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Comparison of Fungi within the *Gaeumannomyces-Phialophora* Complex by Analysis of Ribosomal DNA Sequences

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Four ascomycete species of the genus *Gaeumannomyces* infect roots of monocotyledons. *Gaeumannomyces graminis* contains four varieties, var. *tritici*, var. *avenae*, var. *graminis*, and var. *maydis*. *G. graminis* varieties *tritici*, *avenae*, and *graminis* have *Phialophora*-like anamorphs and, together with the other *Gaeumannomyces* and *Phialophora* species found on cereal roots, constitute the *Gaeumannomyces-Phialophora* complex. Relatedness of a number of *Gaeumannomyces* and *Phialophora* isolates was assessed by comparison of DNA sequences of the 18S rRNA gene, the 5.8S rRNA gene, and the internal transcribed spacers (ITS). *G. graminis* var. *tritici*, *G. graminis* var. *avenae*, and *G. graminis* var. *graminis* isolates can be distinguished from each other by nucleotide sequence differences in the ITS regions. The *G. graminis* var. *tritici* isolates can be further subdivided into R and N isolates (correlating with ability [R] or inability [N] to infect rye). Phylogenetic analysis of the ITS regions of several oat-infecting *G. graminis* var. *tritici* isolates suggests that these isolates are actually more closely related to *G. graminis* var. *avenae*. The isolates of *Magnaporthe grisea* included in the analysis showed a surprising degree of relatedness to members of the *Gaeumannomyces-Phialophora* complex. *G. graminis* variety-specific oligonucleotide primers were used in PCRs to amplify DNA from cereal seedlings infected with *G. graminis* var. *tritici* or *G. graminis* var. *avenae*, and these should be valuable for sensitive detection of pathogenic isolates and for diagnosis of take-all.

Take-all, caused by the soil-borne pyrenomycete fungus *Gaeumannomyces graminis* (Sacc) Arx et Oliver (formerly *Ophiobolus graminis*), is the most damaging root disease of wheat worldwide and is among the most important cereal diseases in the United Kingdom. Wheat and barley are highly susceptible and are frequently affected by take-all when grown successively on the same land, where the fungus infects the roots and tiller bases. Rye and oats are generally more resistant (1). Four varieties of *G. graminis* have been described, var. *tritici*, var. *avenae*, var. *graminis* (1), and var. *maydis* (28).

Isolates of *G. graminis* var. *tritici*, while morphologically similar, can be subdivided into those that are substantially pathogenic to wheat but only slightly pathogenic to rye (termed N isolates) and those that are clearly pathogenic to both wheat and rye (termed R isolates) (9). The degree of adaptation to rye is a consistent characteristic of individual isolates. *G. graminis* var. *avenae* isolates are able to infect oats in addition to wheat and rye, while most *G. graminis* var. *tritici* isolates are unable to infect this host. In addition to causing oat take-all, *G. graminis* var. *avenae* is also the major cause of take-all patch disease of turf grasses. Both *G. graminis* var. *tritici* and *G. graminis* var. *avenae* are capable of infecting a wide range of wild grasses (24). *G. graminis* var. *graminis* is generally a benign parasite, infecting a number of grasses, including Bermuda grass (13), and one species of rice (18). It is usually nonpathogenic to wheat but can colonize wheat roots by means of ectotrophic hyphae and invade the outer cortical layers of the root. *G. graminis* var. *graminis* does not invade, destroy, or occlude vascular tissue as do the pathogenic varieties *G. graminis* var. *tritici* and *G. graminis* var. *avenae*. There is considerable interest in the use of weakly pathogenic or nonpathogenic fungi such as *G. graminis* var. *graminis* as biological control agents for other pathogenic fungi (24). In addition to host differences, *G. graminis* varieties *tritici*, *avenae*, and *graminis* are differentiated by ascospore size and hyphopodial structure (24).

Other species of *Gaeumannomyces* include *G. cylindrosporus*, *G. caricis*, and *G. incrustans*, which infect cereals and grasses, sedges, and turf grass, respectively (10, 12, 24), and are traditionally differentiated by ascospore morphology (24). *G. graminis* varieties *tritici*, *avenae*, and *graminis* have *Phialophora*-like anamorphs and together with other nonpathogenic *Gaeumannomyces* and *Phialophora* species found on cereal roots constitute the *Gaeumannomyces-Phialophora* complex (2, 7, 26). *G. graminis* var. *maydis* has only recently been characterized (28) and was not included in this study. *G. graminis* var. *avenae* and *G. graminis* var. *tritici* have *Phialophora* anamorphs with simple hyphopodia, while the *G. graminis* var. *graminis* anamorph has lobed hyphopodia. The *Phialophora* anamorph of *G. cylindrosporus* is believed to be *Phialophora graminicola*, which has simple or slightly lobed hyphopodia (24). The taxonomy of members of the *Gaeumannomyces-Phialophora* complex is confused because classification is based on complex or unreliable properties, such as colony morphology; the ability to form phialides, hyphopodia, or sexual fruiting bodies in culture; and pathogenicity to cereals and grasses (24).

Traditional methods for detection and identification of *Gaeumannomyces* isolates based on testing host range and examining morphological characteristics (2, 24) are time-consuming and can be inconclusive. In the past, *G. graminis* var. *tritici* has been blamed almost exclusively for losses in wheat yield, while the possible contribution of *G. graminis* var. *avenae* to disease has been neglected, since traditionally *G. graminis* var. *avenae* and *G. graminis* var. *tritici* are distinguishable only by the laborious methods described previously. Identification is also confused by the existence of isolates of *G. graminis* from Australia that are classified as *G. graminis* var. *tritici* on the basis of ascospore length (29) but which are able to infect oats.

These problems have encouraged a number of researchers to characterize *G. graminis* at the molecular level. Hybridiza-

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TABLE 1. Fungal strains and field isolates used in this study

Strain or isolate[a]	Original name	Host of origin	Geographical origin	Source or reference	GenBank accession no.
G. graminis var. *tritici* (N)					
T2	P081/143	Rye	Norfolk, United Kingdom	B. Hollins	U17221
T5	P082/220	Winter wheat	Avon, United Kingdom	B. Hollins	U17222
G. graminis var. *tritici* (R)					
R1	P080/124	Rye	Suffolk, United Kingdom	B. Hollins	U17219
R11	6.1	Wheat	Norfolk, United Kingdom	R. Musker	U17220
R17	P086/406	Oats	Sussex, United Kingdom	B. Hollins	U17220
G. graminis var. *tritici* (oat attacking)					
AT1	74014(274)	Oats	West Australia	29	U17208
AT2	7501(275)	Barley	West Australia	29	U17209
AT3	76002(283)	*Agrostis* sp.	New South Wales, Australia	29	U17210
G. graminis var. *avenae*					
A1	Hornby 61	Spring oats	Wales	D. Hornby	U17206
A3	Gg178			D. Hornby	U17207
G. graminis var. *graminis*					
G1	2033		United States	J. Henson	U17212
G2	1845		Australia	O. Glenn	U17213
G3	077/98		Australia	D. Hornby	U17213
G. cylindrosporus					
C1	1850(101)	Foxtail grass	Poland	J. Henson	U17211
C2	DAR25011	Wheat	United Kingdom	D. Hornby	U17211
G. incrustans					
I1	ATCC 64417	Bermuda grass	Kans.	12	U17214
I3	ATCC 64418	Bluegrass	R.I.	12	U17215
Phialophora sp. (lobed hyphopodia)					
P2	1855	Wheat	Czechoslovakia	J. Henson	U17216
P9	89/5-3		Rothamstead, United Kingdom	D. Hornby	U17216
P. graminicola					
P4	1826	Wild rye grass	R.I.	J. Henson	U17217
P7	89/4-4		Rothamstead, United Kingdom	D. Hornby	U17218
P8	89/4-1		Rothamstead, United Kingdom	D. Hornby	U17218
M. grisea					
2690	0-70			7[b]	U17328
2692	G22			7[b]	U17329

[a] Numbers are our laboratory numbers.
[b] DNA was obtained from J. Henson.

tion of fungal DNA with a cloned mitochondrial DNA fragment from *G. graminis* enabled *Gaeumannomyces* and *Phialophora* spp. to be distinguished from a number of other root-infecting fungi (2, 6). This technique was refined to circumvent the need for isolation of fungi from infected plants by developing PCR primers which amplified a specific region of this mitochondrial DNA fragment. With these primers, *G. graminis* could be detected in infected cereal seedlings and infested soil (8, 21). Restriction fragment length polymorphism (RFLP) analysis of the nuclear or mitochondrial ribosomal DNA of *G. graminis* has allowed identification of *G. graminis* varieties and also gives an indication of the variation within varieties, depending on the restriction enzymes used (15, 23, 26). Ward and Akrofi (25) used PCR primers to amplify ribosomal internal transcribed spacers (ITS) and 5.8S rRNA genes (rDNA) from nuclear DNA and then used restriction enzymes to generate RFLP patterns by digestion of the PCR fragments. This technique was useful for discriminating between *G. graminis* and *P. graminicola* and between *G. graminis* var. *tritici*, var. *avenae*, and var. *graminis*. However, some atypical isolates gave intermediate RFLP patterns, and these researchers found it difficult to assign isolates to taxonomic groups based on these patterns.

We describe here the phylogenetic analysis of members of the *Gaeumannomyces-Phialophora* complex. Additionally, we present evidence suggesting that several oat-attacking *G. graminis* isolates from Australia, previously identified as *G. graminis* var. *tritici* on the basis of ascospore length, are actually more closely related to *G. graminis* var. *avenae*. We report the use of PCR to distinguish between *G. graminis* var. *avenae* and *G. graminis* var. *tritici* and to detect these fungi in infected wheat seedlings. This method is rapid and simple and does not require the isolation of DNA, DNA hybridization procedures, or the use of restriction endonucleases.

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence	Position in sequence:		Source or reference
		ITS1[a]	ITS2[b]	
psnDNA2p	5′-GTCCACACACCGCCCGT-3′			4
pITS2	5′-GCTGCGTTCTTCATCGATGC-3′			24
pITS3	5′-GCATCGATGAAGAACGCAGC-3′			24
pITS4	5′-TTCTTCGCTTATTGATATGC-3′			24
pGa1 (*G. graminis* var. *avenae* 5′ primer)	5′-TGCTTCGGCGGACGATGGT-3′	45–80		This study
pGa2 (*G. graminis* var. *avenae* 3′ primer)	5′-TTACTGCGTTCAGGGTCCTA-3′		81–102	This study
pGt1 (*G. graminis* var. *tritici* 5′ primer)	5′-TTGCTTCGGCGGACGATGGC-3′	44–80		This study
pGt2 (*G. graminis* var. *tritici* 3′ primer)	5′-GTTACTGCGTTCAGGGTCCTG-3′		81–103	This study

[a] Sequence is shown in Fig. 2A.
[b] Sequence is shown in Fig. 2B.

MATERIALS AND METHODS

Fungal isolates. Table 1 lists the fungal isolates used in this study. All isolates were genetically purified by two successive rounds of hyphal-tip isolation prior to this study. The classification of isolates into different varieties and pathogenicity groups was initially based upon morphological characteristics and pathogenicity assays.

Culture conditions. Fungal isolates were maintained as mycelial colonies on potato dextrose agar slopes containing streptomycin (50 μg/ml) under mineral oil at 4°C and as macerated mycelium in 20% glycerol at −80°C. Isolates were recovered by inoculating blocks of mycelium onto potato dextrose agar and incubating at 22°C in the dark for approximately 6 days. No more than three successive subcultures were carried out before returning the isolates to the culture under oil. For liquid cultures, homogenate from a single colony was used to inoculate 100 ml of potato dextrose liquid medium essentially as described by Osbourn et al. (17). Liquid cultures were grown for 5 days at 22°C with shaking at 300 rpm.

DNA purification. Fungal DNA was prepared from lyophilized mycelia harvested from liquid cultures by following the method of Raeder and Broda (19).

Preparation of fungal tissue for PCR. When wheat root tissue or fungal mycelium was to be used directly in a PCR, the samples were prepared by a modification of the method described by Klimyuk et al. (11). Root segments or fungal mycelium (scraped from a colony) was transferred to a microcentrifuge tube containing 80 μl of 0.25 N NaOH and vacuum infiltrated for 2 min. The samples were then incubated in a boiling-water bath for 2 min and subsequently neutralized by addition of 80 μl of 0.25 N HCl and 40 μl of 0.5 M Tris-HCl, pH 8.0–0.25% Nonidet P-40 (Sigma) before being boiled for a further 2 min. Tissue samples were either used immediately or stored at 4°C for up to 1 month. After storage, it was essential to incubate the samples again for 2 min at 100°C prior to PCR analysis. A single 0.5-mm root segment, or approximately 1 to 10 mg of fungal mycelia, was used in the PCR.

PCR amplifications. Oligonucleotide primers used in this study are listed in Table 2 and were synthesized on an Applied Biosystems 391 DNA synthesizer. Oligonucleotides were purified from the columns as described by Sambrook et al. (20). All PCRs were carried out essentially as described by Sambrook et al. (20). Amplification reactions were performed in a volume of 50 μl in the presence of 15 μM each primer and 250 μM deoxynucleoside triphosphates (Pharmacia) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40, and 1.25 U of *Taq* DNA polymerase. Cycling conditions for primers psnDNA2p and pITS4 were 94°C for 5 min and then 94°C for 45 s, 50°C for 30 s, and 72°C for 1 min (35 cycles), followed by a 10-min extension at 72°C. Cycling conditions for primers pGa1, pGa2, pGt1, and pGt2 were 94°C for 5 min and then 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s (35 cycles), followed by a 10-min extension at 72°C.

DNA sequencing. Direct sequencing of PCR products was carried out by using the *f*mol DNA sequencing system (Promega) with primers psnDNA2p, pITS2, pITS3, and pITS4 (27) in a Perkin-Elmer Cetus 9600 Gene Amp machine. Sequencing products were separated on 6% polyacrylamide gels (20).

DNA sequence analysis. DNA sequences were aligned by using the University of Wisconsin Genetics Computer Group program Lineup. Phylogenetic analysis of DNA sequences was performed by using the PAUP (phylogenetic analysis using parsimony) 3.1.1 program (22) with the branch-and-bound algorithm with 500 bootstrap replications. Gaps in the sequence alignment were treated as a fifth base. Insertions or deletions of more than one base were treated as a series of multiple differences rather than as a single difference. Branches having a maximum length of zero were collapsed to reveal polycotomies. The sequences of ITS1 and ITS2 of *Magnaporthe grisea* isolates 2690 and 2692 were obtained in our laboratory.

RESULTS

PCR amplification and sequencing of the ITS and 5.8S DNA. We found it possible to amplify an rDNA PCR fragment consisting of the 3′ 170 nucleotides of the 18S gene, ITS1, the 5.8S gene, and ITS2 (Fig. 1) using the primers psnDNA2p and pITS4, both from purified fungal DNA and directly from fungal tissue, obviating the need to purify fungal DNA. This region was amplified, and the nucleotide sequence obtained in both directions for all isolates is listed in Table 1. At least two

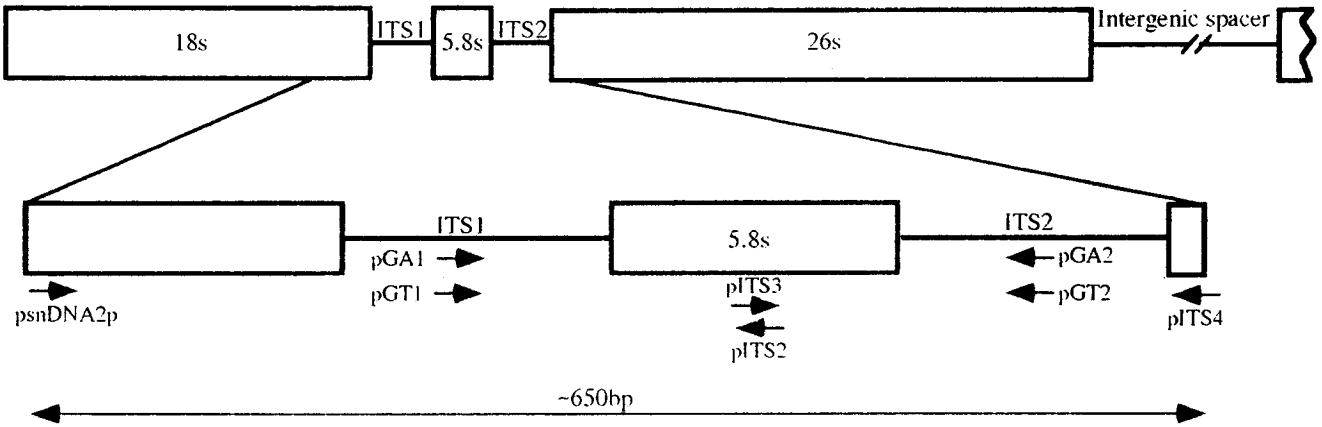

FIG. 1. Schematic representation of the rDNA of *G. graminis*. The open boxes represent the ribosomal subunits. The arrows represent the positions of the PCR primers (psnDNA2p and pITS4), the internal sequencing primers (pITS2 and pITS3), the *G. graminis* var. *tritici*-specific primers (pGt1 and pGt2), and the *G. graminis* var. *avenae*-specific primers (pGa1 and pGa2).

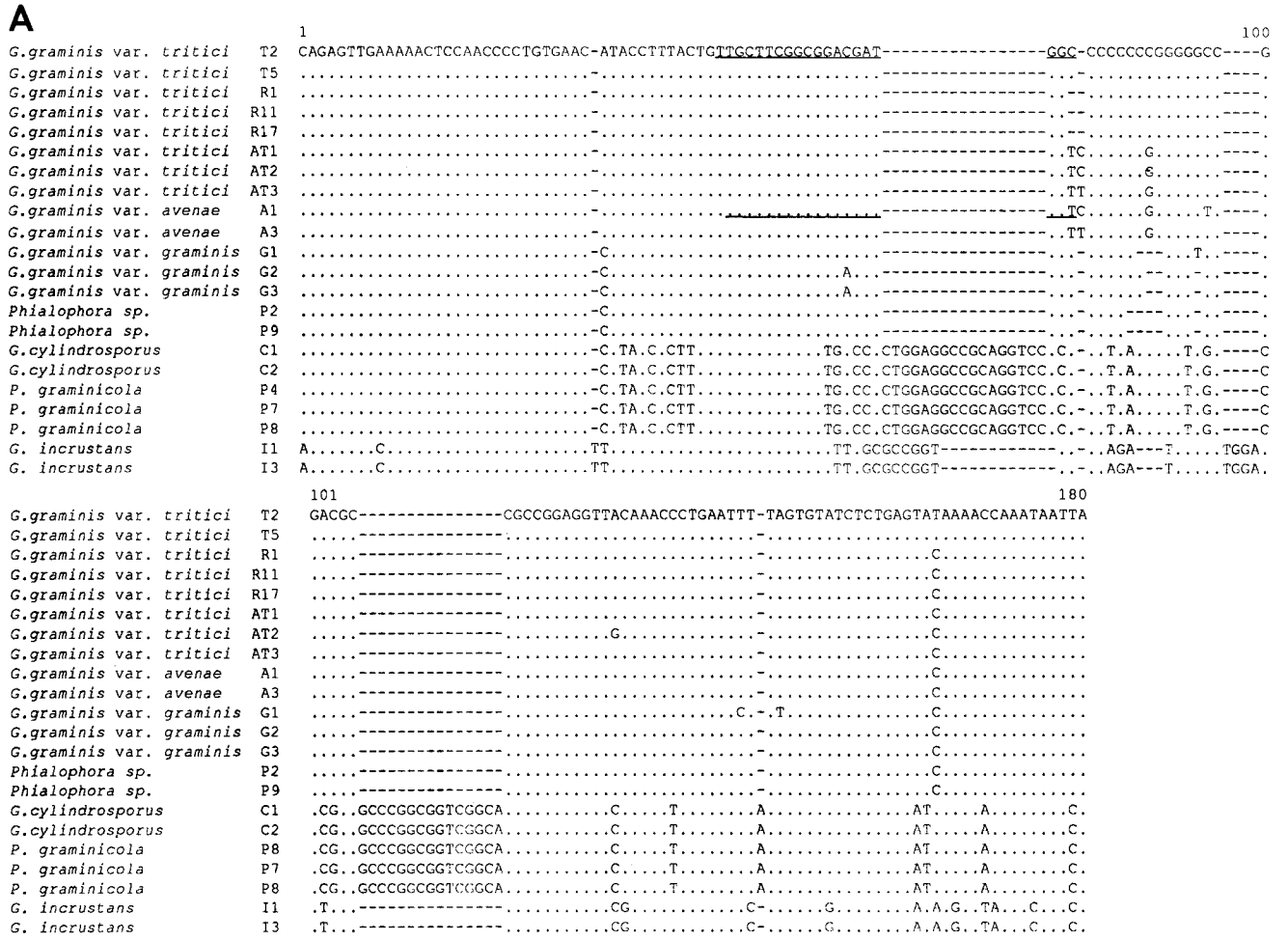

FIG. 2. Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS1 (A) and ITS2 (B). Identity to the reference sequence, that of *G. graminis* var. *tritici* isolate T2, is indicated by dots; alignment gaps (insertion or deletion differences) are indicated by dashes. The regions of ITS1 identical to the variety-specific oligonucleotide primers pGt1 and pGa1 for *G. graminis* var. *tritici* (positions 44 to 80 in T2) and *G. graminis* var. *avenae* (positions 45 to 80 in A1), respectively, are underlined, as are the regions of ITS2 complementary to the variety-specific oligonucleotide primers pGt2 and pGa2 for *G. graminis* var. *tritici* (positions 81 to 103 in T2) and *G. graminis* var. *avenae* (positions 81 to 102 in A1).

isolates per taxonomic group were included in this study. The nucleotide sequences of ITS1 (Fig. 2A) and ITS2 (Fig. 2B) were aligned for comparison with *G. graminis* var. *tritici* isolate T2.

The sequence comparison shows the close relationship between the *Gaeumannomyces* and *Phialophora* isolates included in this study. *G. graminis* var. *tritici* and *G. graminis* var. *avenae* isolates were identical throughout the region of the 18S subunit for which the nucleotide sequence was determined, and they differed by one nucleotide from *G. graminis* var. *graminis*, *P. graminicola*, *G. cylindrosporus*, and *G. incrustans* and by two nucleotides from the two *Phialophora* isolates with lobed hyphopodia, isolates P2 and P9 (data not presented; see Table 1 for the GenBank accession numbers of these sequences). The sequences of the 5.8S subunit were the same for all *G. graminis* varieties, *G. incrustans*, and the *Phialophora* isolates with lobed hyphopodia (P2 and P9). However, the *G. cylindrosporus* and *P. graminicola* isolates all contained the same single nucleotide difference compared with the other isolates. Because only 170 nucleotides of the 18S gene was sequenced, it was not possible to perform a stringent phylogenetic analysis to compare the 18S sequence for members of the *Gaeumannomyces-Phialophora* complex with those of other fungi in the GenBank database. However, a preliminary survey involving comparison with 18S sequences of other ascomycetes (*Ajellomyces capsulatus* [GenBank accession number X58572], *Aspergillus fumigatus* [GenBank accession number X62988], *M. grisea*, *Neurospora crassa* [GenBank accession number M13906], *Ophiostoma ulmii* [GenBank accession number M83259], *Ophiostoma stenoceras* [GenBank accession number M85054], and *Saccharomyces cerevisiae* [GenBank accession number YSCRGEA]) was supportive of the *Gaeumannomyces* isolates being members of the class *Pyrenomycetes*. Of these fungi, *M. grisea* was the most closely related to the genus *Gaeumannomyces* (data not presented).

ITS1 and ITS2 contained higher levels of nucleotide sequence divergence than either the 3′ 170 nucleotides of the 18S subunit or the 5.8S subunit. The sequences for ITS1 and ITS2 were identical within isolates of each taxonomic group, except for the following cases: *G. graminis* var. *avenae* isolates A1 and A3 contained two differences at positions 81 and 94 in ITS1 (Fig. 2A); *P. graminicola* isolates P7 and P8 contained one identical difference from isolate P4 at position 135 in ITS2 (Fig. 2B); *G. graminis* var. *graminis* isolates G2 and G3 con-

B

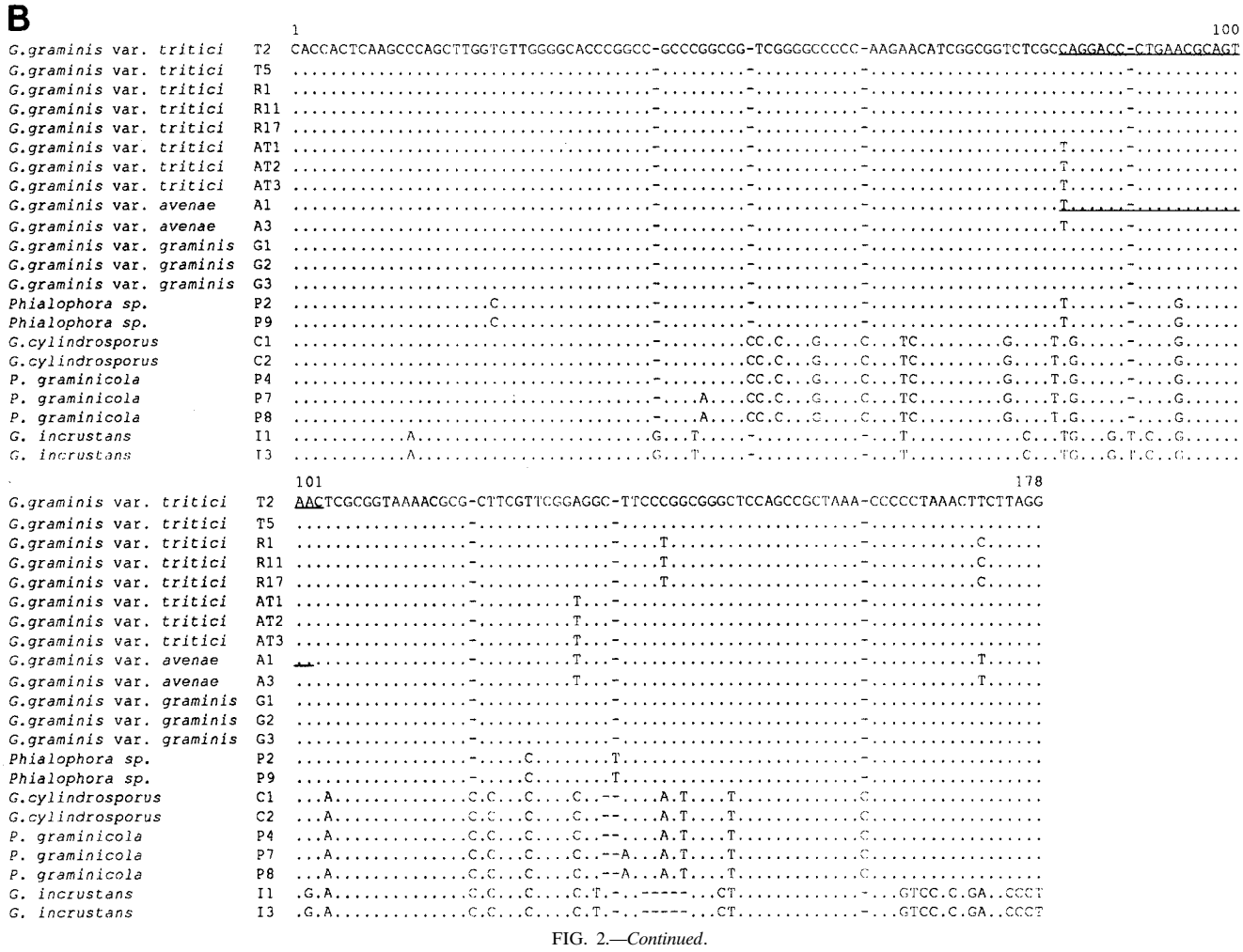

FIG. 2.—*Continued*.

tained identical differences from isolate G1 at positions 87, 93, 145, and 149 in ITS1 (Fig. 2A); and finally, the *G. graminis* var. *tritici* isolates AT1, AT2, and AT3 contained differences at positions 81 and 132 in ITS1 (Fig. 2A), but the taxonomic grouping of these Australian oat-infecting *G. graminis* var. *tritici* isolates is uncertain and they may not be homogeneous.

The ITS1 sequences of *G. graminis* isolates contained between 2 and 11 nucleotide differences and 1 to 6 positions with sequence alignment gaps in a total of 141 positions in comparison with *G. graminis* var. *tritici* isolate T2. *G. cylindrosporus* isolates contained 25 nucleotide differences from *G. graminis* var. *tritici* T2 and four positions with sequence alignment gaps; two of these sequence alignment gaps were insertions of 17 and 15 nucleotides. *G. incrustans* isolates contain 23 nucleotide differences from *G. graminis* var. *tritici* T2 and five positions with sequence alignment gaps; two of these sequence alignment gaps were insertions of 4 and 6 nucleotides (Fig. 2A).

The nucleotide sequence of ITS2 (Fig. 2B) is more conserved than that of ITS1 among *G. graminis* isolates with zero to five nucleotide substitutions in a total of 173 positions. Isolates C1 and C2 differed by 20 identical nucleotide substitutions and one sequence alignment gap compared with *G. graminis* var. *tritici* T2. *G. cylindrosporus* isolates contained 16 nucleotide differences from *G. graminis* var. *tritici* T2 and five positions with sequence alignment gaps. *G. incrustans* isolates contained 28 nucleotide sequence differences from *G. graminis* var. *tritici* T2 and 11 positions with sequence alignment gaps.

Phylogenetic analysis using the ITS1 and ITS2 sequences. The ITS1 and ITS2 regions were chosen for determination of the relationship of the *G. graminis* varieties with each other and with other *Gaeumannomyces* and *Phialophora* species, because these regions contained a higher level of nucleotide sequence divergence than either the 3′ 170 nucleotides of the 18S subunit or the 5.8S subunit. Figure 3 illustrates a 50% majority rule consensus tree generated by using the branch-and-bound algorithm for these data. Isolates of *G. graminis* varieties *tritici* and *avenae* branch together, with 79% bootstrap support. Within this group, the *G. graminis* var. *tritici* N and R isolates branch separately from each other and are shown to be no more closely related to each other than to *G. graminis* var. *avenae*. The *G. graminis* var. *avenae* isolates and the oat-attacking *G. graminis* var. *tritici* isolates branch together with high bootstrap support (98%) to form a distinct subgroup. The *G. graminis* var. *graminis* isolate G1; the two other *G. graminis* var. *graminis* isolates, G2 and G3; and the *Phialophora* isolates P2 and P9, respectively, form three separate branches further back in the tree and are no more closely related to each other than they are to *G. graminis* var. *avenae* or *G. graminis* var. *tritici*. There is good support (82%) for the branching of *G. incrustans* and *M. grisea* with the *G. graminis* varieties. *G.*

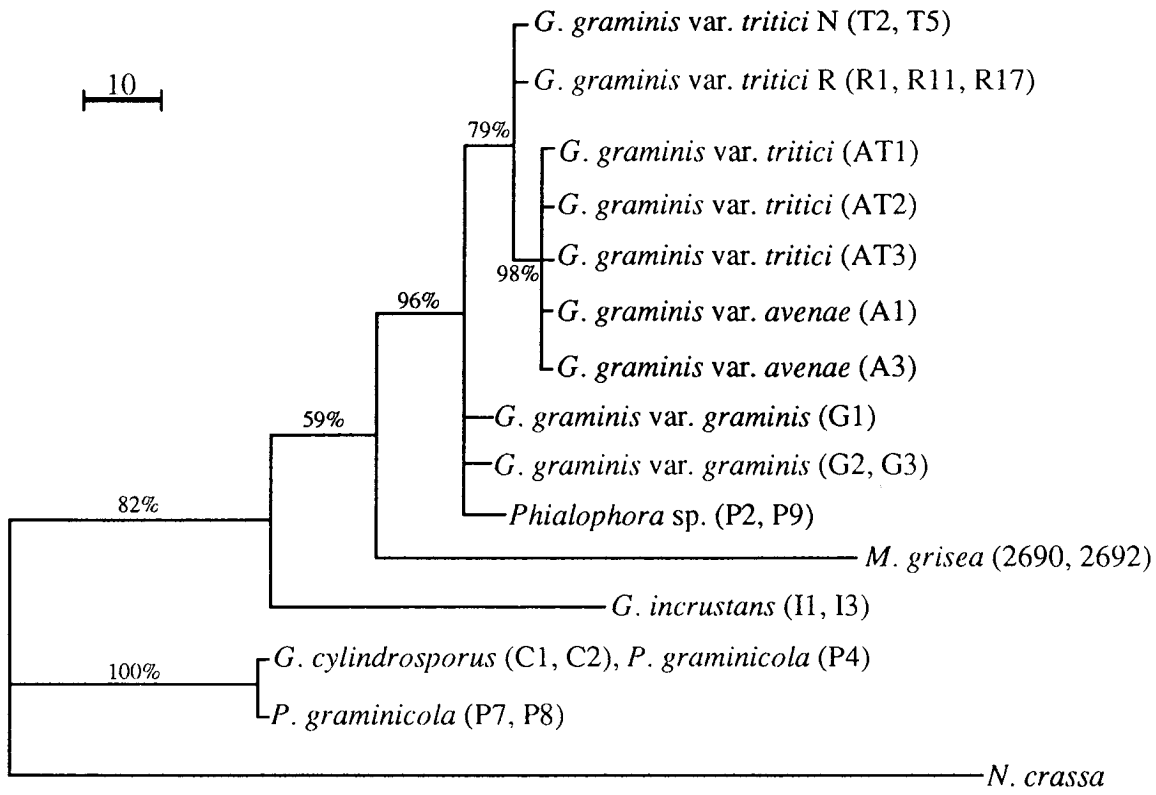

FIG. 3. Fifty percent majority rule consensus tree generated from a branch-and-bound algorithm in PAUP 3.1.1. The tree is based on the aligned nucleotides of ITS1 and ITS2 (Fig. 2). A distance of 10 nucleotide substitution differences is indicated by a bar at the upper left. Branches having a maximum length of zero were collapsed to reveal polycotomies. The percentages are the frequencies with which a given branch appeared in 500 bootstrap replications. The tree was rooted by making *N. crassa* the outgroup.

incrustans clearly diverges from *G. graminis* after this point, while there is relatively weak support (59%) for the branching of *M. grisea* separately from the *G. graminis* varieties. *G. cylindrosporus* and *P. graminicola* clearly form a distinct group.

Evidence that *P. graminicola* may be the asexual state of *G. cylindrosporus*. Nucleotide sequence alignment of the ITS1 and ITS2 regions of the rDNA indicates that *P. graminicola* is closely related to *G. cylindrosporus*. The nucleotide sequences of *G. cylindrosporus* isolates C1 and C2 and *P. graminicola* isolates P4, P7, and P8 are identical to each other in the 3′ 170 nucleotides of the 18S subunit (data not presented), ITS1 (Fig. 2A), and the 5.8S subunit (data not presented), although P7 and P8 both contain the same two nucleotide differences in ITS2 (Fig. 2B). The nucleotide sequences of ITS1 and ITS2 for these isolates diverge significantly when aligned with those of *G. graminis* and *G. incrustans*. This is best illustrated by Fig. 2A, where it can be seen that isolates C1, C2, P4, P7, and P8 contain insertions of 17 nucleotides (positions 61 to 77) and 15 nucleotides (positions 106 to 120) not present in *G. graminis* isolates. Phylogenetic analysis of the ITS1 and ITS2 sequences (Fig. 3) shows that isolates C1, C2, P4, P7, and P8 branch as a monophyletic group. These data are consistent with the possibility that *P. graminicola* is the asexual state of *G. cylindrosporus* (24). Geographical separation (Table 1) could be an explanation for the two nucleotide sequence differences present in United Kingdom *P. graminicola* isolates P7 and P8 in comparison with *G. cylindrosporus* and the *P. graminicola* isolate P4 from the United States. However, information from a larger number of isolates is required in order to confirm this.

***Phialophora* species with lobed hyphopodia.** *Phialophora* isolates with lobed hyphopodia which were included in our analysis (isolates P2 and P9) are clearly related to the *G. graminis* species on the basis of rDNA sequence information. However, these isolates cannot be considered to be any more closely related to *G. graminis* var. *graminis* than they are to *G. graminis* var. *tritici* or *G. graminis* var. *avenae*. Phylogenetic analysis of a larger collection of isolates is required in order to clarify the status of *Phialophora* isolates with lobed hyphopodia.

Oat-infecting *G. graminis* var. *tritici* isolates from Australia are more closely related to *G. graminis* var. *avenae*. The Australian *G. graminis* var. *tritici* isolates AT1, AT2, and AT3 all have the ability to infect oats but have been described as *G. graminis* var. *tritici* (rather than the oat-attacking variety *G. graminis* var. *avenae*) on the basis of ascospore morphology (29). However, from rDNA sequence comparisons, they appear to be more closely related to *G. graminis* var. *avenae*. Parsimony analysis of ITS1 and ITS2 sequences shows the *G. graminis* var. *tritici* isolates AT1, AT2, and AT3 branching as a monophyletic group with *G. graminis* var. *avenae* isolates (Fig. 3).

Specific amplification of *G. graminis* var. *avenae* DNA from infected plants by using variety-specific primers. From the sequence alignments in Fig. 2, *G. graminis* var. *avenae* primers pGa1 and pGa2 and *G. graminis* var. *tritici* primers pGt1 and pGt2 (Table 2) were designed to amplify fragments of *G. graminis* var. *avenae* and *G. graminis* var. *tritici* DNA, respectively. The variety-specific 5′ primers pGa1 and pGt1 anneal in the same position in ITS1 of *G. graminis* var. *avenae* and *G. graminis* var. *tritici*, respectively, and are specific because of a single nucleotide difference at the 3′ end of each primer (Fig.

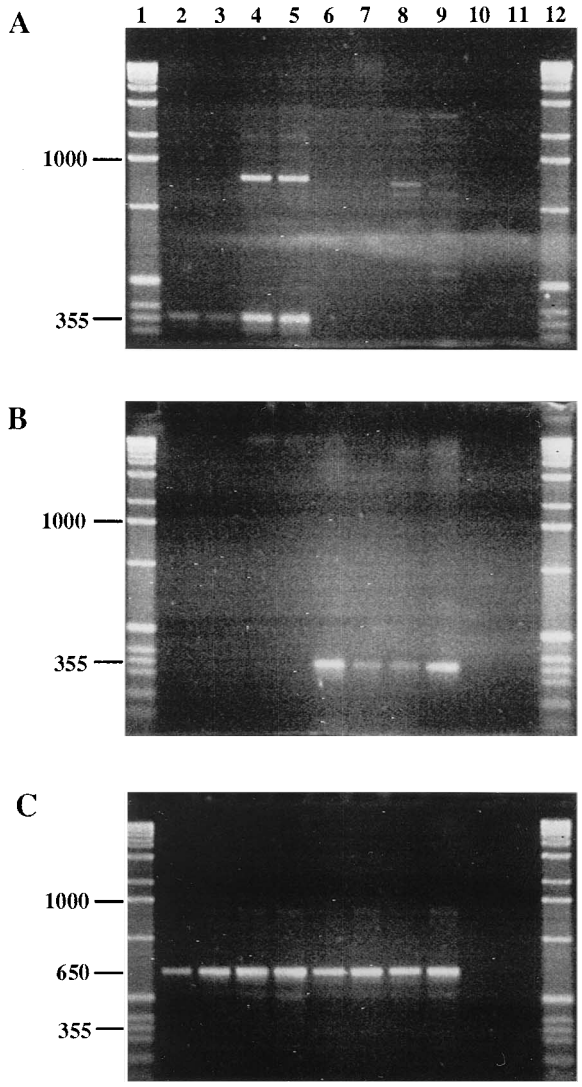

FIG. 4. Examples of PCR amplification products obtained by using nested primers to amplify *G. graminis* var. *avenae* or *G. graminis* var. *tritici* DNA from infected wheat seedlings. (A) Reactions with primers pGa1 and pGa2. (B) Reactions with primers pGt1 and pGt2. (C) Reactions with primers psnDNA2p and pITS4. Reaction mixtures contained DNA of *G. graminis* var. *avenae*-infected seedlings (lanes 2 and 3), and *G. graminis* var. *avenae* DNA (lanes 4 and 5), DNA of *G. graminis* var. *tritici*-infected seedlings (lanes 6 and 7), and *G. graminis* var. *tritici* DNA (lanes 8 and 9), DNA of a healthy wheat seedling (lane 10), and a water control (lane 11). Isolate pairs were *G. graminis* var. *avenae* isolates A1 and A3 and *G. graminis* var. *tritici* isolates T2 and R17. Lanes 1 and 12 contained 1-kb ladder molecular size markers from Bethesda Research Laboratories. Molecular sizes are in base pairs.

2A). The same rationale was used when designing the 3′ specific primers pGa2 and pGt2. Figure 1 also illustrates the annealing positions of these primers. PCR amplification of *G. graminis* var. *avenae* DNA, or wheat root tissue infected with *G. graminis* var. *avenae*, using primers pGa1 and pGa2 gave a 355-bp band which was specific for *G. graminis* var. *avenae* (Fig. 4A). This band was amplified in eight of eight *G. graminis* var. *avenae* isolates tested. In addition, this 355-bp band was also amplified in the oat-infecting *G. graminis* var. *tritici* isolates from Australia, which we have suggested may be more closely related to *G. graminis* var. *avenae*. An additional nonspecific band of 950 bp was also observed for some *G. graminis* var. *avenae* and *G. graminis* var. *tritici* isolates but not for other members of the *Gaeumannomyces-Phialophora* complex. No specific products were observed in PCRs done with DNA from *G. graminis* var. *tritici* isolates, *G. graminis* var. *tritici*-infected wheat root tissue, or healthy wheat root tissue. Primers pGt1 and pGt2 gave a 356-bp band with DNA from isolates of *G. graminis* var. *tritici* and wheat root tissue infected with *G. graminis* var. *tritici* (Fig. 4B). This band was amplified in 11 of 11 *G. graminis* var. *tritici* isolates tested. No products were observed in PCRs done with DNA from *G. graminis* var. *avenae* isolates, *G. graminis* var. *avenae*-infected wheat root tissue, or healthy wheat root tissue. In control PCRs using primers psnDNA2p and pITS4 (Fig. 4C), which should amplify all fungal rDNA sequences, a 650-bp band was observed in all reaction mixtures containing DNA from *G. graminis* var. *avenae*, *G. graminis* var. *tritici*, or *G. graminis* var. *graminis* or *G. graminis* var. *tritici*- or *G. graminis* var. *avenae*-infected wheat root tissue. No products were observed in reaction mixtures containing healthy wheat root tissue. The primers failed to amplify DNA fragments when tested with a number of other fungi, some of which are closely related to *G. graminis* and others of which are taxonomically distinct. These included a number of isolates of related fungi listed in Table 1, *G. cylindrosporus* (C1 and C2), *G. incrustans* (I1 and I3), *P. graminicola* (P4, P7, and P8), and *M. grisea* (2690 and 2692), and also three accessions of *Magnaporthe poae* (1832, 2562, and 2669 [7]) and one accession each of *Phialophora malorum* (1847 [7]), *Leptosphaeria korrae* (1828 [7]), *Septoria avenae* (353.49 [Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands]), and *Septoria lycopersici* (396.52 [Centraalbureau Voor Schimmelcultures]). The primers would not be anticipated to amplify DNA from the rDNA of other plant-pathogenic fungi whose sequences are in the GenBank databases.

DISCUSSION

The two ITS regions in filamentous fungi have been found to contain a higher level of nucleotide sequence variation than either the 18S, 5.8S, or 26S rDNA sequences (27). Therefore, we compared the nucleotide sequences of the ITS1 and ITS2 regions to establish the relationships between the *Gaeumannomyces* and *Phialophora* isolates used in this study. This analysis allowed the clear differentiation of the three *Gaeumannomyces* spp. (*G. graminis*, *G. incrustans*, and *G. cylindrosporus*), the three *G. graminis* varieties (var. *tritici*, var. *avenae*, and var. *graminis*), and *M. grisea* (Fig. 3).

Phylogenetic analysis of rDNA sequences indicates that the *G. graminis* varieties *tritici* and *avenae* are more closely related to each other than either is to *G. graminis* var. *graminis*. This is consistent with conclusions based on the morphologies of the anamorphs of these fungi (*G. graminis* varieties *tritici* and *avenae* have *Phialophora* anamorphs with simple hyphopodia, while the *G. graminis* var. *graminis* anamorph has lobed hyphopodia [24]). The grouping is also consistent with pathogenicity and host range of the varieties (*G. graminis* varieties *tritici* and *avenae* are pathogenic to wheat and other cereals, while *G. graminis* var. *graminis* is a relatively benign parasite of grasses and rice [24]), with RFLP analysis of rDNA (25), and with differences in codon usage (3).

The N- and R-type *G. graminis* var. *tritici* isolates were clearly separated on the basis of ITS DNA sequences, supporting work by O'Dell et al. (15) which differentiated the two groups of isolates on the basis of RFLP analysis of rDNA. The difference between N- and R-type isolates in the ability to infect rye and in their rDNA suggests that they should be

regarded as pathotypes of *G. graminis* var. *tritici*. However, DNA sequence analysis of the rDNA of more isolates is required to confirm that N and R types consistently differ.

Interestingly, three oat-infecting *G. graminis* isolates from Australia initially identified as *G. graminis* var. *tritici* on the basis of ascospore length (29) appear to be more closely related to *G. graminis* var. *avenae*, suggesting that classification of isolates on the basis of ascospore morphology alone may be misleading. This is in agreement with observations of Ward and Akrofi (25) based on RFLP analysis of ribosomal DNA of an oat-attacking *G. graminis* var. *tritici* isolate from Australia, which suggest that this isolate belongs to a subgroup of *G. graminis* var. *avenae*. More isolates need to be studied to establish whether the variation seen between these Australian *G. graminis* var. *tritici* and *G. graminis* var. *avenae* isolates is greater or less than the variation within *G. graminis* var. *avenae* alone. Research in our laboratory indicates that *G. graminis* var. *tritici* isolates AT1 and AT2 do not produce the saponin-detoxifying enzyme avenacinase (3), in contrast to *G. graminis* var. *avenae* isolates (17) and *G. graminis* var. *tritici* AT3 (3). However, *G. graminis* var. *tritici* AT1 and AT2 are avenacin resistant and able to infect oats. Evidently, these isolates have an alternative mechanism for tolerating the toxic effects of avenacin.

The two isolates of *M. grisea* included in the analysis showed a surprising degree of relatedness to *G. graminis* and *G. incrustans*. A comprehensive phylogenetic analysis of rDNA sequences of a range of isolates of *M. grisea* and other *Magnaporthe* species with the sequences of members of the *Gaeumannomyces-Phialophora* complex is required to clarify the relationship of these fungi at the molecular level.

Our results are consistent with the possibility that *P. graminicola* may be the anamorph state of *G. cylindrosporus*. These fungi are clearly distinct from other members of the *Gaeumannomyces-Phialophora* complex, and both *P. graminicola* and *G. cylindrosporus* have common insertions in their ITS sequences. Insertions and deletions in ITS regions are not uncommon in fungi (4, 14, 16). The anamorph of *G. graminis* var. *graminis* has lobed hyphopodia, while those of *G. graminis* varieties *tritici* and *avenae* have simple hyphopodia (24). However, we are unable to conclude whether the *Phialophora* isolates with lobed hyphopodia included in our experiments are likely to represent the anamorph state of *G. graminis* var. *graminis.*

A practical application of the nucleotide sequence comparisons in this study was the design of PCR primers for use in take-all and take-all patch diagnosis. Primers designed to specifically amplify *G. graminis* var. *avenae* and *G. graminis* var. *tritici* DNA were produced, and it was found that it was possible to detect *G. graminis* var. *avenae* and *G. graminis* var. *tritici* directly in infected root tissue of wheat seedlings. These primers have potential for rapid identification and detection of pathogenic isolates and disease diagnosis in cereal crops, in turf grasses, and in soil containing infected root material. While tests involving a limited range of fungi appear promising, it is not yet known how specific these PCR primers would be for *G. graminis* var. *avenae* or *G. graminis* var. *tritici* when tested on infected plants from the field in the presence of many other soil and rhizosphere fungi.

The use of these primers to distinguish between and detect *G. graminis* var. *avenae* and *G. graminis* var. *tritici* has several advantages over other published methods. The method is quick and easy. It relies on the presence or absence of a single specific DNA band on an agarose gel, and further characterization of PCR products by restriction endonuclease digestion is not needed. The sensitivity of PCR-based detection methods circumvents the need to isolate fungi from infected cereal seedlings or to isolate fungal DNA. The information which has been generated in this study of the rDNA of isolates of *Gaeumannomyces* and *Phialophora* should allow the development of further PCR primers which will facilitate identification of the various members of the *Gaeumannomyces-Phialophora* complex. This will be valuable for epidemiological studies of these fungi and may be important, for instance, in the development of biological control agents.

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