A PCR-Based Method For The Detection of *Ophiosphaerella agrostis* In Creeping Bentgrass.

John E. Kaminski, and **Peter H. Dernoeden**, Department of Natural Resource Sciences and Landscape Architecture, University of Maryland, College Park, MD 20742; **Nichole R. O'Neill**, USDA-ARS, Beltsville, MD 20705; and **Henry C. Wetzel III**, Syngenta Crop Protection, Inc., Vero Beach, FL, 32967.

Corresponding author now Assistant Professor, Department of Plant Science, University of Connecticut, Storrs: J.E. Kaminski

E-mail: john.kaminski@uconn.edu

ABSTRACT

Kaminski, J.E., Dernoeden, P.H., O'Neill, N.R., and Wetzel, H.C., III. 2005. A PCR-based method for the detection of *Ophiosphaerella agrostis* in creeping bentgrass. Plant Disease 89:000-000: accepted for publication on XX XXX, 2005.

Dead spot is a relatively new disease of creeping bentgrass and hybrid bermudagrass and is incited by *Ophiosphaerella agrostis*. Initial symptoms are difficult to diagnose and clinicians generally rely on the presence of pseudothecia or isolation of *O. agrostis* on an artificial medium. The main goal of this study was to develop a PCR-based molecular technique capable of quickly identifying *O. agrostis* within infected creeping bentgrass tissues. Oligonucleotide primers specific for *O. agrostis* were developed based on the ITS1 and ITS2 regions of three previously

sequenced isolates of *O. agrostis*. The 22 base-pair (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 asymptomatic, field-grown creeping bentgrass or hybrid bermudagrass plants. Amplification of purified *O. agrostis* DNA was successful at quantities between 50 nanograms and 5 picograms. 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.

INTRODUCTION

Dead spot is a disease of creeping bentgrass (*Agrostis stolonifera* L.), and 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. x *C. transvaalensis* Burtt-Davy) in Texas and Florida (9,11).

On creeping bentgrass putting greens grown in the mid-Atlantic region of the USA, dead spot symptoms may appear as early as May and disease activity 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). 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 one week of initial symptom development (8). Adding to diagnostic difficulties, 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 quickly accomplished through the use of species-specific oligonucleotide primers. 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 include 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, disease information collected from golf courses throughout the USA revealed

that 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 southern regions of the USA (9,11). The sudden appearance of this previously undescribed pathogen on newly seeded putting greens in various regions of the USA 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 molecular technique capable of identifying *O. agrostis* within infected creeping bentgrass tissues. The objectives of this research therefore were to: 1) develop and test oligonucleotide primers specific to *O. agrostis*, and 2) 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. Isolates were collected from 11 states between 1998 and 2003. A total of 80 *O. agrostis* isolates from creeping bentgrass were collected from Illinois (IL) (n=5), Maryland (MD) (n=53), Massachusetts (MA) (n=3), Michigan (MI) (n=2), New Jersey (NJ) (n=5), New York (NY) (n=1), North Carolina (NC) (n=1), Ohio (OH) (n=1), Pennsylvania (PA) (n=5), Texas (TX) (n=1), and Virginia (VA) (n=3), (Table 1 and Table 2). Other common turfgrass pathogens also were collected including *Bipolaris cynodontis* (Marig.) Shoemaker;

Colletotrichum graminicola (Ces.) G.W. Wils.; *G. graminis* var. *avenae*; *G. sorghi*; *Magnaporthe poae* Landschoot and Jackson; *O. korrae*; *O. narmari*; *O. herpotricha*; *R. solani*; *Rhizoctonia zeae* Voorhees; *Rhizoctonia cerealis* Van der Hoeven; and *S. homoeocarpa* (Table 3). Prior to DNA extraction, isolates were stored at -20°C in a 20% glycerol solution or on potato dextrose agar (PDA) slants or in sterile distilled, deionized water (ddH_2O) at 4°C.

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 L^{-1}) on a shaker table (LabLine Orbit Shaker, Lab-Line Instruments Inc., Melrose, IL) set to 90 rpm. Cultures were filtered through Whatman #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 -20°C 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 ground into a fine powder using a micro-pestle (VWR, West Chester, PA). Liquid nitrogen occasionally was used to aid in the grinding process. The DNA was extracted using Qiagen Mini-Prep Kits (Qiagen Inc., Valencia, CA). For the final step, 50 µl of the preheated (65°C) elution buffer were added, the column centrifuged for 1 min at 8000 rpm, and the step repeated. This template DNA was later 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 *O. agrostis* isolates OpOH-1, OpMD-6 and OpVA-1, which had previously 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 compared to other *Ophiosphaerella* species 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 (Figure 1). Primers were synthesized by Qiagen Inc.

Genomic DNA was diluted (1 µl template DNA:99 µl ddH_20) for PCR reactions. The PCR reactions were run using 1 µl of 10x polymerase buffer (New England BioLabs, Inc. (NEB); Beverly, MA); 0.4 µl 100 mM $MgSO_4$ (NEB); 0.2 µL 40 µM dNTPs (NEB); 0.4 µl of each 5 µM primer; 0.1 unit Taq polymerase (NEB); and 1 µl of diluted genomic DNA. DNA-grade distilled, deionized water was added to reach a reaction volume of 10 µl. DNA was amplified using an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 seconds of denaturation at 94°C, 45 s annealing at 65°C, and 90 s elongation at 76°C. 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. 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.

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 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 1x concentration with TE Buffer). Hence, the final DNA

dilution for detection was 1:200 (v/v). 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 28°C. 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 nanograms (ng), 5.0 ng, 0.5 ng, 50 picograms (pg) 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 asymptomatic plant tissues adjacent to dead spot infection centers were used as a negative control. Finally, 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.

Identification of *O. agrostis* in Creeping Bentgrass Seed. Primers were used in 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 Qiagen Mini-Prep Kits, 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 and General Observations. A total of 80 *O. agrostis* isolates were tested in this study (Table 1 and Table 2). Colony morphology varied when grown on PDA and incubated in the dark at 25°C 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). Isolate 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 were previously reported (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 (Figure 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 similar morphology to the aforementioned Ohio isolate contained an additional base pair (bp). The OaITS2 primer developed in this study resided between the two nucleotide differences and therefore amplification of the isolates was not impacted. In addition, 1 bp differences were not detectable when separated on a 1% agarose gel. Primers did not amplify DNA from eleven other turfgrass pathogens tested, including three different *Ophiosphaerella* spp. (Figure 4). 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) (Figure 5). Additionally, primers did not amplify the DNA of asymptomatic, field-grown creeping bentgrass (n=4) or hybrid bermudagrass (n=4) plants. These results indicate the specificity of these primers 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 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. Total purified *O. agrostis* DNA (n=5), therefore, 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 (Figure 6). Amplification of the 5 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 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 molecular technique is very sensitive and results were similar to that reported by Harmon et al. (7) for *Magnaporthe* spp. Amplification was possible with amounts of DNA was as low as 5 pg. Regardless of extracted DNA concentration, amplification of *O. agrostis* with primers OaITS1 and OaITS2 were 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. A total of 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 only was successful 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. Additionally, attempts to culture the fungus directly from seeds 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 x 10^{-4}% to 2.2 x 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 USA 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, personal communication). The possible role of ornamental grasses in the introduction of the pathogen into USA, however, is unknown.

CONCLUSION

Dead spot can be difficult to diagnose from field samples if pseudothecia are not present, and 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.

ACKNOWLEDGEMENTS

We were grateful for the financial support for this study by the United States Golf Association and for the technical assistance of Eva Claire Synkowski. We thank B. Clarke, R. Kane, K. McCann, and N. Tisserat for providing isolates and K. Morris for donating seed.

LITERATURE CITED

1. Câmara, M.P.S., N.R. O'Neill, P. van Berkum, P.H. Dernoeden, and M.E. Palm. 2000. *Ophiosphaerella agrostis* sp. nov. and its relationship to other species of *Ophiosphaerella*. Mycologia 92:317-325.

2. Carling, D.E., S. Kuninaga, and K.A. Brainard. 2002. Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* Anastomosis group-2 (AG-2) and AG-BI. Phytopathology 92:43-50.

3. Chiocchetti, A.L. Sciaudone, F. Durando, A. Garibaldi, and Q. Migheli. 2001. PCR detection of *Fusarium oxysporum* f. sp. *basilici* on basil. Plant Dis. 85:607-611.

4. Dernoeden, P.H., N.R. O'Neill, M.P.S Câmara, and Y. Feng. 1999. A new disease of *Agrostis palustris* incited by an undescribed species of *Ophiosphaerella*. Plant Dis. 83:397.

5. Errampalli, D., J. Saunders, and D. Cullen. 2001. A PCR-based method for detection of potato pathogen, *Helminthosporium solani*, in silver scurf infected tuber tissue and soils. J. Microbiol. Methods 44:59-68.

6. Godfrey, S.A., R.D. Monds, D.T. Lash, and J.W. Marshall. 2003. Identification of *Pythium oligandrum* using species-specific ITS rDNA PCR oligonucleotides. Mycol. Res. 107:790-796.

7. Harmon, P.F., L.D. Dunkle, and R. Latin. 2003. A rapid PCR-based method for the detection of *Magnaporthe oryzae* from infected perennial ryegrass. Plant Dis. 87:1072-1076.

8. Kaminski, J.E. 2004. Biology of *Ophiosphaerella agrostis*, epidemiology of dead spot, and a molecular description of the pathogen. Ph.D. Diss. University of Maryland, College Park.

9. Kaminski, J.E., and P.H. Dernoeden. 2002. Geographic distribution, cultivar susceptibility, and field observations on bentgrass dead spot. Plant Dis. 86:1253-1259.

10. Kaminski, J.E., P.H. Dernoeden, N.R. O'Neill, and B. Momen. 2002. Reactivation of bentgrass dead spot and growth, pseudothecia production, and ascospore germination of *Ophiosphaerella agrostis*. Plant Dis. 86:1290-1296.

11. Krausz, J.P., R.H. White, W. Foerster, N.A. Tisserat, and P.H. Dernoeden. 2001. Bermudagrass dead spot: A new disease of bermudagrass caused by *Ophiosphaerella agrostis*. Plant Dis. 85:1286.

12. O'Gorman, D., B. Xue, T. Hsiang, and P.H. Goodwin. 1994. Detection of *Leptosphaeria korrae* with polymerase chain reaction and primers form the ribosomal internal transcribed spacers. Can. J. Bot. 72:342-346.

13. Rachdawong, S., C.L. Cramer, L.A. Grabae, V.K. Stromberg, G.H. Lacy, and E.L. Stromberg. 2002. *Gauemannomyces graminis* vars. *avenae*, *graminis*, and *tritici* identified using PCR amplification of avenacinase-like genes. Plant Dis. 86:652-660.

14. Singer, V.L., L.J. Jones, S.T. Yue, and R.P. Haugland. 1997. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. Anal. Biochem. 249:228-238.

15. Tisserat, N.A., S.H. Hulbert, and K.M. Sauer. 1994. Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.

16. Wetzel III, H.C., S.H. Hulbert, and N.A. Tisserat. 1999. Molecular evidence for the presence of *Ophiosphaerella narmari* n. comb., a cause of spring dead spot of Bermuda grass, in North America. Mycol. Res. 103:981-989.

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

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

[x] 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 USA.

Isolate designation[x]	Cultivar[y]	City, State	Date Received
OpIL-1[RK]	SR 1119	Glencoe, Illinois	18 Dec. 1998
OpIL-2[RK]	SR1119 + L-93 + Providence	Golf, Illinois	8 Dec. 2000
OpIL-3[RK]	L-93	Olympia Fields, Illinois	8 Dec. 2000
OpIL-4 and OpIL-5[RK]	L-93	Park Ridge, Illinois	1 Aug. 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 Sept. 2000
OpNC-1[HW]	Penncross	Laurinburg, North Carolina	11 Aug. 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 Sept. 2000
OpNY-1	L-93	Altamont, New York	11 Aug. 2000
OpOH-1	L-93	Chardon, Ohio	21 Oct. 1998
OpPA-1	Crenshaw + Southshore	Avondale, Pennsylvania	23 Dec. 1998
OpPA-4	L-93	Honeybrook, Pennsylvania	2 Nov. 1999
OpPA-6 and OpPA-7	SR1120 + L-93 + Providence	Avondale, Pennsylvania	8 Mar. 2000
OpPA-8	L-93	Honeybrook, Pennsylvania	15 Aug. 2000
OpVA-1	Pennlinks	Sterling, Virginia	10 Sept. 1998
OpVA-3 and OpVA-4	Penn A-4	Virginia Beach, Virginia	15 June 2002
#121[NT]	'Champion' bermudagrass	College Station, Texas	Summer 1999

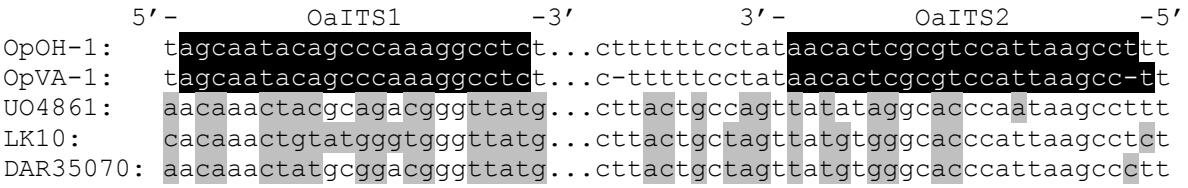
[x] Isolates were collected in this study by author unless otherwise noted as follows; NT = Ned Tisserat, HW = Henry Wetzel, BC = Bruce Clarke, and RK = Randy Kane.

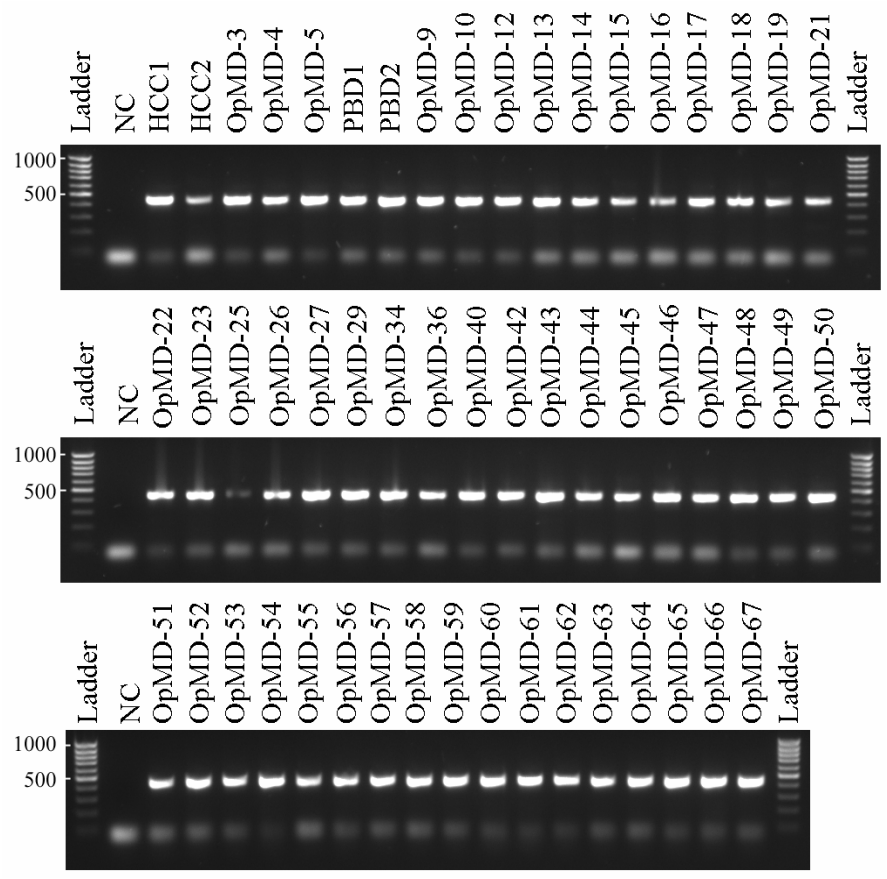
[y] All cultivars are creeping bentgrass (*Agrostis stolonifera* L.) or 'Champion' hybrid bermudagrass (*Cynodon dactylon* [L.] Pers. X *C. transvaalensis* Burtt-Davy).

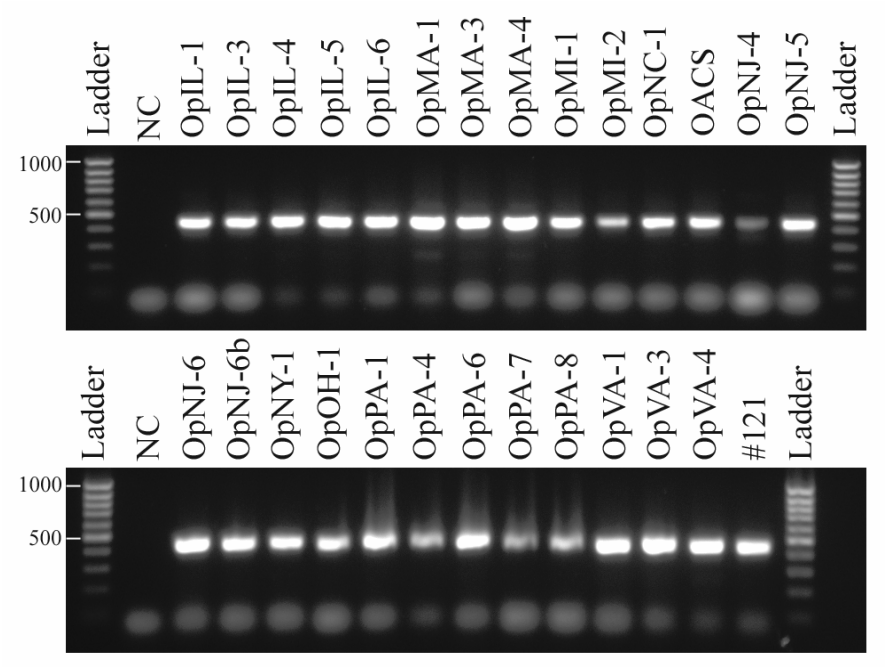
Table 3. Isolate designation; genus and species; and host species of common turfgrass pathogens used to test the specificity of primers OaITS1 and OaITS2.

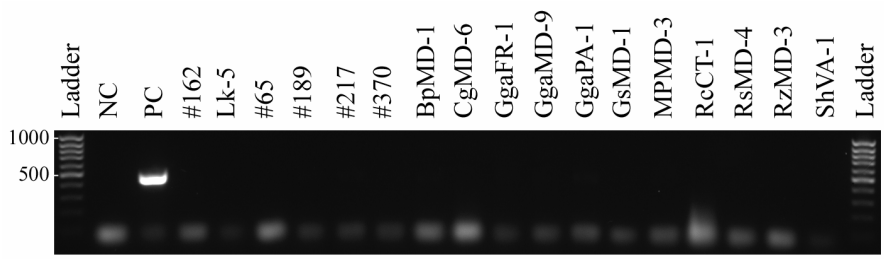
Isolate Designation[x]	Genus species	Host species
#162[NT]	*Ophiosphaerella korrae*	*Cynodon dactylon*
Lk-5[PD]	*O. korrae*	*C. dactylon*
#65[NT]	*O. herpotricha*	*C. dactylon*
#189[NT]	*O. herpotricha*	*C. dactylon*
#217	*O. herpotricha*	*C. dactylon*
#370[KM]	*O. narmari*	*C. dactylon*
BpMD-1	*Bipolaris cynodontis*	*C. dactylon*
CgMD-5	*Colletotrichum graminicola*	*Poa pratensis*
GgaFR-1[PD]	*Gauemannomyces 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*	*Poa 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*

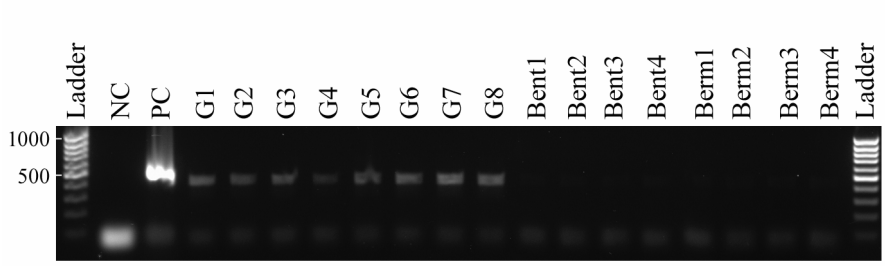
[x] Isolates were collected in this study by author unless otherwise noted as follows; NT = Ned Tisserat, PD = Peter Dernoeden, KM = Kevin McCann, and RK = Randy Kane.


5′- OaITS1 -3′ 3′- OaITS2 -5′
OpOH-1: tagcaatacagcccaaaggcctct...ctttttttcctataacactcgcgtccattaagcctt
OpVA-1: tagcaatacagcccaaaggcctct...c-ttttttcctataacactcgcgtccattaagcc-tt
UO4861: aacaaactacgcagacgggttatg...cttactgccagttatatataggcacccaataagccttt
LK10: cacaaactgtatgggtgggttatg...cttactgctagttatgtgggcacccattaagcctct
DAR35070: aacaaactatgcggacgggttatg...cttactgctagttatgtgggcacccattaagcccctt


Ladder
NC
HCC1
HCC2
OpMD-3
OpMD-4
OpMD-5
PBD1
PBD2
OpMD-9
OpMD-10
OpMD-12
OpMD-13
OpMD-14
OpMD-15
OpMD-16
OpMD-17
OpMD-18
OpMD-19
OpMD-21
Ladder
1000
500
Ladder
NC
OpMD-22
OpMD-23
OpMD-25
OpMD-26
OpMD-27
OpMD-29
OpMD-34
OpMD-36
OpMD-40
OpMD-42
OpMD-43
OpMD-44
OpMD-45
OpMD-46
OpMD-47
OpMD-48
OpMD-49
OpMD-50
Ladder
1000
500
Ladder
NC
OpMD-51
OpMD-52
OpMD-53
OpMD-54
OpMD-55
OpMD-56
OpMD-57
OpMD-58
OpMD-59
OpMD-60
OpMD-61
OpMD-62
OpMD-63
OpMD-64
OpMD-65
OpMD-66
OpMD-67
Ladder
1000
500


Ladder
NC
OpIL-1
OpIL-3
OpIL-4
OpIL-5
OpIL-6
OpMA-1
OpMA-3
OpMA-4
OpMI-1
OpMI-2
OpNC-1
OACS
OpNJ-4
OpNJ-5
Ladder
1000
500
Ladder
NC
OpNJ-6
OpNJ-6b
OpNY-1
OpOH-1
OpPA-1
OpPA-4
OpPA-6
OpPA-7
OpPA-8
OpVA-1
OpVA-3
OpVA-4
#121
Ladder
1000
500


Ladder
NC
PC
#162
Lk-5
#65
#189
#217
#370
BpMD-1
CgMD-6
GgaFR-1
GgaMD-9
GgaPA-1
GsMD-1
MPMD-3
RcCT-1
RsMD-4
RzMD-3
ShVA-1
Ladder
1000
500


Ladder
NC
PC
G1
G2
G3
G4
G5
G6
G7
G8
Bent1
Bent2
Bent3
Bent4
Berm1
Berm2
Berm3
Berm4
Ladder
1000
500

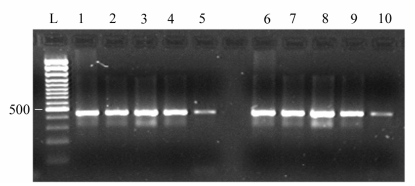

L 1 2 3 4 5 6 7 8 9 10
500

Figure 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. A '-' indicates a single base pair difference between the two *O. agrostis* sequences and '...' indicate partial sequences of the ITS1, 5.8S, and ITS2 regions not shown.

Figure 2. Polymerase chain reaction (PCR) product amplified from the ITS region of DNA from (n=53) *Ophiosphaerella agrostis* isolates collected in Maryland, USA. A negative control (NC) and a 100 bp ladder are shown.

Figure 3. Polymerase chain reaction (PCR) product amplified from the ITS region of DNA from (n=27) *Ophiosphaerella agrostis* isolates from 10 states. A negative control (NC) and a 100 bp ladder are shown.

Figure 4. Polymerase chain reaction (PCR) 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* (#189,#217); *O. narmari* (#370); *Bipolaris cynodontis* (BpMD-1); *Colletotrichum graminicola* (CgMD-6); *Gauemannomyces 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.

Figure 5. Polymerase chain reaction (PCR) 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); hybrid bermudagrass (Berm1-Berm4) plants; and a 100 bp ladder.

Figure 6. Amplification of *Ophiosphaerella agrostis* at various DNA dilutions. From left to right: ladder (L); OpPA-6 (1-5); and OpVA-3 (6-10) at 50 ng, 5 ng, 0.5 ng, 50 pg, and 5 pg DNA.