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Ribosomal DNA sequence comparisons of *Colletotrichum graminicola* from turfgrasses and other hosts

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Abstract

The 5.8S ribosomal gene and the flanking internal transcribed spacers (ITS) 1 and 2 from *Colletotrichum graminicola* isolates causing anthracnose disease of *Agrostis palustris* and *Poa* species were sequenced. Although bootstrap support was not high, two major groups were observed with both UPGMA and parsimony algorithms, one containing isolates from *A. palustris* and another with isolates from *Poa* spp. The ITS sequences were also compared with those of isolates of *C. graminicola* and *C. sublineolum* from *Sorghum* spp., *Zea mays* and *Rottboellia cochinchinesis* as well as other *Colletotrichum* species. Except for one isolate from *P. annua* in Texas, the ITS1 and ITS2 sequences of turfgrass isolates always grouped separately from *C. graminicola* or *C. sublineolum* from non-turfgrass hosts with high bootstrap support. ITS sequences of the turfgrass isolates were more similar to those of other species of *Colletotrichum*, such as *C. coccodes* and *C. dematium*, than they were to *C. graminicola* isolates from other hosts. Turfgrass isolates have ITS sequences which are not identical to those of isolates from *Zea mays* and *Sorghum* species demonstrating diversity among fungi conventionally classified as *C. graminicola*.

Introduction

Anthracnose of turfgrasses is caused by a fungal pathogen identified as *Colletotrichum graminicola*. Two types of disease symptoms are commonly observed. One is a leaf blight that is often referred to as anthracnose foliar blight, and the other is a crown and root rot, which is often called anthracnose basal rot (Smith et al., 1989; Landschoot and Hoyland, 1995). Symptoms of anthracnose have been reported on fescues (*Festuca* spp.), bentgrasses (*Agrostis* spp.), bluegrasses (*Poa* spp.), and perennial ryegrass (*Lolium perenne*). Both types of symptoms require wet or humid conditions, but higher temperatures favour foliar blight, whereas lower temperatures favour basal rot (Jackson and Herting, 1985).

Colletotrichum graminicola was originally described as a pathogen of a wide range of cereals and grasses, but some host specificity appears to exist (Mordue, 1967; Bolton and Cordukes, 1981). Sutton (1980) described *Colletotrichum* isolates from maize (*Zea mays*) and sorghum (*Sorghum* spp.) as two separate species, and designated the maize isolates as *C. graminicola* and the sorghum isolates as *C. sublineolum*. Smiley et al. (1992) suggested that there may also be host specificity among *C. graminicola* isolates that attack different turfgrasses, and other researchers have also noted that in mixed swards of annual bluegrass (*P. annua*) and creeping bentgrass (*A. palustris*), only one of the two species usually becomes severely infected with anthracnose (Backman et al., 1999; Browning et al., 1999).

The taxonomy of *Colletotrichum* species has been based on pathogenicity and features such as conidial shape and size, setae and appressorial morphology. However, morphological characteristics are variable in culture and there is overlap of phenotypes, which has made these criteria not always reliable. DNA sequence comparisons have been used to examine a number of *Colletotrichum* species, and sequences of the internal

transcribed spacer regions (ITS) of the ribosomal DNA have proven to be particularly useful for delimiting members of this genus (Sherriff et al., 1994; 1995; Bailey et al., 1996; Sreenivasaprasad et al., 1996).

For *Colletotrichum graminicola*, isolates from maize, sorghum and rottboellia (*Rottboellia* spp.) have been examined with this approach. Using ITS2 sequences, Sherriff et al. (1995) showed that isolates of *C. graminicola* from maize were distinct from those obtained from sorghum and rottboellia, and recommended that the classification of Sutton (1980) be adopted so that sorghum and rottboellia isolates are regarded as *C. sublineolum*. Sreenivasaprasad et al. (1996) analyzed the ITS1 region and also found that maize and sorghum isolates were distinct, supporting the classification of Sutton (1980). The ITS sequence results are also in agreement with morphological and genetic analyses favouring the distinction of maize isolates from sorghum isolates (Sutton, 1968; Vaillancourt and Hanau, 1992). Sreenivasaprasad et al. (1996) included a single isolate from annual bluegrass, and found that it grouped with isolates from sorghum; however, isolates from other turfgrass species should be examined to test this relationship. Recently, isolates of *C. graminicola* from annual bluegrass and creeping bentgrass were compared to each other by random amplified polymorphic DNA (RAPD) analysis, but there were conflicting results (Backman et al., 1999; Browning et al., 1999). Backman et al. (1999) grouped annual bluegrass isolates with an isolate from sorghum, and creeping bentgrass isolates were grouped with maize isolates, whereas Browning et al. (1999) grouped maize isolates and one sorghum isolate together, apart from turfgrass isolates.

The aim of this study was to assess differences among isolates of *C. graminicola* from different host species by comparing their ITS1 and ITS2 sequences. Comparisons were made between the ITS sequences of *C. graminicola* isolates from bluegrasses, creeping bentgrass and maize, as well as *C. sublineolum* isolates from sorghum and several other *Colletotrichum* species (Sherriff et al., 1995; Sreenivasaprasad et al., 1996; Martin and Garcia-Figueres, 1999)

Materials and methods

Isolates

Tissue from diseased samples of turfgrass was surfaced sterilized for up to 5 min in 0.5% hypochlorite. These were plated onto potato dextrose agar (PDA, Difco, Detroit, MI) amended with 100 mg l^{-1} streptomycin sulphate and incubated at 20 °C. After one week, fungal colonies resembling those of *C. graminicola* with compact, felty and woolly growth (Mordue, 1967) were examined for the presence of typical falcate (crescent shaped with pointed ends) spores of *C. graminicola* (Mordue, 1967). Colonies suspected of being *C. graminicola* were transferred to fresh PDA and incubated at 20 °C and later confirmed as *C. graminicola* based on descriptions by Sutton (1980). Thirteen isolates of *C. graminicola* used in this study (Table 1) were from Ontario, Canada, except for four from British Columbia, Canada (courtesy of J. Elmhirst), and one from Pennsylvania, USA (courtesy of P. Landschoot).

DNA sequencing

Isolates were cultured for two weeks at 10 °C on PDA overlaid with a cellophane membrane sheet (Flexel Inc., Atlanta, GA). After mycelium was harvested and DNA extracted (Edwards et al., 1991), the ITS region of genomic rDNA was amplified by the polymerase chain reaction (PCR) with the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) as described by White et al. (1991). The 30 µl reaction mixture for PCR amplification contained the following: 10 ng DNA, 1 × DNA polymerase buffer (50 mM Tris-HCl at pH 8.5), 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 1 unit *Tsg* DNA polymerase (Biobasic, Scarborough, ON, Canada). Amplifications were performed in a Perkin-Elmer GeneAmp 2400 (PE Applied Biosystems, Mississauga, ON, Canada), with an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Aliquots (3 µl) of amplification products were electrophoresed through 1.4% agarose gels along with a 100 bp ladder size marker (Pharmacia, Mississauga, ON, Canada), stained with ethidium bromide and visualized on a UV transilluminator.

When bands of an appropriate size for the ITS region were observed, the remaining PCR product was electrophoresed through 1.0% agarose gels in a modified TAE buffer (40 mM Tris-acetate, pH 8.0, 0.1 mM Na_2EDTA), and the target bands excised. DNA from excised bands was purified using an Ultrafree®-DA DNA extraction kit (Millipore, Bedford, MA, USA) and sequenced by the dye terminator method on an

Table 1. List of *Colletotrichum* isolates used in this study, their species, host, and origin

Isolate	Species[1]	Host	Origin	Genbank accession[2]	Study[3]	Source
97266	*C. graminicola*	*Agrostis palustris*	Toronto, ON		ITS	This study
99003	*C. graminicola*	*A. palustris*	Pennsylvania, USA		ITS	This study
99323	*C. graminicola*	*Poa pratensis*	Lacombe, AB		ITS	This study
99355	*C. graminicola*	*A. palustris*	Guelph, ON		ITS	This study
99356	*C. graminicola*	*A. palustris*	Guelph, ON		ITS	This study
99369	*C. graminicola*	*P. annua*	Erin, ON		ITS	This study
99370	*C. graminicola*	*P. annua*	Erin, ON		ITS	This study
99372	*C. graminicola*	*P. annua*	Sarnia, ON		ITS	This study
99373	*C. graminicola*	*P. annua*	Sarnia, ON		ITS	This study
99397	*C. graminicola*	*P. annua*	British Columbia		ITS	This study
99398	*C. graminicola*	*P. annua*	British Columbia		ITS	This study
99409	*C. graminicola*	*P. annua*	British Columbia		ITS	This study
99410	*C. graminicola*	*P. sabina*	British Columbia		ITS	This study
CGUW	*C. graminicola*	*P. annua*	Illinois, USA	AF059676	ITS	Genbank
CG671	*C. graminicola*	*P. annua*	Texas, USA	Z32980	ITS1	Sreenivasaprasad et al., 1996
CG221	*C. sublineolum*	*Rottboellia cochinchinesis*	Thailand		ITS2	Sherriff et al., 1995
CG032	*C. graminicola*	*Zea mays*	Zimbabwe	Z32981	ITS1	Sreenivasaprasad et al., 1996
CG091	*C. sublineolum*	*R. cochinchinesis*	Thailand	Z18984	ITS2	Sherriff et al., 1995
CG239	*C. sublineolum*	*Sorghum bicolor*	Brazil		ITS2	Sherriff et al., 1995
CG117	*C. sublineolum*	*S. bicolor*	Texas, USA	Z32975	ITS1	Sreenivasaprasad et al., 1996
CG310	*C. sublineolum*	*S. halpense*	USA		ITS2	Sherriff et al., 1995
CG334	*C. graminicola*	*Z. mays*	USA		ITS2	Sherriff et al., 1995
CS607	*C. sublineolum*	*S. vulgare*	Nigeria	Z32999	ITS1	Sreenivasaprasad et al., 1996
CG668	*C. sublineolum*	*S. vulgare*	Texas, USA	Z32979	ITS1	Sreenivasaprasad et al., 1996
CG670	*C. graminicola*	*Z. mays*	USA	Z33377	ITS1	Sreenivasaprasad et al., 1996
COCCO	*C. coccodes*	*Lycopersicon esculentum*	Bulgaria	Z32929	ITS2	Genbank
COCCO2	*C. coccodes*	*L. esculentum*	Bulgaria	Z32930	ITS1	Sreenivasaprasad et al., 1996
DEMA1	*C. dematium*	*Dianthus* sp.	UK	Z32938	ITS1	Sreenivasaprasad et al., 1996
DEMA2	*C. dematium*	*Medicago sativa*	Netherlands	Z32941	ITS1	Sreenivasaprasad et al., 1996
GLOEO	*C. gloeosporiodes*	*Citrus* sp.	Unknown	AF090855	ITS	Martin and Garcia-Figueres, 1999

[1] *Colletotrichum* isolates from *Sorghum* spp. or *Rottboellia* spp. are identified here as *C. sublineolum*.
[2] Genbank accession number and sequences were obtained from website 'http://www.ncbi.nlm.nih.gov/'.
[3] Study refers to the analysis in which the isolates were involved: ITS = sequence alignment of the complete ITS region, ITS1 = sequence alignment of ITS1, and ITS2 = sequence alignment of ITS2.

Applied Biosystems 377A automated DNA sequencer (Perkin Elmer, Mississauga, ON, Canada) using the primers ITS1 and ITS4 separately. If sequences were ambiguous after alignment of the forward and reverse sequences, new extracts of DNA were made and sequenced as above.

Sequences were aligned with the program CLUSTAL-W (Thompson et al., 1994), using default parameters. In addition to the 13 isolates of *C. graminicola* sequenced in this study, GenBank sequences of 17 other isolates of *Colletotrichum* spp., including seven of *C. sublineolum*, five of *C. graminicola*, two of *C. coccodes*, two of *C. dematium* and one of *C. gloeosporioides*, were also used in alignment of either the complete ITS (15 sequences), the ITS1 (15 sequences) or the ITS2 (12 sequences). Minor adjustments were made to improve alignments following visual inspection. Dendrograms were generated with both distance and parsimony methods, and bootstrap analysis was performed to provide confidence limits on tree topology. For a distance-based phylogeny, the alignment was subjected to bootstrap analysis with the software package PHYLIP (Felsenstein, 1989) using the programs SEQBOOT (1000 bootstrap replications), DNADIST (Jukes-Cantor genetic distance), NEIGHBOR (UPGMA algorithm), and CONSENSE. Parsimony analysis was also applied to the data generated from SEQBOOT using the program DNAPARS, and a majority-rule consensus tree was generated by CONSENSE. In parsimony analysis, *C. gloeosporioides* was used as the outgroup. Finally, dendrograms were produced with the PHYLIP program DRAWGRAM and modified using COREL® PRESENTATIONS.

Results

Thirteen isolates with characteristics typical of *C. graminicola* in culture were obtained from four different turfgrass hosts (Table 1). They were collected from a variety of locations within Canada and the USA, and isolates collected at the same location were taken from separate sites. Isolates from bluegrasses were mostly associated with samples showing typical foliar blight symptoms, whereas isolates from creeping bentgrass were all associated with samples showing basal rot symptoms. The entire ITS comprising the ITS1, the 5.8S and ITS2 regions were sequenced and compared to each other as well as to other ITS sequences reported in GenBank (Table 1). For

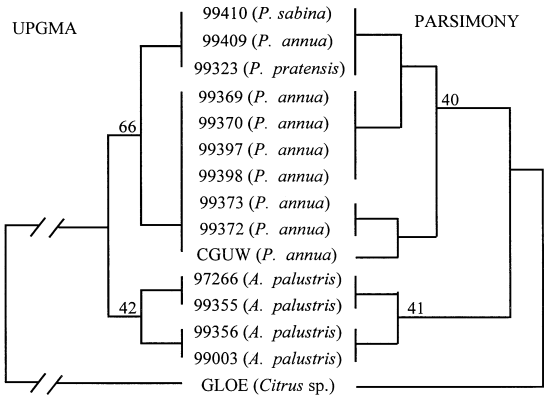

Figure 1. Dendrograms of 14 turfgrass isolates of *Colletotrichum graminicola* and the outgroup *C. gloeosporioides* inferred by genetic distance analysis (left side) or parsimony analysis (right side) of sequence data from DNA of the ITS region (ITS1-5.8S-ITS2). Host species are shown next to isolate names which are described in Table 1. Sequence analysis is described in Methods, and percentages from 1000 bootstrap replications are shown near the corresponding branches. In bootstrap of parsimony analysis only unique sequences were used.

comparisons of the complete ITS region, sequences of 13 turfgrass isolates were aligned with those of an isolate from annual bluegrass from Illinois (CGUW) along with an isolate of *C. gloeosporioides* from a *Citrus* sp. (GLOE) as the outgroup (Figure 1). In both UPGMA and parsimony analyses, all of the isolates collected from turfgrass formed two main groups with isolates from bluegrass species separated from creeping bentgrass isolates. However, the bootstrap values were low to moderate (40–66) for these main branches. The nucleotide identity was high among sequences, and there was only a small number of informative sites in the ITS alignment of turfgrass isolates of *C. graminicola*. From this analysis of the complete ITS region, isolates 99370, 99323, 97266 and 99003 as well as the isolate CGUW reported from annual bluegrass in Illinois were chosen for comparison to other *C. graminicola* and *Colletotrichum* spp.

Most ITS sequences previously reported for various *Colletotrichum* spp. comprised only the ITS1 or the ITS2 region, and so separate analyses of the ITS1 and the ITS2 sequences were conducted. A comparison of ITS1 sequences using UPGMA and parsimony analyses showed high bootstrap support (86 and 93, respectively) for the branch containing a cluster of isolates from maize, sorghum and one isolate from annual bluegrass (Figure 2). Within this cluster of maize and sorghum isolates, there was further stratification

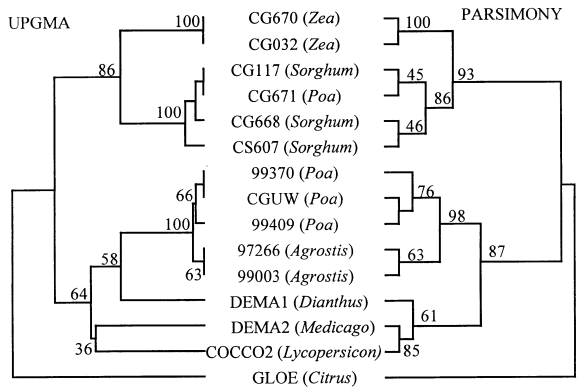

Figure 2. Dendrograms of 15 isolates of *Colletotrichum* spp. inferred by genetic distance analysis (left side) or parsimony analysis (right side) of sequence data from DNA of the ITS1 region. Host genera are shown next to isolate names which are described in Table 1. Sequence analysis is described in Materials and methods, and percentages from 1000 bootstrap replications are shown near the corresponding branches.

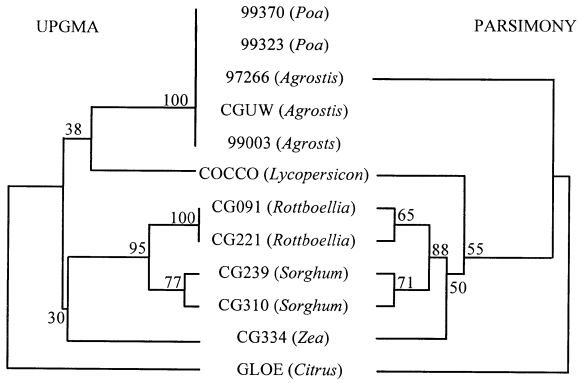

Figure 3. Dendrograms of 12 isolates of *Colletotrichum* spp. inferred by genetic distance analysis (left side) or parsimony analysis (right side) of sequence data from DNA of the ITS2. Host genera are shown next to isolate names which are described in Table 1. Sequence analysis is described in Materials and methods, and percentages from 1000 bootstrap replications are shown near the corresponding branches. In bootstrap of parsimony analysis, only one sequence of the five identical ITS2 sequences from turfgrasses was used.

based on host species (sorghum or maize) with very high bootstrap support for this separation (Figure 2). Turfgrass isolates were grouped together with very high bootstrap support ($\geq$98, Figure 2), except for the isolate from annual bluegrass in Texas (CG671), which was most similar to a sorghum isolate (CG117). In ITS1 analyses, isolates from annual bluegrass and creeping bentgrass from Canada and the northern USA clustered with isolates of *C. dematium* and *C. coccodes* with moderate (64, UPGMA) to high (87, parsimony) bootstrap support (Figure 2). Surprisingly, in both parsimony and UPGMA analyses, one isolate of *C. dematium* (DEMA2) was found to be more similar to *C. coccodes* (COCCO2) than to the other isolate of *C. dematium* (DEMA1).

A comparison of ITS2 sequences also showed that isolates from annual bluegrass and creeping bentgrass clustered separately from maize and sorghum isolates with high bootstrap support (Figure 3). There was no variation in ITS2 sequences of turfgrass isolates, and to avoid complications in bootstrap for parsimony analysis, only a single ITS2 sequence from turfgrass isolates was used for parsimony analysis. In UPGMA analysis, the *C. coccodes* isolate (COCCO) clustered with the turfgrass isolates, while it clustered with the maize and sorghum isolates in parsimony analysis, in both cases without high bootstrap support (Figure 3). For both types of analyses, the sorghum isolates were distinguished from rottboellia isolates with moderate to high bootstrap support (Figure 3). The single maize isolate (CG334) was more similar to the sorghum and rottboellia isolates but with low bootstrap support ($\leq$50, Figure 3). This bootstrap value increased to 65 when the COCCO was omitted from parsimony analysis (data not shown).

Discussion

The results showed that isolates of *C. graminicola* from creeping bentgrass differed from isolates from bluegrasses in ITS sequence, but the bootstrap values were not high for the grouping based on the ITS sequences. Although it appears that there is differentiation among these fungi related to their host origin, supporting the RAPD results of Backman et al. (1999), these differences are not large.

In growth room tests, both Backman et al. (1999) and Browning et al. (1999) found that creeping bentgrass isolates caused foliar blight and basal rot on both creeping bentgrass and annual bluegrass, whereas annual bluegrass isolates caused little and often no disease on creeping bentgrass. Annual bluegrass isolates generally appeared to have the most limited host ranges of any *C. graminicola* isolates. It is unknown how this might affect their distribution in the field, but some degree of host specialization may explain the genetic differences between *C. graminicola* isolates from bluegrasses and creeping bentgrass.

ITS1 and ITS2 sequence analyses of isolates of *Colletotrichum* from turfgrasses and a variety of other hosts showed that the turfgrass isolates formed their own clade with relatively high bootstrap support. In most previous literature, nearly all *Colletotrichum* isolates on gramineae have been assigned to *C. graminicola* (Sutton, 1980). Sutton (1968) demonstrated differences in appressoria of isolates from *Zea, Sorghum*, and *Saccharum*, and Sutton (1980) proposed the transfer of sorghum isolates to *C. sublineolum*. Our results based on ITS sequence data support the distinction between isolates from maize or sorghum. However, our data do not support the classification of bluegrass and creeping bentgrass isolates with the *C. graminicola* or *C. sublineolum* isolates described by Sherriff et al. (1995) and Sreenivasaprasad et al. (1996), and the results suggest that further division of *C. graminicola* may be necessary for turfgrass isolates.

An exception to the differentiation of turfgrass isolates of *C. graminicola* from maize and sorghum isolates by ITS sequence analysis was an isolate from annual bluegrass in Texas which was most similar to sorghum isolates based on ITS1 sequences. Annual bluegrass was the most susceptible host compared to maize, sorghum and creeping bentgrass, and could be infected by any *Colletotrichum* isolates from turfgrasses, maize or sorghum (Backman et al., 1999; Browning et al., 1999). In Texas, there is considerable commercial cultivation of sorghum, but sorghum is not grown near the origins of the northern *C. graminicola* isolates. It is not known if sorghum fields in Texas were close enough to the infected annual bluegrass to allow for cross infection, but based on inoculation studies by Browning et al. (1999), it is certainly possible that isolates originating from sorghum are capable of infecting annual bluegrass.

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