A PCR-Based Method for the Detection of *Ophiosphaerella agrostis* in Creeping Bentgrass

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ABSTRACT

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Dead spot is a relatively new disease of creeping bentgrass and hybrid bermudagrass that is incited by *Ophiosphaerella agrostis*. Initial symptoms are difficult to diagnose and clinicians generally rely on the presence of pseudothecia within infected tissue or isolation of *O. agrostis* on an artificial medium. The main goal of this study was to develop a polymerase chain reaction-based technique capable of quickly identifying *O. agrostis* within infected creeping bentgrass tissues. Oligonucleotide primers specific for *O. agrostis* were developed based on the internal transcribed spacer (ITS) rDNA regions (ITS1 and ITS2) of three previously sequenced isolates of *O. agrostis*. The 22-bp primers amplified a 445- or 446-bp region of 80 *O. agrostis* isolates collected from creeping bentgrass and bermudagrass in 11 states. Primers did not amplify DNA from other common turfgrass pathogens, including three closely related species of *Ophiosphaerella*. Selective amplification of *O. agrostis* was successful from field-infected creeping bentgrass samples and primers did not amplify the DNA of noninfected, field-grown creeping bentgrass or hybrid bermudagrass plants. Amplification of purified *O. agrostis* DNA was successful at quantities between 50 ng and 5 pg. The entire process, including DNA isolation, amplification, and amplicon visualization, may be completed within 4 h. These results indicate the specificity of these primers for assisting in the accurate and timely identification of *O. agrostis* and the diagnosis of dead spot in both bentgrass and bermudagrass hosts.

Additional keywords: *O. herpotricha*, *O. korrae*, *O. narmari*

Dead spot is a disease of creeping bentgrass (*Agrostis stolonifera* L.) that is caused by *Ophiosphaerella agrostis* Dernoeden, M. P. S. Câmara, N. R. O'Neill, van Berkum et M. E. Palm (1,4). The pathogen first was isolated from a golf course in Maryland in 1998 and since has been isolated from creeping bentgrass in at least 11 states (9). The pathogen also has been isolated from hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burtt-Davy) in Texas and Florida (9,11).

On creeping bentgrass putting greens grown in the mid-Atlantic region of the United States, dead spot symptoms may appear as early as May and disease severity often peaks between July and August (8). Initially, the disease appears as small, copper or reddish-brown spots approximately 1 to 2 cm in diameter, which slowly increase to approximately 8 to 10 cm throughout the summer months (4,8). Initial symptoms are difficult to diagnose and often are mistaken for damage caused by other common turfgrass diseases and pests such as dollar spot (*Sclerotinia homoeocarpa* F. T. Bennett), copper spot (*Gloeocercospora sorghi* Bain & Edgerton ex Deighton), Microdochium patch (*Microdochium nivale* (Fr.) Samuels & I. C. Hallett), and black cutworms (*Agrotis ipsilon* Hufnagel). New disease symptoms also may be confused with mechanical damage from ball marks, which typically are found on bentgrass putting greens. Once infection occurs, turfgrass in the center of dead spots dies, forming pits or depressions which adversely affect the playability of the putting surface. Recovery of bentgrass into infected spots is slow and dead spots often remain present throughout the winter until bentgrass growth resumes in the spring. Due to this slow recovery and the limited ability to manage the disease curatively, early identification of dead spot is critical.

A key diagnostic aide used to identify *O. agrostis* is the presence of pseudothecia, which often are found embedded in necrotic leaf tissue and stolons (10). These sexual fruiting bodies may develop quickly, and viable ascospores may be present within 1 week of initial symptom development (8). However, pseudothecia are not always present and isolation of *O. agrostis* on an artificial medium often is necessary for a positive laboratory diagnosis. Isolation of the pathogen, however, may take several days to weeks, and variation in colony color and morphology among *O. agrostis* isolates can make identification of the fungus difficult (8,10).

Polymerase chain reaction (PCR) is a molecular technique routinely used in the identification of various fungal pathogens (3,5,6). Positive identification of diseased plants may be accomplished quickly through the use of species-specific oligonucleotide primers (3,5). PCR primers capable of detecting common turfgrass pathogens present at low concentrations have been developed from various regions of fungal genomic DNA. Harmon et al. (7) designed primers from the Pot2 transposon of *Magnaporthe grisea* (Herbert) Barr and *M. oryzae* Couch. Another region of genomic DNA used in the development of species-specific primers is the avenacinase gene from *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *avenae* (E. M. Turner) Dennis (13). Conserved sequences within the internal transcribed spacer (ITS) region also have been developed to identify *Rhizoctonia solani* Kühn AG-2-2 as well as several subsets within this anastomosis group (2). Additionally, ITS regions (ITS1 and ITS2) previously were used in the development of primers for three other *Ophiosphaerella* spp. found in association with diseases of turfgrass, including *O. herpotricha* J. C. Walker, *O. korrae* Walker and Smith, and *O. narmari* Wetzel, Hulbert, and Tisserat (12,15,16).

To date, based on information collected from golf courses throughout the United States, dead spot generally develops on newly constructed creeping bentgrass putting greens or renovated greens that had been fumigated with methyl bromide (9). Additionally, dead spot found on hybrid bermudagrass occurs on greens that have been overseeded with roughstalk bluegrass (*Poa trivialis* L.), a cool-season turfgrass species often seeded into dormant hybrid bermudagrass putting greens in the south-

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ern regions of the United States (9,11). The sudden appearance of this previously undescribed pathogen on newly seeded putting greens in various regions of the United States raises the question of the pathogens' origin. The role of seed in the spread of *O. agrostis* is unknown. The development of species-specific oligonucleotide primers, however, may be useful in identifying *O. agrostis* in seed.

The main goal of this study was to develop a PCR-based technique capable of identifying *O. agrostis* within infected creeping bentgrass tissues. Therefore, the objectives of this research were to (i) develop and test oligonucleotide primers specific to *O. agrostis* and (ii) use species-specific primers to test for the presence of the pathogen in plants and in commercially available creeping bentgrass and roughstalk bluegrass seed.

MATERIALS AND METHODS

Fungal isolates. In all, 80 *O. agrostis* isolates from creeping bentgrass and hybrid bermudagrass were collected from putting greens in 11 states between 1998 and 2003 (Tables 1 and 2). Other common turfgrass pathogens also were collected, including *Bipolaris cynodontis* (Marig.) Shoemaker; *Colletotrichum graminicola* (Ces.) G. W. Wils.; *Gaeumannomyces graminis* var. *avenae*; *Gloeocercospora sorghi*; *M. poae* Landschoot and Jackson; *O. korrae*; *O. narmari*; *O. herpotricha*; *R. solani*; *R. zeae* Voorhees; *R. cerealis* Van der Hoeven; and *S. homoeocarpa* (Table 3). Prior to DNA extraction, isolates were stored on potato dextrose agar (PDA) slants or in sterile distilled, deionized water (ddH_2O) at 4ºC.

DNA isolation. To isolate the DNA from fungal cultures, isolates were grown at room temperature for 7 to 10 days in 100 ml of potato dextrose broth (PDB) (24 g liter^{-1}) on a shaker table (LabLine Orbit Shaker, Lab-Line Instruments Inc., Melrose, IL) set to 90 rpm. Cultures were filtered through Whatman no. 1 filter paper, rinsed in ddH_2O, and lyophilized in a Freezemobile 6 (The VirTis Company, Gardiner, NY) for 24 to 48 h. Freeze-dried mycelial mats were stored at –20ºC or immediately prepared for DNA extraction. Approximately 20 to 30 mg of freeze-dried mycelia were placed in a 1.8-ml microcentrifuge tube (VWR, West Chester, PA) and

Table 1. Isolate designation, cultivar affected, collection location, and date of isolation of *Ophiosphaerella agrostis* isolates collected from Maryland

Isolate designation	Bentgrass cultivar[a]	City	Isolation date
HCC1 and HCC2	L-93 + Crenshaw	Hunt Valley	21 October 1998
OpMD-3 and OpMD-19	Penncross	Ocean City	16 October 1998
OpMD-4 and OpMD-10	L-93 + Crenshaw	Upper Marlboro	26 October 1998
OpMD-5	L-93 + Crenshaw	Aberdeen	15 October 1998
PBD1 and PBD2	Penn G-2	Urbana	21 August 1998
OpMD-9	Providence	Silver Spring	13 November 1998
OpMD-12	L-93	Havre de Grace	14 August 1999
OpMD-13	'Bardot' colonial	College Park	8 September 1999
OpMD-14 and OpMD-15	L-93	Joppa	26 July 2001
OpMD-16	Penn A-4	Laytonsville	30 August 2001
OpMD-17	Providence	College Park	26 October 2001
OpMD-18	Providence	College Park	9 November 2001
OpMD-21 through OpMD-23	L-93	College Park	20 June 2002
OpMD-25 through OpMD-27	L-93	College Park	28 October 2002
OpMD-29, OpMD-34, and OpMD-40	L-93	College Park	25 November 2002
OpMD-34	L-93	College Park	25 November 2002
OpMD-36	'Bavaria' velvet	College Park	25 November 2002
OpMD-40	L-93	College Park	25 November 2002
OpMD-42	L-93	College Park	28 January 2003
OpMD-43 through OpMD-47	L-93	College Park	26 March 2003
OpMD-48 through OpMD-67	L-93	College Park	1 October 2003

[a] All bentgrass cultivars listed are creeping bentgrass (*Agrostis stolonifera* L.) unless otherwise specified.

Table 2. Isolate designation, cultivar affected, and collection location and date of various *Ophiosphaerella agrostis* isolates from the United States

Isolate designation[a]	Cultivar[b]	City, state	Date received
OpIL-1^{RK}	SR 1119	Glencoe, Illinois	18 December 1998
OpIL-2^{RK}	SR1119 + L-93 + Providence	Golf, Illinois	8 December 2000
OpIL-3^{RK}	L-93	Olympia Fields, Illinois	8 December 2000
OpIL-4 and OpIL-5^{RK}	L-93	Park Ridge, Illinois	1 August 2002
OpMA-1	L-93	West Bridgewater, Massachusetts	27 July 2002
OpMA-3 and OpMA-4	Penn A-4	Hingham, Massachusetts	Summer 2003
OpMI-1 and OpMI-2	Providence	East Tawas, Michigan	29 September 2000
OpNC-1^{HW}	Penncross	Laurinburg, North Carolina	11 August 2000
OACS^{BC}	L-93	Englishtown, New Jersey	Summer 2001
OpNJ-4	L-93	New Brunswick, New Jersey	27 July 1999
OpNJ-5, OpNJ-6, and OpNJ-6b	Penn A-4	Northfield, New Jersey	28 September 2000
OpNY-1	L-93	Altamont, New York	11 August 2000
OpOH-1	L-93	Chardon, Ohio	21 October 1998
OpPA-1	Crenshaw + Southshore	Avondale, Pennsylvania	23 December 1998
OpPA-4	L-93	Honeybrook, Pennsylvania	2 November 1999
OpPA-6 and OpPA-7	SR1120 + L-93 + Providence	Avondale, Pennsylvania	8 March 2000
OpPA-8	L-93	Honeybrook, Pennsylvania	15 August 2000
OpVA-1	Pennlinks	Sterling, Virginia	10 September 1998
OpVA-3 and OpVA-4	Penn A-4	Virginia Beach, Virginia	15 June 2002
#121^{NT}	'Champion' bermudagrass	College Station, Texas	Summer 1999

[a] Isolates were collected in this study by author unless otherwise noted as follows; NT = Ned Tisserat, HW = Henry Wetzel, BC = Bruce Clarke, and RK = Randy Kane.

[b] All cultivars are creeping bentgrass (*Agrostis stolonifera* L.) or 'Champion' hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burtt-Davy).

ground into a fine powder using a micropestle (VWR). Liquid nitrogen occasionally was used to aid in the grinding process. The DNA was extracted using DNEasy Plant Mini Kits (Qiagen Inc., Valencia, CA). For the final step, 50 µl of the preheated (65ºC) elution buffer were added, the column centrifuged for 1 min at 8,000 rpm, and the step repeated. Extracted DNA then was visualized on an agarose gel and template DNA later was diluted for PCR analysis.

Development of species-specific oligonucleotide primers. Oligonucleotide primers specific for *O. agrostis* were developed based on the ITS1 and ITS2 regions of isolates OpOH-1, OpMD-6, and OpVA-1, which previously had been sequenced and deposited in the GenBank database under the accession numbers AF191550, AF191549, and AF191548, respectively (1). The primers were OaITS1 (5′-AGCAATACAGCCCAAAGGCCTC-3′) and OaITS2 (5′-AAAGGCTTAATGGACGCGAGTG-3′). These primers were chosen based on nucleotide differences when ITS1 and ITS2 sequence alignments were compared among *O. agrostis*, *O. herpotricha*, *O. korrae*, and *O. narmari* and were designed to amplify a portion of the ITS1 region, the entire 5.8s rDNA, and a portion of the ITS2 region of all *O. agrostis* isolates (Fig. 1). Primers were synthesized by Qiagen Inc.

Genomic DNA was diluted (1 µl of template DNA:99 µl ddH_20) for PCR reactions. The PCR reactions were run using 1 µl of 10× polymerase buffer (New England BioLabs, Inc. [NEB]; Beverly, MA), 0.4 µl of 100 mM $MgSO_4$ (NEB), 0.2 µl of 40 µM dNTPs (NEB), 0.4 µl of each 5 µM primer, 0.1 unit of Taq polymerase (NEB), and 1 µl of diluted genomic DNA. DNA-grade ddH_2O was added to reach a reaction volume of 10 µl. DNA was amplified using an initial denaturation step at 94ºC for 5 min, followed by 30 cycles of 30 s of denaturation at 94ºC, 45 s of annealing at 65ºC, and 90 s of elongation at 76ºC. Reactions were run in an Eppendorf Mastercylcer (Hamburg, Germany), and amplification products were visualized on a 1 or 2% agarose gel stained with ethidium bromide (0.5 to 1.0 µg ml^{-1}). Gels were run at 125 V for 30 to 45 min in a 1× Tris-acetate-EDTA (TAE) Buffer (Invitrogen, Carlsbad, CA) containing 40 mM Tris-acetate (pH 8.3) and 1 mM EDTA. Results were confirmed in a replication run using a different thermal cycler (PTC-0220 DNA Engine Dyad Peltier Thermal Cycler; MJ Research, Inc., Waltham, MA) with the reaction mixture and cycling profile previously described. To rule out a possible inhibition of the PCR reaction or lack of template DNA, primers specifically designed to amplify the ITS regions of *O. korrae* (15), *O. narmari* (16), *O. herpotricha* (15), and *R. solani* (2) also were used to amplify fungal DNA of the respective turfgrass pathogens.

DNA quantification and primer sensitivity. The DNA concentrations of *O. agrostis* isolates (n = 80) were determined using the PicoGreen dsDNA Quantification Kit (Molecular Probes Inc., Eugene, OR; 14). Genomic DNA from each isolate was diluted (1:100) in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and 50 µl of diluted DNA were added to 50 µl of the PicoGreen reagent (diluted to 1× concentration with TE buffer). Hence, the final DNA dilution for detection was 1:200 (vol/vol). Six calibration samples containing DNA stock solutions of previously determined concentrations were used to develop a standard curve. The DNA quantification was performed using a Fluoroskan Ascent Microplate Fluorometer (Thermo Labsystems, Franklin, MA). Prior to quantification, samples were shaken for 10 s and incubated for 5 min at 28ºC. Immediately following incubation, DNA concentrations were quantified at an excitation (485 nm) and emission (538 nm) wavelength specific for the fluorescent dye.

To assess the sensitivity of the developed primers for detecting *O. agrostis*, stock DNA was diluted and PCR performed on known DNA concentrations. Primers developed in this study were tested against DNA quantities of 50, 5.0, and 0.5 ng and 50 and 5 pg. Previously isolated DNA from five *O. agrostis* isolates were selected for the sensitivity assay and the experiment was repeated twice.

Detection of *O. agrostis* in creeping bentgrass. Field samples of creeping bentgrass exhibiting symptoms of dead spot were collected from a research putting green at the Paint Branch Turfgrass Research Facility (PBTRF) located in College Park, MD. Additionally, primers were tested on *O. agrostis*-infected creeping bentgrass from Black Rock Country Club (BRCC) located in Massachusetts. The presence of *O. agrostis* was confirmed either by isolation or the presence of pseudothecia and ascospores characteristic of the species. Samples of putatively noninfected plant tissues adjacent to dead spot infection centers were used as a negative control. In addition, healthy hybrid bermudagrass plants were collected from a National Turfgrass Evaluation Program (NTEP, Beltsville, MD) variety trial located at PBTRF and used as a negative control for the primers. Isolation of DNA from all plant material was performed using the aforementioned DNEasy extraction kit and procedures described above. The DNA from a single *O. agrostis* isolate (OpVA-4) served as a positive control and was included in all PCR reactions involving diseased plants and seeds.

Identification of *O. agrostis* in creeping bentgrass seed. Primers were used in

Table 3. Isolate designation, genus and species, and host species of common turfgrass pathogens used to test the specificity of primers OaITS1 and OaITS2[a]

Isolate designation[b]	Genus, species	Host species
#162^{NT}	*Ophiosphaerella korrae*	*Cynodon dactylon*
Lk-5^{PD}	*O. korrae*	*C. dactylon*
#65^{NT}	*O. herpotricha*	*C. dactylon*
#189^{NT}	*O. herpotricha*	*C. dactylon*
#217^{NT}	*O. herpotricha*	*C. dactylon*
#370^{KM}	*O. narmari*	*C. dactylon*
BpMD-1	*Bipolaris cynodontis*	*C. dactylon*
CgMD-5	*Colletotrichum graminicola*	*Poa pratensis*
GgaFR-1^{PD}	*Gaeumannomyces graminis* var. *avenae*	*Agrostis stolonifera*
GgaMD-9	*G. graminis* var. *avenae*	*A. stolonifera*
GgaPA-1	*G. graminis* var. *avenae*	*A. stolonifera*
GsMD-1	*Gloeocercospora sorghi*	*A. stolonifera*
MpMD-3	*Magnaporthe poae*	*P. annua*
RcCT-1	*Rhizoctonia cerealis*	*P. annua*
RsMD-4	*R. solani*	*P. pratensis*
RzMD-3	*R. zeae*	*Lolium perenne*
ShVA-1	*Sclerotinia homoeocarpa*	*A. stolonifera*

[a] ITS = internal transcribed spacer.
[b] Isolates were collected in this study by author unless otherwise noted as follows; NT = Ned Tisserat, PD = Peter Dernoeden, and KM = Kevin McCann.

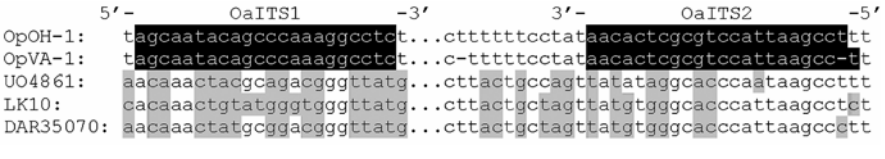

Fig. 1. Location of primers OaITS1 and OaITS2 (black background with white text) within the internal transcribed spacer (ITS) region of *Ophiosphaerella agrostis* isolates OpOH-1 and OpVA-1. Sequence differences among the *O. agrostis* isolates and *O. korrae* (LK10), *O. herpotricha* (OU4861), and *O. narmari* (DAR 03570) are shaded in gray; – indicates a single base pair difference between the two *O. agrostis* sequences and … indicates partial sequences of the ITS1, 5.8S, and ITS2 regions not shown.

an attempt to amplify *O. agrostis* DNA from commercial creeping bentgrass seed. In addition to creeping bentgrass, primers were evaluated against the DNA from seed of four roughstalk bluegrass cultivars, including 'Snowbird', 'Sabre', 'Winterplay', and 'Bariviera'. All roughstalk bluegrass seed was provided by Kevin Morris of the NTEP. Seed DNA were extracted using the DNEasy Plant Mini Kit, with the same procedural specifications previously described with the following modifications. A total of 200 to 300 mg of seed was ground in autoclaved mortars and pestles with liquid nitrogen at the start of the extraction procedure. The DNA extraction generally was replicated twice for each seed sample and amplification with primers OaITS1 and OaITS2 was attempted twice for each DNA sample. Only seed collected from Philadelphia Country Club (PCC) and BRCC were known to have been used to establish putting greens that were later diagnosed with dead spot. A total of 10 DNA isolations were made from seed collected from PCC and BRCC, and PCR again tested twice per sample. Extracted DNA was confirmed by running a sample aliquot on an agarose gel and visualizing the DNA fragment under UV light. Amplification procedures were identical to those described previously.

RESULTS AND DISCUSSION

Fungal isolation. In all, 80 *O. agrostis* isolates were tested in this study (Tables 1 and 2). Colony morphology varied when grown on PDA and incubated in the dark at 25ºC for 10 days. Most isolates collected exhibited a typical rose-quartz or pink colony color previously described (1,4,10). Several other isolates appeared olive-gray or buff and were similar in colony color to the original description of the Ohio isolate (OpOH-1) (1,10). Isolates OpNC-1 and OpNJ-6 fit into a previously undescribed mycelial color class, and were gray when grown on PDA under the aforementioned conditions. A complete description of most of the isolates tested was reported previously (8).

Development of species-specific oligonucleotide primers. The selected primers amplified a putative 445- or 446-bp amplicon in each of the 80 *O. agrostis* isolates tested in this study (Figs. 2 and 3). The 446-bp amplicon resulted from amplification of the OpOH-1 isolate, which had an additional 2 nucleotides in the ITS sequences reported by Câmara et al. (1). It is unknown if other isolates with morphology similar to the aforementioned Ohio isolate contained an additional base pair. The OaITS2 primer developed in this study resided between the two nucleotide differences and, therefore, amplification of the isolates was not impacted. Primers did not amplify DNA from 11 other turfgrass pathogens tested, including three different *Ophiosphaerella* spp. (Fig. 4). The speci-

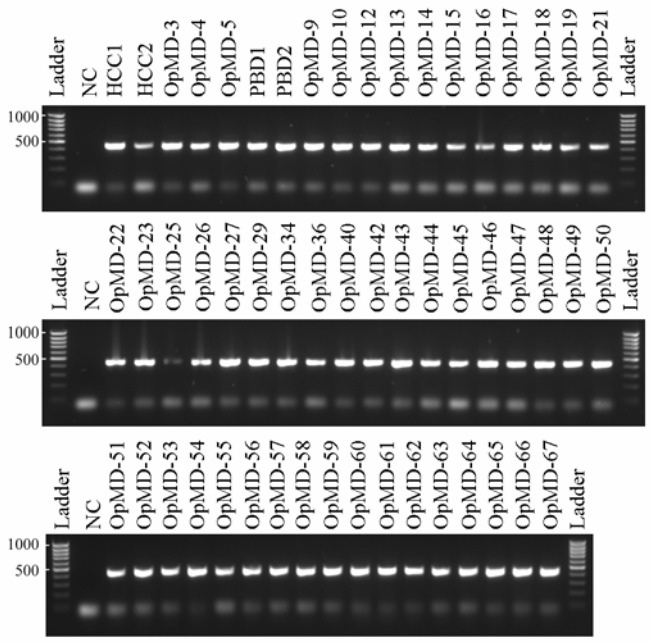

Fig. 2. Polymerase chain reaction product amplified from the internal transcribed spacer region of DNA from (n = 53) *Ophiosphaerella agrostis* isolates collected in Maryland. A negative control (NC) and 100-bp ladder are shown.

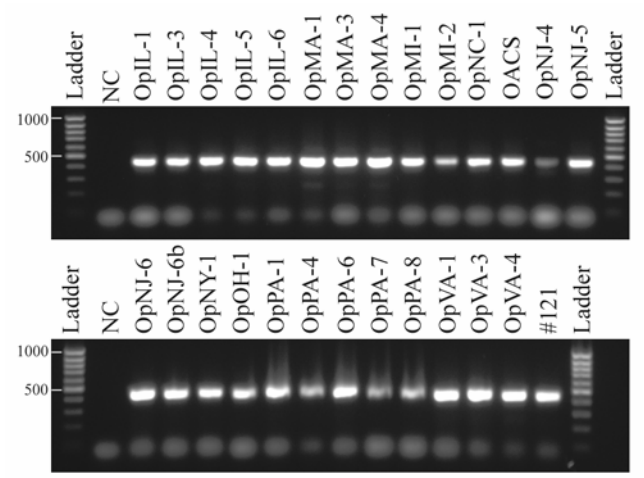

Fig. 3. Polymerase chain reaction product amplified from the internal transcribed spacer region of DNA from (n = 27) *Ophiosphaerella agrostis* isolates from 10 states. A negative control (NC) and 100-bp ladder are shown.

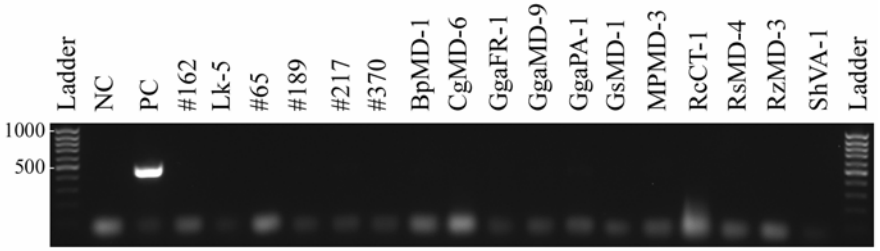

Fig. 4. Polymerase chain reaction amplification of fungal DNA from various turfgrass pathogens. From left to right: 100-bp ladder, negative control (NC), positive control (PC; OpVA-4), *Ophiosphaerella korrae* (#162, Lk-5), *O. herpotricha* (#65, #189, #217), *O. narmari* (#370), *Bipolaris cynodontis* (BpMD-1), *Colletotrichum graminicola* (CgMD-6), *Gaeumannomyces graminis* var. *avenae* (GgaFR-1, GgaMD-9, GgaPA-1), *Gloeocercospora sorghi* (GsMD-1), *Magnaporthe poae* (MpMD-3), *Rhizoctonia cerealis* (RcCT-1), *R. solani* (RsMD-4), *R. zeae* (RzMD-3), *Sclerotinia homoeocarpa* (ShVA-1), and a 100-bp ladder.

ficity of the *O. agrostis* primers was based on the unique sequences of the ITS1 and ITS2 regions chosen when compared with *O. herpotricha*, *O. korrae,* and *O. narmari*. The annealing temperature of 65ºC was chosen to closely match the melting temperature of the primers and to eliminate the potential of priming a reaction with the three closely related *Ophiosphaerella* spp. There were no differences in the selective amplification of *O. agrostis* DNA when reactions were run in either thermal cycler. Selective amplification of *O. agrostis* was successful from each of the field-infected creeping bentgrass samples (n = 8; Fig. 5). Primers did not amplify the DNA of uninfected, field-grown creeping bentgrass (n = 4) or hybrid bermudagrass (n = 4) plants. These results indicate the specificity of this assay for assisting in the identification of *O. agrostis* and the diagnosis of dead spot in both bentgrass and bermudagrass species.

DNA quantification and primer sensitivity. Quantification of the six calibration samples resulted in the linear equation $y = 0.090x - 0.017$ (R^2 = 0.99), where y = strength of the PicoGreen emission wavelength (nm) and x = DNA concentration (µg ml^{-1}). The DNA concentrations of 13 isolates were considered to be outside the desired range for detection; therefore, concentrations for these samples were extrapolated from the regression equation. Total DNA extracted from *O. agrostis* isolates (20 to 30 mg of freeze-dried mycelium) using the DNeasy DNA isolation kit averaged 18.03 ng µl^{-1} (range = 0.73 to 224.26 ng µl^{-1}; *data not shown*).

Early attempts to amplify portions of *O. agrostis* DNA were erratic when total extracted stock DNA was used in the amplification procedure. Therefore, total purified *O. agrostis* DNA (n = 5) was diluted to varying levels to determine the sensitivity to the developed primers. Amplification of purified *O. agrostis* DNA was successful at quantities ranging between 50 ng and 5 pg (*data not shown*). Amplification of the five isolates generally was detected with all quantities of DNA. Although attempts to amplify the pathogen generally were successful, DNA amplification of isolates OpMD-16 and OpMD-25 resulted in varying inconsistencies. The characteristic amplicon, however, always was present for the aforementioned isolates when 5 ng of DNA was used in the assay. Amplification of the other isolates generally resulted in the presence of the distinctive amplicon at all concentrations analyzed in this study. This PCR-based technique is very sensitive and results were similar to that reported by Harmon et al. (7) for *Magnaporthe* spp. Amplification of *O. agrostis* was possible with amounts of DNA as low as 5 pg. Regardless of extracted DNA concentration, amplification of *O. agrostis* with primers OaITS1 and OaITS2 was successful when stock DNA from pure cultures or infected creeping bentgrass plants was diluted 1:100.

Identification of *O. agrostis* in creeping bentgrass seed. Primers were used in an attempt to amplify DNA of *O. agrostis* from commercially available creeping bentgrass and roughstalk bluegrass seed. Due to the appearance of dead spot in the years following seeding, many golf course managers did not have seed available for testing. In all, 20 seed samples were collected from various locations, however, only seed from PCC and BRCC was known to have been planted into greens in which dead spot occurred. Amplification of *O. agrostis* DNA was successful only on a single attempt in seed from PCC (*data not shown*). Attempts to repeat this amplification from the template DNA of the aforementioned sample and from additional DNA extractions of all collected seed were unsuccessful. Although not examined, it is unclear whether inhibitors within the extracted seed DNA were present that may have prevented successful amplification during the PCR reaction. Attempts to culture the fungus directly from seed plated on water agar yielded several unknown fungal species, but *O. agrostis* was not isolated.

Although the pathogen was not consistently detected in seed tested in this study, the role of seed in the spread of *O. agrostis* remains unclear. In this study, very small quantities of seed were tested for the presence of *O. agrostis*. Seeded at a standard rate of 50 kg ha^{-1}, a total of 2.8 kg of bentgrass seed would be needed to establish an average-sized putting green (e.g., 557 m^2). Based on the quantity of seed tested in this study (400 to 600 mg sample^{-1}), only 1.4×10^{-4} to 2.2×10^{-4}% of the seed used to establish an average-sized putting green was evaluated for the presence of *O. agrostis*. Information on the introduction of *O. agrostis* into the United States is still limited. *O. agrostis* was identified on *Schizostachyum lima* (Blanco) Merr., a bamboo-like plant native to Indonesia that was quarantined by the Animal and Plant Health Inspection Services in 2000 (N. R. O'Neill, *unpublished*). However, the possible role of ornamental grasses in the introduction of the pathogen into United States is unknown.

Dead spot can be difficult to diagnose from field samples if pseudothecia are not present; diagnosis often requires isolation of the pathogen to obtain a positive identification. Isolation in pure culture, however, may take several days, and variation in colony color makes accurate identification of the pathogen difficult (10). Primers developed in this study were capable of detecting *O. agrostis* in pure culture and within infected creeping bentgrass in as little as 4 h. Due to the novelty of this pathogen and the sometimes difficult diagnosis of the disease, these primers will assist diagnostic labs in the identification of dead spot. Molecular techniques, PCR in particular, continue to improve the accuracy and speed of diagnosing plant pathogens. Early diagnosis will assist turf managers in implementing management strategies that help reduce damage caused by dead spot.

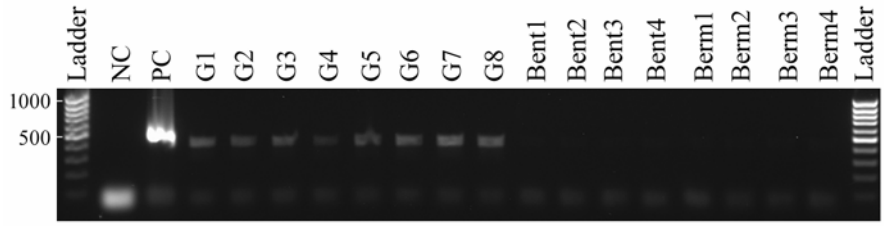

Fig. 5. Polymerase chain reaction amplification of fungal DNA from field-infected creeping bentgrass. From left to right: 100-bp ladder, negative control (NC), positive control (PC; OpVA-4), *Ophiosphaerella agrostis*-infected bentgrass plants (G1-G8), asymptomatic creeping bentgrass (Bent1-Bent4), and hybrid bermudagrass (Berm1-Berm4) plants, and a 100-bp ladder.

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