Detection of *Gaeumannomyces graminis* Varieties Using Polymerase Chain Reaction with Variety-Specific Primers

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ABSTRACT

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The polymerase chain reaction (PCR) was used for detection of *Gaeumannomyces graminis*, the causal agent of take-all disease in wheat, oats, and turfgrass. NS5 and NS6 universal primers amplified the middle region of 18S ribosomal DNA of *Gaeumannomyces* species and varieties. Primers GGT-RP (5′ TGCAATGGCTTCGTGAA 3′) and GGA-RP (5′ TTTGTGTGTGAC CATAC 3′) were developed by sequence analysis of cloned NS5-NS6 fragments. The primer pair NS5:GGT-RP amplified a single 410-bp fragment from isolates of *G. graminis* var. *tritici*, a single 300-bp fragment from isolates of *G. graminis* var. *avenae*, and no amplification products from isolates of *G. graminis* var. *graminis* or other species of *Gaeumannomyces*. The primer pair NS5:GGA-RP amplified a single 400-bp fragment from isolates of varieties *tritici* and *avenae*. Two sets of primer pairs (NS5:GGT-RP and NS5:GGA-RP) were used in PCR reactions to detect and identify the varieties *tritici* and *avenae* either colonizing wheat, oats, or grass roots, or in culture. No amplification products were observed using DNA extracted from plants infected with eight other soilborne fungal pathogens or from uninoculated plants.

Gaeumannomyces graminis (Sacc.) Arx & D. Olivier, a filamentous soilborne fungus, parasitizes the roots and crowns of susceptible members of the *Gramineae* (23,25). *Gaeumannomyces graminis* var. *tritici* J. Walker is the etiologic agent of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) take-all disease, a major root disease that limits yield in many areas of the world (25,26). *Gaeumannomyces graminis* var. *avenae* (E.M. Turner) Dennis can cause take-all of oats (*Avena sativa* L.) and take-all patch of bentgrass (*Agrostis palustris* Huds.), and occasionally is isolated from barley and wheat. *Gaeumannomyces graminis* var. *graminis* infects and colonizes wheat, but in contrast to varieties *tritici* or *avenae*, it causes little take-all. However, *G. graminis* var. *graminis* causes Bermudagrass (*Cynodon dactylon* (L.) Pers.) decline (5), spring dead spot (15), take-all root rot of St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) (6,13,31), crown sheath rot of rice (*Oryza sativa* L.) (26), root rot of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) (28), and root rot of zoysiagrass (*Zoysia japonica* Steudel) (30).

Diagnosis of take-all disease is generally based on visual symptoms, host identification, predisposing environmental conditions, and the presence of darkly pigmented, ectotrophic runner hyphae on plant roots and/or crowns. Identification of *G. graminis* as the possible etiological agent in diseased plants generally is attempted by culturing the isolated fungus in vitro, and then observing both the colony morphology and the teleomorphic state. The formation of perithecia in field-infected plants is rare, and in vivo formation of this teleomorph usually occurs in about half of the isolates cultured.

The polymerase chain reaction (PCR) assay is highly sensitive and reproducible as a tool for detection and identification of fungi when species-specific primers are carefully designed. This approach has been used to identify fungi, including *Fusarium* spp. (22), *G. graminis* (10), *Rhizoctonia oryzae* Ryker & Gooch (14), *Magnaporthe poae* Landschoot & Jackson (3), *Leptosphaeria korrae* J.C. Walker & A.M. Sm. (24), *Ophiosphaerella herpotricha* (Fr.:Fr.) J.C. Walker (24), *Phialophora gregata* (Allington & D.W. Chamberlain) W. Gams (4), and *Verticillium* spp. (16,18). Nuclear rDNA of fungi consists of the small and large subunits, a 5.8S region, and the internal transcribed spacer (ITS) region(s). Each subunit and region base sequence is variable among the genera and species of fungi (1). The ITS region of *Gaeumannomyces* is highly variable among its species (8) and less variable among its varieties (2). However, the small subunit of nuclear rDNA is distinctly variable among the varieties of *G. graminis* (8). The copy number of the rDNA repeat is more than 50 per genome, suggesting a possible high sensitivity for detection if used as a target gene for PCR. Thus, the small subunit of nuclear rDNA would be valuable for detection of *G. graminis* varieties using PCR amplification. In previous studies with *G. graminis*, oligonucleotide primer pairs were derived from clone pMSU315 of *G. graminis* (21) and from direct sequencing of the ITS region of *G. graminis* var. *tritici* and *G. graminis* var. *avenae* (2). These primer pairs amplified multiple-sized DNA fragments in *G. graminis* varieties, but *G. graminis* var. *graminis* could not be differentiated from other *G. graminis* varieties (*tritici* and *avenae*). In a previous report (8), we showed that the middle region (NS5-NS6 fragment) of the small subunit of nuclear rDNA was variable in size among the species and varieties of *G. graminis*. The objective of our research was to develop variety-specific primers from the middle region of the small subunit of nuclear rDNA (18S rDNA) for the identification and differentiation of *G. graminis* varieties infecting plant tissue.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Isolates of *G. graminis* var. *tritici*, *G. graminis* var. *graminis*, *G. graminis* var. *avenae*, *G. incrustans*, *G. cylindrosporus*, and *G. leptosporus* used in this study are listed in Table 1. Cultures were maintained on one-fifth strength potato dextrose agar (PDA) (29) plates. To obtain mycelia for DNA extraction, the isolates were grown in GYP medium (glucose 2%, yeast extract 0.5%, and peptone 0.5%) (9), filtered, and ground in liquid nitrogen using a mortar and pestle. DNA was extracted according to the methods used by Murray and Thompson (17).

PCR. Primers NS5 and NS6 (27) were used to amplify part of the small subunit of nuclear rDNA (18S rDNA). Primers were synthesized at the Genetic Engineering Facility of the Biotechnology Center at the University of Illinois at Urbana-Champaign. Amplification reactions were made in a total volume of 50 µl containing: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.0 mM $MgCl_2$; 200 µM each of dATP, dCTP, dGTP, and dTTP; 0.4 µM each of the primers; 50 ng of genomic DNA; and 1.0 unit of *Taq* DNA polymerase (Promega Corporation, Madison, WI), under 60 µl of mineral oil (Sigma Chemical Co., St. Louis, MO). Negative controls, in which

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the genomic DNA template was replaced by water, were used in all experiments to test for contamination. Temperature cycling was carried out in a DNA thermal cycler (Gene Amp PCR 2400; Perkin-Elmer Cetus, Foster, CA). An initial denaturation step of 93°C for 3 min was followed by 35 amplification cycles of denaturation, annealing, and extension. Temperatures and times for these steps were 93°C for 1 min, 52°C for 1 min, and 72°C for 1 min, respectively. After 35 cycles were completed, the samples were incubated at 72°C for 5 min. Ten µl of the amplification products were separated by electrophoresis in 1.4% agarose gels, stained with ethidium bromide, and photographed under UV light. All assays were performed at least two times.

DNA sequencing and analysis. PCR products (called N5-N6 fragments) were purified using a QIAquick DNA purification kit (QIAGEN Inc., Valencia, CA). The purified N5-N6 fragments from isolates WF9039, WF9040 (*G. graminis* var. *tritici*); WF922, WF937 (*G. graminis* var. *avenae*); WF921, WF9238 (*G. graminis* var. *graminis*); WF914 (*G. incrustans*); WF912 (*G. cylindrosporus*); and WF9427 (*G. leptosporus*) were cloned using a TA Cloning Kit and pCRII vector (Invitrogen Corporation, Carlsbad, CA). PCR amplified products were sequenced using an ABI automated sequencer with fluorescent-dye-labeled dideoxy terminators at the Genetic Engineering Facility of the Biotechnology Center at the University of Illinois at Urbana-Champaign.

Selection and specificity of *G. graminis* var. *tritici*– and *G. graminis* var. *avenae*–specific primers. The DNA sequences were edited by SeqEd version 1.0.3 (Applied Biosystems Division, Foster, CA). Sequence alignments were done with the CLUSTAL V program (11). Two PCR reverse primers, GGT-RP and GGA-RP, were designed specifically for varieties *tritici* and *avenae* of *G. graminis* based on the sequence of the N5-N6 region of 18S rDNA. The design of the primers was aided by use of the software OLIGO, version 4.1 (National Bioscience, Plymouth, MN). Primer NS5 was used with each of the reverse primers (GGT-RP and GGA-RP) to assess their specificity among samples of *G. graminis* var. *tritici* and *G. graminis* var. *avenae*. PCR amplifications were conducted with DNA preparations from *Gaeumannomyces* from various sources (Table 1) and from other genera of fungi including: *Colletotrichum graminicola* (Ces.) Wilson, *Fusarium solani* (Mart.) Sacc., *F. graminearum* Schwabe, *Leptosphaeria korrae*, *Magnaporthe poae*, *Phialophora* sp., *Pythium ultimum* Trow, *Rhizoctonia solani* Kühn, *Sclerotinia homoeocarpa* F.T. Bennett, and *Trichoderma viride* Pers.:Fr.

Detection and identification of *G. graminis* var. *tritici* or *G. graminis* var.

Table 1. Isolates of *Gaeumannomyces* species and varieties used to screen the polymerase chain reaction primers (NS5:GGT-RP and NS5:GGA-RP)

Species Isolate	Host	Source (collector/state)	Primer GGT-RP[a]	Primer GGA-RP[b]
G. graminis var. *tritici*				
WF861	Wheat	Bockus/KS	+	+
WF9036 (GH7)	Wheat	Bockus/KS	+	+
WF9037 (RL-4)	Wheat	Bockus/KS	+	+
WF9039 (GH-90)	Wheat	Bockus/KS	+	+
WF9040 (P-L)	Wheat	Bockus/KS	+	+
WF9041 (STV-1)	Wheat	Bockus/KS	+	+
WF9419	Wheat	Bockus/KS	+	+
WF9420 (Jo-1)	Wheat	Bockus/KS	+	+
WF9421 (RL-287)	Wheat	Bockus/KS	+	+
WF9422	Wheat	Bockus/KS	+	+
WF9423 (TA-85)	Wheat	Bockus/KS	+	+
WF964 (550)	Wheat	Mathre/MT	+	+
WF965 (528)	Wheat	Mathre/MT	+	+
WF966 (558)	Wheat	Mathre/MT	+	+
WF967 (568)	Wheat	Mathre/MT	+	+
WF968 (698)	Wheat	Mathre/MT	+	+
WF969 (801)	Wheat	Mathre/MT	+	+
WF9610 (802)	Wheat	Mathre/MT	+	+
WF9611 (804)	Wheat	Mathre/MT	+	+
WF9634 (GGT-8)	Wheat	Huber/IN	+	+
WF9635 (GGT-11)	Wheat	Huber/IN	+	+
WF9637 (GGT-12)	Wheat	Huber/IN	+	+
G. graminis var. *avenae*				
WF9022	Bentgrass	Wilkinson/IL	+	+
WF9024	Bentgrass	Wilkinson/IL	+	+
WF9025	Bentgrass	Wilkinson/IL	+	+
WF9026	Bentgrass	Wilkinson/IL	+	+
WF9032	Bentgrass	Wilkinson/IL	+	+
WF922	Bentgrass	Jackson/RI	+	+
WF934	Bentgrass	Wilkinson/France	+	+
WF936	Bentgrass	Wilkinson/IL	+	+
WF937 (93BRWI)	Bentgrass	Wilkinson/WI	+	+
WF939 (93RHF)	Bentgrass	Wilkinson/IL	+	+
WF9310 (93-SOA)	Bentgrass	Wilkinson/IL	+	+
WF9311 (RL1)	Bentgrass	Wilkinson/IL	+	+
WF9312 (93GLK18G)	Bentgrass	Wilkinson/IL	+	+
WF9424 (255)	Bentgrass	Wilkinson/WA	+	+
WF9426 (260)	Bentgrass	Wilkinson/WA	+	+
WF9445 (POCO-4)	Bentgrass	Sanders/PA	+	+
WF9448	Bentgrass	Sanders/PA	+	+
WF9449 (FR- 1)	Bentgrass	Wilkinson/France	+	+
WF951 (OV-2)	Bentgrass	Wilkinson/IL	+	+
WF953 (NETC75)	Bentgrass	Wilkinson/IL	+	+
G. graminis var. *graminis*				
WF9014	Zoysiagrass	Wilkinson/IL	–	–
WF9236 (FL-177)	St. Augustine	Elliott/FL	–	–
WF9237 (FL-167)	Bermudagrass	Elliott/FL	–	–
WF9238 (FL- 199)	St. Augustine	Elliott/FL	–	–
WF9450	Bermudagrass	Wilkinson/FL	–	–
WF9451	Bermudagrass	Wilkinson/FL	–	–
WF9452 (FL-173)	Rice	Elliott/FL	–	–
WF9454 (FL-195)	St. Augustine	Elliott/FL	–	–
WF9455 (FL-198)	St. Augustine	Elliott/FL	–	–
WF9457 (FL-208)	Bermudagrass	Elliott /FL	–	–
WF9458	Bermudgrass	Wilkinson/FL	–	–
WF9462 (FL-221)	Rice	Elliott/FL	–	–
WF9463	St. Augustine	Wilkinson/CA	–	–
WF9469 (92-8186-2B)	Rice	Elliott/AR	–	–
WF9470 (TX-9 I - 1)	Rice	Elliott/TX	–	–
WF9471 (PPRI-4754)	Millet	South Africa	–	–
WF9472	Soybean	Australia	–	–
G. incrustans				
WF867	Zoysiagrass	Wilkinson/MO	–	–
WF914	Zoysiagrass	Wilkinson/KS	–	–
WF9341	St. Augustine	Wilkinson/FL	–	–
WF9342	Bermudagrass	Wilkinson/FL	–	–

(*continued on next page*)

[a] A single 410-bp product was amplified from *G. graminis* var. *tritici* isolates and a single 300-bp product was amplified from *G. graminis* var. *avenae* isolates.

[b] A single 400-bp product was amplified from isolates of *G. graminis* varieties *tritici* and *avenae*.

Table 1. (*continued*)

Species Isolate	Host	Source (collector/state)	Primer GGT-RP[a]	Primer GGA-RP[b]
G. incrustans (continued)				
WF9343	Bermudagrass	Wilkinson/FL	–	–
WF9344	Bermudagrass	Wilkinson/FL	–	–
WF9416	Zoysiagrass	Wilkinson/KS	–	–
WF9417	Zoysiagrass	Wilkinson/KS	–	–
WF9418	Zoysiagrass	Wilkinson/KS	–	–
WF9446	Annual bluegrass	Jackson/RI	–	–
WF9447	Annual bluegrass	Wilkinson/NY	–	–
G. cylindrosporus				
WF912	Wheat	Wilkinson/KS	–	–
G. leptosporus				
WF9427		(ATCC24161)	–	–
G. graminis-like isolates				
WF9023	bentgrass	Wilkinson/IL	+	+
WF9024	Bentgrass	Wilkinson/IL	+	+
WF9033	Bentgrass	Wilkinson/IL	+	+
WF9124	St. Augustine	Wilkinson/CA	–	–
WF9250	Wheat	Fouly/Egypt	–	–
WF932	Zoysiagrass	Wilkinson/IL	–	–
WF933	Bentgrass	Wilkinson/France	+	+
WF935	Bentgrass	Wilkinson/IL	+	+
WF938	Bentgrass	Wilkinson/IL	+	+
WF9435	Bermudagrass	Wilkinson/GA	–	–
WF9232	Bermudagrass	Elliott/FL	–	–
WF9465	Bermudagrass	Elliott/FL	–	–
WF9467	Bermudagrass	Elliott/FL	–	–
WF9468	Bermudagrass	Elliott/FL	–	–
WF9453	Bermudagrass	Elliott/FL	–	–
WF9456	Bermudagrass	Elliott/FL	–	–
WF9459	Bermudagrass	Elliott/FL	–	–
WF9460	Bermudagrass	Elliott/FL	–	–
WF9461	Bermudagrass	Elliott/FL	–	–
WF9464	Bermudagrass	Elliott/FL	–	–
WF9432	Centipedegrass	Wilkinson/GA	–	–

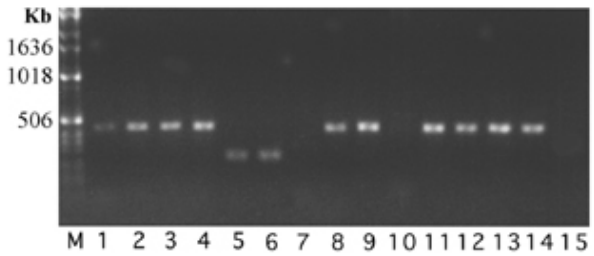

Fig. 1. Specific detection of *Gaeumannomyces graminis* var. *tritici* and *G. graminis* var. *avenae* isolates using polymerase chain reaction (PCR) with NS5:GGT-RP primer pair (lanes 1 to 7) and with NS5: GGA-RP primer pair (lanes 8 to 15). Lane M, 1 kb DNA ladder; lanes 1 to 4, 11, and 12 are *G. graminis* var. *tritici* isolates WF9039, WF9040, WF9419, WF9421, WF964, and WF969, respectively; lanes 5, 6, 8, 9, 13, and 14 are *G. graminis* var. *avenae* isolates WF9024, WF9026, WF922, WF934, WF936, and WF937, respectively; and lanes 7, 10, and 15 are *G. graminis* var. *graminis* isolates WF9014, WF9236, and WF9238, respectively.

***avenae* infecting wheat, oats, and turfgrass roots.** Both field-infected and inoculated wheat, oats, and bentgrass plants were used as potential sources of *Gaeumannomyces*. Plants were established in Cone-tainers (Ray Leach "Cone-tainers"; Stuewe & Sons, Inc., Corvallis, OR) using sterilized seed according to Wilkinson et al. (29). One 0.5 cm^2 piece of fungal culture grown on one-fifth PDA was placed on top of the vermiculite and covered with additional vermiculite prior to seeding. Each Cone-tainer received 10 ml of sterile distilled water and was incubated for 1 week prior to placing three to five surface-sterilized cereal or 200 bentgrass seeds on top of the vermiculite. The seeds were covered with 5 cm^3 of sterile vermiculite, and each Cone-tainer received 10 ml of sterile distilled water every other day for 30 days. The Cone-tainers were incubated in growth chambers at 15°C for 4 weeks. Whole plants were extracted from the Cone-tainers, and the roots were washed free of growth medium. Noninfected roots were recovered 4 weeks after planting from Cone-tainers not receiving the fungus. Upon sampling, both noninfected and infected roots were immediately frozen to –80°C. All field-infected plants displayed take-all symptoms and were collected in Illinois, Wisconsin, Kansas, Oregon, and Washington. Field infected plants were stored at –80°C.

DNA was extracted from plants by grinding root tissue (about 0.1 g) in liquid nitrogen and using a DNA extraction kit (QIAGEN). The extracted DNA was used in PCR assays with primer pairs NS5:GGT-RP or NS5:GGA-RP. PCR reactions and conditions for detecting *G. graminis* var. *tritici* and *G. graminis* var. *avenae* in plants were the same as described above. All experiments (except DNA isolations and sequencing reactions) were performed at least twice.

RESULTS

DNA sequences and design of *G. graminis* var. *tritici*– and *G. graminis* var. *avenae*–specific primers. The PCR assay with primers NS5 and NS6 amplified fragments from 280 to 1,100 bp for *Gaeumannomyces* species and varieties. Isolates of *G. graminis* var. *tritici* and *G. graminis* var. *avenae* had unique fragments of 627 and 607 bp, respectively. The nucleotide sequences of these two amplified fragments were similar, except for a 23-bp insertion in the amplified fragment of *G. graminis* var. *tritici* isolates. The insertion was located at base 384, downstream from the NS5 primer sequence. After multiple sequence alignment analysis of the N5-N6 fragments, the GGT- and GGA-specific primers that were used with the NS5 forward primer were designed with the following sequences: GGT-RP (5′ TGCAAT GGCTTCGTGAA 3′) and GGA-RP (5′ TTTGTGTGTGACCATAC 3′). The primer GGT-RP was selected from the 23-bp insertion sequence of *G. graminis* var. *tritici* isolates that was not present in *G. graminis* var. *avenae* isolates. The primer GGA-RP was selected from nucleotide sequences in *G. graminis* var. *avenae* isolates that had no homology with those of *G. graminis* var. *tritici* isolates and was located at base 367, downstream from the NS5 primer sequence.

Specificity of the primers GGT-RP and GGA-RP. A 410-bp fragment from isolates of *G. graminis* var. *tritici* and a 300-bp fragment from isolates of *G. graminis* var. *avenae* were amplified using the NS5 and GGT-RP primers. No fragments were amplified from purified DNA of *G. graminis* var. *graminis* isolates (Figs. 1 and 2A). Only a 400-bp fragment was amplified from isolates of *G. graminis* var. *tritici* and *G. graminis* var. *avenae* using the NS5 and GGA-RP primers (Figs. 1 and 2A). No fragments were amplified using the primer pairs NS5:GGT-RP or NS5:GGA-RP from purified DNA of the other *Gaeumannomyces* species tested (Fig. 2A) nor from any of the other 10 fungi tested (Fig. 2B).

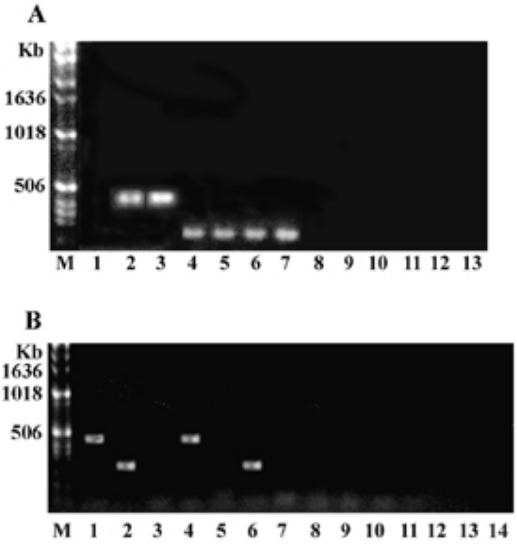

Fig. 2. Amplification of purified genomic DNA from different isolates of *Gaeumannomyces* species and varieties and other fungal species by polymerase chain reaction (PCR) with primers NS5:GGT-RP. **(A)** Lane M, 1 kb DNA ladder; lane 1, no DNA template; lanes 2 and 3, *G. graminis* var. *tritici* isolates WF965 and WF9610, respectively; lanes 4 to 7, *G. graminis* var. *avenae* isolates WF936, WF939, WF9312, and WF9424, respectively; lanes 8 and 9, *G. graminis* isolates WF9236 and WF9450, respectively; lanes 10 and 11, *G. incrustans* isolates WF914 and WF867, respectively; lane 12, *G. cylindrosporus* isolate WF912; lane 13, *G. leptosporus* isolate WF9427. **(B)** Lane M, 1 kb DNA ladder; lane 1, *G. graminis* var. *tritici* isolate WF969; lane 2, *G. graminis* var. *avenae* isolate WF9311; lane 3, *G. graminis* var. *graminis* isolate WF9451; lane 4, *G. graminis* var. *tritici* isolate WF9423; lane 5, *G. graminis* var. *graminis* isolate WF9455; lane 6, *G. graminis* var. *avenae* isolate WF9426; lane 7, *Sclerotinia homoeocarpa* isolate WF946; lanes 8 and 9, *Magnaporthe poae* isolates WF9119 and WF841, respectively; lane 10, *Fusarium solani*; lane 11, *F. graminearum*; lane 12, *Colletotrichum graminicola*; lane 13, *Rhizoctonia solani* isolate WF9222; and lane 14, *Trichoderma viride*.

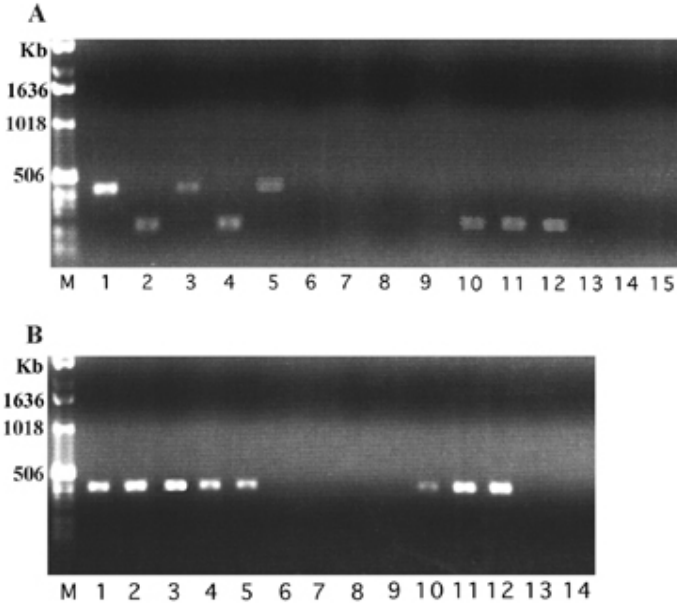

Fig. 3. (A) Polymerase chain reaction (PCR) amplification of DNA from plant tissue with primers NS5 and GGT-RP. Lane M, 1 kb DNA ladder; lanes 1, 3, and 5, wheat roots infected with *G. graminis* var. *tritici* isolates WF9039, WF9040, and WF9041, respectively; lanes 2 and 4, oat and bentgrass roots inoculated with *G. graminis* var. *avenae* isolates WF922 and WF937, respectively; lanes 10, 11, and 12, bentgrass roots naturally infected with *G. graminis* var. *avenae*; lanes 6 to 9, and 13 to 15, bentgrass roots inoculated with *G. graminis* var. *graminis* isolate WF9238, *Magnaporthe poae* isolate WF9119, *Phialophora* sp., *Sclerotinia homoeocarpa* isolate WF946, *Leptosphaeria korrae* isolate WF9674, *Rhizoctonia solani* isolate WF9222, and *Pythium ultimum*, respectively. **(B)** PCR amplification of DNA from plant tissue with primers NS5 and GGA-RP. Lane M, 1 kb DNA ladder; lanes 1 and 2, wheat roots infected with *G. graminis* var. *tritici* isolates WF9039 and WF9040, respectively; lanes 3 and 4, oat roots inoculated with *G. graminis* var. *avenae* isolates WF922 and WF937, respectively; lane 5, bentgrass roots inoculated with *G. graminis* var. *avenae* isolate WF937; lanes 6 to 9, 13, and 14, bentgrass roots inoculated with *G. graminis* var. *graminis* isolate WF9238, *M. poae* isolate WF9119, *Phialophora* sp., *S. homoeocarpa* isolate WF946, *L. korrae* isolate WF9674, and *R. solani* isolate WF9222, respectively; lanes 10 to 12, bentgrass roots naturally infected with *G. graminis* var. *avenae*.

Detection of *G. graminis* var. *tritici* and *G. graminis* var. *avenae* in infected wheat, oats, or turfgrass roots. *G. graminis* var. *tritici* and var. *avenae* were detected and identified from seedlings inoculated with these fungi. No amplification products were detected after PCR of DNA extracted from plants inoculated with *G. graminis* var. *graminis*, *G. incrustans*, *G. cylindrosporus*, *G. leptosporus*, *M. poae*, *L. korrae*, *S. homoeocarpa*, *R. solani*, *P. ultimum*, *F. solani*, *F. graminearum*, and *Phialophora* sp. The PCR assay with primer NS5 and either the GGT-RP or GGA-RP primer amplified no DNA fragments from noninoculated plants, although the DNA did act as a template for primers NS5 and NS6. A 410-bp fragment was amplified repeatedly from DNA extracted from wheat infected with *G. graminis* var. *tritici* when the NS5 and GGT-RP primer pair was used (Fig. 3A). A 300-bp fragment was amplified from DNA extracted from oats and bentgrass infected with *G. graminis* var. *avenae* when this primer pair was used (Fig. 3A). No DNA sequences extracted from root tissue inoculated with *G. graminis* var. *graminis*, *Phialophora* sp., *M. poae*, *L. korrae*, *S. homoeocarpa*, *R. solani*, *P. ultimum* (Fig. 3A), *G. incrustans*, *F. solani*, or *F. graminearum* were amplified when the NS5 and GGT-RP primer pair was used. The presence of *Gaeumannomyces* species and varieties in inoculated plants was confirmed by culturing them on selective medium MS-7 (10) and comparing culture morphology to that of the original culture used as inoculum. Only a 400-bp fragment was amplified from DNA extracted from root tissue infected with *G. graminis* var. *tritici* or *G. graminis* var. *avenae* when the NS5 and GGA-RP primers were used (Fig. 3B). No DNA sequences extracted from root tissue inoculated with *G. graminis* var. *graminis*, *Phialophora* sp., *M. poae*, *L. korrae*, *S. homoeocarpa*, *R. solani* (Fig. 3B), *P. ultimum*, *G. incrustans*, *F. solani*, or *F. graminearum* were amplified by PCR using these primers.

DISCUSSION

A new and definitive system for identifying and separating *G. graminis* vars. *tritici*, *avenae*, and *graminis* has been developed. Further, using specific primers for PCR amplification, it is now possible to identify and delineate the *G. graminis* varieties from field-infected tissue or cultures. In addition, these two sets of primer pairs (NS5:GGT-RP and NS5:GGA-RP) show no homology with *G. graminis* var. *graminis*, other species of *Gaeumannomyces*, or other tested fungi that are known pathogens of cereals and turfgrass. We suggest that this simple procedure can be extended to the investigation of fungi that are known to infect other grass species (7). The primers were identified from the N5-N6 region of 18S rDNA. Ribosomal DNA

(rDNA) provides a powerful tool for fungal identification and detection in plant tissue, because it is highly conserved and universally found in living cells (12). The ITS region was previously used to differentiate the species of *Gaeumannomyces* (2,8). Because the ITS region is highly variable among species of *Gaeumannomyces*, it is a useful tool to differentiate these species (8). The ITS region is less variable among the varieties of *G. graminis*, thus precluding its use as a tool for differentiation (8). For *Gaeumannomyces* varieties, using larger rDNA fragments (18S [32] and 28S [19]) is more practical than using the ITS region. The development of techniques for rapid and easy sequencing of large stretches of 18S and/or 28S rDNA facilitated the exploitation of these fragments for evaluating phylogenetic relationships and developing specific primers for species and subspecies identification (20).

In this study the specific primers GGT-RP and GGA-RP were used with the forward universal primer NS5 to (i) identify and differentiate isolates of *G. graminis* var. *tritici*, *G. graminis* var. *avenae*, and *G. graminis* var. *graminis*, and (ii) detect *G. graminis* var. *tritici* and *G. graminis* var. *avenae* infecting wheat, oats, or grasses.

This technique offers a major advantage over other published techniques. Bryan et al. (2) reported the development of a PCR assay and primers to detect *G. graminis* var. *tritici* and *G. graminis* var. *avenae* in artificially inoculated wheat and oats. The assay was tested on a small number of isolates, and the specificity of the primers was not established against *G. graminis* var. *graminis*, other soil fungi, or naturally infected plants. In addition, multiple-sized DNA fragments from *G. graminis* var. *tritici* were amplified using these primers (2). The reported use of nested primers for identifying *G. graminis* did not allow for differentiation among varieties (21).

The use of PCR to detect pathogens in plant tissue becomes more effective once inhibitors of PCR, which are contained in plant tissue, are removed (4,10,24). To address the presence of substances inhibitory to PCR in host tissue, we tried three methods for the isolation of DNA from infected plant tissue. Two of the methods included grinding root tissue in liquid nitrogen, followed by either suspending in CTAB buffer at 65°C for 15 min with subsequent phenol/chloroform extraction (24), or boiling ground root tissue in Tris-EDTA buffer (14). These methods resulted in DNA suspensions containing a brown substance(s), which could be phenol or lignin (4). No DNA fragments were amplified when these substances were present. The only method effective in obtaining DNA suitable for PCR was the QIAGEN DNA extraction method. This procedure was efficient in removing the PCR inhibitors from plant tissue and was cost-effective when used for large numbers of samples (~U.S. $1.50 per sample).

LITERATURE CITED

1. Apples, J. B., and Honeycutt, R. L. 1996. rDNA: Evolution over a billion years. Pages 81-135 in: DNA Systematics. Vol. 2. S. K. Dutter, ed. CRC Press, Boca Raton, FL.
2. Bryan, G. T., Daniels, M. J., and Osbourn, A. E. 1995. Comparison of fungi within the *Gaeumannomyces-Phialophora* complex by analysis of ribosomal DNA sequences. Appl. Environ. Microbiol. 61:681-689.
3. Bunting, T. E., Plumley, K. A., Clarke, B. B., and Hillman, B. I. 1996. Identification of *Magnaporthe poae* by PCR and examination of its relationship to other fungi by analysis of their nuclear rDNA ITS-1 regions. Phytopathology 86:398-404.
4. Chen, W., Gray, L. E., and Grau, C. R. 1996. Molecular differentiation of fungi associated with brown stem rot and detection of *Phialophora gregata* in resistant and susceptible soybean cultivars. Phytopathology 86:1140-1148.
5. Elliott, M. L. 1991. Determination of an etiological agent of Bermudagrass decline. Phytopathology 81:1380-1384.
6. Elliott, M. L., Hagan, A. K., and Mullen, J. M. 1993. Association of *Gaeumannomyces graminis* var. *graminis* with a St. Augustinegrass root rot disease. Plant Dis. 77:206-209.
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. Fouly, H. M., Wilkinson, H. T., and Chen, W. 1997. Restriction analysis of internal transcribed spacers and the small subunit gene of ribosomal DNA among four *Gaeumannomyces* species. Mycologia 89:590-597.
9. Gill, H. S., and Zentmeyer, G. A. 1978. Identification of *Phytophthora* species by disc electrophoresis. Phytopathology 68:163-167.
10. Henson, J. M., Goins, T., Grey, W., Mathre, D. E., and Elliott, M. L. 1993. Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. Phytopathology 83:283-287.
11. Higgins, D. G., Bleasby, A. J., and Fuchs, R. 1992. CLUSTAL V: Improved software for multiple sequence alignment. Comput. Appl. Biosci. 8:189-191.
12. Hori, H., and Osawa, S. 1987. Origin and evaluation of organisms as deduced from 5S ribosomal RNA sequences. Mol. Biol. Evol. 4:445-472.
13. Krausz, J. P. 1991. Take-all patch suspected in Texas warm-season turf. South. Turf Managmt. 2:12.
14. Mazzola, M., Wong, O. T., and Cook, R. J. 1996. Virulence of *Rhizoctonia oryzae* and *R. solani* AG-8 on wheat and detection of *R. oryzae* in plant tissues by PCR. Phytopathology 86:354-360.
15. McCarty, L. B., and Lucas, L. T. 1989. *Gaeumannomyces graminis* associated with spring dead spot of Bermudagrass in the southeastern United States. Plant Dis. 73:659-661.
16. Moukhamedov, R., Hu, X., Nazar, R. N., and Robb, J. 1994. Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84:256-259.
17. Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.
18. Nazar, R. N., Hu, X., Schmidt, J., Culham, D., and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. Physiol. Mol. Plant Pathol. 39:1-11.
19. Qu, T. H., Michot, B., and Bachellerie, J. P. 1983. Improved methods for structure probing in large RNAs: A rapid "heterologous" sequencing approach is coupled to the direct mapping of nuclease accessible sites. Application to the 5′ terminal domain of eukaryotic 28S rRNA. Nucleic Acids Res. 11:5903-5920.
20. Qu, T. H., Nicoloso, M., and Bachellerie, J. P. 1988. Phylogenetic calibration of the 5′ terminal domain of large rRNA achieved by determining twenty eukaryotic sequences. J. Mol. Evol. 28:113-124.
21. Schesser, K., Luder, A., and Henson, J. M. 1991. Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis*, in infected wheat plants. Appl. Environ. Microbiol. 57:553-556.
22. Schilling, A. G., Moller, E. M., and Geiger, H. H. 1996. Polymerase chain reaction-based assay for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86:515-522.
23. Smiley, R. W., Dernoeden, P. H., and Clarke, B. B. 1992. Compendium of Turfgrass Diseases. 2nd ed. American Phytopathological Society, St. Paul, MN.
24. 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.
25. Walker, J. 1980. *Gaeumannomyces*, *Linocarpon*, *Ophiobolus*, and several other genera of scolecospored Ascomycetes and *Phialophora* conidial states, with a note on hyphopodia. Mycotaxon 11:1-129.
26. Walker, J. 1981. Taxonomy of take-all fungi and related genera and species. Pages 15-74 in: Biology and Control of Take-all. M. J. C. Asher and P. J. Shipton, eds. Academic Press, London, UK.
27. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols, A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego.
28. Wilkinson, H. T. 1994. Root rot of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) caused by *Gaeumannomyces graminis* ((Sacc.) Arx & Olivier) var. *graminis*. Plant Dis. 78:1220.
29. Wilkinson, H. T., Cook, R. J., and Alldredge, J. R. 1985. Relation of inoculum size and concentration to infection of wheat roots by *Gaeumannomyces graminis* var. *tritici*. Phytopathology 75:98-103.
30. Wilkinson, H. T., and Kane, R. T. 1993. *Gaeumannomyces graminis* infecting zoysiagrass in Illinois. Plant Dis. 77:100.
31. Wilkinson, H. T., and Pedersen, D. 1993. *Gaeumannomyces graminis* var. *graminis* infecting St. Augustinegrass in southern California. Plant Dis. 77:536.
32. Woese, C. R., Stackebrandt, E., Macke, T. J., and Fox, G. E. 1985. Phylogenetic definition of the major eubacteria taxa. Syst. Appl. Microbiol. 6:143-151.