Identification of Darkly Pigmented Fungi Associated with Turfgrass Roots by Mycelial Characteristics and RAPD-PCR

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ABSTRACT

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A select group of darkly pigmented turfgrass pathogens are referred to as ectotrophic root-infecting (ERI) fungi. Identification of ERI fungi, as well as darkly pigmented saprophytes, cannot be rapidly or reliably determined by traditional methods. The objectives of this study were (i) to determine if these darkly pigmented fungi could be presumptively identified by mycelial growth rates and characteristics, and (ii) to definitively identify these fungi based on DNA products generated through random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR). Previously identified isolates of *Gaeumannomyces cylindrosporus, G. graminis* var. *avenae, G. graminis* var. *graminis, G. incrustans, Magnaporthe poae, Ophiosphaerella herpotricha, O. korrae,* and *Phialophora graminicola* were obtained. The species were separated into two groups based on their growth rate on half strength potato dextrose agar at 25°C. Slower-growing species [$\bar{X}$ = 2.5; range = 1.6 to 3.4 mm per day] were *G. cylindrosporus, P. graminicola, O. herpotricha,* and *O. korrae,* whereas fast-growing species [$\bar{X}$ = 5.5; range = 3.2 to 6.6 mm per day] were *G. graminis* var. *avenae, G. graminis* var. *graminis, G. incrustans,* and *M. poae*. Colony characteristics and growth rate were valuable in presumptively distinguishing most species. For example, *O. korrae* colonies had distinctively raised or dome-shaped mycelium, whereas *O. herpotricha* isolates produced a brownish black colored exudate in the center of 2-week-old colonies. *Gaeumannomyces graminis* var. *avenae* isolates were separated from *G. incrustans* and *M. poae* based on their very slow growth at 30°C. Initially, only single isolate of *G. cylindrosporus, G. graminis* var. *avenae, G. graminis* var. *graminis, G. incrustans, M. poae, O. herpotricha, O. korrae,* and *P. graminicola* were evaluated against 20 oligonucleotide primers (10-mers) for identifiable and reproducible DNA products. Primers were found that reliably produced bands unique to each species, excluding *G. graminis* var. *graminis*. Then, an additional 52 known ERI fungal isolates were screened against the species-specific 10-mers to confirm reproducibility and clarity of the DNA fingerprints. When fungal isolates were obtained from diseased turfgrass roots, clinical identification of ERI fungi was achieved in 2 to 3 weeks.

Additional keywords: *G. graminis* var. *tritici, L. narmari, M. rhizophila*

Precise identification of darkly pigmented fungi that infect or inhabit roots of turfgrasses is difficult and time consuming. Many of these darkly pigmented fungi are pathogens and are referred to as ectotrophic root-infecting (ERI) fungi (10). These pathogens cause diseases that produce a patch symptomatology and include the following: *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *avenae* (E. M. Turner) Dennis; *G. graminis* (Sacc.) Arx & D. Olivier var. *graminis*; *Ophiosphaerella korrae* (J. C. Walker & A. M. Sm.) R. Shoemaker & C. Babcock (*Leptosphaeria korrae* J. C. Walker & A. M. Sm.) (22); *L. narmari* J. C. Walker & A. M. Sm.; *Magnaporthe poae* Landschoot & Jackson; and *O. herpotricha* (Fr.:Fr.) J. C. Walker. Darkly pigmented saprophytes (i.e., *G. cylindrosporus* D. Hornby, D. Slope, R. Gutteridge, & Sivanesan; *G. incrustans* Landschoot & Jackson; and *Phialophora graminicola* (Deacon) J. Walker) also are frequently isolated from roots of diseased turfgrass plants. *Magnaporthe rhizophila* Scott & Deacon, a darkly pigmented fungus, has recently been reported on Kentucky bluegrass in North America (11). Its distribution among ERI fungi that cause patch diseases throughout the United States is unknown.

With the exception of *G. graminis* var. *graminis*, which produces deeply lobed hyphopodia, there are no distinctive features that allow for the precise identification of any of these ectotrophs. To identify a disease incited by these fungi, the pathogen must be isolated, and its ascocarp must be induced to form in the laboratory. From 4 to 10 weeks are required before mature ascocarps of these Ascomycetes are produced (3,5,13,14). Production of ascocarps is often unsuccessful because the isolates may be attenuated biotypes or incompatible mating types. Other physiologically important factors, known or unknown, may not be reproducible in the laboratory.

Due to the difficulties associated with traditional diagnostic techniques, several molecular-based methods for identification of ERI fungi have been studied. Nameth et al. (18) developed a monoclonal antibody for the detection of *O. korrae* from necrotic ring spot affected root tissue. Henson (8) cloned a 4.3-kilobase (kb) piece of mitochondrial DNA of *G. graminis* var. *tritici* to use as a probe to identify the casual agent of take-all of wheat (*Triticum aestivum* L. emend. Thell.). However, because this probe hybridized with DNA from all three *G. graminis* varieties (i.e., *G. graminis* var. *avenae, G. graminis* var. *graminis* and *G. graminis* var. *tritici*), it cannot be used to distinguish among them. A DNA probe for *O. korrae*, which detects this pathogen in diseased Kentucky bluegrass (*Poa pratensis* L.) and bermudagrass (*Cynodon dactylon* (L.) Pers.), also was developed (24). Bunting et al. (2) developed polymerase chain reaction (PCR) primers from cloned DNA of *M. poae* that specifically produced a 450 base pair amplification product from isolates grown in vitro. Most recently, internal transcribed spacer (ITS) regions have proven useful for the development of specific DNA probes for *O. korrae*, (20,25) and *O. herpotricha* (25).

We investigated the potential of the random amplified polymorphic DNA (RAPD) PCR technology (28,29) to provide an expeditious and accurate identification of these fungi. To reduce the number of PCR

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reactions, mycelial growth rate and colony characteristics were examined to determine if they were useful in presumptively identifying unknown isolates. Landschoot (10) reviewed the literature as it relates to ERI fungi, and provided a summary of growth rates, colony morphologies, and sexual characteristics of these fungi. This investigation extends the information base on the colony characteristics of several of these fungi and compares for the first time the growth rates of all species, except *L. narmari* and *M. rhizophila*, together on the same medium. Portions of this investigation involved a greater number of isolates than previously described or compared.

The investigative approach involved the following steps: (i) we obtained a group of known reference ERI fungi (i.e., *G. cylindrosporus*, *G. graminis* var. *avenae*, *G. graminis* var. *graminis*, *G. incrustans*, *M. poae*, *O. herpotricha*, *O. korrae*, and *P. graminicola*); (ii) we described colony morphology and quantified growth rates among isolates; (iii) we identified primers that reliably produced distinct DNA products unique to each species or variety; (iv) we employed RAPD-PCR against numerous reference isolates to ensure reproducibility of results; and (v) we matched species- or variety-specific DNA fingerprints with those from fungi presumptively identified based on colony morphology.

MATERIALS AND METHODS

Reference isolates were obtained from various researchers who confirmed their identity based on ascospore measurements and ascocarp characteristics. These isolates included 3 *G. cylindrosporus*, 5 *G. graminis* var. *avenae*, 4 *G. graminis* var. *graminis*, 2 *G. graminis* var. *tritici*, 5 *G. incrustans*, 9 *O. korrae*, and 13 *M. poae*. Exceptions included one *Phialophora* sp. and 7 *P. graminicola* isolates that were identified by colony morphology, and 6 *O. herpotricha* isolates that were identified by dot blot hybridization and DNA amplification using *O. herpotricha*–specific oligonucleotide primers (N. A. Tisserat, personal communication). Four isolates of *L. narmari* and one isolate of *M. rhizophila*, originally identified by ascocarp characteristics, were only subjected to RAPD-PCR, since they were obtained at the end of the study. Numerous unknown, darkly pigmented fungi were isolated from symptomatic turfgrass plants sent to the University of Maryland for disease diagnosis. Presumptive identifications of these unknowns were made by comparing their colony characteristics with those of the reference isolates as well as with descriptions found in the literature.

The unknown fungi were obtained from turfgrasses by using two surface sterilization techniques. For the first technique, root tissue (3 to 5 mm) was submersed in 1% (wt/vol) $AgNO_3$ solution in sterile deionized H_2O (dH_2O) for 30 to 60 s, then submersed in 5% (wt/vol) NaCl solution in sterile dH_2O for 1 min to precipitate the $AgNO_3$. The tissue was then rinsed in sterile dH_2O and blotted dry with sterile filter paper (15). For the second technique, root tissue (3 to 5 mm) was submersed in 10% (vol/vol) Clorox (sodium hypochlorite) solution in sterile dH_2O for 2 min, then rinsed in sterile dH_2O, and blotted dry with sterile filter paper. The second technique was only used when *O. korrae* was a suspected pathogen. Following surface sterilization, root tissues were placed onto either a selective medium for *Gaeumannomyces*-like fungi (SM-GGT7) (6) or water agar plus streptomycin sulfate (50 mg per liter). Colonies were then transferred to half-strength potato dextrose agar (PDA/2) (Difco, Detroit, MI) for use in subsequent DNA isolation and to compare colony characteristics and growth rates among isolates.

Another group of unrelated fungi that are commonly associated with soil and turfgrass roots were collected and assessed for the possibility of containing DNA sequences similar to the reference ERI fungi. These were termed "non-ERI fungi" and included a single isolate of each of the following: *Aspergillus* sp. Link., *Colletotrichum graminicola* (Ces.) G. W. Wils., *Fusarium solani* (Mart.) Appel & Wollenweb. emend. W. C. Snyder & H. N. Hans., *Pythium aphanidermatum* (Edson) Fitzp., and *Rhizoctonia solani* Kühn 'AG1-1A'.

Colony morphology and growth rates. Colony morphology was assessed for 2 weeks. Additional assessments were made periodically over the following 4 weeks.

Growth rates were obtained by incubating isolates in darkness for 7 to 8 days at 20, 25, and 30°C, ± 1°C. There were three petri dish subsamples of three known reference isolates of *G. cylindrosporus*, *G. graminis* var. *avenae*, *G. graminis* var. *graminis*, *G. incrustans*, *M. poae, O. herpotricha*, *O. korrae,* and *P. graminicola*. Two measurements were taken at right angles using a ruler every 24 h and averaged. Growth rate studies were conducted twice using different incubators, and data were analyzed as a completely randomized design combined over growth chambers (17). Replicated data were analyzed by the analysis of variance using SAS PROC GLM (16) and significantly different means were separated by Fisher's protected least significant difference at $P = 0.05$.

DNA extraction. A 7-mm-diameter disk of mycelium was transferred from PDA/2 to 50 ml of synthetic potato dextrose yeast broth (per liter, 24 g of Difco potato dextrose broth and 1.5 g of Difco yeast extract). Flasks were maintained at room temperature (22 to 26°C) in the dark for 2 to 3 weeks until a dense mycelial mat was formed. Mycelia were then harvested on sterile filter paper in a Buchner funnel, rinsed three times in 30 ml of dH_20, and vacuum-dried for 5 min. Mats were transferred to 50-ml plastic centrifuge tubes, weighed, and stored at –70°C until DNA was extracted.

Mycelial mats were ground into fine pieces with a mortar and pestle previously stored at –70°C. If mycelium was harvested just prior to DNA extraction, liquid nitrogen was used to aid in the grinding process. Ground mycelium (200 mg) was divided evenly between two 1.7-ml microfuge tubes. Cell lysis was accomplished by re-grinding with a micropestle for 2 min at room temperature in 400 µl of LETS buffer (100 mM LiCl, 10 mM EDTA, 20 mM Tris-HCL, pH 8.0, 0.5% sodium dodecyl sulfate, and 500 µg/ml Proteinase K). The lysed mycelial mixture was de-proteinated with 500 µl/tube of phenol/chloroform/isoamyl alcohol (25:24:1) for 30 s using a vortex, and centrifuged 10 min at 14,000 rpm in a microfuge (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY); 250 µl of supernatant was removed from each tube and combined with 1.0 ml of absolute ethanol and mixed by inverting gently; finally, the supernatant was centrifuged for 10 min at 14,000 rpm. The DNA pellet was either air or vacuum dried and resuspended in 20 µl of sterile, distilled dH_20 and stored at –20°C.

PCR conditions. The PCR conditions employed were similar to those described by Williams et al. (29), and were based on master mix formulations recommended by the Perkin-Elmer Cetus Co. (Norwalk, CT). Briefly, the master mix consisted of the following: 0.1 µM of primer (ten-base oligonucleotide, Operon Technologies, Inc., Alameda, CA); 0.25 unit of *Amplitaq* DNA polymerase; 200 µM each of dATP, dCTP, dGTP, and dTTP in GeneAmp 10 X PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; and 0.001% (wt/vol) gelatin). Sterile, distilled dH_20 (GIBCO BRL, Gaithersburg, MD) was used to bring the master mix to a 90-µl volume and to make working dilutions of dNTPs, primers, and template DNA. For individual PCR reactions, 9 µl of the master mix was combined with 1 µl of genomic DNA (containing 0.6 to 15 ng template DNA) and covered with 25 µl of mineral oil.

Polymerase chain reactions were performed in GeneAmp PCR reaction tubes (Perkin-Elmer Cetus) using a thermal cycler (MJ Research, PTC-100, Watertown, MA) programmed for an initial denaturation of 1.5 min at 95°C. The following regime was then employed: 26 cycles of 30 s at 95°C; 1 min at 30°C; and 1 min at 72°C. Amplification continued as follows: 16 cycles of 30 s at 95°C; 2 min at 34°C; and 1 min at 72°C; and a final extension of 8 min at 72°C. The latter 16 cycles were changed to increase primer specificity by increasing the stringency of the reaction conditions.

Following PCR, the entire 10-µl reaction was electrophoresed at 70V for 1.5 h in a 1.4% agarose gel with an ethidium bromide (EtBr) concentration of 0.08 µg per ml of agarose using a chilled Minnie the Gel-Cicle (Hoefer Scientific Instruments, San Francisco, CA) horizontal electrophoresis unit. When greater than 10 amplification reaction products were electrophoresed simultaneously, EtBr at 0.16 µg per ml of agarose was used with an ISS Intermediate Device (12 × 14 cm gel bed) (Integrated Separation Systems, Natick, MA) horizontal electrophoresis unit at room temperature. Gels were photographed with Polaroid film 57 under UV light.

Operon's 10-mer primer kit A, which consists of 20 primers, initially was assessed against one reference isolate each of *G. cylindrosporus, G. graminis* var. *avenae*, *G. graminis* var. *graminis*, *G. incrustans*, *M. poae*, *O. herpotricha, O. korrae*, and *P. graminicola*. Additionally, 52 reference isolates were screened against species- or variety-specific 10-mers to confirm reproducibility and clarity of the DNA products. Another group of 49 presumptively identified fungi also were subjected to RAPD-PCR and results were matched with the DNA products of reference isolates. All PCR reactions were done at least twice, but only the brighter and more intensely stained bands were compared for reproducibility.

RESULTS AND DISCUSSION

Growth rates and mycelial characteristics. The species were separated into two groups based on their growth rate at 25°C (Table 1). Slow-growing species had an average growth rate of 2.5 mm per day (range 1.6 to 3.4 mm per day) and included *G. cylindrosporus*, *P. graminicola*, *O. herpotricha*, and *O. korrae*. Fast-growing species had an average growth rate of 5.5 mm per day (range 3.2 to 6.6 mm per day) and included *G. graminis* var. *avenae*, *G. graminis* var. *graminis*, *G. incrustans,* and *M. poae*. Except for *M. poae* WIL A-2 at 20 and 25°C, most isolates exhibited statistically similar rates of growth within slow and fast groups. The latter isolate was among the oldest stored isolates and this may have influenced its growth rate.

Phialophora graminicola and *G. cylindrosporus* were indistinguishable, based on growth rates (Table 1) and colony characteristics. These observations support the view that *P. graminicola* is the anamorph of *G. cylindrosporus* (9). When initially isolated from roots, the colony color of *P. graminicola* was creamy white. After transfer, the isolates of both species changed color, which was variable. After 2 weeks of growth, colony pigments of both species were either brownish yellow, orange-yellow, gray, or olive green. The majority of isolates produced a creamy-white band in the new growth zone at the perimeter of the colony. This creamy-white band and relatively darker colony pigmentation distinguished these species from *O. korrae* and *O. herpotricha*. Furthermore, the advancing hyphae of *P. graminicola* and *G. cylindrosporus* on PDA/2 exhibited a slight curling back, whereas *O. korrae* and *O. herpotricha* grew straight outward from the seeded plug.

Ophiosphaerella korrae produced distinctively raised mycelium that appeared domelike in the center of all colonies (n = 10). Newest growth was white, while older growth became dark gray. *Ophiosphaerella herpotricha* was not isolated from turfgrasses sent to the university, so comparisons were only made with the six isolates provided by N. A. Tisserat. Colonies produced aerial mycelium and the center of the colony exuded a brownish-black liquid within 2 weeks. This exudate was unique to *O. herpotricha*, but was not reported by Tisserat et al. (26). Isolates were initially white, and mycelium color changed to tan or brown with age.

Walker and Smith (27) describe *L. narmari* as producing aerial mycelium whose color changes from white to buff (with the buff color persisting for several days) and eventually turns to dark gray to black, resembling mycelium on the agar surface. Optimum growth rate of *L. narmari* is 4 to 5 mm per day at 25°C on PDA (27). Based on Walker and Smith's results, *L. narmari* could be grouped with the slow-growing species in this study. Based on mycelial characteristics, no isolates of *L. narmari* were recovered in this study, nor has this species been reported associated with turfgrass species in the U.S.

The leading mycelium of all fast-growing species distinctively curled back to the center of the colony. Except for isolate MACO-5, all isolates *G. graminis* var. *avenae* (n = 10) had similar colony characteristics. Isolate MACO-5 produced pure white mycelium that turned light brown with age, whereas all others produced light gray mycelium that darkened with age and blackened PDA/2. All isolates produced hyphae that initially grew from the seeded plug in an S-shape, which was very pronounced when observed from the underside of the petri dish. Young hyphae at the periphery of the colony were appressed, but older mycelium (i.e., ≥1 week) was aerial. The three isolates listed in Table 1 as well as an additional 7 isolates that were examined later (data not shown) grew at ≤2.4 mm per day at 30°C. The very slow growth of these isolates at 30°C distinguished *G. graminis* var. *avenae* from other fast-growing species. Dernoeden and O'Neill (5) also noted very little growth of *G. graminis* var. *avenae* at 30°C.

Table 1. Mean radial growth rates of eight darkly pigmented root-infecting fungi grown at 20, 25 and 30°C on half-strength potato dextrose agar (PDA/2) (Difco, Detroit, MI)

		Mean radial growth rate (mm per day)[x]		
Fungus	**Isolate**	**20°C**	**25°C**	**30°C**
Gaeumannomyces graminis var. *avenae*	ENG-4 / LL[y]	5.2 a[z]	5.9 b	0.4[y]
G. graminis var. *avenae*	FR-1	5.0 a	5.4 bc	0.8 ef
G. graminis var. *avenae*	WASH-4	5.2 a	6.4 a	2.4 de
G. graminis var. *graminis*	561	4.0 b	6.4 a	5.0 bc
G. graminis var. *graminis*	FL-36	4.0 b	5.6 bc	7.3 a
G. graminis var. *graminis*	RI	4.1 b	6.6 a	7.7 a
G. incrustans	SYD	3.2 cd	5.5 bc	6.8 ab
G. incrustans	WHEAT	3.0 cde	5.4 c	6.6 abc
G. incrustans	ZOY A-5	3.2 cd	5.7 bc	6.1 abc
Magnaporthe poae	73-1a	3.5 bc	5.7 b	5.9 abc
M. poae	OT A-1 / NAV A-5[y]	3.5[y]	4.5[y]	4.5 cd
M. poae	WIL A-2	2.6 def	2.9 def	4.9 bc
G. cylindrosporus	GC-2	2.2 f–i	2.8 efg	0.9 ef
G. cylindrosporus	Hiland	2.3 e–h	3.2 de	0.2 ef
G. cylindrosporus	Ledgemont	2.1 f–j	2.6 fgh	1.3 ef
Phialophora graminicola	Camp	2.4 e–h	3.2 de	1.2 ef
P. graminicola	Prior	2.5 d–g	3.4 d	1.0 ef
P. graminicola	Purdue	2.2 f–i	2.9 def	0.2 ef
Ophiosphaerella herpotricha	KS28	1.6 i–k	1.8 jk	0.1 f
O. herpotricha	KS115	1.5 i–k	1.8 jk	0.4 ef
O. herpotricha	KS189	1.2 k	1.6 k	0.2 f
O. korrae	MD-II	1.8 g–k	2.4 ghi	0.1 f
O. korrae	NOVI	1.7 h–k	2.2 hij	0.3 ef
O. korrae	WA-99	1.4 jk	2.0 ijk	0.0 f

[x] Two measurements were taken at right angles on three individual petri dishes for each isolate every 24 h for 7 to 8 days. The value presented is an average over the measurement collection time.

[y] Growth rate data were not used in analyses due to death of some isolates. Replacement isolates used as measurements were repeated as follows: isolate LL replaced ENG-4 in the second measurement at 30°C; NAV A-5 replaced OT A-1 in the second measurement at 20 and 25°C, and for both measurements at 30°C.

[z] Means in a column followed by the same letter are not significantly different at P = 0.05 according to Fisher's protected least significant difference.

Gaeumannomyces graminis var. *graminis* was not isolated from turfgrasses sent to the university, so comparisons only were made with the four reference isolates. Isolates RI, FL-36, and FL-39 initially were olive green, but eventually turned gray. Hyphae appeared black when viewed from the underside of a petri dish. Isolates RI, FL-36. and FL-39 produced dense, aerial mycelium, but 561 was feltlike in appearance. As noted by Cunningham (4), isolates produced lobed hyphopodia on petri dish surfaces.

All *G. incrustans* isolates (n = 8) obtained directly from root tissue readily produced black crusts of mycelium, and most blackened PDA/2. Crusts usually formed on the surface of PDA/2, but some were embedded. Several weeks were required before some older, stored isolates would produce the black crusts.

The majority of *M. poae* isolates (n = 17) aggregated mycelium together into thick mycelial strands (producing the dominant color of the colony), which seemed to separate this fungus from *G. incrustans*. Colony and strand color were extremely variable as previously discussed by Landschoot et al. (12). Most were initially white then darkened with age to a yellowish brown or olive green integrated with white mycelium. Many isolates (70%) produced black crusts of mycelium embedded in PDA/2, but few isolates blackened PDA/2. These crusts were described by Landschoot et al. (12) as "aggregates of fused darkly pigmented mycelium."

Scott and Deacon (21) described *M. rhizophila* colonies as initially thin, later becoming woolly, and grayish brown to olive green black in color, with irregular margins and leading hyphae tending to curl back toward the center of the colony. *Magnaporthe rhizophila* colonies attained a diameter of 80 mm in 10 days at 28°C on PDA (21). Based on Scott and Deacon's results, *M. rhizophila* could be grouped with the fast-growing species in this study. Based on mycelial characteristics, no isolates of *M. rhizophila* were recovered in this study.

Many saprophytes or facultative parasites also were isolated from roots of turfgrasses. Other common, darkly pigmented fungi that were isolated included *Menisporella* spp. (n = 3) and unidentified *Phialophora*-like spp. (n = 6). These *Phialophora*-like spp. were extremely slow growing (1.1 to 2.2 mm day at 25°C) and had very compact, felted, and slightly raised mycelium on PDA/2. Colony color of these *Phialophora*-like fungi ranged from gray to olive green to reddish orange, with the newest growth appearing white at the margin. Colony characteristics of these *Phialophora*-like spp. were unlike those of the slow-growing fungi previously described. Elliott and Landschoot (7) found several isolates of an unidentified *Phialophora* sp. associated with roots of bermudagrass and bermudagrass-perennial ryegrass (*Lolium perenne* L.) mixtures. *Ophiosphaerella korrae* was difficult to distinguish from a *Menisporella* sp. based on colony morphology and growth rate (data not shown). The *Menisporella* sp., however, was easily identified based on distinctive setulate conidia from conidiophores with conspicuous collarettes in culture (1). Numerous isolates (n = 18) of sterile, darkly pigmented fungi that grew >3.5 mm per day at 25°C on PDA/2 isolated from turfgrass roots could not be identified, and some of these isolates may represent undescribed species.

Table 2. Summary of the most reliable primers and expected amplification product(s) for ectotrophic root-infecting (ERI) fungi

Fungus	Primer	Product (kb)
Slow-growing species		
G.c./P.g.[y]	OPA-02	0.37
O.k.	OPA-02	0.49 and 1.35
	OPA-08	None
O.h.	OPA-02	0.49
	OPA-08	0.30
L.n.	OPA-02	0.49
	OPA-08	None
Fast-growing species[z]		
G.g.a.	OPA-11	1.23
G.g.g.	None	. . .
G.i.	OPA-09	1.23
	OPA-18	0.98 and/or 1.35
M.p.	OPA-09	1.11
	OPA-18	0.92
M.r.	OPA-09	0.86 and 1.97

[y] Fungi abbrevations are as follows: *G. cylindrosporus* [G.c.], *G. incrustans* [G.i.], *G. graminis* var. *avenae* [G.g.a.], *G. g. graminis* [G.g.g.], *L. narmari* [L.n.], *M. poae* [M.p.], *M. rhizophila* [M.r.], *O. herpotricha* [O.h.], *O. korrae* [O.k.], and *P. graminicola* [P.g.].

[z] If lobed hyphopodia are present, the isolate is probably *G. graminis* var. *graminis*.

Polymerase chain reaction. Several oligonucleotide primers were used to distinguish among the ERI fungi (Table 2). *Gaeumannomyces cylindrosporus* and *P. graminicola* are believed to be saprophytes (15). Because their hyphae and growth habits appear similar to other ERI fungi on field-grown turfgrass roots, it is important to be able to distinguish them from the pathogenic ectotrophs. A unique, 0.37-kb product was generated using (hereinafter "product from" will refer to "product generated by means of primer . . .") primer OPA-02 with all isolates of *G. cylindrosporus* (n = 3) and *P. graminicola* (n = 9) (Fig. 1). The 0.37-kb product was not amplified from other ERI or non-ERI fungi. These molecular data and morphological characteristics reinforce the recognized relationship between *G. cylindrosporus* and *P. graminicola* as noted by Hornby et al. (9).

Primer OPA-11 yielded a unique, 1.23-kb product that separated *G. graminis* var. *avenae* from the other known ERI fungi evaluated (Table 2). This product was not amplified from the non-ERI fungi.

No primers were found that produced identifying DNA products for all four of the *G. graminis* var. *graminis* isolates evaluated. Fortunately, *G. graminis* var. *graminis* can be visually identified by its deeply lobed hyphopodia (10). *Gaeumannomyces graminis* var. *tritici* isolates 568 and FR shared a 0.86-kb product from OPA-18 (data not shown); however, these results should be considered preliminary since they were based on only two isolates. *Gaeumannomyces graminis* var. *tritici* is indistinguishable from *G. graminis* var. *avenae* in culture (4), but is not commonly

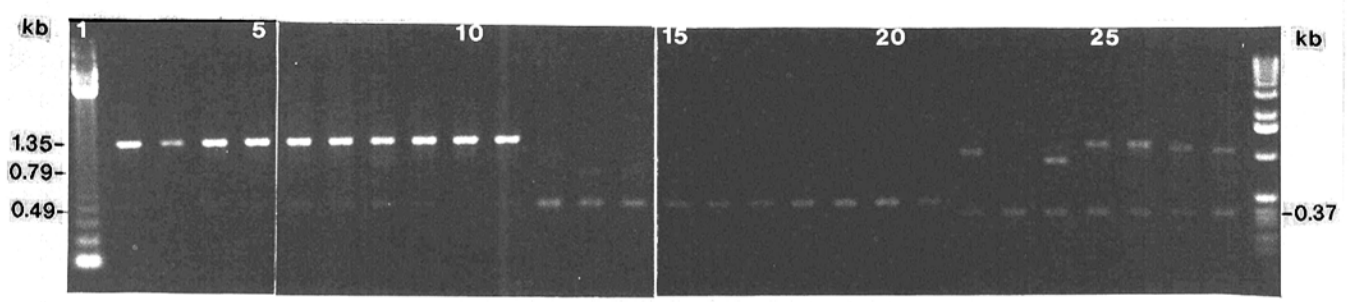

Fig. 1. Amplification products using primer OPA-02: lanes 1 and 29 are 123 bp and 1 kb DNA ladders; lanes 2 to 11 are *Ophiosphaerella korrae* isolates #63, JACK, LMRC, MDII, MDIV, NJ, NOVI, RI, WA-99, and WOOD; lanes 12 to 15 are *Leptosphaeria narmari* isolates 17497, 20806, 41447, and 51197; lanes 16 to 21 are *O. herpotricha* isolates 28, 65, 66, 115, 184, and 189; lanes 22 to 24 are *Gaeumannomyces cylindrosporus* isolates GC-2, Hiland C.C., and Ledgemont C.C.; lanes 25 to 28 are *Phialophora graminicola* isolates Prior, Camp, New York, and Walnut Hills. Amplification products resolved in a 1.4% agarose gel at 70 V for 1.5 h.

found on the roots of turfgrasses. Nilsson and Smith (19) discuss the possible carryover of *G. graminis* var. *tritici* from cereal/forage crops into newly seeded turfgrass areas. Based on our results, no isolates of *G. graminis* var. *tritici* were recovered from turfgrass.

Gaeumannomyces incrustans and *M. poae* are among the most difficult to separate from one another based on colony characteristics. They were, however, clearly separated using primers OPA-09 (Fig. 2) and OPA-18 (Table 2). None of the non-ERI fungi (*C. graminicola* not assessed against these primers) shared any amplification products with *G. incrustans* or *M. poae* using OPA-09 or OPA-18.

Gaeumannomyces incrustans and *M. poae* are heterothallic and therefore require two strains (mating types A and a) to reproduce sexually (13,14). The two mating types of *G. incrustans* could not be distinguished. Primer OPA-04 may provide evidence of mating type (i.e., "a" and "A") differences for *M. poae*. With the exception of *M. poae* "73-1 a," all reference "a" isolates of *M. poae* had a 0.49-kb product from OPA-04. All reference "A" isolates had a 0.52-kb product from OPA-04. *Magnaporthe poae* "73-1 a" also shared the 0.52-kb product. Further comparisons of more isolates may yield additional primers that effectively separate the two *M. poae* mating types.

The identity of reference isolates of *Phialophora* spp., as noted previously, was based on colony or other characteristics and not on ascocarp data. *Phialophora* sp. Orchard Lake appeared to be *M. poae*. This isolate was identified in the early 1980s when Smiley (23) reported that *P. graminicola* was the casual agent of summer patch. Later, Landschoot and Jackson (14) reported that Smiley had misidentified the summer patch causal agent as *P. graminicola*. The pathogen was an undescribed species of *Magnaporthe*, which they named *M. poae* (14). Smiley's *P. graminicola* isolates (i.e., "#197," "60," and "57-84") also were shown to be *M. poae* based on RAPD-PCR products from OPA-09 and OPA-18.

Magnaporthe rhizophila was recently reported to be associated with the roots of Kentucky bluegrass in Pennsylvania (11). This isolate was obtained to determine if the species shared similar DNA products with *M. poae* and to see if any of the unknown isolates collected in this study were *M. rhizophila*. Figure 2 demonstrates, as a result of using primer OPA-09, that *M. rhizophila* "Mr#2" has a unique DNA profile, different from that of *G. incrustans* and *M. poae*. None of the unidentified fast-growing, sterile, darkly pigmented fungi isolated in this study exhibited a DNA profile similar to that of *M. rhizophila* "Mr#2" or produced perithecia on PDA/2 or PDA as described by Landschoot and Gullino (11) and Scott and Deacon (21), respectively.

Primer OPA-02 produced two discrete products, 0.49- and 1.35-kb, for *O. korrae* (Fig. 1). *Leptosphaeria narmari* and *O. herpotricha* shared the 0.49-kb product with *O. korrae*, but not the 1.35-kb product. Thus, *L. narmari* could not be effectively separated from *O. herpotricha* by means of primer OPA-02. The only non-ERI fungus to share the 1.35-kb product with *O. korrae* was *C. graminicola,* which did not exhibit the 0.49-kb product.

A 0.30-kb product from OPA-08 separated *O. herpotricha* from *O. korrae* and *L. narmari* (Table 2) (*C. graminicola* was not assessed against this primer). None of the other ERI or non-ERI fungi evaluated shared this 0.30-kb product.

Presumptively identified fungi. If the DNA product generated from a presumptively identified ERI-fungal isolate was totally different from the reference DNA fingerprint, it was tested against one or two other suspected species. Not all possible combinations of primers were evaluated for all unidentifiable "presumptive" isolates. Of the 52 presumptively identified ectotrophs from diseased turfgrasses, 23 were positively identified based on RAPD-PCR products and mycelial characteristics and 29 were not identified. The 23 isolates included 5 *G. graminis* var. *avenae*, 8 *G. incrustans*, 1 *O. korrae*, 4 *M. poae*, and 5 *P. graminicola.* The 29 unidentified isolates were reexamined and colonies were inspected for conidia. Of these, 2 had died, 3 were *Menisporella* spp., 6 were slow-growing species (i.e., ≤2.2 mm per day on PDA/2) and 18 were fast-growing, sterile, and darkly pigmented. No unique DNA product linked all of the slow-growing (≤2.2 mm per day on PDA/2) species. Furthermore, the 18 fast-growing, sterile, darkly pigmented unidentified isolates were shown not to be *G. incrustans, M. poae, M. rhizophila* or any of the 3 *G. graminis* varieties. This was based on comparisons of DNA products generated from known reference isolates of the aforementioned species. However, these isolates did have some mycelial characteristics in common with the reference isolates. Some of these ectotrophs may represent undescribed species.

In summary, results of these investigations, in combination with screening additional isolates, could be used to develop species- or variety-specific DNA probes to detect specific ectotrophs directly on turfgrass root and crown tissue. With the current methodology described in our experiments, growing time of the fungus in liquid culture could be reduced to 1 week to obtain just enough mycelium for one DNA extraction. This would provide enough DNA to perform 2,000 10-µl assays. This would reduce identification time, which is critical for most disease management programs. Several samples were processed with DNA from 1-week-old colonies, and it was found that DNA extraction was cleaner and easier with these younger colonies. The combined assessment process (i.e., presumptive identification based on mycelial characteristics followed by RAPD-PCR) will allow for the precise identification of most of these fungi in a reasonable amount of time. The ultimate goal is to be able to isolate an unknown darkly pigmented ectotroph and make a presumptive identification within 2 weeks and a positive identification with RAPD-PCR within a week thereafter. Once the pathogen is identified, turfgrass managers would be able to respond with the most appropriate chemical and cultural options in a timely manner.

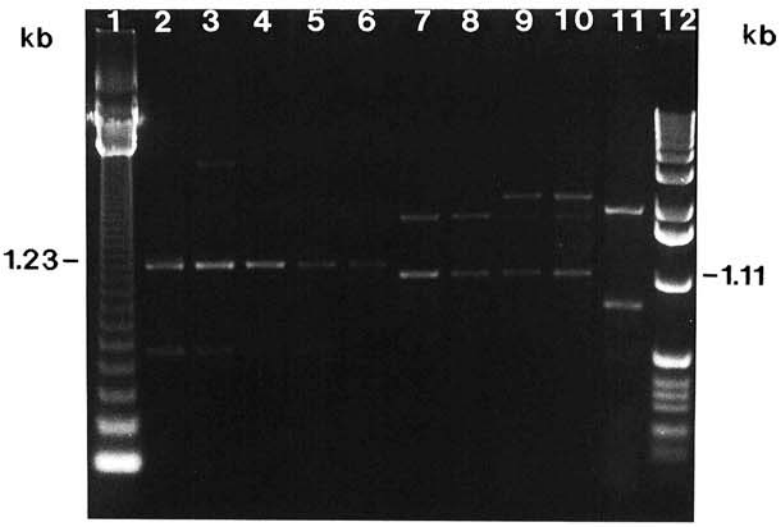

Fig. 2. Amplification products using primer OPA-09: lanes 1 and 12 are 123 bp and 1 kb DNA ladders; lanes 2 to 6 are *Gaeumannomyces incrustans* isolates A#91, SYD, ZOY A-5, CHTF CRF (1992), and Glade Valley C.C. (1993); lanes 7 to 10 are *Magnaporthe poae* isolates NAV A-1, OAK A-5, SIL a-5, and SIL a-8; lane 11 is *M. rhizophila* isolate MR#2. Amplification products resolved in a 1.4% agarose gel at 70 V for 1.5 h.

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LITERATURE CITED

1. Barron, G. L. 1968. The Genera of Hyphomycetes From Soil. Robert E. Krieger Publishing Co., Inc., New York.
2. Bunting, T. E., Plumley, K. A., Clarke, B. B., and Hillman, B. I. 1994. Utilization of the polymerase chain reaction to identify *Magnaporthe poae*. (Abstr.) Phytopathology 84:542.
3. Crahay, J. N., Dernoeden, P. H., and O'Neill, N. R. 1988. Growth and pathogenicity of *Leptosphaeria korrae* in bermudagrass. Plant Dis. 72:945-949.
4. Cunningham, P. C. 1981. Isolation and culture. Pages 103-123 in: Biology and Control of Take-all. M. J. C. Asher and P. J. Shipton, eds. Academic Press, New York.
5. Dernoeden, P. H., and O'Neill, N. R. 1983. Occurrence of Gaeumannomyces patch disease in Maryland and growth and pathogenicity of the casual agent. Plant Dis. 67:528-532.
6. Elliott, M. L. 1991. A selective medium for *Gaeumannomyces*-like fungi. Plant Dis. 75: 1075.
7. Elliott, M. L., and Landschoot, P. J. 1991. Fungi similar to *Gaeumannomyces* associated with root rot of turfgrasses in Florida. Plant Dis. 75:238-241.
8. Henson, J. M. 1989. DNA probe for identification of the take-all fungus, *Gaeumannomyces graminis*. Appl. Environ. Microbiol. 55: 284-288.
9. Hornby, D., Slope, D. B., Gutteridge, R. J., and Sivanesan, A. 1977. *Gaeumannomyces cylindrosporus*, a new ascomycete from cereal roots. Trans. Br. Mycol. Soc. 69:21-25.
10. Landschoot, P. J. 1993. Taxonomy and biology of ectotrophic root-infecting fungi associated with patch diseases of turfgrasses. Pages 41-71 in: Turfgrass Patch Diseases Caused by Ectotrophic Root-Infecting Fungi. B. B. Clarke and A. B. Gould, eds. American Phytopathological Society, St. Paul, MN.
11. Landschoot, P. J., and Gullino, M. L. 1993 First report of *Magnaporthe rhizophila* on Kentucky bluegrass in North America. Plant Dis. 77:1169.
12. Landschoot, P. J., Hoyland, B. F., and Clarke, B. B. 1993. Variation in cultural characteristics and pathogenicity within a local population of *Magnaporthe poae*. Int. Turfgrass Soc. Res. J. 7:335-341.
13. Landschoot, P. J., and Jackson, N. 1989. *Gaeumannomyces incrustans* sp. nov., a root-infecting hyphopodiate fungus from grass roots in the United States. Mycol. Res. 93: 55-58.
14. Landschoot, P. J., and Jackson, N. 1989. *Magnaporthe poae* sp. nov., a hyphopodiate fungus with a *Phialophora* anamorph from grass roots in the United States. Mycol. Res. 93: 59-62.
15. Landschoot, P. J., and Jackson, N. 1990. Pathogenicity of some ectotrophic fungi with *Phialophora* anamorphs that infect the roots of turfgrasses. Phytopathology 80: 520-526.
16. Littell, R. C., Freund, R. J., and Spector, P. C. 1991. SAS system for linear models. 3rd ed. SAS Institute., Cary, NC.
17. McIntosh, M. S. 1983. Analysis of combined experiments. Agron. J. 75:153-155.
18. Nameth, S. T., Shane, W. W., and Stier, J. C. 1990. Development of monoclonal antibody for detection of *Leptosphaeria korrae,* the casual agent of necrotic ringspot disease of turfgrass. Phytopathology 80:1208-1211.
19. Nilsson, H. E., and Smith, J. D. 1981. Take-all of grasses. Pages 433-451 in: Biology and Control of Take-all. M. J. C. Asher and P. J. Shipton, eds. Academic Press, New York.
20. O'Gorman, D., Xue, B., Hsiang, T., and Goodwin, P. H. 1994. Detection of *Leptosphaeria korrae* with the polymerase chain reaction and primers from the ribosomal internal transcribed spacers. Can. J. Bot. 72: 342-346.
21. Scott, D. B., and Deacon, J. W. 1983. *Magnaporthe rhizophila* sp. nov., a dark mycelial fungus with a *Phialophora* conidial state, from cereal roots in South Africa. Trans. Br. Mycol. Soc. 81:77-81.
22. Shoemaker, R. A., and Babcock, C. E. 1989. Phaeosphaeria. Can. J. Bot. 67:1500-1599.
23. Smiley, R. W. 1984. "Fusarium blight syndrome" re-described as a group of patch diseases caused by *Phialophora graminicola*, *Leptosphaeria korrae*, or related species. (Abstr.) Phytopathology 74:811.
24. Tisserat, N. A., Hulbert, S. H., and Nus, A. 1991. Identification of *Leptosphaeria korrae* by cloned DNA probes. Phytopathology 81: 917-921.
25. Tisserat, N. A., Hulbert, S. H., and Sauer, K. M. 1994. Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.
26. Tisserat, N. A., Pair, J. C., and Nus, A. 1989. *Ophiosphaerella herpotricha*, a cause of spring dead spot of bermudagrass in Kansas. Plant Dis. 73:933-937.
27. Walker, J., and Smith, A. M. 1972. *Leptosphaeria narmari* and *L. korrae* spp. nov., two long-spored pathogens of grasses in Australia. Trans. Br. Mycol. Soc. 58:459-466.
28. Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.
29. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.