag.* 72:293-298.