Identification of *Ophiosphaerella herpotricha* by Cloned DNA Probes

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Research was supported in part by grants from the Heart of America Golf Course Superintendents Association, the Kansas Turfgrass Foundation, and the National Turfgrass Evaluation Program.

We appreciate the assistance of all who supplied fungal isolates or DNA.

Contribution 92-684-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan.

Accepted for publication 21 September 1992.

ABSTRACT

Sauer, K. M., Hulbert, S. H., and Tisserat, N. A. 1993. Identification of *Ophiosphaerella herpotricha* by cloned DNA probes. Phytopathology 83:97-102.

DNA of *Ophiosphaerella herpotricha*, a cause of spring dead spot of bermudagrass, was digested with *Xba*I and was cloned. A 1.5-kb clone (pOH29) was selected from a genomic library for its specificity and strong hybridization to the total DNA of 29 *O. herpotricha* isolates. pOH29 did not hybridize to other fungi commonly associated with roots and stolons of bermudagrass, including *Leptosphaeria korrae, L. narmari, Gaeumannomyces graminis* var. *graminis,* and *G. incrustans*. The probe detected *O. herpotricha* DNA isolated from 200 mg (wet weight) of infected bermudagrass roots and from 1 μg of lyophilized mycelium. DNA hybridization techniques, with pOH29 as a probe, provide methods of identifying nonsporulating cultures of *O. herpotricha* and of detecting the pathogen in root tissue. Oligonucleotide primers, developed from pOH29 and pOH20, amplified numerous DNA fragments, ranging in size from 0.2 to 6 kb of total DNA from *O. herpotricha* as well as several other ectotrophic fungi. Even though no primer pairs that amplified only *O. herpotricha* DNA were identified, pOH29 may be useful in studying geographic variation among *O. herpotricha* isolates.

Additional keywords: diagnostics, restriction fragment length polymorphism, taxonomy.

Spring dead spot is a destructive root disease of bermudagrass (*Cynodon dactylon* (L.) Pers.), which is used for amenity purposes in North America (1,2) and Australia (10). The disease results in circular or arc-shaped patches of dead turf, ranging in size from a few centimeters to more than a meter in diameter. The spots appear in early spring as bermudagrass breaks dormancy, and they often reappear in the same location in successive years (12).

Several ectotrophic, root-rotting fungi are known to cause symptoms of spring dead spot. These include *Ophiosphaerella herpotricha* (Fr.:Fr.) J. C. Walker (= *Ophiobolus herpotrichus* (Fr.:Fr.) Sacc. & Roum.) (12), *Leptosphaeria korrae* J. C. Walker & A. M. Sm. (14), *L. narmari* J. C. Walker & A. M. Sm. (14), and *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *graminis* J. C. Walker (5). Recent isolations from spring dead spot-affected bermudagrass indicate some of these ectotrophic fungi may have overlapping geographic distributions along the northern climatic-transition zone in the eastern United States, where spring dead spot is severe. For example, both *L. korrae* and *O. herpotricha* have been found in Kentucky (P. Vincelli, *personal communication*), while *L. korrae, O. herpotricha,* and *G. g.* var. *graminis* have been isolated from diseased bermudagrass in North Carolina (5) (W. Shane, The Ohio State University, Columbus, *personal communication*). Whether more than one spring dead spot pathogen may occur in the same location or even the same diseased patch is unknown.

Information on the distribution, ecology, and potential interactions of *L. korrae, G. g.* var. *graminis,* and *O. herpotricha*, in association with spring dead spot, has been limited by the inability to accurately and rapidly identify these ectotrophic pathogens. Although these fungi can be differentiated on the basis of ascospore morphology, ascocarps rarely occur on naturally infected turfgrass and cannot be induced easily in the laboratory. Other physiological and morphological characteristics of these fungi in culture, including colony color and texture, are not unique and cannot be consistently used for differentiation. Recent development of monoclonal antibodies (6) and DNA probes (11) for *L. korrae* and DNA amplification techniques for *G. g.* var. *graminis* (9) has improved identification of these fungi. The purpose of our research was to identify and clone highly repetitive DNA sequences from *O. herpotricha* that could be used to detect

the fungus in infected plant tissue and to determine whether oligonucleotide primers developed from these clones could be used in DNA-amplification techniques to identify *O. herpotricha*.

MATERIALS AND METHODS

Fungal isolates. Twenty-eight isolates of *O. herpotricha* and twenty-nine cultures or DNA of other fungal and bacterial species were obtained from various sources for DNA-hybridization analysis (Table 1). Many of the species tested were those difficult to differentiate morphologically from *O. herpotricha* and those responsible for, or associated with, patch diseases of turfgrasses. All fungal isolates were maintained on potato-dextrose agar at 25 C in the dark or were stored at −80 C in a 5% sterile glycerol solution.

DNA isolation. DNA was extracted from fungal isolates following the method described by Tisserat et al (11). DNA from many of the *O. herpotricha* isolates could not be digested completely with *Xba*I after the initial DNA extraction. DNA from these isolates was further purified on cesium chloride density gradients (8).

DNA digests and gel blot hybridization. Genomic DNA (2–6 μg per lane) was digested with *Xba*I restriction enzyme (Promega, Madison, WI) and was fractionated on 0.7% agarose gels. The DNA was transferred to an MSI blotting membrane (Micron Separations Inc., Westboro, MA) by capillary action, baked at 80 C in a vacuum oven for 2 h or cross-linked in a UV Stratalinker 2400 (Stratagene, LaJolla, CA), and hybridized as previously described (11).

Cloning DNA fragments. DNA samples from *O. herpotricha*

TABLE 1. Host plants and contributors of fungal and bacterial cultures or DNA preparations used in dot blot or Southern hybridization assays

Isolate	Host	Location	Source
Agrobacterium tumefaciens A348		Kansas	White
Colletrotrichum sp. KS128	*Zoysia*	Kansas	Tisserat
Curvularia sp.	*Triticum*	Kansas	Bockus
Drechslera poae	*Festuca*	Kansas	Bockus
Fusarium avenaceum	*Triticum*	Kansas	Bockus
Fusarium equiseti	*Triticum*	Kansas	Bowden
Fusarium graminearum	*Triticum*	Kansas	Bowden
Fusarium moniliforme		Kansas	Bowden
Fusarium oxysporum	*Medicago*	Kansas	Bowden
Fusarium solani	*Medicago*	Kansas	Bowden
Gaeumannomyces graminis var. *avenae* WF 8619-1	*Agrostis*	Illinois	Wilkinson
Gaeumannomyces graminis var. *graminis* KS2	*Cynodon*	North Carolina	Lucas
Gaeumannomyces graminis var. *graminis* FL19	*Cynodon*	Florida	Elliot
Gaeumannomyces graminis var. *tritici* KS19	*Triticum*	Kansas	Bockus
Gaeumannomyces incrustans KS13	*Poa*	Kansas	Tisserat
Leptosphaeria korrae 60259	*Poa*	New York	ATCC
Leptosphaeria narmari 13726	*Cynodon*	Australia	Walker
Macrophomina phaseolina	*Glycine*	Kansas	Schwenk
Magnaporthe grisea	*Oryza*	Philippines	Leung
Magnaporthe poae NY197	*Poa*	New York	Dernoeden
Neurospora crassa		Kansas	Leslie
Ophiosphaerella herpotricha KS1	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS3	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha-like KS4	*Zoysia*	Kansas	Tisserat
Ophiosphaerella herpotricha KS5	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS6	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS10	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS11	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS20	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS23	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS27	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS28	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS29	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS35	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS65	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS66	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS67	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS68	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS70	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha KS71	*Cynodon*	Kansas	Tisserat
Ophiosphaerella herpotricha TX75	*Cynodon*	Texas	Tisserat
Ophiosphaerella herpotricha TX76	*Cynodon*	Texas	Tisserat
Ophiosphaerella herpotricha OK107	*Cynodon*	Oklahoma	Tisserat
Ophiosphaerella herpotricha OK108	*Cynodon*	Oklahoma	Tisserat
Ophiosphaerella herpotricha OK112	*Cynodon*	Oklahoma	Tisserat
Ophiosphaerella herpotricha OK115	*Zoysia*	Oklahoma	Tisserat
Ophiosphaerella herpotricha OK145	*Cynodon*	Oklahoma	Tisserat
Ophiosphaerella herpotricha OK153	*Cynodon*	Oklahoma	Tisserat
Pseudomonas andropogonis	*Zea*	Kansas	Claflin
Pythium aphanidermatum	*Triticum*	Kansas	Bockus
Pythium ultimum	*Medicago*	Kansas	Bowden
Rhizoctonia sp. (binucleate) KS116	*Agrostis*	Kansas	Tisserat
Rhizoctonia solani (AG-1) KS124	*Festuca*	Kansas	Tisserat
Stagonospora sp.	*Tripsacum*	Kansas	Bowden
Stagonospora nodorum	*Triticum*	Kansas	Bockus
Stemphylium alfalfae	*Medicago*	Kansas	Stuteville
Xanthomonas campestris pv. *oryzae*	*Oryza*	Kansas	Leach

ascospore isolates, KS65 and KS71, were digested completely with the restriction enzyme *Xba*I. DNA fragments were ligated into the *Xba*I-cut pUC18 plasmid vector and used to transform *E. coli* strain DH5α. Recombinant clones were screened by colony hybridization to labeled *O. herpotricha* genomic DNA (8). Thirty-three recombinant clones were initially selected based on their intense hybridization to DNA of *O. herpotricha*. To verify their high-copy number and the size of the cloned DNA fragment, individual plasmid-DNA preparations were digested with *Xba*I, fractionated on an agarose gel, transferred to an MSI membrane, and probed with labeled genomic DNA from *O. herpotricha*. Five of the recombinant clones that gave strong hybridization signals were selected for further analysis. Several clones showing little or no hybridization were also selected as representative probes for low copy-number genomic sequences.

Detection of *O. herpotricha* in turfgrass roots. Stolons of bermudagrass selections, Midfield, Arizona Common, NM375, NMS3, and NMS4, were placed into 6-cm-diameter × 25-cm-deep plastic pots (McConkey Co., Sumner, WA) containing a steam-sterilized soil/perlite/peat mixture (1:1:1, v/v) and were allowed to grow for 3–6 mo on a greenhouse bench. Kentucky bluegrass was seeded into a similar potting medium. Plants were then inoculated with either *O. herpotricha, G. g.* var. *graminis, G. incrustans,* or *L. korrae* in a manner described by Tisserat et al (12). Plants were incubated for at least 90 days and then were washed with water to remove soil from the roots. Root segments were removed and processed for DNA extraction. Root samples from each inoculation were also surface-sterilized and placed on acidified PDA.

Field samples of bermudagrass showing symptoms of spring dead spot were collected from Kansas, Oklahoma, Texas, Georgia, Maryland, and Kentucky and were processed as previously described. Isolations were made from each sample to determine whether *L. korrae, O. herpotricha,* or *G. g.* var. *graminis* was present.

Total DNA from root-tissue samples (200–400 mg) was extracted and transferred to an MSI membrane as described by Tisserat et al (11). Hybridization with labeled DNA from pOH29 was performed, as described above for gel blots.

Sensitivity assay. Sensitivity of the DNA probe was determined by hybridization to serial dilutions of total DNA extracted from 0.05 g of lyophilized mycelium of *O. herpotricha*. The extraction procedure was the same as that used for infected root material. DNA dilutions were made in TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) to correspond with mycelial dry weights of 10,000, 1,000, 100, 10, and 1 μg. Samples were transferred to membranes using the slot blot apparatus as previously described and were probed with a DNA clone from *O. herpotricha*. The sensitivity assay was repeated two times.

Primer selection and DNA amplification. Sequencing was performed on the previously constructed DNA clones from *O. herpotricha*, using a Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH). Oligodeoxyribonucleotide primers OHPR5 (5′-CGTAAGCCAGCTCGCAGTTG-3′) and OHNEW8 (5′-TTCGGTAAAGATAGCGGCCC-3′) were selected from pOH29, a 1.5-kb high-copy DNA probe specific for *O. herpotricha*. Primers POH20-A (5′-TATAGCGCTAGAAGATTACC-3′) and POH20-B (5′-GGATAGAAGTACCACCTGCC-3′) were derived from sequences from pOH20. The pOH20 probe carried a low copy-number, 1.4-kb genomic fragment. Primers were obtained from Operon Technologies, Inc. (Alameda, CA).

Amplification reactions were performed in a programmable thermal cycler (MJ Research, Inc., Watertown, MA). A 25-μl reaction mixture contained 25 pmol of each primer, 0.25 U of Replitherm DNA polymerase (Epicentre Technologies, Madison, WI), and 200 μM 2′-deoxynucleoside 5′-triphosphates (U.S. Biochemical Corp.). Template DNA, in 1 μl volumes, included approximately 1–10 ng of pOH20 and pOH29 and 10–100 ng of total fungal DNA. Reactions using primers OHPR5 and OHNEW8 were amplified for 30 cycles (1 min at 92 C, 1.5 min at 55 C, and 2.5 min at 72 C). Primers POH20-A and POH20-B were used in 35 cycle reactions (30 s at 94 C, 45 s at 58 C, and 90 s at 72 C).

Amplification products (10–20 μl per lane) were electrophoresed in a 1% agarose gel at 60 V for 3–4 h in TBE buffer (0.44 M boric acid, 0.44 M trizma base, and 10 mM EDTA), were stained with ethidium bromide, and were viewed under ultraviolet light.

RESULTS

Verification of *O. herpotricha* isolates. Many of the putative *O. herpotricha* isolates used were cultured from infected root material and were tentatively identified by morphological similarities to known ascospore isolates in culture. Attempts to induce ascocarp formation in these field isolates were unsuccessful. As a result, it was necessary to verify their identity before selecting a multicopy, species-specific DNA probe. This was done by restriction fragment length polymorphism (RFLP) analysis using the low-copy clones pOH13 (0.8 kb), pOH20 (1.6 kb), and pOH32 (0.2 kb). The pOH20 probe hybridized to identical *Xba*I-digested fragment sizes in all ascospore and field isolates tested, except KS4 and KS33 (Fig. 1). The pOH13 and pOH32 probes also distinguished KS4 and KS33 from the other isolates (data not shown). Isolates KS4 and KS33, tentatively identified as *O. herpotricha*, failed to hybridize to the probes or gave dissimilar banding patterns, and thus were determined not to be *O. herpotricha*. DNA of *L. korrae* and *L. narmari* also gave dissimilar banding patterns or did not hybridize to the three probes. Although the low-copy probes were useful in taxonomic identification of *O. herpotricha* isolates, they hybridized too weakly to be useful in rapid-detection assays and were not used to develop DNA probes.

Specificity of pOH29 to *O. herpotricha*. A 1.5-kb *Xba*I-digested genomic DNA fragment (pOH29) was selected for further study,

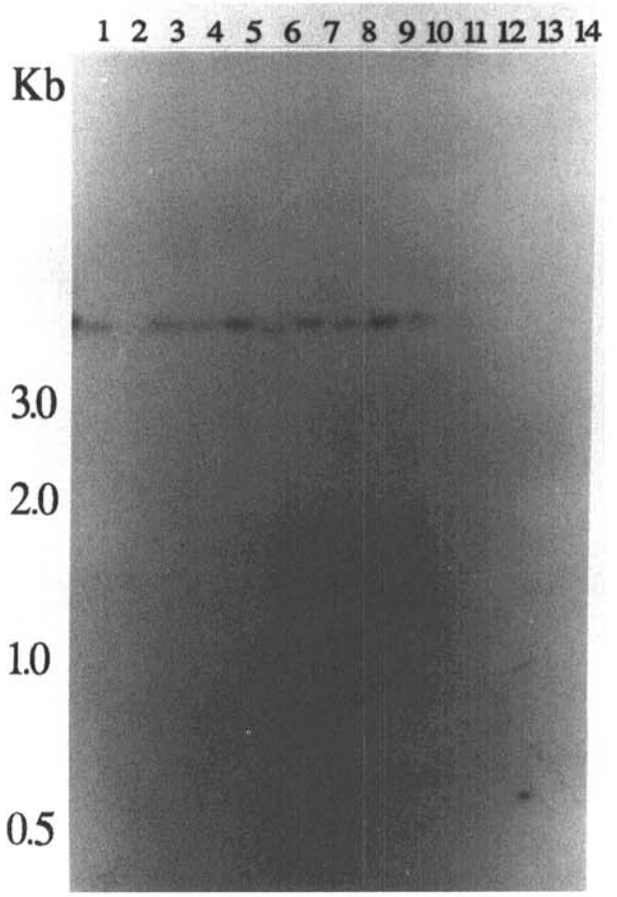

Fig. 1. Hybridization of ^{32}P-labeled insert pOH20 to *Xba*I-digested fungal DNA of *Ophiosphaerella herpotricha* isolates, KS3, KS11, KS23, KS29, KS66, KS68, TX75, TX76, OK112, OK115 (lanes 1–10), and *Leptosphaeria narmari* and *L. korrae* isolates, KS4 and KS33 (lanes 11–14). Ascospores and field isolates (KS29, KS66, and KS68) of *O. herpotricha* had identical hybridization patterns.

based on its apparent specificity and strong hybridization to DNA of *O. herpotricha*. pOH29 hybridized strongly to a number of various-sized fragments of *Xba*I-digested DNA of *O. herpotricha* isolates (Fig. 2). Polymorphisms among isolates were detected.

Specificity of the multicopy clone pOH29 was determined by dot blot hybridization to total DNA of *O. herpotricha* and 29 other fungal and bacterial species (Fig. 3). The clone hybridized to all isolates of *O. herpotricha*, including those identified by hybridization patterns to the low-copy probes (pOH13, pOH20, and pOH32). pOH29 did not hybridize to DNA of the unidentified isolates KS4 and KS33 (data not shown) or to DNA of other fungi and bacteria listed in Table 1, except for a slight hybridization to DNA of *Xanthomonas campestris* pv. *oryzae* (Fig. 3). We speculate that this was the result of minor contamination of plasmid-vector sequences during the preparation of the genomic probe.

Detection of *O. herpotricha* in root tissue. The ability of the multicopy probe pOH29 to detect *O. herpotricha* in diseased plants was tested by probing slot blots of total DNA from field- and greenhouse-grown bermudagrass roots colonized by the fungus (Fig. 4). The pOH29 probe did not hybridize to DNA of healthy Kentucky bluegrass, tall fescue, bermudagrass, or to bermudagrass inoculated with *L. korrae, G. g.* var. *graminis,* or *G. incrustans* in the greenhouse (Fig. 4, Table 2). Hybridization to DNA of four bermudagrass selections inoculated with *O. herpotricha* was detected.

The pOH29 probe hybridized to DNA extracted from 93% (38 of 41) of the spring dead spot-affected bermudagrass field

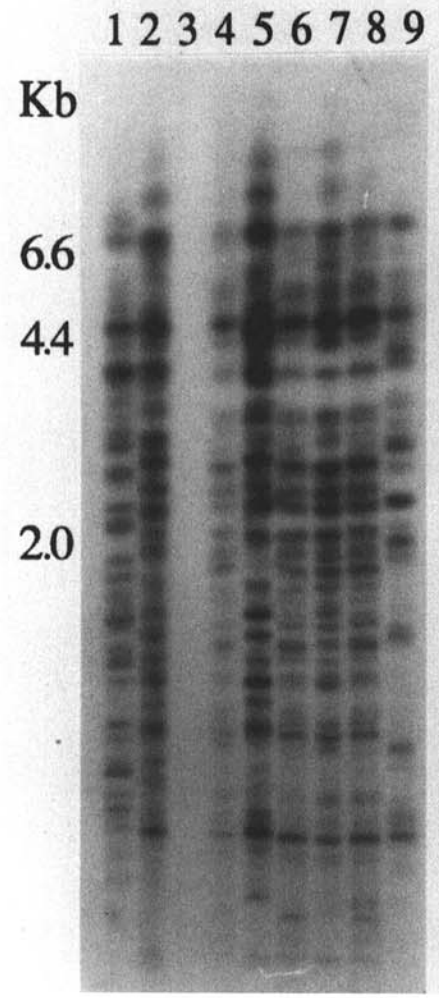

Fig. 2. Hybridization of ^{32}P-labeled insert pOH29 to *Xba*I-digested DNA of *Ophiosphaerella herpotricha* isolates, KS1, KS3 (lanes 1 and 2), KS11, KS29, KS67, KS68, KS70, and TX75 (lanes 4–9). No hybridization was detected to KS4 (lane 3), a morphologically similar, but unidentified, ectotrophic fungus isolated from zoysia grass.

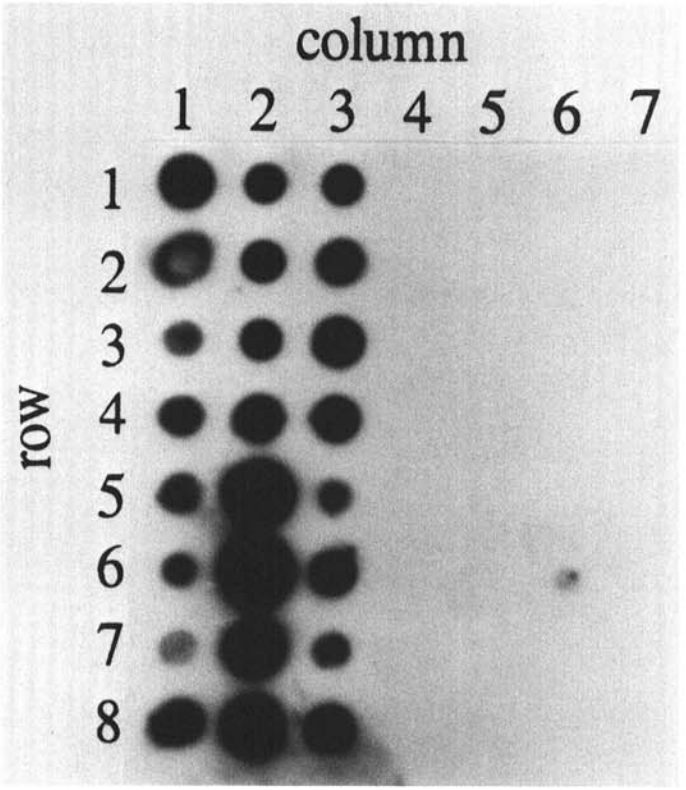

Fig. 3. Dot blot hybridization of ^{32}P-labeled pOH29 to total DNA of 24 *Ophiosphaerella herpotricha* isolates (columns 1–3) and 29 other fungal and bacterial species (columns 4–7). pOH29 hybridized to all *O. herpotricha* isolates and hybridized slightly to *Xanthomonas campestris* pv. *oryzae* (column 6, row 6), but did not hybridize to DNA of other fungi and bacteria.

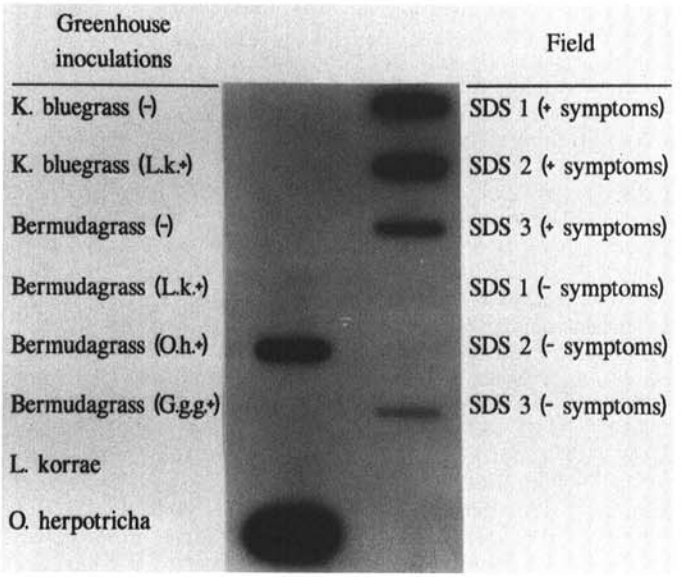

Fig. 4. Slot blot hybridization of pOH29 to fungal and plant DNA extracted from 200 mg (wet weight) of bermudagrass root samples. Bluegrass (L.k.+) and bermudagrass (L.k.+, O.h.+, and G.g.g.+) = turfgrass colonized by *Leptosphaeria korrae, Ophiosphaerella herpotricha,* or *Gaeumannomyces graminis* var. *graminis*. Bluegrass and bermudagrass (−) = noninfected root material. Sodium dodecyl sulfate (SDS) 1, 2, and 3 (+ symptoms) represent root samples collected from three distinct spring dead spot patches at the same location in Kansas. Sodium dodecyl sulfate (SDS) 1, 2, and 3 (− symptoms) represent samples from nonsymptomatic turf at the same location. *O. herpotricha* was isolated from SDS 3 (− symptoms). The bottom two slots in lane 1 represent the degree of hybridization associated with 1 μg of DNA from *O. herpotricha* and *L. korrae*.

samples collected in Kansas, Oklahoma, Texas, and Kentucky, where *O. herpotricha*, but not *L. korrae*, was isolated from at least one sample (Table 2). In the same locations, pOH29 also hybridized to four of 31 samples showing no symptoms of spring dead spot. *O. herpotricha* was subsequently isolated from root segments of one of the nonsymptomatic plant samples. In one sampling location (Hutchinson, KS), pOH29 hybridized to DNA from 20 of 22 samples collected from the margins of spring dead spot patches, whereas *O. herpotricha* was only isolated from roots of 11 of 22 samples.

Total DNA extracted from spring dead spot-affected bermudagrass collected in Georgia, Maryland, and Kentucky did not hybridize to pOH29 but did hybridize to the *L. korrae* probe, pLK88, developed by Tisserat et al (11) (Table 2). *L. korrae*, but not *O. herpotricha*, was subsequently isolated from these samples.

Sensitivity of the pOH29 probe. Sensitivity of the pOH29 probe was determined by hybridization to serial dilutions of total DNA extracted from 0.05 g of lyophilized mycelium of *O. herpotricha*. pOH29 hybridized to DNA equivalent to that found in 1 μg of lyophilized mycelium. The intensity of hybridization was similar to 10 pg of purified *O. herpotricha* genomic DNA (Fig. 5).

TABLE 2. Hybridization of pOH29 to DNA extracted from roots of turfgrass species inoculated with ectotrophic fungi in the greenhouse or from field samples of symptomless or spring dead spot-affected bermudagrass

Location	Number of samples	pOH29[a]	Isolation[b]
Greenhouse samples[c]			
Bermudagrass-			
G. g. var. *graminis*	3	0	
Bermudagrass-*G. incrustans*	3	0	
Bermudagrass-*L. korrae*	4	0	
Bermudagrass-*O. herpotricha*			
Selection NM375	4	4	
Selection NMS3	4	4	
Selection NMS4	4	4	
Midfield	4	4	
Bermudagrass healthy	5	0	
Bluegrass healthy	5	0	
Tall fescue	3	0	
Bermudagrass field samples[d]			
Georgia diseased	1	0	*L. korrae*
Kansas			
Independence diseased	1	1	*O. herpotricha*
Wichita diseased	3	3	*O. herpotricha* (3/3)
Wichita healthy	3	2	None
Manhattan diseased	10	9	*O. herpotricha* (9/10)
Manhattan healthy	17	1	*O. herpotricha* (2/17)
Hutchinson diseased	22	20	*O. herpotricha* (11/22)
Hutchinson healthy	11	1	None
Kentucky			
Diseased	1	0	*L. korrae*
Healthy	1	0	None
Maryland diseased	1	0	*L. korrae*
Oklahoma			
Tulsa diseased	3	3	*O. herpotricha* (3/3)
Grove diseased	1	1	*O. herpotricha*
Texas diseased	1	1	*O. herpotricha*
Other field samples			
Manhattan, Kansas			
Zoysia healthy	7	0	None
Tall fescue healthy	7	0	None

[a] Number of samples that hybridized strongly to pOH29 in slot hybridization assays following DNA extraction from root samples.

[b] Isolation of *Ophiosphaerella herpotricha* or *Leptosphaeria korrae* from root samples and the proportion ( ) of samples in which these fungi were successfully isolated. Field samples in which *L. korrae* was isolated hybridized strongly to the *L. korrae*-specific probe, pLK88.

[c] Turfgrass samples inoculated with *L. korrae, Gaeumannomyces graminis* var. *graminis, G. incrustans*, or *O. herpotricha* 1–3 mo prior to DNA extraction from root samples and hybridization.

[d] Diseased samples collected from margins of turf showing symptoms of spring dead spot or healthy samples collected from areas showing no symptoms at the same location.

Amplification of O. herpotricha DNA. The oligonucleotide primers OHPR5 and OHNEW8, sequenced from pOH29, amplified a 1.4-kb segment from the pOH29 template and also amplified DNA fragments of various sizes from genomic DNA templates of *O. herpotricha* isolates (Fig. 6). Amplification products among isolates, even those from different geographic regions, were similar, but not identical in size. None of the polymerase chain reaction (PCR) products were common to all tested isolates. The primers also amplified fragments from total DNA templates of several other fungi, including *L. narmari, Fusarium equiseti,* and *Pythium aphanidermatum*, which could not be distinguished easily from those of *O. herpotricha* (data not shown).

A second set of primers (POH20-A and POH20-B) from pOH20 amplified a prominent 0.7-kb fragment from genomic DNA of all *O. herpotricha* isolates tested (data not shown). The PCR products were approximately half the size of the original pOH20 insert. The primers also amplified DNA of nine of 17 other fungal species. Several fungi, including *L. narmari* and *G. g.* var. *graminis*, had a 0.7-kb PCR product similar to that found in *O. herpotricha* isolates.

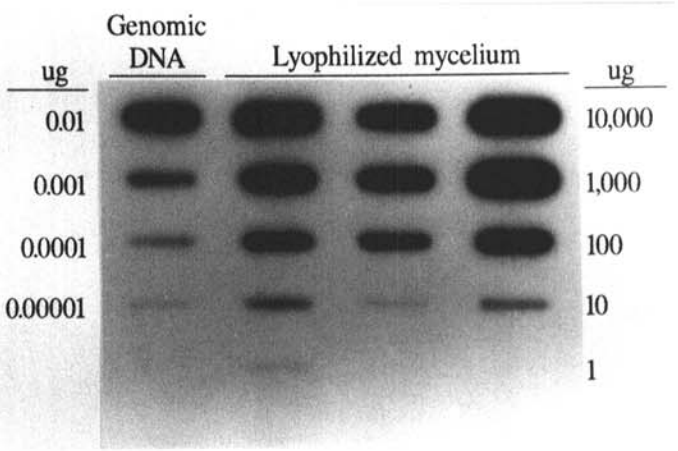

Fig. 5. Sensitivity of the pOH29 probe was determined by hybridization to serial dilutions of genomic DNA (column 1) and DNA extracted from lyophilized mycelium of *Ophiosphaerella herpotricha* (columns 2–4).

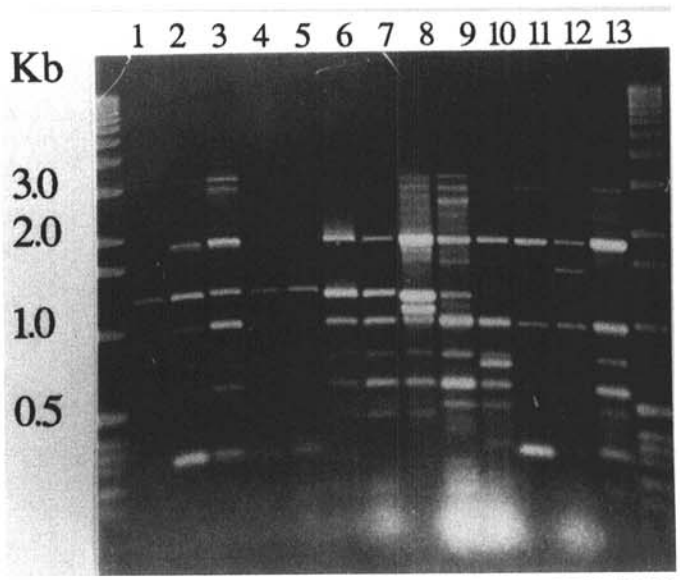

Fig. 6. Amplified DNA of pOH29 (lane 1) and isolates KS11, KS66, KS67, KS68, OK112, OK115, KS29, OK153, OK145, TX75, TX76, and KS23 (lanes 2–13) of *Ophiosphaerella herpotricha* using primers OHPR5 and OHNEW8 derived from the sequence of the pOH29 clone of *O. herpotricha*.

DISCUSSION

We cloned several DNA fragments from *O. herpotricha* that can be used for diagnostic or taxonomic identification of this fungal species. The low-copy clones, pOH13, pOH20, and pOH32, hybridized to either one or a few unique restriction fragments, whereas the 1.5-kb fragment of pOH29 hybridized to various-sized fragments of *Xba*I-digested DNA of all *O. herpotricha* isolates tested. pOH29 provided the best sensitivity in dot blot hybridization assays. Our results agree with previous studies (4,11) that demonstrated the advantages of sensitive DNA probes that recognize multicopy sequences. Although the low-copy probes were not used in dot blot hybridizations, they were useful in initially verifying that the isolates were *O. herpotricha* and in distinguishing the morphologically similar, but unidentified, fungal isolates KS4 and KS33.

The root and thatch environment of bermudagrass supports the growth of numerous fungi and bacteria. As a result, any DNA probe used to detect the pathogen in field material must be species specific. In our studies, pOH29 did not hybridize to DNA extracted from cultures of numerous fungi commonly found on amenity turfgrass species. Furthermore, pOH29 hybridized to 93% of the plant samples collected from spring dead spot patches in locations where *O. herpotricha* was isolated but not to DNA extracted from several noninfected turfgrass species. The DNA extracted from these field samples presumably contained DNA from many microorganisms, including those tested in our study. Nevertheless, we did not detect nonspecific hybridization of pOH29 to these samples.

The pOH29 probe hybridized to DNA extracted from four symptomless plants collected from locations adjacent to spring dead spot patches, even though the fungus could only be isolated from two of the samples. *O. herpotricha* is often difficult to isolate, especially from lightly colonized or severely rotted roots in early spring. For example, *O. herpotricha* was isolated from only 50% of the Hutchinson, KS, field samples that showed symptoms and that tested positive using the dot blot hybridization assay (Table 2). The sensitivity of the dot blot hybridization assay (1 μg of lyophilized mycelium or 10 pg of DNA) may allow the detection of *O. herpotricha* in sparsely colonized root samples, which may explain the positive hybridization to symptomless field samples collected adjacent to spring dead spot patches

Dot blot hybridization assays using ^{32}P-labeled probes were highly sensitive. However, this labeling procedure may have limitations for routine diagnostic use in laboratories not set up to use radioisotopes. A nonradioactive label (Gene Images Kit, U.S. Biochemical Corp.) was attempted with pOH29, but it was not as sensitive as the radioactive label. Further testing of nonradioactive-labeling techniques is needed.

DNA-amplification techniques have been successfully used to identify fungal plant pathogens (7,9). In our studies, primers derived from the low-copy pOH20 clone amplified a single fragment of identical size in all *O. herpotricha* isolates, but also identified fragments of similar size in several other fungi. Primers derived from the multicopy pOH29 probe amplified multiple fragments that were polymorphic in size. These primers also amplified fragments of various sizes in several other fungi. The lack of complete specificity of either primer pair in amplifying only DNA of *O. herpotricha* complicates their diagnostic use; neither pair generates a single fragment or single pattern of fragments unique to the species. Furthermore, specificity is not improved by increasing the annealing temperature nor is it improved by other methods attempted to insure nonspecific priming. It may be possible to generate a single unique banding pattern for *O. herpotricha* with these primers by digesting the PCR products with a restriction enzyme or to reamplify the PCR products using a nested set of primers similar to the technique reported by Schesser et al (9).

DNA probes such as pOH29, which are highly polymorphic and which hybridize to multiple genomic fragments, can be particularly useful genetic markers because polymorphisms at numerous genomic sites can be assayed with a single hybridization. Potential uses include differentiating among genotypes in a population and estimating the amount of genetic variation within or between populations derived from different hosts or geographic locations. Because the sexual cycle of *O. herpotricha* has not been observed on bermudagrass field samples and because fungal populations may be predominantly clonal, it could be possible to determine the origin of new isolates. The MGR element of *Magnaporthe grisea* (3,13) is a recent example of such a probe. The pOH29 probe identifies roughly 20–30 bands in *Xba*I-digested genomic *O. herpotricha* DNA, most of which are polymorphic. Preliminary analysis of 26 isolates has indicated that ascospore cultures derived from a single ascocarp may have nearly identical banding patterns, while isolates from different regions differ widely. A sample of this variation can be assayed using the pOH29 primers. The primers amplify two to nine polymorphic bands in the 0.2- to 2-kb range, and the banding patterns are highly repeatable. The PCR approach might be favored over a hybridization analysis when large numbers of isolates must be classified.

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