Identification of *Magnaporthe poae* by PCR and Examination of Its Relationship to Other Fungi by Analysis of Their Nuclear rDNA ITS-1 Regions

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ABSTRACT

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Magnaporthe poae is the causal agent of summer patch disease in turfgrasses. Identification of this heterothallic, root-infecting fungus has been difficult because of the lack of diagnostic structures in nature and the time required for the fungus to produce perithecia in culture. Potential probes for the identification of *M. poae* were obtained by producing a partial genomic library of *M. poae* isolate 73-15 in the pGEM3Zf$^+$ plasmid vector and subsequently screening cloned DNA for strong hybridization to 73-15 isolate genomic DNA. Using Southern blot analysis, a 2.7-kb DNA clone (pMp2-7) that hybridized to six confirmed isolates of *M. poae* from NJ, PA, NY, and RI was identified. Except for one isolate of *Colletotrichum graminicola*, the probe did not hybridize to 42 other fungal isolates that commonly inhabit the turfgrass environment. Oligonucleotides that would prime amplification of a 453-nucleotide (nt) fragment from *M. poae* DNA, but not from *C. graminicola* DNA, were developed based on sequence from one end of pMp2-7. These oligonucleotides also primed amplification of a similar-sized fragment of one isolate of *M. rhizophila* (PREM 45952) DNA. DNA from another isolate of *M. rhizophila* (Mr-2 from PA) did not amplify using these primers. To examine the relationship of *M. poae* to other fungal species, the internal transcribed spacer region (ITS-1) of nuclear ribosomal DNA for members of the *Magnaporthe* genus and several other fungi was sequenced. Sequences of three isolates of *M. poae*, identified through mating, and isolate PREM 45952 were very similar, having only five variable sites of 236. Isolate Mr-2 had seven sites variable to the *M. poae* isolates, two that were identical to variable sites in PREM 45952. Using maximum parsimony analysis, the *M. rhizophila* and *M. poae* isolates grouped in 75% of bootstrap replications. *M. rhizophila* isolates formed a monophyletic group away from the *M. poae* isolates in four of five equally parsimonious trees, but this grouping was not supported by more than 50% of bootstrap replications.

The heterothallic ascomycete, *Magnaporthe poae* Landschoot & Jackson, was identified in 1989 as the primary causal agent of summer patch, a devastating disease of turfgrasses in North America (14). This disease is most prevalent on hard fescue (*Festuca longifolia* Thuill.), blue fescue (*F. glauca* Lam.), Kentucky bluegrass (*Poa pratensis* L.), and annual bluegrass (*P. annua* L.) in late summer when temperatures exceed 28°C during the day and night temperatures remain above 20°C (20). Foliar symptoms include characteristic rings and crescent patterns of blighted turf that may coalesce into large necrotic areas. In the later stages of infection, resistant grasses and weeds may recolonize the centers of infected patches, resulting in a "frog-eye" or "doughnut-shaped" patch. Summer patch has been reported primarily in temperate regions of the United States (19). Because *M. poae* can infect turfgrass roots 6 to 8 weeks prior to the development of visual foliar symptoms (20), a rapid and definitive method for the detection of this pathogen from infected roots could enable turfgrass managers to initiate control measures prior to symptom expression.

Current diagnostic procedures for summer patch are based on visual symptom expression, host identification, environmental parameters, and the presence of darkly pigmented, ectotrophic runner hyphae on turfgrass roots and leaf sheaths. Definitive diagnosis can only be attained, however, by producing the teleomorph of *M. poae* in vitro. This may be accomplished by isolating the fungus from infected tissues and pairing suspected isolates with known mating types of *M. poae* on surface-sterilized wheat seedlings (12). Unfortunately, perithecia may take several months to form in culture. Because of the time required to obtain a positive identification of *M. poae* using conventional laboratory procedures, patch areas in the field may continue to expand until environmental conditions become less favorable for disease. The disease also may be misdiagnosed by turf managers, resulting in excessive fungicide usage and poor disease control.

Polymerase chain reaction (PCR) amplification can be used to identify specific DNA fragments from very small amounts of template DNA, and has been used to identify other fungal pathogens that infect members of the *Poaceae* family (6,8,17). Because of the great sensitivity of PCR and because the amount of fungal tissue at the site of infection is quite small, PCR would be preferable to Southern blot analysis or dot blots as a detection method. Therefore, one of our objectives was to develop an identification procedure using sequence-specific PCR to give a timely, definitive disease diagnosis.

Our second objective was to address the phylogenetic relationship of *M. poae* to other *Magnaporthe* spp. and to fungi that have similar growth or pathogenic characteristics. This was important

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because species of the *Magnaporthe* genus are biologically diverse and include foliar pathogens (*M. grisea* (Hebert) Barr and *M. salvinii* (Cattaneo) R. Krause & R. K. Webster) and root-infecting pathogens (*M. poae* and *M. rhizophila* Scott & Deacon). In addition, *M. poae* grows similarly to species in the genus *Gaeumannomyces* in that they have a dark, ectotrophic, root-infecting habit. Bryan et al. (3) used rDNA spacer regions for phylogenetic analysis of *Gaeumannomyces* and similar fungi, but no *Magnaporthe* spp. were included. DNA sequences such as the ribosomal RNA genes are used frequently to study phylogenetic relationships among organisms (23), and could, in this case, either support the current taxonomy of these species or suggest a need for taxonomic reevaluation. The noncoding spacer regions of this locus offer the most variation, since they evolve most rapidly (23), and could be most informative for closely related species. To better understand the taxonomic placement of *M. poae* relative to other *Magnaporthe* members and other similar fungi, we analyzed sequences of the nuclear rDNA first internal transcribed spacer region (ITS-1).

MATERIALS AND METHODS

Fungal isolate cultures and mating. Fungal isolates (Tables 1 and 2) were grown on potato-dextrose agar (PDA; Difco Laboratories, Detroit) and stored on cellulose filters at –80°C (22) or in a skim milk/glycerol solution maintained in liquid nitrogen (4). Except for isolate Ken-1, *M. poae* isolates listed in Table 1 were isolated from turf in NY, PA, RI, and NJ by P. Landschoot and have been maintained in culture since 1989. The two *M. rhizophila* isolates from South Africa (PREM 45953 and PPRI 4757) were obtained from a source in North America for this study. Isolate Ken-1 was identified from a turf sample in 1992 using the P_1/P_2 primers. Development of the teleomorph by the traditional wheat seedling method confirmed its identity. Mating of Ken-1 was also successful on oatmeal agar (OMA). Prior to 1992, *M. poae* isolates were recycled through wheat seedlings in the greenhouse on a yearly basis. Since 1992, cultures of *M. poae* have been stored on cellulose filters at –80°C. DNA from *M. grisea* isolates Pg15022 and Pg74 was supplied by J. Correll. Tables 1 and 2 list the source, location, and host of isolates used in the PCR and Southern blot analysis.

The identification of *M. poae* isolates was confirmed by mating individual isolates with ATCC tester isolates 64411 and 64412 on wheat seedlings according to Landschoot (12). Additional mating studies were performed by pairing opposite mating types on full-strength oatmeal agar (Difco Laboratories) in the absence of wheat seedlings. Plates were stored at room temperature in normal laboratory lighting. Perithecia were observed in a minimum of 20 days.

DNA extraction. Approximately 1 g of mycelial tissue was obtained by growing the isolate on a thin disk of cellophane (Flexel, Covington, IN) placed on a PDA plate. DNA was extracted from the mycelium following the method described by Hillman et al. (9) with these alterations: after the phenol/chloroform extraction step, the aqueous phase was extracted once more in phenol/chloroform/isoamyl alcohol (25:24:1) and once in chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated using 0.1 volume of 3 M sodium acetate and 2.5 to 3 volumes of ethanol. After resuspension in distilled water and RNase digestion, the DNA was precipitated with polyethylene glycol and washed in 70% ethanol before final resuspension in distilled water.

For small-scale extractions, 1.5 to 50 mg of tissue was ground in a microfuge tube in liquid nitrogen and 600 µl of extraction buffer was added. This mixture was then extracted in one volume phenol/chloroform/isoamyl alcohol (25:24:1), followed by extraction with one volume of chloroform/isoamyl alcohol (24:1). Total nucleic acid was precipitated with 0.1 volume of 3 M sodium acetate and three volumes of 95% ethanol. After collecting the precipitate by centrifugation, the pellet was washed with 70% ethanol and resuspended in distilled water.

Cloning and screening of *M. poae* DNA libraries. A partial genomic library was made of *M. poae* DNA isolate 73-15 in the plasmid vector pGEM3Zf$^+$ (Promega Corp., Madison, WI). DNA was digested with *Bam*HI and fractionated in an agarose gel. DNA fractions were extracted from the agarose using Geneclean (Bio 101, Inc., La Jolla, CA). The fraction representing DNA sized 3 to 5 kb was ligated in the plasmid vector. Screening of clones was performed by randomly labeling total genomic DNA from isolate 73-15 with $\alpha[^{32}P]$-dATP and hybridizing to plasmid DNA spotted to Zetaprobe membrane (Bio-Rad Laboratories, Hercules, CA).

Southern blots were performed by separating approximately 5 µg of *Bam*HI-digested DNA through a 1% agarose gel and blotting to Zetaprobe nylon membrane (Bio-Rad Laboratories) using a pressure transfer system (Stratagene Inc., La Jolla, CA). The clone pMp2-7 was $[^{32}P]$-labeled by a random primer labeling kit (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). Blots were treated according to the Zetaprobe protocol (Bio-Rad Laboratories). The fungal isolates tested are presented in Tables 1 and 2.

A total genomic library was prepared in the phage vector λ/Fix II (Stratagene Inc.). Genomic DNA of isolate 73-15 was partially digested with *Sau*3AI. DNA was then ligated to prepared phage arms and collected according to manufacturer protocols. Average insert size was 9 to 12 kb. Approximately 8,000 clones were collected representing about 97% of the genome.

PCR primers and reactions. Sequencing of pMp2-7 was initiated from the universal forward and reverse direction primers using the Sequenase kit (U.S. Biochemical, Cleveland). Primers were obtained from the DNA Synthesis Network Laboratory at the Uni-

TABLE 1. The 34 isolates of *Magnaporthe poae* recovered from *Poa* spp. and evaluated in Southern blot analysis and polymerase chain reaction (PCR)

Isolate	Source/location	Southern blot analysis	PCR analysis
73-15	ATCC[a] 64411	+	+
73-1	ATCC 64412	NT[b]	+
Cra A-1	West Windsor, NJ	+	+
Bal A-7	Springfield, NJ	NT	+
Ken-1	Lexington, KY	NT	+
Nav A-3	Middletown, NJ	NT	+
Nav A-5	Middletown, NJ	NT	+
Med A-8	Medford Lakes, NJ	NT	+
Oak A-5	Ambler, PA	+	+
OT A-2	Ambler, PA	NT	+
OT A-3	Ambler, PA	NT	+
OT A-5	Ambler, PA	+	+
OT A-12	Ambler, PA	NT	+
Pit A-2	Pittsburgh, PA	+	+
Pit A-11	Pittsburgh, PA	+	+
Rid A-7	Paramus, NJ	NT	+
Rid A-9	Paramus, NJ	NT	+
Roc A-2	Sparkhill, NJ	NT	+
Roc A-10	Sparkhill, NJ	NT	+
San A-6	Oreland, PA	NT	+
San A-7	Oreland, PA	NT	+
Sil A-1	Spring Lake Heights, NJ	NT	+
Sil A-6	Spring Lake Heights, NJ	+	+
Std A-4	Wayne, PA	NT	+
Std A-7	Wayne, PA	NT	+
Wil A-1	Morristown, NJ	NT	+
Wil A-2	Morristown, NJ	+	+
Wil A-3	Morristown, NJ	NT	+
Wil A-6	Morristown, NJ	NT	+
Wil A-8	Morristown, NJ	NT	+
Wil 1-1	Morristown, NJ	NT	+

[a] ATCC = American Type Culture Collection, Rockville, MD.
[b] NT = not tested.

versity of Medicine and Dentistry of New Jersey and Ransom Hill Bioscience (Ramona, CA). Sequences of the two oligonucleotides used for identification were: P_1 = 5′-ATATTATTCCCATCACCG-3′ and P_2 = 5′-TGTTCAAGCTGTCGATGT-3′. Amplification of the 453-nucleotide (nt) fragment was achieved in reactions containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 160 nM dNTPs, 250 nM each primer, 1.5 units of Perkin-Elmer Cetus (Foster City, CA) *Taq* polymerase/50 µl of reaction, and approximately 250 ng of template DNA. Reactions were heated to 95 to 100°C for 3 min without the polymerase, which was added during the denaturation step of the first cycle. The cycle conditions were 95°C for 1 min, 55°C for 2 min, 75°C for 3 min, and cycled 30 times with a final step for 5 min at 75°C.

ITS-1 sequencing and analysis. The ITS-1 region of the nuclear ribosomal DNA was amplified using the ITS-1 and -2 primers described by White et al. (23). These primers were obtained from L. Lee (Rutgers University, New Brunswick, NJ). Amplified DNA was then cloned into the pGEM-T vector (Promega Corp.), transformed into *E. coli* INV cells (Invitrogen, San Diego, CA), and sequenced as above. Sequences of cloned ITS-1 regions for the three isolates of *M. rhizophila* (Mr-2, PPRI 4757, and PREM 45953) were obtained by cloning the ITS-1 PCR products into vector pGEM-T and purifying the DNA using Quiagentips (Quiagen, Inc., Chatsworth, CA). Cycle sequencing reactions were performed using Prism dye-terminator cycle sequence ready reaction kit (Applied Biosystems, Inc., Foster City, CA). Reactions were run on a 373A DNA sequencer (Applied Biosystems, Inc.) following the removal of unincorporated dye using Centrisep columns (Princeton Separations, Adelphia, NJ). Sequences were aligned using the CLUSTAL W (7) program and analyzed by branch-and-bound parsimony analysis using PAUP (21). Support for the trees was evaluated by bootstrap analyses.

The fungal sequences analyzed represented *M. poae*, *M. rhizophila*, *M. grisea*, *M. salvinii*, *Gaeumannomyces incrustans* Landschoot & Jackson, *G. graminis* (Sacc.) Arx & D. Olivier var. *graminis*, *Colletotrichum graminicola* (Ces.) G. W. Wils. (teleomorph *Glomerella graminicola* Politis), and *Leptosphaeria korrae* Walker & Smith.

RESULTS

Selectivity of the DNA probe. Following the fractionation of DNA from *M. poae* isolate 73-15, fractions representing sizes 1 to 2 kb, 3 to 5 kb, and 6 to 8 kb were cloned into pGEM3Zf+. DNA from 150 clones was screened using ^{32}P-labeled genomic DNA from isolate 73-15. A strongly hybridizing clone with an insert of

TABLE 2. The 42 non-*Magnaporthe poae* isolates evaluated in Southern blot analysis and polymerase chain reaction (PCR)

Isolate	Host	Source/location	Southern blot analysis	PCR analysis
Acremonium starii (Lf3)	*Festuca rubra*	C. R. Funk, NJ	NT[a]	–
Acremonium starii (St3)	*Festuca ovina*	C. R. Funk, NJ	NT	–
Acremonium typhinum (blue3)	*Festuca rubra*	C. R. Funk, NJ	NT	–
Acremonium typhinum (1211)	*Festuca rubra*	C. R. Funk, NJ	NT	–
Alternaria alternata	*Malus* sp.	T. Joaquim, ADA[b]	–	–
Bipolaris sorokiniana	*Festuca* sp.	T. Joaquim, ADA	–	–
Cladosporium cladosporioides	Soil	T. Joaquim, ADA	–	–
Colletotrichum acutatum	Fruit	T. Joaquim, ADA	NT	–
Colletotrichum fragariae	*Fragaria* sp.	T. Joaquim, ADA	NT	–
Colletotrichum gloeosporioides	*Fragaria* sp.	T. Joaquim, ADA	NT	–
Colletotrichum graminicola (1)	*Poa annua*	T. Joaquim, ADA	+	–
Colletotrichum graminicola (2)	*Poa annua*	T. Joaquim, ADA	NT	–
Colletotrichum graminicola (6)	*Poa annua*	T. Joaquim, ADA	NT	–
Colletotrichum graminicola (8)	*Poa annua*	T. Joaquim, ADA	NT	–
Cryphonectria parasitica	*Castanea dentata*	B. Hillman, NJ	–	–
Curvularia lunata	Unknown	T. Joaquim, ADA	–	–
Curvularia lunata	*Oryza sativa*	Texas	–	–
Epicoccum nigrum	*Triticum aestivum*	T. Joaquim, ADA	–	–
Fusarium culmorum	Unknown	T. Joaquim, ADA	NT	–
Gaeumannomyces incrustans (Kan)	*Cynodon dactylon*	ATCC[c] 64417	–	–
Gaeumannomyces incrustans (PR)	*Poa annua*	ATCC 64416	–	–
Gaeumannomyces incrustans (Zoy5)	*Zoysia* sp.	P. Landschoot, IL	–	–
Gaeumannomyces graminis var. *graminis* (FL104)	*Cynodon dactylon*	M. Elliott, FL	NT	–
Gaeumannomyces graminis var. *graminis* (FL180)	*Cynodon dactylon*	M. Elliott, FL	NT	–
Laetisaria fuciformis	*Poa pratensis*	T. Joaquim, ADA	–	–
Leptosphaeria korrae	*Poa pratensis*	T. Joaquim, ADA	NT	–
Leptosphaerulina sp.	*Lolium perrene*	T. Joaquim, ADA	–	–
Magnaporthe grisea (IG-1)	*Oryza sativa*	I. Raskin	NT	–
Magnaporthe rhizophila	*Triticum aestivum*	PREM 45952	NT	+
Magnaporthe rhizophila (Mr-2))	*Poa pratensis*	P. Landschoot, PA	NT	–
Magnaporthe rhizophila	*Setaria italica*	PREM 45953	NT	–
Magnaporthe rhizophila	*Setaria* sp.	PPRI 4757	NT	–
Magnaporthe salvinii	*Oryza sativa*	ATCC 44754	NT	–
Microdochium (Gerlachia) *nivale*	*Agrostis palustris*	T. Joaquim, ADA	–	–
Mortierella epigama	Compost	T. Joaquim, ADA	–	–
Penicillium sp.	*Vitis* sp.	T. Joaquim, ADA	–	–
Pyricularia grisea (15022)	*Digitaria sanguinalis*	ATCC 15022	NT	–
Pyricularia grisea (74)	*Digitaria sanguinalis*	J. Correll, AK	NT	–
Pythium irregulare	Unknown	T. Joaquim, ADA	–	–
Rhizoctonia cerealis	*Zoysia* sp.	T. Joaquim, ADA	–	–
Rhizoctonia solani	Turf	T. Joaquim, ADA	–	–
Rhizoctonia zeae	*Festuca arundinacea*	T. Joaquim, ADA	–	–
Sclerotinia homoeocarpa	Turf	T. Joaquim, ADA	–	–
Typhula incarnata	Turf	T. Joaquim, ADA	–	–

[a] NT = not tested.
[b] ADA = Agri-Diagnostics Associates, Cinnaminson, NJ.
[c] ATCC = American Type Culture Collection, Rockville, MD.

approximately 3 kb, pMp2-7, was selected to check for cross-hybridization to other fungi encountered in the turfgrass and soil environment. Southern blot analysis using various *Bam*HI-digested DNAs revealed that pMp2-7 hybridized to a single fragment of DNA from all of the *M. poae* isolates tested and to a larger *Bam*HI fragment of one isolate of *C. graminicola* (Fig. 1; Tables 1 and 2).

Sensitivity and selectivity of the P_1 and P_2 amplification. Two primers were designed to amplify a 453-nt fragment of *M. poae* DNA by PCR. All 31 *M. poae* isolates tested (Table 1), as well as one isolate of *M. rhizophila* (Fig. 2B), resulted in amplification of a fragment of the expected size. DNA from *M. rhizophila* isolate Mr-2 did not amplify using the P_1/P_2 primers. The isolate of *C. graminicola* that tested positive in Southern blot analysis did not produce a detectable 0.45-kb product. Amplification of as little as 0.2 ng (11.5 pmol) of 73-15 DNA could be detected in an ethidium bromide-stained agarose gel (Fig. 2A).

Although several different methods for extracting total DNA from infected roots were evaluated, no 0.45-kb fragment was amplified. Moreover, adding 1 µl of this root/fungal DNA mix to a 100-µl positive control PCR reaction inhibited amplification.

To examine the origin of clone pMp2-7, its nucleotide sequence was determined. Figure 3 represents pMp2-7 with restriction sites mapped for *Ava*I, *Dde*I, *Hae*II, *Sac*I, *Sty*I, and *Xmn*I. Complete sequencing of pMp2-7 revealed an insert length of 2,748 bp, and one apparent large open reading frame (ORF) capable of encoding a protein of 271 amino acid residues with a predicted molecular mass of 30.3 kDa. A possible upstream promoter region was also identified. The putative ORF contained a slightly hydrophobic N-terminus and a hydrophyllic, proline-rich internal region. No proteins in the database showed convincing homology to spans of greater than 40 amino acids (30% identities). The clone also contained regions of repetitive DNA encoding lengths of repeated amino acids in all reading frames. None of these repeats were part of any putative ORFs. The putative, complete ORF and the primers designed for PCR identification of *M. poae* are indicated in Figure 3.

Analysis of ITS-1 sequences. Sequences of the ITS-1 region of the nuclear rDNA of the 13 fungal isolates, including the ITS-1 and -2 primer sequences and the adjacent 5.8S sequence, varied in length from 202 to 244 residues. Isolates obtained as *M. rhizophila* PPRI 4757 and PREM 45953 were initially included in alignment and maximum parsimony analyses, but were omitted from the reported study because of their questionable identity. PREM 45953 had very different morphological characteristics from other *M. rhizophila* isolates and neither produced perithecia readily on OMA or PDA with cellophane. Both exhibited very different ITS-1 sequences from the *Magnaporthe* spp. Clustal W

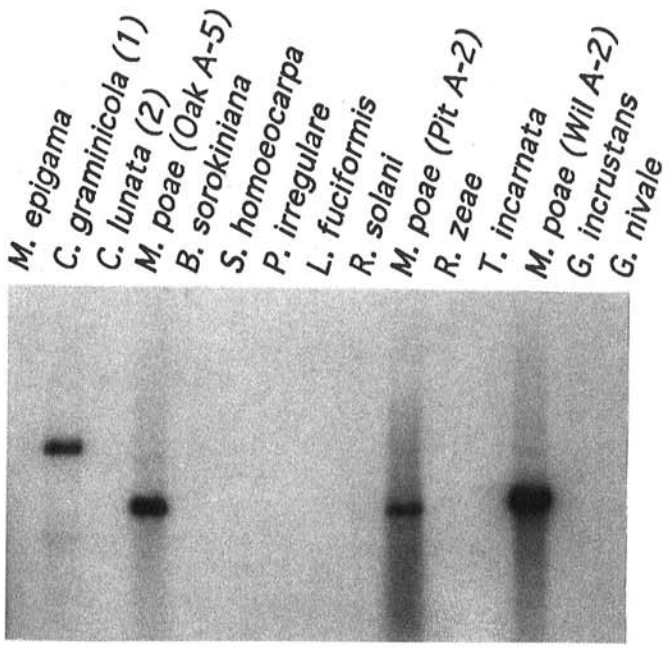

Fig. 1. Southern blot analysis of various fungi using the ^{32}P-labeled clone, pMp2-7, from *Magnaporthe poae* isolate 73-15.

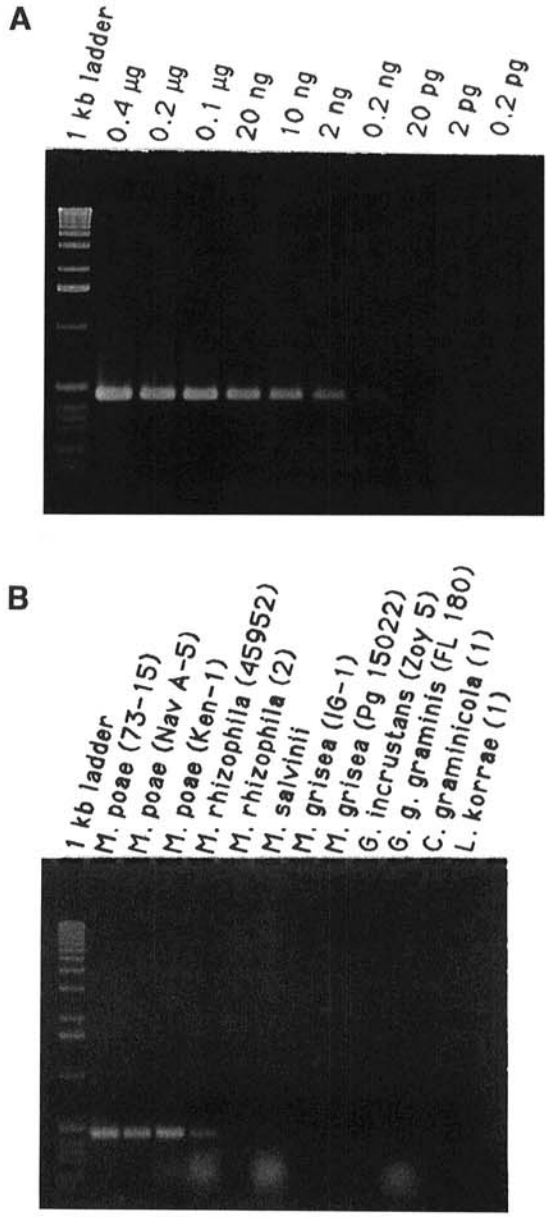

Fig. 2. Polymerase chain reaction (PCR) amplification using the primers P_1 and P_2 described in Materials and Methods. **A,** Amplification of *Magnaporthe poae* isolate 73-15 DNA using varying amounts of template. Quantities are given in nanograms of isolated DNA. **B,** Products of amplification reactions for several fungi listed in Tables 1 and 2.

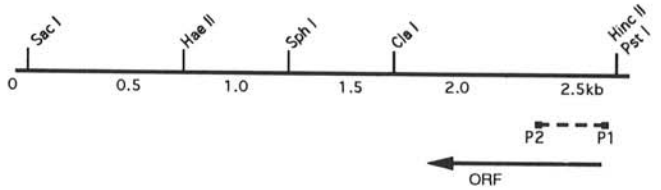

Fig. 3. Schematic representation of the full clone (pMp2-7) depicting some restriction sites, the location of the putative complete open reading frame, and the placement of the designed primers (P_1 and P_2).

alignments of all sequences were 250 characters long (Fig. 4A). Of the 250 characters, 152 were variable, of which 48 were informative. *M. poae* (Ken-1) had one character difference from the other two *M. poae* isolates (73-15 and Nav A-5), which were identical. *M. rhizophila* (PREM 45952) had five variable sites from the *M. poae* isolates (73-15 and Nav A-5). The differences were at three locations and could be explained as two substitutions and a deletion (Fig. 4A). *M. rhizophila* (Mr-2) had seven character differences from the 73-15 and Nav A-5 isolates, two of which were identical to the two substitutions described for PREM

A

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M.poae 73-15          GGGATCATTACAGAGTTGCAAAACTCCAACCCCT-GTGAACCTTACCTTTACTGTTG-CTTCGGCG
M.poae NavA-5         .................................-....................-........
M.poae Ken-1          .................................-....................-........
M.rhizophila 45952    .................................-....................-......--
M.rhizophila 2        .................................-....................-........
M.salvinii            ................A................-...T............TC....-........
M.grisea IG-1         ..........T.....A................-......A.A....C.GTC....-........
M.grisea 15022        ..........C.....A................-......A.A....C.GTC....-........
G.incrustans Zoy5     ..........A.....................-......T..............-........
G.g.graminis FL104    ..........A.....A................-........-.............-........
G.g.graminis FL180    ................A................-........-............A........
C.graminicola Cog2    ..........TC.....A.CGCT..A.....T.T......A.--A.C.A.......-........
L.korrae 1            A...........CGA.A.T.C.GGC..C.AGTGCA.CAC.AAC.GTA.GGG.G.G.TATG..TATT

73-15       G-----------------------CGCCCC-------------GTCGCCGCCGGAGGTTCAAAACCCTGAATT
NavA-5      .-----------------------......-------------.............................
Ken-1       .-----------------------......-------------.............................A....
M.r. 45952   ------------------------.....-------------.C..............T.............
M.r. 2      .------------------------.....--------------.C.............TGG...........
M.salvinii  .AGG---------------------A.T...CCTGGGGGAGGTC.C.TG.C-T......CAC....T.......
IG-1        .-------------------------..A.----------------GC..................T..T.T..
15022       .-------------------------..T.----------------GC............AC....T..TGTG.
Zoy5        .TTGG--------------CGCCGGT....AGATGGGCCTGGAG......CG.C.GA....G............
FL104       .AATA--------------T-----G.....-------CCGGCC.A..T..-.C.GA...AC............
FL180       .ACGA--------------T-----G....---------AGGGC.A.....-........AC............
Cog2        .GCAGGGGGTCCCCGGGCCCGGCCC......CTACCAGGGACGT.GGC..........A.ACCC.A.TCT.T..
Lk-1        ACCCTTGTTTATTGAGTACCTATGTTT..TT-------GGTGGCT.GC.T...AA.A.GA..CCC.AT....CC

73-15       -TT-AGTGTATCTCTGAGTACGAAAAGAACCTGAAAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGC
NavA-5      -..-.....................................................................
Ken-1       -..-.....................................................................
M.r. 45952  -..-.....................................................................
M.r. 2      -..-.A.............T.....................................................
M.salvinii  -.AC......C.......G.AA....C..AT-----.....................................
IG-1        -..TCCA............CCT....GAC.A----.T...C.....................................
15022       A..TTTA.C.........CCTA...GAC.A----.T...C.....C.....................................
Zoy5        -C.-.....G........A.AAG.T.A...------...C.....................................
FL104       -..-.............C..A...CC..AT-----.....................................
FL180       -..T.............C..A....C..AT-----.....................................A
Cog2        -.AAC.AACG.T...TCTG.GTGGC.C..G.-A..T............T.....................-
Lk-1        -.ATTTAT.T..AA.C..CGTCTG..T...AAT..T.....C.....................................
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B

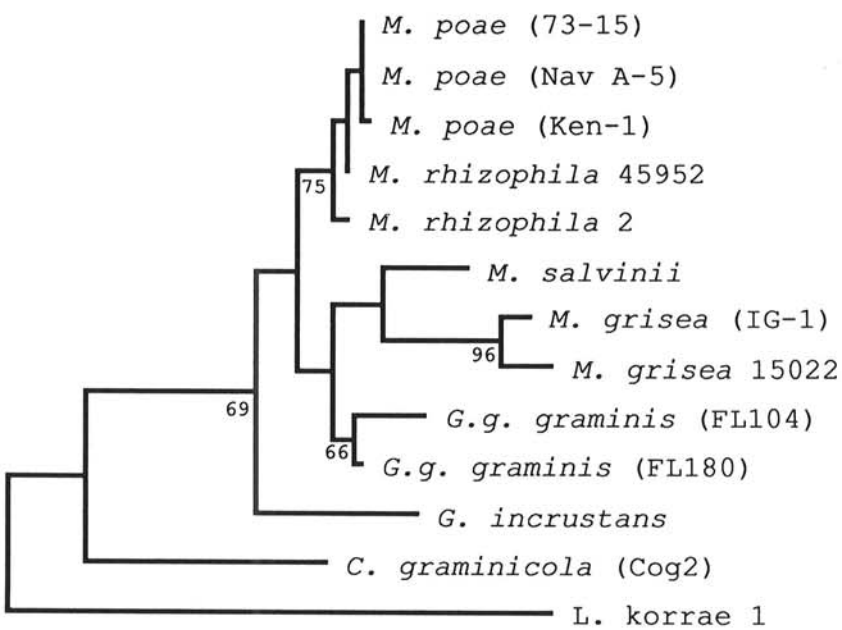

Fig. 4. A, Sequence alignment (nucleic acids 30-232) for all 13 isolates. **B,** Phylogram of one of the five trees resulting from the branch-and-bound analysis for all 13 sequences. *Leptosphaeria korrae* was designated as the outgroup. Numbers represent bootstrap replication percentages when the values were greater than 50%.

45952. These sites were the unique characters that supported the *M. rhizophila* clade.

Branch-and-bound analysis of all sequences using PAUP with *Leptosphaeria korrae* (Lk-1) as the outgroup resulted in five trees of 253 steps, consistency index = 0.87 and homopasy index = 0.13. Bootstrap values were noted on branches when greater than 50%. The median transition:transversion ratio of unambiguous changes for the five trees was 8:10.

The tree in Figure 4B is one of the five trees resulting from the PAUP branch-and-bound analysis. The other four trees differed in the arrangements of *M. poae*/*M. rhizophila* spp. and grouping of *G. graminis* var. *graminis* (FL104) with *G. graminis* var. *graminis* (FL180). Four of the five trees placed the *M. rhizophila* isolates in a monophyletic group. Two of the five trees grouped the *M. poae* isolates as a monophyletic group separate from the *M. rhizophila* group/individuals. Three of the five trees grouped isolates FL104 and FL180 together. Bootstrap values strongly supported the *M. grisea* clade (96%) and the *M. poae*/*M. rhizophila* clade (75%). From this analysis, the grouping of all of the *Magnaporthe* and *Gaeumannomyces* isolates away from the *Colletotrichum* and *Leptosphaeria* isolates was supported in 69% of the bootstrap replications.

DISCUSSION

Prior to this report, DNA or protein-based methods of detecting a variety of turfgrass pathogens, including *Pythium* spp., *Rhizoctonia solani* Kühn, *Sclerotinia homoeocarpa* F. T. Bennett, *G. graminis*, and *L. korrae* have been presented (6,8,17). Our report of a sequence-specific PCR method for identification of the summer patch pathogen, *M. poae*, expanded that list and provided an alternative to the more time-consuming, classical identification method (14). Diagnosis of summer patch may be confirmed through the presence of symptoms on a susceptible host during appropriate environmental conditions and amplification of fungal DNA using the primers we described. Recently, random amplified polymorphic DNA (RAPD) analysis has been used to examine populations within *M. poae* isolates (10). This technique can also be used to identify non-*M. poae* isolates, but requires a longer preparation time and is more difficult to interpret than the method described in this study. Although *M. rhizophila* DNA may, in some instances, be amplified by P_1 and P_2 to produce a product of the same size as *M. poae*, it has only once been reported in areas where summer patch is a problem (11,13). The Pennsylvanian isolate Mr-2, that did not amplify, is not believed to be pathogenic on the source plant, Kentucky bluegrass. The pathogenicity of the South African isolate PREM 45952 was not tested on turfgrass using a unsubcultured inoculum. It is important to note the potential amplification of nonpathogenic isolates of both *M. poae* and *M. rhizophila* in identification and use this information to only confirm the disease diagnosis.

Because of the presence of inhibitors to PCR in host tissue, we were unable to detect *M. poae* directly from root/fungal DNA extracts. We, therefore, isolated fungi before DNA extraction by plating the surface-sterilized roots on a semiselective medium (SM-7) (5) with a cellophane overlay. In 1 to 2 weeks, enough mycelium could be scraped for PCR identification. Although using nested primers may enhance specificity and sensitivity, problems with inhibition from turfgrass host tissues continue to exist in other systems using nested primers (6). For example, similar results have been reported by Henson et al. (8) for DNA extractions of turfgrasses. DNA extraction and purification methods to eliminate these inhibitors have thus far been unsuccessful.

Analysis of the physical characters used to describe *Magnaporthe* spp. taxonomically reveals basic similarities and fundamental sexual differences. *M. poae* and *M. rhizophila* have similar ascospores and perithecial shapes (14), and both will infect and cause vascular discoloration of wheat roots (15,18). *M. rhizophila* is distinct from *M. poae* in that it is homothallic (*M. poae* is heterothallic) and its ascospores are, on average, shorter (26 µm) than those of *M. poae* (30 µm) (14). Moreover, *M. rhizophila* enters its sexual phase much more readily than *M. poae*. *M. rhizophila* takes 2 to 3 weeks to produce perithecia, whereas *M. poae* requires several months (T. E. Bunting, *personal communication*).

The ITS-1 region provides a highly variable sequence that does not allow for useful comparisons between distantly related organisms (2,23). The various fungi were included to give perspective to the *M. poae*/*M. rhizophila* analysis. Sequence alignments indicated the ITS-1 region was very conserved for *M. poae* and *M. rhizophila* isolates. Greater variability can be seen within the *M. grisea* spp. as isolates IG-1 and Pg15022 differ at 12 sites. Although there is clearly a sexual distinction between the *M. poae* and *M. rhizophila*, the sequence analysis presented in our study provided evidence supporting their close relationship.

In parsimony analysis, only two characters supported a distinct *M. rhizophila* clade. Stronger support for a *M. rhizophila* clade or a conspecific *M. rhizophila*/*M. poae* relationship requires additional *M. rhizophila* isolates and more sequence data. This analysis can also help to explain the amplification of *M. rhizophila* (PREM 45952) DNA using primers P_1 and P_2. Similarities between these species at ITS-1 and detection amplification loci suggested that they may have only recently diverged. In addition, they may or may not still have sufficient genetic similarity to successfully mate, had their mating phenotype not deviated. Bruns et al. (1) demonstrated that phylogenetic distance and the degree of morphological differences do not always correlate. A recent study analyzing the ribosomal DNA sequences of *Penicillium* spp. and *Talaromyces* spp. concluded that the imperfect *Penicillium* spp. have evolved amongst the perfect *Talaromyces* spp. (16). This suggests that the genes controlling sporulation and absence of the perfect state can evolve rapidly and independently within a genus. We believe that the different mating systems of *M. poae* and *M. rhizophila* may also involve an evolutionarily rapid change, supported by their genomic similarities.

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