Identification of *Leptosphaeria korrae* by Cloned DNA Probes

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ABSTRACT

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Leptosphaeria korrae was identified by a unique banding pattern of *Eco*RI-digested total DNA that was fractionated by gel electrophoresis and stained with ethidium bromide. Discrete and intensely stained bands of DNA that migrated at 1.1, 1.3, and 2.4 kb were noted on 38 isolates of *L. korrae*, but not in 26 other fungal species. DNA fragments were isolated from the 1.1- and 1.3-kb bands of *Eco*RI-digested DNA and were cloned. Two clones, pLK66 and pLK88, hybridized to multiple-sized *Eco*RI fragments of *L. korrae*, and occurred at an estimated 10–100 copies per genome. The two clones did not cross-hybridize. The clone pLK88 was specific to *L. korrae* and hybridized to DNA of the fungus isolated from 200 mg (wet weight) of infected Kentucky bluegrass and bermudagrass roots and from 1 μg of lyophilized mycelium. DNA hybridization techniques with pLK88 as a probe provide ways of identifying *L. korrae*, an ectotrophic, root-infecting fungus of turfgrasses, and of detecting the pathogen in root tissue.

Additional keywords: *Cynodon*, *Gaeumannomyces*, *Ophiosphaerella herpotricha*, *Poa pratensis*.

Leptosphaeria korrae J. C. Walker & A. M. Sm., an ectotrophic, root- and crown-infecting fungus, is a cause of spring dead spot of bermudagrass (*Cynodon dactylon* (L.) Pers.) in Australia (19) and certain geographic areas in the United States (1,2). The fungus also is responsible for a patch disease of Kentucky bluegrass (*Poa pratensis* L.) in North America called necrotic ringspot (15). In both spring dead spot and necrotic ringspot, the pathogen colonizes roots, crowns and/or stolons of turfgrass plants. During periods of suboptimal turfgrass growing conditions, such as temperature stress or dormancy periods, much of the root system on infected plants is killed, resulting in the development of circular, arclike, or "doughnut" patches of dead turf (1,2,15). These patterns often reappear yearly in affected turfgrass at the same location.

Accurate field identification of spring dead spot and necrotic ringspot is difficult because other soilborne fungi are known to cause similar patch-type symptoms. For example, *L. narmari* J. C. Walker & A. M. Sm., *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *graminis* J. C. Walker, and *Ophiosphaerella herpotricha* (Fr.:Fr.) J. C. Walker also cause spring dead spot in different geographic regions (9,18,19). Symptoms caused by these pathogens on bermudagrass are identical to those reported for *L. korrae*. Furthermore, another ectotrophic fungus, *Magnaporthe poae* Landschoot & Jackson, causes symptoms on Kentucky bluegrass similar to those of *L. korrae* (15).

Diagnosis of diseases caused by *L. korrae* is often attempted by isolating the fungus from symptomatic plants. However, isolating and differentiating *L. korrae* from other ectotrophic fungi in culture is difficult. Most ectotrophic fungi, including *L. korrae*, have relative slow growth rates and do not readily produce ascocarps on artificial media. Ascocarp development in some isolates of *L. korrae* can be induced by infesting sterile oat or rye seed, but only after incubating the infested seed for several weeks or months. Other physiological and morphological characteristics in culture, including colony color and texture, are not definitive for differentiating *L. korrae* from certain other ectotrophic fungi.

Recently, more reliable techniques for identifying *L. korrae* have been attempted. Hawkes and Harding (7) were able to distinguish cultures of *L. narmari* from *L. korrae* by isoelectric focusing of protein extracts. Nameth et al (10) reported the development of a monoclonal antibody specific to *L. korrae*. The purpose of our research was to identify and clone highly repetitive DNA sequences in *L. korrae* that could be used to detect the fungus in plant tissue. Preliminary reports on this research have been published (17).

MATERIALS AND METHODS

Fungal isolates. Total DNA was extracted from 38 isolates of *L. korrae*. Isolates from Wisconsin, provided by G. Worf, University of Wisconsin, Madison, were 2BB-85, 25-85, 1-85, 5-85, 21-85, 22-85, 18BR-85, 24-85, Hartman, 27-85, 23-85, 4BR-85, 7BR-85, 16BR-85, and WI (not numbered). Isolates from Michigan, provided by J. Vargas, Michigan State University, East Lansing, were Wood, Novi, and Mich-5. Isolate MD II from Maryland was provided by P. Dernoeden, University of Maryland, College Park. Isolates from Minnesota, provided by the University of Minnesota (St. Paul) Diagnostic Laboratory, were MN-1 and MN-2. Isolates from Washington, provided by G. Chastagner, Washington State University Research Center, Puyallup, were WA-8, WA-19, WA-64, WA-99, WA-110, WA-113, and WA-236. Isolates from Colorado, provided by W. Brown, Colorado State University, Ft. Collins, were CO-236, CO-701, and CO-775. Isolates from Idaho, provided by D. Thompson, University of Idaho, Moscow, were Henderson, Ward, Schultz, Dean, and Vickens. Isolate 51197 from Australia was provided by J. Walker, Biological and Chemical Research Institute, New South Wales, Australia, and isolate 60259 (New York) was obtained from the ATCC, Rockville, MD.

Other isolates of ectotrophic and soil-inhabiting fungi were obtained for DNA hybridization analysis. Many of the species tested were those that are difficult to differentiate from *L. korrae* morphologically and are responsible for or are associated with patch diseases of turfgrasses. Isolates or genomic DNA of *Gaeumannomyces graminis* var. *avenae* (E. M. Turner) Dennis (WF 8619-1), *G. graminis* var. *graminis* (unnumbered), *L. narmari* (13726), *M. poae* (NY-197), and *M. grisea* (Hebert) Barr comb. nov. (Guy-11) were provided by H. Wilkinson, University of Illinois, Urbana; L. Lucas, North Carolina State University, Raleigh; J. Walker, Australia; N. Jackson, University of Rhode Island, Kingston; and H. Leung, Washington State University, Pullman, respectively. Isolates of *Curvularia* sp., *Drechslera poae* (Baudys) Shoemaker, *Fusarium avenaceum* (Fr.:Fr:) Sacc., *F. equiseti* (Corda) Sacc., *F. graminearum* Schwabe, *F. moniliforme* J. Sheld., *F. oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.), *F. solani* (Mart.) Sacc., *G. graminis* var. *tritici* J. Walker (JO-1), *Macrophomina phaseolina* (Tassi) Goidaninch, *Neurospora crassa* Shear & Dodge, *Pythium aphanidermatum* (Edson) Fitzp., *P. ultimum* Trow, *Septoria nodorum* (Berk.) Berk., *S. andropogonis* J. J. Davis, and *Stemphylium* sp. were provided by W. Bockus, R. Bowden, J. Leslie, W. Pfender, F. Schwenk, and D. Stuteville, Kansas State University, Manhattan. Isolates of *O. herpotricha* (KS3, KS27, and KS70), *Rhizoctonia cerealis* Van der Hoeven (KS116), *R. solani* Kühn AG-4 (KS124), *G. incrustans* Landschoot & Jackson (KS13 and KS69), and *Colletotrichum* sp. were isolated from turfgrass in Kansas by the first author. All fungal isolates were maintained on potato-dextrose agar at 25 C in the dark.

DNA isolation. Fungal isolates except *Pythium* spp. were grown in stationary plastic petri plates (8.5 cm diameter) that contained 15–20 ml of modified yeast extract broth (5 g of sucrose, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$-$7H_2O$, 0.5 g of KCl, 10 mg of $FeSO_4$-$7H_2O$, 1 g of yeast extract, and 0.2 g of asparagine per liter H_2O) at 25 C for 1–4 wk. Pythium isolates were grown in potato-carrot broth (broth extracted after boiling 40 g of potato and 40 g of carrot in 1 L H_2O). Mycelium of each isolate was harvested onto sterile filter paper in a Buchner funnel, transferred immediately to 50-ml plastic centrifuge tubes, and stored at −80 C.

DNA was purified by a procedure similar to that used by Saghai-Maroof et al (13). Frozen mycelium was ground to a fine powder with dry ice in an electric coffee grinder or in liquid nitrogen with a mortar and pestle. Samples were then suspended in 2× CTAB buffer (2× CTAB = 1.4 M NaCl, 2% hexadecyltriethylammoniumbromide (Sigma Chemical Co., St. Louis, MO), 1% β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0) and heated to 65 C for 30 min in a water bath. After extraction with chloroform/isoamyl alcohol (24:1), 1:200 vol of RNase solution (10 mg/ml) was added to the aqueous phase, which was incubated for 30 min at 37 C. After a second chloroform/isoamyl alcohol extraction, the DNA was precipitated by the addition of 0.8 vol of isopropanol. DNAs were washed in 70% ethanol at room temperature and resuspended in TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA).

Only highly purified genomic or plasmid DNA samples were used in the copy number reconstruction experiments (see below). For these experiments, genomic DNAs extracted by the above procedures or plasmid preparations made by standard alkaline lysis protocols were further purified on cesium chloride density gradients (14).

DNA digests and gel blot hybridization. Restriction enzymes, purchased from Bethesda Research Labs (BRL, Gaithersburg, MD) or Promega (Madison, WI), were used in a fivefold excess under conditions specified by the manufacturer. Genomic DNAs (2–6 μg/lane) were digested with the appropriate restriction enzymes and fractionated on 0.8% agarose gels. The DNA was transferred to an MSI blotting membrane (Micron Separations Inc., Westboro, MA) by capillary action and incubated for 3 h at 65 C in prehybridization solution (5× SSC, 1% sodium dodecyl sulfate, 50 mM sodium phosphate, pH 6.5, 10 mM Tris, pH 7.0, 10 mg/ml of herring sperm DNA; 1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). ^{32}P-labeled probes were added to the prehybridization solution and the blots were incubated an additional 12–24 h at 65 C. Cloned DNA fragments to be used as probes were isolated from vector sequences by separation on agarose gels, cutting the cloned sequences from the gel and purifying the DNA by the freeze-squeeze method (16). Cloned DNA fragments were labeled with ^{32}P by the random primer (14) or nick translation (12) methods. After hybridization, membranes were washed for 2 h with four rinses of 0.1× SSC, 0.2% sodium dodecyl sulfate solution at 65 C before autoradiographic exposure.

Genomic copy number reconstructions. By comparing the intensity with which one microgram of genomic DNA of *L. korrae* and known amounts of cloned fragments hybridized to labeled DNA of the same clone, the approximate number of copies that hybridized to the cloned fragments were estimated in the genome. A genome size of 5×10^7 base pairs was assumed for *L. korrae*; this is approximately the size of the *N. crassa* genome (11). If 40 copies of a 1.3-kb sequence exist in the genome of *L. korrae*, this sequence accounts for 52 kb of DNA or approximately 0.1% of the total genome. In this case, labeled DNA from a clone of the 1.3-kb sequence should hybridize with similar intensity to 1 μg of total *L. korrae* genomic DNA as it does to 0.001 μg of the cloned sequence itself. Hybridization intensities were visually compared by probing slot blots carrying genomic DNA (1 μg) and various amounts of cloned DNA (pLK66 or pLK88). One microgram of maize carrier DNA was added to samples of cloned DNA fragments so that each slot on the slot blot carried approximately 1 μg of DNA. One slot carried 1 μg of *L. korrae* DNA. One microgram of maize DNA was included in a separate slot as a negative control. All DNA samples were denatured by adding 100 μl of 0.4 M NaOH and were incubated at room temperature for 15 min. The samples were neutralized by adding 100 μl of 2.0 M ammonium acetate, pH 7.0, and transferred to a MSI membrane with a Bio-Slot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA). Membranes were baked at 80 C in a vacuum oven for 2 h before hybridization. Reconstruction experiments were repeated twice.

Cloning of DNA fragments. DNA from *L. korrae* was digested to completion with the restriction enzyme *Eco*RI and was size

fractionated by electrophoresis on low melting temperature agarose (Fisher Scientific, Pittsburgh, PA). Intensely stained bands of DNA that migrated at 1.1 and 1.3 kb were cut from the gel. The DNA was recovered by melting the agarose followed by extraction with phenol and chloroform and by precipitation (12). DNA fragments were ligated into the *Eco*RI-cut pUC18 plasmid vector and used to transform the *Escherichia coli* strain DH5α by the procedure of Hanahan (6). Forty recombinant clones from each of the two ligations with the 1.1- and 1.3-kb fractions were examined for the presence of high copy sequences. Individual plasmid DNAs were cut with *Eco*RI, fractionated on agarose gels, transferred to nylon membranes, and probed with labeled genomic DNA from *L. korrae*. Only sequences with multiple copies in the genome gave strong hybridization signals.

Detection of *L. korrae* in turfgrass roots. Arizona common bermudagrass and Kentucky bluegrass cultivar Park were seeded (about 0.1 g/pot) into 6- × 25-cm plastic pots (McConkey Co., Sumner, WA) that contained a steamed soil/perlite/peat mixture (1:1:1 v/v). Bermudagrass was inoculated 3–4 mo after seeding with 2 g of sterile oats or oats infested with an isolate of *L. korrae* or *O. herpotricha* (18). Kentucky bluegrass was inoculated with *L. korrae* only. Pots were placed in the greenhouse and were watered and fertilized as needed. Plants were incubated for about 90 days, then washed with water to remove soil from roots. Visible discoloration of roots was noted on all plants inoculated with *L. korrae* and *O. herpotricha*. Infected root tissue (200–400 mg) was frozen in 1.5 ml-microfuge tubes by the addition of liquid nitrogen and ground with a steel rod made by rounding the tip of a Phillips screwdriver with a grinder. Ground samples were either stored frozen or processed immediately. Frozen samples were suspended in 600 μl of 2× CTAB buffer, extracted twice with chloroform, and precipitated by the addition of 0.8 vol of isopropanol. DNA was resuspended in 40 μl of TE buffer. Twenty microliters from each sample were transferred to nylon membranes with the slot blot apparatus and were hybridized with labeled DNA from the cloned fragments as previously described. DNA hybridization experiments were repeated four times.

Sensitivity of the DNA probes was determined by hybridization to serial dilutions of total DNA extracted from 0.05 g of lyophilized mycelium of *L. korrae*. The extraction procedure was the same as that used for infected root material. Dilutions of the total DNA extracted were made in TE buffer to correspond with mycelial dry weights of 1,000, 100, 10, 1, and 0.1 μg. Samples were transferred to membranes by using the slot blot apparatus as previously described and were probed with DNA clones from *L. korrae*. The sensitivity assay was repeated once.

RESULTS

Comparison of isolates of *L. korrae* by gel electrophoresis. Total DNA from 37 North American isolates and one Australian isolate of *L. korrae* was digested with the *Eco*RI restriction enzyme, fractionated by agarose gel electrophoresis, and stained with ethidium bromide. Intensely stained bands of DNA that corresponded to sizes of 1.1, 1.3, and 2.4 kb were noted for all 38 *L. korrae* isolates tested (Fig. 1). Several other less intensely stained bands of various sizes were also detected and were consistent among isolates. The banding pattern of *Eco*RI-digested DNA of *L. korrae* was unique and could easily be differentiated from *Eco*RI digests of total DNA of 26 other fungal species by agarose gel electrophoresis (data not shown).

Identification of clones. DNA fragments, from the 1.1- and 1.3-kb bands noted on agarose gels of *Eco*RI-digested total DNA of *L. korrae*, were cloned into the multicopy vector pUC18. Ten of the 80 recombinant plasmids tested, six of which contained 1.1-kb inserts and four 1.3-kb inserts, showed strong hybridization to DNA of *L. korrae*. The four 1.3-kb inserts were distinct clones (i.e., they did not cross-hybridize with one another in dot blot tests). Only three of six 1.1-kb inserts hybridized to one another. Furthermore, none of the 1.1-kb DNA inserts hybridized to the 1.3-kb inserts. Nevertheless, inserts of the same size (e.g., all 1.1-kb inserts) gave similar hybridization patterns to *Eco*RI DNA digests of *L. korrae*.

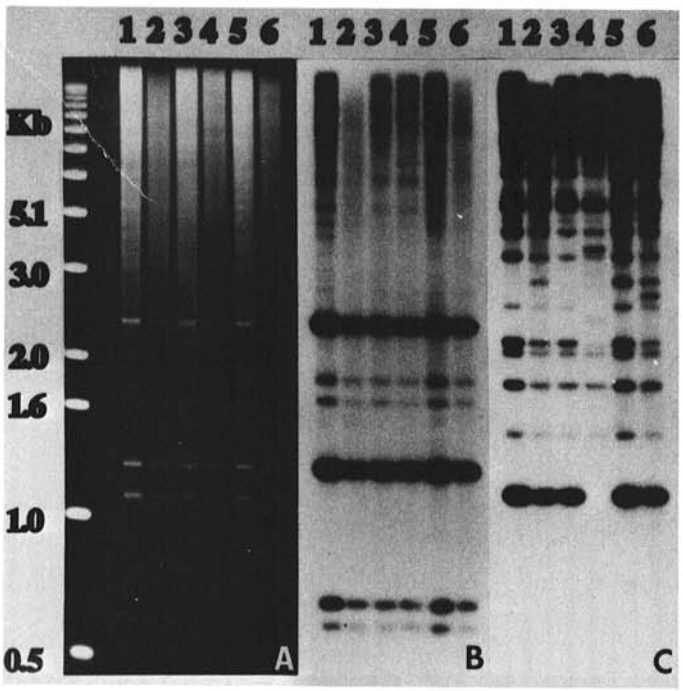

Fig 1. A, Agarose gel electrophoresis of *Eco*RI digests of total DNA from six representative isolates of *Leptosphaeria korrae*. Thirty-two additional isolates (not shown) had identical banding patterns. Lanes 1–6 are DNA from isolates WA-99, WA-126, WA-8, CO-236, Idaho-Henderson, and WI (not numbered). Autoradiographs of identical DNA blots after hybridization with ^{32}P-labeled inserts from clones **B,** pLK66 (1.3 kb) and **C,** pLK88 (1.1 kb).

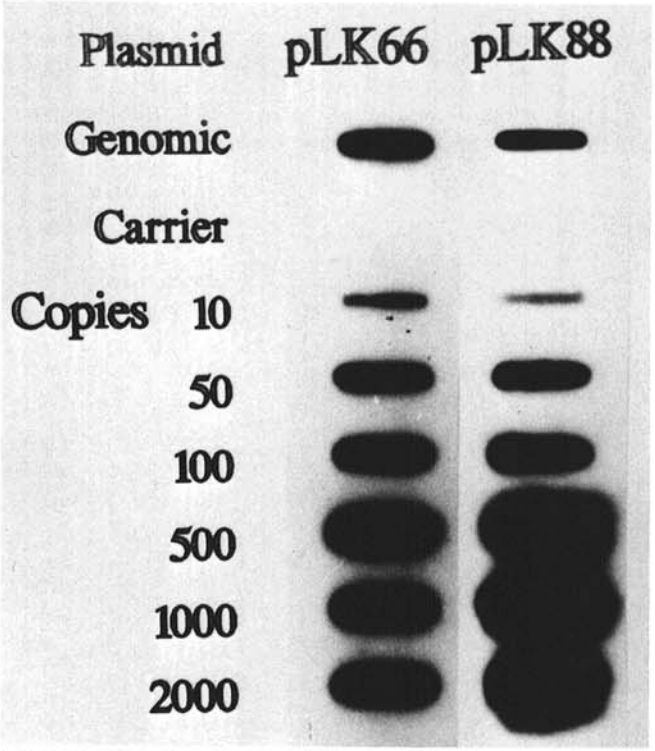

Fig 2. Estimation of sequence copy number of inserts from pLK66 and pLK88 in the genome of *Leptosphaeria korrae* by reconstruction analysis. Hybridization intensities were compared by probing slot blots carrying genomic DNA and various amounts of cloned DNA (10–1,000 copies) (pLK66 or pLK88). One microgram of maize carrier DNA was added to samples of cloned DNA fragments so that each slot carried approximately 1 μg of DNA.

Two plasmids, pLK88 and pLK66, that contained 1.1- and 1.3-kb inserts respectively, were selected for further study based on their apparent specificity and strong hybridization to DNA of *L. korrae*. The clone pLK66 hybridized strongly to DNA in the 1.3- and 2.4-kb bands, and to a lesser extent other fragment sizes of *Eco*RI-digested genomic DNA of *L. korrae* isolates (Fig. 1), but not to DNA in the 1.1-kb band. Hybridization patterns among 26 isolates of *L. korrae* were identical (not all 38 isolates were tested). The clone pLK88 also hybridized strongly to multiple-sized DNA fragments of all 26 isolates tested, but in a pattern distinct from pLK66 (Fig. 1). The insert hybridized strongly to the 1.1-kb band (except CO-236 and CO-701), but not to the 1.3- or 2.4-kb bands. Other differences in the hybridization pattern were also detected among isolates.

Genomic copy number reconstructions. An estimate of the sequence copy number represented by the inserts in pLK66 and pLK88 was obtained from quantitative filter hybridization experiments. We determined that approximately 50–100 copies of the pLK66 insert, and 10–50 copies of the pLK88 insert were present within the genome of *L. korrae* (Fig. 2).

Specificity of probes to *L. korrae*. Specificity of pLK66 and pLK88 was determined by hybridization to *Eco*RI-digested total DNA of *L. korrae* and 26 other fungal species. The clone pLK66 hybridized intensely to multiple-sized DNA fragments of *L. korrae*, slightly to large-sized DNA fragments of *O. herpotricha*, and not at all to the other fungal species tested (Fig. 3). Clone pLK88 did not hybridize to DNA of any fungal species tested except *L. korrae* (Fig. 3).

The ability of pLK66 and pLK88 to detect *L. korrae* in diseased plants was tested by isolating total DNA from Kentucky bluegrass and bermudagrass roots colonized by the fungus, and probing with the DNA clones in a slot blot hybridization test. Because slight hybridization of pLK66 to *O. herpotricha* was previously detected in Southern blot analysis, DNA from bermudagrass roots colonized by *O. herpotricha* was also included in the tests. pLK66 and pLK88 did not hybridize to DNA from noninfected bermudagrass or Kentucky bluegrass roots, but did hybridize to DNA from root samples of both turfgrasses colonized by *L. korrae* (Fig. 4). pLK66 also hybridized to total DNA of bermudagrass roots colonized by *O. herpotricha*, whereas the insert from pLK88 did not.

In sensitivity assays, pLK88 hybridized to DNA equivalent to that found in 1 μg of lyophilized mycelium. The intensity of hybridization was similar to 1 ng of purified DNA from the same isolate of *L. korrae*.

DISCUSSION

Isolates of *L. korrae* can be definitively identified by a unique banding pattern of *Eco*RI-digested DNA fractionated by gel electrophoresis. This technique may be useful in identifying isolates that do not produce ascocarps in culture or in differentiating cultures of *L. korrae* from other fungal species with similar morphology and growth habits. Nevertheless, the practical use of this technique for rapid diagnosis of diseased turf samples is limited because of the length of time required to isolate and grow the fungus for DNA extraction.

DNA probes have been successfully used to differentiate fungal species in taxonomically complex genera (4,5,8) and for detection of plant pathogens in host tissue (3). We have cloned a 1.1-kb DNA fragment (pLK88) that can be used to differentiate isolates of *L. korrae* from morphologically similar species of ectotrophic fungi that also cause patch diseases of turfgrasses. Furthermore, pLK88 can be used to detect *L. korrae* in artificially infected plant tissue. Preliminary testing (N. A. Tisserat and S. H. Hulbert, data not shown) indicates pLK88 will also detect *L. korrae* in naturally infected roots of Kentucky bluegrass (samples courtesy of W. Shane, Ohio State University, Columbus). Extensive testing of naturally infected bermudagrass and Kentucky bluegrass has been limited because *L. korrae* has not been isolated from any turfgrass patch disease in Kansas.

Southern blot and reconstruction analysis indicated that both pLK66 and pLK88 occurred in multiple copies (10–100) within the genome of *L. korrae*. DNA probes that recognize multiple copy sequences should increase sensitivity in plant assays over single copy probes (8). Interestingly, the 1.1- and 1.3-kb bands observed in *Eco*RI digests of *L. korrae* are not made up of homogeneous DNA fragments. Instead, each band is composed of DNA fragments of similar size but apparently variable sequence. Only three of six high copy DNA fragments cloned

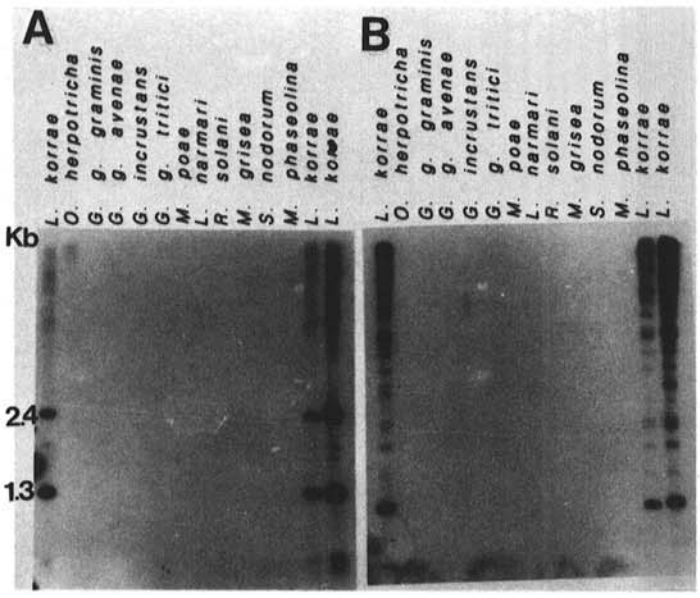

Fig. 3. Autoradiographs of identical DNA blots after hybridization with ^{32}P-labeled inserts from clones (**A**) pLK66 (1.3 kb) and (**B**) pLK88 to total DNA of *Leptosphaeria korrae* and 11 other fungal species. pLK66 and pLK88 did not hybridize to DNA of 15 additional fungal species (not shown) in dot blot hybridization tests.

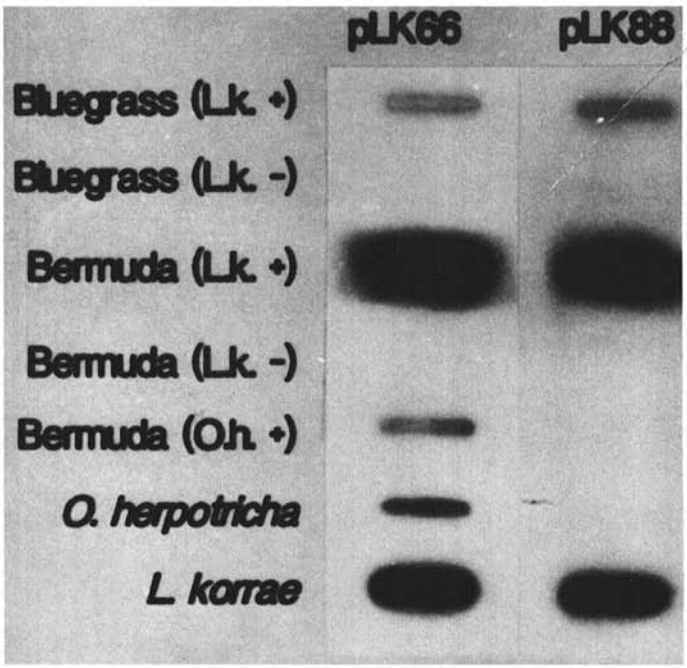

Fig 4. Slot hybridization of pLK66 and pLK88 to fungal and plant DNA extracted from 200 mg (wet weight) of Kentucky bluegrass and bermudagrass root samples. Bluegrass (Lk +) and Bermudagrass (Lk +) = turfgrass roots colonized by *Leptosphaeria korrae*; Bluegrass (Lk −) and Bermudagrass (Lk −) = noninfected turfgrass roots; and Bermudagrass (Oh +) = bermudagrass roots colonized by *Ophiosphaerella herpotricha*. Bottom slots represent the degree of hybridization associated with 1 μg of DNA from *O. herpotricha* and *L. korrae*.

from the 1.1-kb band and none of four clones from the 1.3-kb band hybridized to one another. It is possible that certain isolates of *L. korrae* may not contain a 1.1-kb fragment homologous to pLK88. This may explain the lack of hybridization of pLK88 to the 1.1-kb DNA band of isolate CO-236 of *L. korrae* (Fig. 3). Nevertheless, pLK88 did hybridize to other, larger-sized DNA fragments of this isolate as well as to total, undigested DNA of all 38 isolates of *L. korrae* tested in dot blot hybridization tests (data not shown). Therefore, polymorphisms among isolates should not affect the usefulness of this probe in detecting *L. korrae*. The apparent abundance of distinct multiple copy sequences suggests that DNA from these "repetitive families" could account for a significant portion of the total genome of *L. korrae*.

The use of DNA probes for diagnostics offers advantages in specificity and sensitivity. In our studies, pLK88 hybridized to DNA of *L. korrae* extracted from 200 mg wet weight of Kentucky bluegrass and bermudagrass roots colonized by the fungus and from 1 μg of lyophilized mycelium. This sensitivity is similar to the monoclonal antibody ELISA test for *L. korrae* developed by Nameth et al (10). The sensitivity of the hybridization assay might be enhanced by improving DNA extraction techniques or by concentrating DNA from larger sized samples. Furthermore, sensitivity may be increased by nucleotide sequencing of the insert from pLK88, developing oligonucleotide primers, and amplifying complementary DNA sequences in root samples by polymerase chain reaction techniques.

Etiological studies of turfgrass patch diseases have been hampered by the inability to identify the sometimes numerous, nonsporulating isolates of ectotrophic fungi isolated from diseased roots. DNA hybridization techniques, such as the use of pLK88 to identify *L. korrae*, offer a new tool for diagnosis and ecological and epidemiological studies of patch diseases. Identification of DNA probes specific to other species of ectotrophic fungi, and development of nonradioactive labeling techniques will be useful in diagnosing patch diseases of turfgrasses.

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