A Species-Specific PCR Assay for Detection of *Diplodia pinea* and *D. scrobiculata* in Dead Red and Jack Pines with Collar Rot Symptoms

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ABSTRACT

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A polymerase chain reaction (PCR)-based assay was developed for the specific detection of the fungal pathogens *Diplodia pinea* and *D. scrobiculata* from pine host tissues. Variation among mitochondrial small subunit ribosome gene (mt SSU rDNA) sequences of *Botryosphaeria* species and related anamorphic fungi was exploited to design primer pairs. Forward primer DpF and forward primer DsF, each when used with the nonspecific reverse primer BotR, amplified DNA of *D. pinea* or *D. scrobiculata*, respectively. Specificity was confirmed using multiple isolates of each of these two species and those of closely related fungi including *Botryosphaeria obtusa*. The detection limits for DNA of each pathogen in red and jack pine bark were 50 to 100 pg μl^{-1} and 1 pg μl^{-1} in red and jack pine wood. The assay was tested using naturally occurring red and jack pine seedlings and saplings exhibiting symptoms of *Diplodia* collar rot. Samples from lower stems/root collars of 10 dead trees of each species from each of three sites at each of two locations were tested. Results were positive for *D. pinea* or *D. scrobiculata* for the large majorities of symptomatic bark and wood samples from both locations. For positive samples, however, there were effects of location and host species on detection of *D. pinea* (more frequent on red pine) and *D. scrobiculata* (more frequent on jack pine) ($P < 0.01$ in both cases). These results indicate that these new primers are potentially useful for studies in areas or hosts in which both pathogens may be present.

Additional keywords: *Pinus banksiana*, *P. resinosa*, *Sphaeropsis sapinea*

The variation exhibited by fungi comprising the conifer shoot blight and canker pathogen complex *Sphaeropsis sapinea* *sensu lato* has prompted studies that have clarified their identity. Palmer et al. (20) recognized two forms: isolates of the A group were described as producing abundant white to gray-green aerial mycelium in culture, compared with dark gray and more appressed mycelium of B group isolates. These A and B groups were suggested to differ in radial growth rate, conidial size, texture of conidial walls, production of spermatia or microconidia, and the ability to penetrate nonwounded hosts (20,37,38), but each of these characters has been shown to either vary substantially within groups or to be similar for isolates of different groups (5,34,35,39). However, analyses using a variety of molecular techniques, including isozymes, RAPD and ISSR markers, and sequences of the nuclear rDNA ITS and 5.8s rDNA, microsatellite loci, and protein coding

genes (9,33,40,42), allow clear differentiation of the two groups. Thus, the B group has been recognized as a distinct species and assigned the name *Diplodia scrobiculata* (9). Insufficient justification for the separation of *Sphaeropsis* from *Diplodia* led to the recent suggestion of reversion to the name *D. pinea* for the A group (9).

Both *D. pinea* and *D. scrobiculata* are pathogens of native and introduced pines grown in the north central United States as ornamentals or for production of Christmas trees, pulp, poles, and lumber. Inoculation of intact shoot tips of red pine (*Pinus resinosa*) and jack pine (*P. banksiana*) seedlings with conidia of either *D. pinea* or *D. scrobiculata* resulted in similar symptoms (5). However, in that experiment, and in subsequent studies using seedlings of pines and conifers in several other genera that were wounded and inoculated with mycelium on agar plugs, *D. pinea* isolates generally caused greater incidence and/or severity of symptoms than did isolates of *D. scrobiculata* (1,3–6,27,30). *D. pinea* also has been frequently isolated from stems of asymptomatic red pine nursery seedlings in Wisconsin and Minnesota (25,28,31) and shown to proliferate to cause collar rot and rapid mortality of trees under water stress (25). Virulent isolates of *D. pinea* also have been obtained from asymptomatic tissues of Aus-

trian pine (*P. nigra*) in Kentucky (11). Most recently, isolates of *D. scrobiculata* also were obtained from asymptomatic red pine nursery seedlings, but the ability of these isolates to induce symptoms was not tested (28).

In addition to the locations and tree species mentioned above, the reported geographic and host ranges of *D. pinea* and *D. scrobiculata* are broad and overlapping. *D. pinea* is known to be widely distributed in the natural ranges of pines in the Northern Hemisphere and where these trees have been introduced in the Southern Hemisphere. Well-characterized isolates (i.e., by means of analysis of molecular markers) of *D. pinea* have been obtained from Africa, Asia, Australasia, Europe, and from both eastern and western North America (including Mexico). These isolates have been obtained from many species of pine, as well as species of cedar (*Cedrus*), larch (*Larix*), spruce (*Picea*), and Douglas-fir (*Pseudotsuga*) (7,9,13,20,23,29,33). The geographic area in which *D. scrobiculata* is known to occur is more limited. However, well-characterized isolates have been collected in the United States from California, Florida or Georgia, Michigan, Minnesota, Wisconsin, Kentucky, and West Virginia, and also from Canada, France, Israel, Italy, and Mexico. These isolates have been obtained from at least 10 pine species, cedar, and spruce (7,9,13,33).

Not only do geographic distributions and host ranges overlap, *D. pinea* and *D. scrobiculata* also are known to occur together. For example, both species have been obtained from individual red pine plantations in Minnesota (18) and from red pine seedlings in a Minnesota nursery (28). Other reports include detection of these two species from a single tree. The isolates of Morelet and Chandelier (17) that corresponded to *D. pinea* and *D. scrobiculata* (33) were each obtained from a single deodar cedar (*Cedrus deodara*) tree in France. Finally, attempts by Palmer et al. (20) to reisolate from plantation red pines previously inoculated with *D. pinea* also sometimes yielded *D. scrobiculata*.

Overlapping host and geographic ranges and similarities in symptoms and morphology can make identification of *D. pinea* and *D. scrobiculata* difficult and complicate studies of disease etiology. Investigation of these pathogens and diseases they cause would be facilitated by methods to rapidly detect each species,

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especially directly from host tissues collected from the field. A two-step polymerase chain reaction (PCR) assay using primers based on nuclear rDNA ITS sequence was developed by Flowers et al. (10) to identify *D. pinea* in Austrian pine tissues. Due to high similarities in ITS sequences (42), that assay cannot differentiate among *D. pinea*, *D. scrobiculata*, and a third pathogen that also can be associated with conifers, *Botryosphaeria obtusa* (9,13,21). A previous phylogenetic analysis of the mitochondrial small subunit ribosome gene (mt SSU rDNA) revealed a region of substantial sequence variation among *Botryosphaeria* species and related anamorphic fungi, including *Diplodia* species (41). Our objectives were to further explore sequence variation in this region, and to exploit this variation to develop specific primers for detection of *D. pinea* and *D. scrobiculata*. The widespread occurrence of dead red and jack pine seedlings with collar rot symptoms in central Wisconsin in 2004 offered the potential to test DNA extraction procedures and mt

SSU rDNA primers using diseased host tissues, and to further investigate the association of these pathogens with their hosts.

MATERIALS AND METHODS

Fungal isolates. Fungal isolates used in designing and testing mt SSU rDNA primers were acquired by the authors or cooperators from host plants or were purchased from collections (Table 1).

DNA sequencing and primer design. Procedures, materials, and equipment for genomic DNA extraction from fungal cultures were similar to those previously described (23). All DNA extractions and PCR analyses were carried out using standard procedures to avoid cross-contamination of DNA. Negative controls (no DNA) were always included. Most mt SSU rDNA sequences were acquired in a previous study (41). Other sequences were obtained by amplifying the variable region of the mt SSU rDNA using primers MSU1 and BotR (*Botryosphaeria*-specific primer derived from Zhou and Stanosz [41] sequences; Table 2) or MSU1 and NMS2 (15). DNA

was amplified in a 50- μ l reaction with 0.8 μ M each primer (University of Wisconsin-Madison Biotechnology Center), 600 μ M dNTPs, 1 $\times$ buffer, 2.0 mM MgCl₂, 1 U *Taq* DNA polymerase (all purchased from Promega Corp., Madison, WI), and 2 to 10 ng of fungal DNA. The following temperature profile was used in a Biometra T1 Thermocycler (Whatman Biometra, Goettingen, Germany): 1 cycle of 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 53°C, 1 min at 72°C, followed by 1 cycle of 5 min at 72°C. Fragment sizes were verified on 0.7% agarose gels in Tris-boric acid-EDTA buffer (TBE). The fragments were purified using the QIAGEN QIAquick PCR Purification Kit (QIAGEN Inc., Chatsworth, CA), labeled with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), then analyzed by the University of Wisconsin Biotechnology Center on an Applied Biosystems 3730XL DNA sequencer. The sequenced regions of the mt SSU rDNA were aligned using ClustalX 1.64b (36) with gap penalty = 25 and gap

Table 1. Isolates used in designing and testing mt SSU rDNA primers for species-specific polymerase chain reaction (PCR) assay to detect *Diplodia pinea* and *D. scrobiculata*

Species	Isolate no.	Host	Location	Collector	SSU GenBank accession no.	Other collection no.
<i>Diplodia pinea</i>	411	<i>Pinus resinosa</i>	Minnesota	M. Palmer	AF051636	DAOM 229440
	92-19	<i>P. resinosa</i>	Wisconsin	G. R. Stanosz		
	92-50	<i>P. radiata</i>	South Africa	W. Swart	AF051636	ATCC 64714
	93-23	<i>P. radiata</i>	Australia	E. Davison		
	94-19	<i>Cedrus deodara</i>	France	P. Chandelier		
	94-139	<i>P. sylvestris</i>	Netherlands	B. van Dam		
	96-40	<i>P. halepensis</i>	Israel	Z. Solel		
<i>D. scrobiculata</i>	98-6	<i>P. elliotii</i>	China	G. Peterson		
	113	<i>P. banksiana</i>	Michigan	M. Palmer	AF051637	DAOM 229441
	124	<i>P. banksiana</i>	Wisconsin	M. Palmer		DAOM 222531
	474	<i>P. resinosa</i>	Minnesota	M. Palmer		
	94-17	<i>C. deodara</i>	France	P. Chandelier		
	96-39	<i>P. halepensis</i>	Israel	Z. Solel		
	96-46	<i>P. elliotii</i>	Florida or Georgia	S. Fraedrich		
<i>D. pinea</i> f. sp. <i>cupressi</i>	97-73	<i>Pinus</i> sp.	Italy	L. Sparapano		
	98-1	<i>P. taeda</i>	Georgia	C. Rodriguez		
	94-158	<i>Cupressus sempervirens</i>	Israel	Z. Solel	AF271131	DAOM 229439
<i>Botryosphaeria rhodina</i>	96-3	<i>Quercus</i> sp.	California	E. Hecht-Poinar	AF051641	ATCC 208824
	96-172	<i>Theobroma cacao</i>	Sri Lanka	E. Müller	AF271142	CBS 356.59
<i>D. mutila</i>	94-6	<i>Malus</i> sp.	New Zealand	N. Tisserat		
	97-85	<i>Malus pumila</i>	Unknown	H. Boeswinkel	AF271140	ATCC 60259
<i>B. obtusa</i>	95-81	<i>Prunus armeniaca</i>	Wisconsin	E. Smalley		
	96-2	<i>Cercis canadensis</i>	Washington, D.C.	G. Samuels	DQ023299	ATCC 208826
	96-115	<i>Prunus persica</i>	Georgia	K. Britton	DQ023298	ATCC 60851
<i>B. quercuum</i>	96-186	<i>Q. cerris</i>	Italy	A. Vannini	AF271141	CBS 177.89
<i>B. tsugae</i>	96-174	<i>Tsuga heterophylla</i>	Canada	A. Funk	AF271137	CBS 418.64
<i>B. dothidea</i>	96-6	<i>Prunus persica</i>	Georgia	P. L. Pusey		
<i>B. ribis</i>	96-8	<i>Rhizophora mangle</i>	Florida	M. Rayacchetry	DQ023300	ATCC 208827
	96-9	<i>C. canadensis</i>	Washington, D.C.	K. Jacobs		ATCC 208822
<i>Fusarium tricinctum</i>	96-145	<i>Pinus banksiana</i>	Wisconsin	D. R. Smith		
<i>Cladosporium</i> sp.	96-146	<i>P. banksiana</i>	Wisconsin	D. R. Smith		
<i>Chaetomium</i> sp.	96-147	<i>P. resinosa</i>	Wisconsin	D. R. Smith		
<i>Schlerophoma pithyophila</i>	96-154	<i>P. banksiana</i>	Wisconsin	D. R. Smith		
<i>Epicoccum</i> sp.	96-155	<i>P. banksiana</i>	Wisconsin	D. R. Smith		
<i>Alternaria</i> sp.	97-3	<i>P. banksiana</i>	Wisconsin	D. R. Smith		
<i>Pestalotiopsis</i> sp.	00-2	<i>P. resinosa</i>	Wisconsin	J. Blodgett		
	03-01	<i>P. resinosa</i>	Wisconsin	D. R. Smith		
<i>Trichoderma</i> sp.	00-1	<i>P. resinosa</i>	Wisconsin	J. Blodgett		
	03-02	<i>P. resinosa</i>	Wisconsin	D. R. Smith		
<i>Sirococcus conigenus</i>	00-47	<i>P. resinosa</i>	Wisconsin	J. Blodgett		
	01-04	<i>Picea abies</i>	Germany	A. Wulf		
<i>Pseudorobillarda</i> sp.	03-05	<i>Pinus resinosa</i>	Michigan	D. R. Smith		

extension penalty = 5. Potential primer sequences were chosen manually and checked for primer-dimer, hairpins, and other sequences that would interfere with PCR using NetPrimer (online).

Primer specificity testing. Initially, seven *D. pinea* (92-19, 92-50, 93-23, 94-19, 94-139, 96-40, and 98-6) and seven *D. scrobiculata* isolates (124, 474, 94-17, 96-39, 96-46, 97-73, and 98-1) from diverse hosts and geographic locations were used to test the specificity of the primers (Table 1). DNA was extracted from liquid cultures (23) and amplified as described above.

Specificity of the primers also was tested with isolates of close relatives of *D. pinea* and *D. scrobiculata*. These species included *B. dothidea*, *B. obtusa*, *B. quercuum*, *B. rhodina*, *B. ribis*, *B. tsugae*, *D. mutila*, and a fungus referred to as *D. pinea* f. sp. *cupressi* (previously shown to be distinct from *D. pinea*) (24,32,40) (Table 1). We also tested the primers using a number of other fungi we frequently have cultured from pine tissue including *Fusarium tricinatum*, *Cladosporium* sp., *Chaetomium* sp., *Schlerophoma pythiophylla*, *Epicoccum* sp., *Alternaria* sp., *Pestalotiopsis* sp., *Trichoderma* sp., *Sirococcus conigenus*, and *Pseudorobillarda* sp. (Table 1). Amplification of the DNA from all of these fungi was also attempted with the universal mt SSU rDNA primers MSU1 and NMS2 to verify that this region could be amplified even though the specific primers failed to produce amplification products.

Finally, to verify that the primers did not amplify any DNA from the host tissue, amplification was attempted on DNA extracted from bark, wood, and needles from red pine and jack pine trees that had been kept in the greenhouse and were symptom-free for 3 years. DNA was extracted from the pine tissue using the procedure of Cubero et al. (8) with slight modification. A small piece of bark (4 to 16 mm²) or needle (~1 cm long) was placed in a 1.5-ml microcentrifuge tube with ~2 mm³ of acid-washed sand (0.25 mm industrial quartz, Unimin Corp., Portage, WI) and 250 µl of extraction buffer (1% wt/vol hexadecyltrimethylammonium bromide [CTAB], 1

M NaCl, 100 mM Tris, pH 8, 20 mM EDTA, 1% polyvinylpyrrolidone [PVP]). The plant material was ground with a sterile Kontes polypropylene pestle (Kimble/Kontes, Vineland, NJ), then incubated for 30 min at 70°C. One volume chloroform:isoamyl alcohol (24:1) was added, mixed, then centrifuged for 5 min at 10,000 rpm. The aqueous phase was transferred to a new tube. Two volumes of precipitation buffer were added (1% CTAB, 50 mM Tris, pH 8, 10 mM EDTA, 40 mM NaCl), mixed by inversion for 2 min, then centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 350 µl of 1.2 M NaCl. One volume chloroform:isoamyl alcohol (24:1) was added, mixed, then centrifuged for 5 min at 10,000 rpm. The aqueous phase was transferred to a new tube, and 0.6 volume of ice-cold isopropanol was added, mixed, and incubated at -20°C for 15 min. The samples were then centrifuged at 13,000 rpm for 20 min. The supernatant was removed, and 500 µl of 70% ethanol was added and centrifuged at 10,000 rpm for 5 min. The ethanol was removed and the nucleic acid pellet dried and resuspended in 50 µl of sterile water.

DNA extraction procedures. Three DNA extraction procedures were tested on red pine needles and red pine bark bearing *D. pinea* pycnidia and conidia. The first was a relatively simple extraction using polyethylene glycol and sodium chloride to precipitate the DNA (14). The second procedure (8) was the one detailed above. A third procedure commonly used on plant material was also tested (10). Briefly, the plant material was ground in a CTAB buffer with sand (1% CTAB, 0.7 M NaCl, 0.1 M Tris, pH 8.0, 10 mM EDTA), incubated 1 h at 60°C, proteins were removed with chloroform:isoamyl alcohol (24:1), and DNA precipitated by ice-cold isopropanol. This procedure differed from the second procedure in the absence of PVP in the extraction buffer and the absence of an extra precipitation step followed by chloroform extraction of the resuspended pellet. For all three procedures, the extracted DNA was amplified using the species-

specific primers, and presence of fragments was verified on 0.7% agarose gels in TBE buffer.

Detection limit. DNA was extracted from mycelial cultures of *D. pinea* and *D. scrobiculata* (23) and quantified using a TKO 100 Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) and DNA-specific dye Hoechst 33258 (Sigma-Aldrich, St. Louis, MO). For each pathogen, six DNA dilution series were made: (i) red pine needle DNA extract as the diluent; (ii) red pine bark DNA extract as the diluent; (iii) red pine wood DNA extract as the diluent; (iv) jack pine needle DNA extract as the diluent; (v) jack pine bark DNA extract as the diluent; and (vi) jack pine wood DNA extract as the diluent. Plant DNA was extracted using the slightly modified method of Cubero et al. (8) described above. A dilution series with water as the diluent also was made for DNA of each fungus. The highest concentration of DNA tested was 10 ng/µl and the lowest was 1 pg/µl. PCR reactions were performed as described above.

Tree sampling and testing. Trees were sampled during the summer of 2004 at three sites in each of two locations, one in Adams County (44°10'N, 90°41'W) and the other in the Black River State Forest (BRSF) in Jackson County (44°22'N, 89°53'W), both in central Wisconsin. The soil type in each location is deep, well-drained to excessively well-drained sand, and the average annual precipitation is approximately 750 mm. Prior to European settlement, barrens of jack pine and prairie grasses would have commonly occupied such locations. Red and jack pine seedlings and saplings (usually <1 m tall) present at all six sites had developed naturally from seed. Recently killed seedlings and saplings of each species with symptoms of collar rot including blue to black discoloration of the inner bark and wood were common at each site.

Ten plots were located at intervals of at least 10 m along a transect across each site. Usually four seedlings and saplings were sampled at each plot; these were selected to be located as close together as possible, and included one recently killed

Table 2. Primer sequences with alignment to mt SSU rDNA sequences from *Diplodia pinea*, *D. scrobiculata*, and several related species

Identities	Species-specific forward primer ^a 5'----->3'	<i>Botryosphaeria</i> -specific reverse primer 5'----->3'
Primer BotR		GCTTACACTTTTCATTTATAGACC
Primer DpF	CTTATATATCAAACTATGCTTTG- TA	
<i>Diplodia pinea</i>	CTTATATATCAAACTATGCTTTG- TA	GCTTACACTTTTCATTTATAGACC
Primer DsF	CTTATATATCAAACTAATGTTTG- CA	
<i>D. scrobiculata</i>	CTTATATATCAAACTAATGTTTG- CA	GCTTACACTTTTCATTTATAGACC
<i>Botryosphaeria obtusa</i> (96-2 and 96-115)	CTTATAAATCAATCTAAGCTTTG- TA	GCTTACACTTTTCATTTATAGACC
<i>D. pinea</i> f. sp. <i>cupressi</i> (94-158)	CTTATA--TCATACTATGATTGGTA	GCTTACACTTTTCATTTATAGACC
<i>B. rhodina</i> (96-3 and 96-172) ^b	CTTATAT TATACAAAAA ATTGG- TA	GCTTACACTTTTCATTTATAGACC
<i>B. ribis</i> (96-8)	CTTATATA AATACAAAAA TATC- TA	GCTTACACTTTTCATTTATAGACC

^a Bold type indicates nucleotides that differ across species.

^b Because *B. rhodina* has a large insertion in this region, this sequence occurs further downstream and the fragment generated using these primers is much larger than for the other species.

red pine, one recently killed jack pine, one live red pine without symptoms, and one live jack pine without symptoms. Due to the absence of a live red or jack pine seedling or sapling in proximity to the other sampled trees in some plots, fewer than 10 live trees of each species were collected from some sites. A segment of the lower stem and/or root collar exhibiting symptoms of collar rot was cut from each sampled tree with surface-disinfested tools and individually bagged. These segments were placed on ice until returning to the laboratory, where they were stored at -20°C until DNA extraction.

From each segment, a 4.5×4.5 mm sample of bark (tissues from the vascular cambium outward) was removed with a sterile scalpel and placed in a sterile 1.5-ml microcentrifuge tube. A similarly sized sample of underlying wood was removed with a sterile scalpel and placed in a separate sterile 1.5-ml microcentrifuge tube. Samples from dead trees were taken from an area with discolored wood. Samples were ground, DNA was extracted using the modified Cubero procedure (8), and PCR reactions were performed as described above. All samples were tested for the presence of both *D. pinea* and *D. scrobiculata* using the respective specific primers. At least one negative control (water) and one positive control (DNA extracted from a *D. pinea* or *D. scrobiculata* culture) was included with each PCR reaction. Fragment sizes were verified on 0.7% agarose gels in TBE buffer.

The mean frequencies of trees positive with either pathogen, and for these positive trees the mean frequencies with *D. pinea* and with *D. scrobiculata*, were calculated for dead trees of each host at each location (Table 3). Analyses of variance were used to test for effects of location, host species, and interactions on these frequencies. Percentage data were converted to proportions, and the arcsine of the square root transformation was applied before analyses were performed using Minitab for Windows version 14 (Minitab Inc., State College, PA).

RESULTS

DNA sequencing, primer design, and specificity testing. The 19-bp region of diversity among mt SSU rDNA sequences (41) allowed design of primers pairs specific for *D. pinea* and *D. scrobiculata*. We designed a *D. pinea*-specific forward primer (DpF; Table 2), a forward primer that would amplify *D. scrobiculata* (DsF; Table 2), and a reverse primer (BotR; Table 2) that would amplify most *Botryosphaeria* species and some other fungi. The primer pair DpF/BotR amplified a 700-bp fragment from *D. pinea*, but did not yield any fragments from *D. scrobiculata* or any other fungal species tested. The primer pair DsF/BotR amplified a 700-bp fragment from *D. scrobiculata*, but did not yield any fragments from *D. pinea* or any other fungal species tested. Neither primer pair yielded amplification products from red or jack pine tissues.

DNA extraction procedures. Fragments of the correct size were most likely to be amplified from DNA extracted using the procedure of Cubero et al. (8) (90% positive for infected red pine bark; 100% for infected needles). The simpler CTAB extraction yielded 80% positive for infected bark, while the PEG precipitation yielded 50% positive from infected red pine needles and 0% positive for infected bark.

Detection limit. The detection limit for *D. pinea* DNA diluted in red and jack pine needle or bark extract using primers DpF/BotR was 100 pg/ μl . For *D. pinea* DNA diluted in red or jack pine wood extract, the detection limit was 1 pg/ μl . The detection limit for *D. scrobiculata* using primers DsF/BotR varied: it was 50 pg/ μl when diluted in red and jack pine bark and red pine needle extracts; 100 pg/ μl for jack pine needles extracts; and 1 pg/ μl for red and jack pine wood extracts. For DNA of *D. pinea* diluted in water, the detection limit with DpF/BotR was 1 pg/ μl . For DNA of *D. scrobiculata* diluted in water, the detection limit with DsF/BotR was 1 pg/ μl .

Tree sampling and testing. Both pathogens were detected from samples collected at each of the six sites. Test results were positive for either *D. pinea* or *D. scrobiculata* for large majorities of bark and wood samples from dead red pine and dead jack pines from both locations (Table 3; example of gel in Figure 1). For example, 90% of red pine bark samples from Adams Co. tested positive and 93% of jack pine wood samples from Adams Co. tested positive. Analysis of variance of transformed data did not indicate effects of location, pine species, tissue (bark or wood), or interactions on the percentages

Fig. 1. Polymerase chain reaction products from DNA extracted from red and jack pine tissues. **A**, Products using primers DpF and BotR to detect *Diplodia pinea*. **B**, Products using primers DsF and BotR to detect *D. scrobiculata*. For both **A** and **B**: M, 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA); J, jack pine; R, red pine; B, bark; W, wood; D, dead; L, live (no symptoms); number, plot from which seedling was collected; +, positive control DNA (*D. pinea* isolate 411 for **A**; *D. scrobiculata* isolate 124 for **B**); -, negative control (no DNA).

Table 3. Frequencies of detection of *Diplodia pinea* (Dp) and *D. scrobiculata* (Ds) in dead red and jack pines^a with collar rot symptoms that were tested using species-specific polymerase chain reaction (PCR) assay

Location	Pine species	Trees positive with either pathogen ^b						Positive trees with Dp (%) ^c						Positive trees with Ds (%) ^d					
		Bark			Wood			Bark			Wood			Bark			Wood		
		Mean	Range	SE	Mean	Range	SE	Mean	Range	SE	Mean	Range	SE	Mean	Range	SE	Mean	Range	SE
Adams Co.	Red	90	70-100	10	83	70-90	7	92	86-100	4	89	78-100	6	15	0-30	9	11	0-22	6
	Jack	83	60-100	12	93	80-100	7	52	50-56	2	61	50-70	6	62	50-70	6	43	30-60	9
BRSF	Red	100	100	0	80	70-90	6	60	30-100	20	69	50-100	15	40	0-70	21	36	0-63	19
	Jack	83	70-100	9	67	30-90	19	26	10-43	9	12	0-25	7	87	71-100	8	88	75-100	7

^a Recently killed seedlings and saplings that had developed naturally from seed were sampled at three sites (replicates) in each of the two locations. One dead seedling of each species was sampled in each of 10 plots located at intervals of at least 10 m along a transect across each site (30 trees of each species per location).

^b Analysis of variance of transformed data did not indicate effects of location, pine species, tissue (bark or wood), or interactions on percentages of dead trees that tested positive for either pathogen (values of $P > 0.17$).

^c Analysis of variance of transformed data indicated effects of location ($P < 0.01$) and pine species ($P < 0.01$), but not tissue or interaction (values of $P > 0.36$) on percentages of positive dead trees with *D. pinea*.

^d Analysis of variance of transformed data indicated effects of location ($P = 0.01$) and pine species ($P < 0.01$), but not tissue or interaction (values of $P > 0.46$) on percentages of positive dead trees with *D. scrobiculata*.

of dead seedlings or saplings that tested positive for either pathogen ($P > 0.17$ in all cases).

Results differed, however, for frequency of detection of *D. pinea* among positive trees (Table 3). Both bark and wood of dead red and jack pine seedlings or saplings from Adams Co. tested positive for *D. pinea* more frequently than bark and wood of the respective species from BRSF ($P < 0.01$ for effect of location). Also, both bark and wood of dead red pines from each location tested positive for *D. pinea* more frequently than bark and wood from dead jack pines from the respective locations ($P < 0.01$ for effect of pine species). Analysis of variance did not indicate effects of tissue (bark or wood) or interactions on frequencies of positive dead trees with *D. pinea* ($P > 0.36$ in all cases).

Results also varied for frequency of detection of *D. scrobiculata* among positive trees (Table 3). Both bark and wood of dead red and jack pine seedlings or saplings from BRSF tested positive for *D. scrobiculata* more frequently than bark and wood of the respective species from Adams Co. ($P = 0.01$ for effect of location). Also, both bark and wood of dead jack pines from each location tested positive for *D. scrobiculata* more frequently than bark and wood from dead red pines from the respective locations ($P < 0.01$ for effect of pine species). Analysis of variance did not indicate effects of tissue (bark or wood) or interactions on frequencies of positive dead trees with *D. scrobiculata* ($P > 0.46$ in all cases).

Individual bark and wood samples from a dead tree usually were positive for only one pathogen and usually the bark and wood samples contained the same pathogen. However, of the 60 samples of bark and 60 samples of wood from dead trees of each host, two red pine bark samples, seven jack pine bark samples, one red pine wood sample, and one jack pine wood sample tested positive for both pathogens. Bark samples were positive (for at least one pathogen) and respective underlying wood samples were negative for 11 dead red pines and 10 dead jack pines. Bark samples were negative and respective underlying wood samples were positive (for at least one pathogen) for two dead red pines and eight dead jack pines.

Test results for samples from live trees rarely were positive. Of the samples collected from the live trees at both locations, three of 39 red pine bark samples and none of 43 jack pine bark samples were positive for *D. pinea*. Also, one of 39 red pine bark samples and three of 43 jack pine bark samples were positive for *D. scrobiculata*. None of the 39 red pine wood samples and none of the 43 jack pine wood samples from live trees at the two locations were positive for either pathogen. Water controls included with PCR reactions never were positive for either pathogen.

DISCUSSION

The ability to directly test host tissues to unambiguously confirm the species of pathogen that is present is an improvement over previous PCR assays for identification of *D. pinea* and *D. scrobiculata*. Although species-specific RAPD markers and ISSR fingerprint markers had been identified previously, these have been used after pure cultures of pathogens have been obtained (7,33,40). As mentioned previously, Flowers et al. (10) developed a nested PCR assay using nuclear rDNA ITS primers that should detect *D. pinea*, *D. scrobiculata*, and *B. obtusa* in pine tissues, but results of that assay do not differentiate among these three species. These pathogens have wide geographic distributions and broad, overlapping host ranges (21,22). For example, routine conifer disease surveys in Ontario yielded not only *D. pinea* and *D. scrobiculata*, but also isolates from jack pine, red pine, and white spruce (*Picea glauca*) that ultimately were identified as *B. obtusa* (7,13). Therefore, our new primers should be particularly useful during further studies in areas where *D. pinea*, *D. scrobiculata*, and *B. obtusa* or other closely related fungi could all be present.

Specificity of the new primer pairs can be attributed to the substantial variation in the 19-bp region of mt SSU rDNA that the forward primers span. We examined this region of the genome because, although nuclear rDNA ITS sequences have been used to design primers for identification of many species of fungi, as well as prokaryotes, plants, and animals, sequences in the ITS region vary little for some *Botryosphaeria* species and related anamorphic fungi (42). The ITS sequences for *D. pinea* and *D. scrobiculata* differ by only two nucleotides, and differ from the sequence of their close relative *B. obtusa* by only two other nucleotides. In theory, these minor differences could be sufficient to allow design of useful specific primers. However, due to the location of these particular nucleotides, primers cannot be designed to permit differential PCR reactions. Similarly, Luchi et al. (16) found no differences in a partial sequence of nuclear SSU rRNA of *D. pinea* and *D. scrobiculata*. They did, however, develop a nonspecific primer pair to allow real-time quantitative PCR assay of *D. pinea* in pine shoots inoculated with only this pathogen.

The method by which DNA is extracted from the plant tissues may affect the results of the PCR-based assays. Although it is more labor intensive and time-consuming, the extraction method of Cubero et al. (8) produced fewer negative results from red pine needles and red pine bark containing *D. pinea* pycnidia and spores than simpler DNA extraction methods. Even though others have used the standard CTAB plant DNA extraction procedure on Austrian pine tissues success-

fully (10), simpler extraction procedures may fail to remove or inactivate some chemicals present in red pine tissues that could interfere with PCR or damage DNA. The ability to detect 50 to 100 times less fungal DNA when dissolved in water rather than red or jack pine DNA extract indicates that some interference with the PCR is still occurring even when using Cubero's DNA extraction method.

Because fungal hyphae can contain numerous mitochondria (50 to 90 copies of the mitochondrial genome per cell for yeast [12]), mt SSU rDNA should exist in a high enough copy number to have a detection limit similar to nuclear rDNA ITS. For some tissues, however, our assay was not as sensitive as the assay of Flowers et al. (10). Their nested PCR assay using ITS primers allowed detection of five to ten times less DNA in bark than our assay. Again, possible differences in quantity or quality of interfering compounds in bark of Austrian pine (assayed by Flowers et al. [10]) as compared to red pine and jack pine could explain this difference. However, greater sensitivity might result from the nested nature of their assay, in which universal ITS primers are used first, followed by the primers for more specific DNA amplification. In our own assay, greater sensitivity in wood extract as compared to bark or needle extracts is probably due to interfering compounds in the latter. Despite greater sensitivity in wood extracts, however, our assay occasionally failed to detect either pathogen in wood underlying a positive bark sample. Although in these cases neither pathogen may have been present in the wood, this result also might have occurred due to difficulty in always completely grinding wood samples.

In contrast to the negative results for some symptomatic tissues, a small number of samples from healthy trees were positive for one or the other pathogen. Because these positive results were always from bark, they could be explained by the presence of spores on the surface of the stem. An alternative explanation is that we detected latent infections, a common occurrence in the basal stem area of nursery red and jack pine seedlings and branches of forest trees (28,31).

Similar mean frequencies of detection of the respective pathogens from both bark and wood suggest that only one tissue would need to be collected and tested using these methods in future surveys. For example, field sampling could be limited to symptomatic bark, or these pathogens could be detected from materials lacking bark (such as stained lumber). Unfortunately, the small size of the sample that can be easily ground and extracted could limit the usefulness of this detection method. Cultural assay methods using stem segments (2,28,31) have the potential to detect the pathogen's presence any-

where in or on a much greater volume of host material, potentially increasing efficiency compared to the very small bit of tissue used for the PCR-based assay. Conversely, the ability to detect these fungi in very small amounts of host material may allow dissection of samples into very small pieces to investigate relative abundance and distribution of these pathogens within different host tissues and organs.

In contrast to current knowledge of the broad-scale geographic distribution of *D. pinea* and *D. scrobiculata*, relatively little is known about their local co-occurrence. Reports of Palmer et al. (20) and Palmer (18) include recognition of both species in the same county or even the same red pine plantation in Michigan, Minnesota, and Wisconsin. Similarly, we detected both *D. pinea* and *D. scrobiculata* at all six sampled sites. Our results and earlier reports also confirm the potential for intimate association between *D. pinea* and *D. scrobiculata* within host tissues. Consistent with our detection of both species from some individual samples, Morelet and Chandelier (17) isolated both *D. pinea* and *D. scrobiculata* from the collar of a declining *Cedrus deodara* tree. Attempts by Palmer et al. (20) to reisolate *D. pinea* from jack pines artificially inoculated with that species also yielded isolates of *D. scrobiculata*. The implications of co-occurrence of these pathogens in the same forests, trees, and tissues for the production of inoculum, disease development, and subsequent survival are possible subjects for future study.

The location effect indicated by our data also demonstrates the potential for one species to be more prevalent, or at least detected more frequently, than the other species within the geographic range in which they both occur. This tendency also was indicated by results of Palmer (18). During that investigation of isolate types associated with top kill of red pines in Minnesota and Wisconsin, plantations tended to yield only one, or predominantly one, of these two fungi. For example, although both species were present, *D. pinea* was much more common among isolates from a Hubbard Co., MN red pine plantation, and *D. scrobiculata* was much more common among isolates from a Wadena Co., MN red pine plantation. Although results from inoculation trials in both greenhouse and field experiments indicate that *D. pinea* can be more aggressive than *D. scrobiculata* (1,3–6), differences in capacity for sporulation, survival, or responses of these two species to host and environmental conditions that could also affect prevalence in a particular location or region are not known.

The particular pathogen species (i.e., *D. pinea* or *D. scrobiculata*) associated with collar rot symptoms observed in field situations have not been determined previously. Although collar rot has been noted

infrequently on small numbers of red pine seedlings in the nursery by Palmer and Nicholls (19) and commonly on large numbers of planted red pine seedlings and saplings by Stanosz and Cummings Carlson (26), identification of the associated fungus (or fungi) as *Sphaeropsis sapinea* *sensu lato* was made only on the basis of the gross morphology of conidia. Identification of both *D. pinea* and *D. scrobiculata* on trees sampled in this study suggests the possibility that each of these species could play a role in collar rot development and subsequent mortality. This potential has been proven for *D. pinea*, identified by Stanosz et al. (25) (on the basis of analysis of RAPD markers) as the pathogen that was detected from asymptomatic red pine nursery seedlings and proliferated to cause collar rot and kill these seedling when subjected to drought stress in greenhouse experiments. In contrast, whether or not *D. scrobiculata* is actually capable of causing collar rot and killing trees is unknown. However, *D. scrobiculata* recently was identified among isolates obtained from surface-disinfested lower stem segments of asymptomatic red pine nursery seedlings in Minnesota (28). In addition, Blodgett and Bonello (1) recently confirmed the ability of an isolate of *D. scrobiculata* to colonize the lower stems of wounded and then inoculated Austrian pines in a greenhouse trial. Further studies are necessary to prove the potential of *D. scrobiculata* as an aggressive pathogen capable of causing the mortality with which it was associated in our current survey.

The previous association of *Sphaeropsis sapinea* *sensu lato* with collar rot symptoms occurring in the field also was limited to occurrence on planted red pine seedlings and saplings (26). Detection of *D. pinea* and *D. scrobiculata* in our study has proven that naturally occurring seedlings of both pine species can acquire the fungus from natural inoculum sources. The potential for these pathogens to persist in forests and interfere with natural regeneration of these native species may present a great challenge to managers attempting to maintain these ecologically and economically important species in forests of the Great Lakes region.

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