Unraveling Evolutionary Relationships Among the Divergent Lineages of *Colletotrichum* Causing Anthracnose Disease in Turfgrass and Corn

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ABSTRACT

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Colletotrichum species cause anthracnose diseases on a number of grass hosts and are common inhabitants of many others. They are divided into four species: *C. sublineolum* is pathogenic to *Sorghum* spp.; *C. caudatum* is found on C4 grasses such as indiagrass and big bluestem; *C. falcatum* causes red rot of sugarcane; and *C. graminicola* sensu lato is a broadly defined species including isolates that attack maize, wheat, oats, and many forage, turf, and amenity grasses of the subfamily Pooideae. In this paper, a combination of hierarchical- and nonhierarchical-

based analyses were employed to examine evolutionary relationships among the grass-infecting *Colletotrichum* species, with special emphasis on isolates from turf and other grasses in the subfamily Pooideae. Reconstructions performed with data sets from over 100 *Colletotrichum* isolates at three variable loci using phylogenetic and network-based methodologies unambiguously supported the taxonomic separation of maize-infecting isolates of *C. graminicola* from the pooid-infecting strains of *Colletotrichum*. To reflect the evolutionary relationships that exist between these distinct lineages, we propose the resurrection of the species name *C. cereale* to describe the pooid-infecting isolates. There was also support for further subdivision of *C. cereale*, but the current data are insufficient to confidently subdivide the species, as there was some evidence of recombination between lineages of this species.

When confronted with fungi that rapidly emerge as destructive pathogens in cultivated plant communities, an understanding of how genetic variation is organized often allows us to reconstruct the sequence of events influencing both the onset and trajectory of disease epidemics. By considering fungal phytopathogen life histories and diversity, it has been possible to deduce the events that have shaped many of the most destructive plant disease outbreaks during the past century. For example, the manifestation of the Dutch elm disease pandemics caused by *Ophiostoma* spp. is known to be characterized by a series of rapid intercontinental migratory events, introgressive gene transfers resulting in novel hybrid genotypes, and, significantly, by the replacement of one pathogen (*O. ulmi*) by a new, more destructive species (*O. novo-ulmi*) (11 for review). Similarly, by reconstructing phylogenetic relationships among host-specific populations of the rice blast pathogen (*Magnaporthe oryzae*), a recent study suggests that host shifts of the pathogen from non-rice plants to rice crops occurred during the era of rice domestication (17). Pursuit of this knowledge is not merely academic: with enough information, plant pathologists have a greater probability of implementing successful disease control measures.

In cereals and grasses, anthracnose disease is caused by four distinct *Colletotrichum* species, each delimited partly by host

specificity, but primarily differentiated by morphological features. *C. sublineolum* Henn. is pathogenic to *Sorghum* spp.; *C. caudatum* Peck is found on a number of C4 grasses such as indiagrass and big bluestem (*Sorghastrum nutans* (L.) Nash and *Andropogon gerardii* Vitman, respectively); and *C. falcatum* Went causes red rot of sugarcane (*Saccharum officinarum* L.). *C. graminicola* (Ces.) G.W. Wilson is generally considered to inhabit a wide range of hosts including corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), and many forage, turf, and amenity grasses of the Poaceae subfamily Pooideae (71). Sutton formally proposed in 1980, however, that the species circumscription is properly applied only to *Colletotrichum* on corn; therefore, valid publication of *C. graminicola* sensu stricto Sutton rendered illegitimate the application of the name *C. graminicola* to any *Colletotrichum* from hosts other than corn (61). As no diagnoses of a new species taxon for the fungi distributed on pooid grasses has been established, *C. graminicola* sensu lato G.W. Wilson is still routinely, but inappropriately, employed to describe these strains (10).

Historically, *C. graminicola* sensu lato G.W. Wilson was known as a plant pathogen of minor importance (18,20), and in almost all grasses this is still true. But toward the end of the 20th century, two devastating anthracnose disease epidemics caused by *C. graminicola* occurred quite suddenly in North America: first, in corn crops and later in turfgrasses of the subfamily Pooideae cultivated as golf course greens (especially annual bluegrass [*Poa annua* L.] and creeping bentgrass [*Agrostis stolonifera* L.]). The recent disease upsurge caused by *C. graminicola* has devastated turfgrasses maintained as golf course greens since the early 1990s and shows no signs of abating. In turf, changing cultivation practices are likely a contributing factor to disease development, as stands of grass are exposed to increasingly harsh management regimes designed to enhance playability for golfers. Factors such as minimal nitrogen fertilization, decreased cutting heights, variability in fungicide efficacy, and the increased usage of plant growth regulating chemicals have all been implicated in the enhancement of

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All sequences have been deposited in GenBank (accession nos. DQ126157 to DQ126262, DQ131924 to DQ132027, DQ132028 to DQ132051, and DQ133257 to DQ133340).

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains supplemental material not included in the print edition. The online version contains maximum parsimony trees of ITS, HMG box, and SOD2 regions, and a representative sample of the sequence alignments that were the basis for those trees.

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anthracnose disease levels (22; J. Inguagiato and B. B. Clarke, unpublished data).

As anthracnose disease in turfgrasses attributed to *C. graminicola* became increasingly problematic throughout the 1990s, a number of research groups conducted analyses of molecular genetic diversity or inferred gene genealogies intended to characterize variability in the *C. graminicola* species group. Several conflicting hypotheses were proposed concerning the evolution and diversity of *C. graminicola*, but no theory has generally been accepted as an accurate representation of the species history. Molecular data were used to suggest a close connection between *C. sublineolum* and isolates of *Colletotrichum* causing anthracnose in annual bluegrass turf (6,30), whereas another study supported an association between isolates from creeping bentgrass turf and those from corn (6). Results from other research groups supported a separate lineage of turfgrass pathogens, diverged from corn and sorghum isolates, which share a single ancestor (12,19,25,31).

In this study, we jointly consider three complementary data sets to investigate the patterns of evolutionary relationships among *Colletotrichum* isolates responsible for anthracnose disease in turfgrasses, corn, and other grass hosts. We tested the following expectations: (i) *C. graminicola* sensu lato G.W. Wilson is actually an assemblage of divergent lineages, rather than a single cohesive species; and (ii) although this fungus appears to reproduce clonally through asexual conidia (16), with the sexual state (*Glomerella*) not yet observed in a natural setting, lineages of these fungi may still be structured to some degree by reticulate evolution. To consider these concepts, data from over 100 *Colletotrichum* isolates from grasses and cereals were collected from more than 88 populations throughout the United States and Canada. A combination of hierarchal- and nonhierarchal-based approaches that were used to test the limits of species boundaries rigorously provides compelling evidence of sympatric, species-level phylogenetic divergence between *C. graminicola* pathogenic to maize and *Colletotrichum* populations causing disease in grasses of the subfamily Pooideae, including cultivated C3 turfgrass species. In particular, these analyses demonstrated that *Colletotrichum* spp. isolated from maize and pooid grasses are each more closely related to *C. sublineolum* from sorghum than these two lineages are to one another. To reflect the evolutionary relationships that exist between these distinct organisms, we formally propose to resurrect and revise the species *C. cereale* Manns (54) to describe the unique lineages isolated from pooid grasses, while *C. graminicola* sensu stricto Sutton uniquely describes *Colletotrichum* from corn hosts. To aid communication throughout this narrative and to emphasize the fundamental distinction to be made between these highly divergent lineages, we will henceforth refer to any *Colletotrichum* specimen isolated from pooid grasses as a member of the *C. cereale* species group, and *C. graminicola* as the species of *Colletotrichum* pathogenic to corn.

MATERIALS AND METHODS

Taxon sampling for molecular analysis. A total of 107 specimens of *C. graminicola* isolated from North American grasses (Table 1) were assessed for this study, with the majority of individuals (76%) isolated from diseased stands of turfgrass maintained as golf course greens. This sample collection includes specimens from 88 localities, and closely reflects the geographic regions where anthracnose disease on golf course greens has been most prevalent in North America; not all known localities were sampled.

Collected fungi were established in pure culture on potato dextrose agar (PDA; Fisher Scientific, Hampton, NH) and then single-spore purified to ensure isogenic lines. Cultures were preserved as dehydrated mycelia on Whatman glass fiber at -20°C . Identification of fungal colonies as *Colletotrichum* was performed using spore and setae morphological characters and later confirmed by

performing a BLAST search (3) of the partial ribosomal DNA (rDNA) sequences against the National Center for Biotechnology Information (NCBI) database.

Outgroup taxa. Phylogenetic relationships within the genus *Colletotrichum* are currently ill-defined, making the choice of an appropriate outgroup to root our phylogenetic tree uncertain based upon previously published hypotheses. Traditional *Colletotrichum* systematics relies heavily upon host plant association (13); this criterion has been repeatedly shown to be unreliable for the genus; in some instances several species are capable of infecting a single host plant (37,42) or a single species may be associated with an extremely broad host range (28 for review). Morphological characters in the genus may also be systematically uninformative, and are often inadequate to resolve even interspecific relationships with any level of confidence (62). Likewise, molecular investigations of the group conducted to date fail to infer evolutionary relationships between the *Colletotrichum* taxa robustly. Instead, unresolved, minimally supported topologies from which no significant conclusions can be drawn have been found (45). Because of the ambiguous state of *Colletotrichum* taxonomy, we constructed a phylogenetic tree using the nucleotide sequence from the rDNA internal transcribed spacer (ITS) region to select an appropriate species empirically as outgroup taxa for this study. BLAST searches of the NCBI database GenBank using the ITS sequence from strains MO-1001178, PA-50005, and NJ-6340 identified a number of *Colletotrichum* species likely to be closely related to *C. graminicola*. Twenty-four sequences were analyzed (AY536046, AF272786, AF489567, AJ301951, AJ301922, AF272783, AF272782, AB042305, AB042304, AY376530, AB196301, AJ301954, AF411770, AJ536231, AF534469, AJ301975, AJ301968, and AJ311391) along with a representative selection of *C. graminicola* ingroup taxa. Based upon the resultant 75% consensus tree topology (data not shown), four isolates of *C. sublineolum* from sorghum were included in this study, since this species appeared to be a closely related sister taxon to *C. graminicola*. In addition, two isolates of *C. acutatum* J.H. Simmonds were included as outgroup taxa because they are clearly distinct from *C. graminicola* both morphologically and on the molecular level, yet still show a close enough relationship to allow for unambiguous nucleotide sequence alignments.

DNA isolation, amplification and sequencing. Total genomic DNA was extracted from fungal cultures using a standard phenol/chloroform protocol as described (19). Polymerase chain reaction (PCR) products for the ITS region of the *ITS1*, 5.8S, and *ITS2* rDNA and the conserved HMG-box of the *MAT1-2* mating-type locus (HMG) were generated using published primer pairs and amplification conditions: ITS4 and ITS5 (70) for the ITS region, NcHMG 1 and 2 (4) or CgHMG 1 and 2 (66) for the HMG sequence. New primer pairs were designed to amplify a 625- or 505-bp portion of a single copy manganese-type superoxide dismutase gene (*SOD2*) (26): SOD625F/R (5'GCCACAGTAC-ATATTGCCTAAGC3' and 5'TCATCCCGGGAGCCAGAAAAC-CT3') or SOD507F/R (5'ATGGCAGCCTTTCCGTTGAGATAC-3' and 5'AGTTGACATGAAGCCACCTACAGC3'). PCR primers were synthesized by Sigma Genosys (The Woodlands, TX), and all amplifications were performed in a Biometra UNO thermoblock (Whatman Biometra, Goettingen, Germany) in 25- μl reactions using 37.5 ng of genomic DNA, 1 unit of *Taq* DNA polymerase (Promega, Madison, WI) in 10 $\times$ PCR buffer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, and 12.5 ng of each primer. *SOD2* products were amplified from genomic DNA using an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min; with a final extension at 72°C for 10 min. Negative controls were included in all amplifications to check for possible contamination. Amplified fragments were visualized on 0.8% agarose gels, and then excised and purified using the Gene Clean III Kit (Qbiogene, Irvine, CA). Nucleotide sequences were

generated from the forward and reverse strands directly from the amplicon using the corresponding PCR primers and BigDye Terminator cycle-sequencing chemistry (Applied Biosystems, Foster City, CA) on an ABI 3100 Capillary Sequencer following the manufacturer's protocol but using a quarter of the suggested reaction volume.

Nucleotide sequences were assembled and edited using the Lasergene Sequence Analysis Software package (DNASTAR, Inc., Madison, WI). Three hundred eighteen DNA sequences were deposited in GenBank under accession nos. DQ126157 to DQ126262 (ITS), DQ131924 to DQ132027 (HMG), and DQ132028 to

DQ132051 and DQ133257 to DQ133340 (*SOD2*). The remaining 15 sequences were not reported because they overlapped data already placed by Du et al. (25) (DQ003109-12, DQ003114, DQ003116-17, DQ002855-59, DQ002826, DQ002862, DQ002865, and DQ002868). Sequences were aligned using the Clustal W algorithm (65) and then manually adjusted in Microsoft Word (Microsoft Corp., Redmond, WA). The alignment of protein coding regions was refined according to amino acid sequences and intron positions. Gaps were removed from the data set, coded as single multi-state characters, and then reintroduced as coded data for the phylogenetic analyses.

TABLE 1. Sources of *Colletotrichum* isolates used in this study

Clade ^a	Haplotype (frequency)	Isolate name	Host species	Origin	Source	Original reference
...	1 (1)	NJ-CACA (outgroup)	<i>Calamagrostis acutifolia</i> (<i>C. acutatum</i>)	New Jersey	...	This study
B	2 (1)	ALB-99325	<i>Poa pratensis</i>	Alberta, Canada	T. Hsiang	This study
A	3 (1)	MA-6722	<i>Poa annua</i>	Massachusetts	...	This study
A	4 (1)	CT-6956	<i>Poa annua</i>	Connecticut	...	This study
A	5 (22)	CA-62	<i>Poa annua</i>	California	F. Wong	This study
A	5 (22)	CA-1049	<i>Poa annua</i>	California	F. Wong	This study
A	5 (22)	CA-1143	<i>Poa annua</i>	California	F. Wong	This study
A	5 (22)	CA-CL9	<i>Poa annua</i>	California	F. Wong	This study
A	5 (22)	CA-SH29	<i>Poa annua</i>	California	F. Wong	This study
A	5 (22)	CT-2	<i>Poa annua</i>	Connecticut	N. Jackson	(12)
A	5 (22)	IL-P6G	<i>Poa annua</i>	Illinois	N. Jackson	(12)
A	5 (22)	IL-PT	<i>Poa annua</i>	Illinois	R. Kane	This study
A	5 (22)	IL-PV1	<i>Poa annua</i>	Illinois	R. Kane	This study
A	5 (22)	IL-PV2	<i>Poa annua</i>	Illinois	R. Kane	This study
A	5 (22)	IL-RCC	<i>Poa annua</i>	Illinois	R. Kane	This study
A	5 (22)	NJ-6795	<i>Poa annua</i>	New Jersey	...	This study
A	5 (22)	NJ-7284	<i>Poa annua</i>	New Jersey	...	This study
A	5 (22)	NY-8422	<i>Poa annua</i>	New York	...	This study
A	5 (22)	NY-USGA	<i>Poa annua</i>	New York	...	This study
A	5 (22)	ONT-00176	<i>Agrostis stolonifera</i>	Ontario, Canada	T. Hsiang	This study
A	5 (22)	PA-50014	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
A	5 (22)	PA-50111	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
A	5 (22)	PA-50231	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
A	5 (22)	PA-V1	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
A	5 (22)	PA-WH3	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
A	5 (22)	RI-8	<i>Poa annua</i>	Rhode Island	N. Jackson	(12)
A	6 (1)	NBR-13	<i>Poa annua</i>	New Brunswick, Canada	N. Jackson	(12)
A	7 (1)	CA-540	<i>Poa annua</i>	California	F. Wong	This study
A	8 (1)	CO-8910	<i>Poa annua</i>	Colorado	...	This study
A	9 (4)	KS-20DGU	<i>Dactylis glomerata</i>	Kansas	...	This study
A	9 (4)	KS-20DGY	<i>Dactylis glomerata</i>	Kansas	...	This study
A	9 (4)	KS-20EVD	<i>Elymus virginicanus</i>	Kansas	...	This study
A	9 (4)	KS-20EVM	<i>Elymus virginicanus</i>	Kansas	...	This study
A	10 (1)	NJ-CA1	<i>Calamagrostis acutifolia</i>	New Jersey	...	This study
A	11 (1)	NJ-DG1	<i>Dactylis glomerata</i>	New Jersey	...	This study
A	12 (1)	NJ-8627	<i>Poa annua</i>	New Jersey	...	This study
A	13 (1)	KS-10EC1A	<i>Elymus canadensis</i>	Kansas	...	This study
A	14 (1)	NY-8900	<i>Poa annua</i>	New York	...	This study
A	15 (1)	NJ-8626	<i>Poa annua</i>	New Jersey	...	This study
A	16 (1)	NJ-6340	<i>Poa annua</i>	New Jersey	...	This study
A	17 (2)	CA-EG15	<i>Poa annua</i>	California	F. Wong	This study
A	17 (2)	CA-SC44	<i>Poa annua</i>	California	F. Wong	This study
A	18 (1)	MA-11	<i>Poa annua</i>	Massachusetts	N. Jackson	(12)
A	19 (1)	CT-14	<i>Poa annua</i>	Connecticut	N. Jackson	(12)
A	20 (1)	KS-20BIG	<i>Bromus inermis</i>	Kansas	...	This study
A	17 (2)	NJ-9582	<i>Poa annua</i>	New Jersey	...	This study
A	17 (2)	PA-50183	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
A	22 (1)	CA-SC32	<i>Poa annua</i>	California	F. Wong	This study
A	23 (1)	CA-1715	<i>Poa annua</i>	California	F. Wong	This study
A	24 (1)	RI-22	<i>Agrostis stolonifera</i>	Rhode Island	N. Jackson	(12)
B	25 (1)	PA-50002	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	26 (1)	CT-28	<i>Agrostis stolonifera</i>	Connecticut	N. Jackson	(12)
B	27 (1)	NJ-6607	<i>Poa annua</i>	New Jersey	...	This study
B	28 (1)	MA-17	<i>Agrostis stolonifera</i>	Massachusetts	N. Jackson	(12)
B	29 (1)	ONT-00133	<i>Agrostis stolonifera</i>	Ontario, Canada	T. Hsiang	This study
B	30 (1)	CT-25	<i>Agrostis stolonifera</i>	Connecticut	N. Jackson	(12)
B	31 (3)	MA-20	<i>Agrostis stolonifera</i>	Massachusetts	N. Jackson	(12)

(Continued on next page)

^a Clades A to D are inferred from multilocus sequence analyses, as shown in Figure 1.

Phylogenetic analyses. We used both Bayesian maximum likelihood (ML) and maximum parsimony (MP) methods to infer phylogenetic relationships from each gene region and for the combined data set. Models of evolution and ML parameters were estimated individually for each data set using the Akaike information criterion (AIC) (1) as implemented in Modeltest version 3.06 (47). Bayesian analyses were performed using MrBayes version 3.0b4 (34), which allowed the different data partitions to be modeled separately using the evolutionary models and informative priors that best described the data. The optimal model selected under the AIC implemented in Modeltest was specified as prior for each gene. One cold and three incrementally heated

Metropolis-coupled Monte Carlo Markov chains were started from random trees and run simultaneously for 14,000,000 generations each in order to sample the phylogenies according to their posterior probabilities. Three replicate runs were performed, and tree topologies and stationarity levels were compared for convergence (34). To determine the point at which stationarity was achieved in each run, log likelihood scores were plotted against generation time; the initial generations sampled before convergence were discarded as burn-in. We sampled trees from every 500 generations across all four independent analyses to calculate posterior probabilities for each branch in the ML tree. Trees sampled from the posterior distribution were imported into

TABLE 1. (Continued from preceding page)

Clade ^a	Haplotype (frequency)	Isolate name	Host species	Origin	Source	Original reference
B	31 (3)	MA-21	<i>Agrostis stolonifera</i>	Massachusetts	N. Jackson	(12)
B	31 (3)	ONT-00128	<i>Agrostis stolonifera</i>	Ontario, Canada	T. Hsiang	This study
B	32 (2)	ONT-00124	<i>Agrostis stolonifera</i>	Ontario, Canada	T. Hsiang	(16)
B	32 (2)	ONT-00126	<i>Agrostis stolonifera</i>	Ontario, Canada	T. Hsiang	This study
B	33 (1)	PA-50621	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	34 (1)	NY-19	<i>Agrostis stolonifera</i>	New York	N. Jackson	(12)
B	35 (1)	PA-4410	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	36 (1)	NJ-6491	<i>Poa annua</i>	New Jersey	...	This study
B	37 (2)	NJ-4990	<i>Poa annua</i>	New Jersey	...	This study
B	37 (2)	PA-1112	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	38 (2)	PA-50005	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	38 (2)	PA-50181	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	39 (1)	MA-24	<i>Agrostis stolonifera</i>	Massachusetts	N. Jackson	(12)
B	40 (1)	PA-50623	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	41 (1)	CT-18	<i>Agrostis stolonifera</i>	Connecticut	N. Jackson	(12)
A	42 (1)	TX-26	<i>Agrostis stolonifera</i>	Texas	N. Jackson	(12)
A	43 (1)	VA-8977	<i>Poa annua</i>	Virginia	...	This study
A	44 (1)	ONT-99359	<i>Agrostis stolonifera</i>	Ontario, Canada	T. Hsiang	This study
A	45 (1)	NH-23	<i>Agrostis stolonifera</i>	New Hampshire	N. Jackson	(12)
A	46 (1)	NJ-8467	<i>Poa annua</i>	New Jersey	...	This study
A	47 (5)	NJ-7130	<i>Agrostis stolonifera</i>	New Jersey	...	This study
A	47 (5)	NJ-8400	<i>Lolium perenne</i>	New Jersey	...	This study
A	47 (5)	NJ-RWCC	<i>Poa annua</i>	New Jersey	...	This study
A	47 (5)	NJ-HF2B	<i>Agrostis stolonifera</i>	New Jersey	...	This study
A	47 (5)	PA-211	<i>Poa annua</i>	Pennsylvania	W. Uddin	This study
B	48 (1)	NJ-6553	<i>Poa annua</i>	New Jersey	...	This study
B	49 (1)	CT-27	<i>Agrostis stolonifera</i>	Connecticut	N. Jackson	(12)
B	50 (1)	NY-16	<i>Agrostis stolonifera</i>	New York	N. Jackson	This study
...	51 (1)	NJ-10BB (outgroup)	<i>Vaccinium corymbosum</i> (<i>C. acutatum</i>)	New Jersey	P. Oudemans	This study
C	52 (1)	S3001	<i>Sorghum bicolor</i> (<i>C. sublineolum</i>)	Burkina Fasso	L. Vaillancourt	(67)
D	53 (1)	NY-15182	<i>Zea mays</i>	New York	G. Bergstrom	This study
C	54 (2)	S12001	<i>Sorghum bicolor</i>	Brazil	L. Vaillancourt	(67)
C	54 (2)	S19001	<i>Sorghum bicolor</i>	South Africa	L. Vaillancourt	(25)
C	55 (1)	S17001	<i>Sorghum bicolor</i>	Texas	L. Vaillancourt	(25)
D	56 (2)	MO-478	<i>Zea mays</i>	Missouri	L. Vaillancourt	This study
D	56 (2)	MO-978	<i>Zea mays</i>	Missouri	L. Vaillancourt	This study
D	57 (18)	BZ-500190	<i>Zea mays</i>	Brazil	L. Vaillancourt	(67)
D	57 (18)	IN-10472	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-10570	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-10670	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-10970	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-12270	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-12475	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-300170	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	IN-DUB90	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	57 (18)	KY-197	<i>Zea mays</i>	Kentucky	L. Vaillancourt	This study
D	57 (18)	KY-297	<i>Zea mays</i>	Kentucky	L. Vaillancourt	This study
D	57 (18)	KY-397	<i>Zea mays</i>	Kentucky	L. Vaillancourt	This study
D	57 (18)	KY-398	<i>Zea mays</i>	Kentucky	L. Vaillancourt	(25)
D	57 (18)	MO-178	<i>Zea mays</i>	Missouri	L. Vaillancourt	This study
D	57 (18)	MO-878	<i>Zea mays</i>	Missouri	L. Vaillancourt	This study
D	57 (18)	MO-1001178	<i>Zea mays</i>	Missouri	L. Vaillancourt	This study
D	57 (18)	NC-200170	<i>Zea mays</i>	North Carolina	L. Vaillancourt	This study
D	57 (18)	NY-AU80	<i>Zea mays</i>	New York	L. Vaillancourt	(25)
D	57 (18)	NY-400180	<i>Zea mays</i>	New York	L. Vaillancourt	This study
D	58 (1)	IN-D77	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study
D	59 (1)	IN-900190	<i>Zea mays</i>	Indiana	L. Vaillancourt	This study

PAUP* version 4.0b10 (63) and, after excluding the burn-in, a 75% majority-rule consensus tree was constructed, with the percentage of samples recovering a particular clade representing that clade's posterior probability (33). Bayesian posterior probabilities correspond to the statistical probability that a clade is present in the true tree, given the specified priors, the likelihood model, and the data examined (33,39); a final 75% consensus tree was then generated from all trees in the posterior distribution sampled from the three runs (83,539 trees).

MP analyses were performed using PAUP* under the heuristic search option, with starting trees obtained from 100 random-addition replicates, and using a tree-bisection reconnection (TBR) branch swapping strategy. Multiple MP trees for any data set were combined into single strict consensus trees. Nonparametric bootstrap resampling was conducted to evaluate relative levels of support for individual nodes (27) using 1,000 bootstrap pseudo-replicates with 100 random additions and TBR branch swapping. MP analyses were conducted for both the individual data sets and the total combined data set.

Estimating recombination and its impact on phylogenetic inference. Although *C. graminicola* is thought to reproduce in nature almost exclusively by clonally generated conidia, populations of this fungus still may be structured to some extent by recombination, either through historical events or through the exchange of genetic material by means of vegetative anastomosis between different individuals. Since the presence of recombination has the potential to produce conflicting phylogenetic signal, and may lead to incorrect evolutionary inferences, we investigated whether relationships between the *C. graminicola* taxa are more accurately represented by a reticulating network rather than a bifurcating phylogenetic tree topology. First, topologies of individual gene genealogies and the multilocus gene tree were visually inspected for the presence of incongruence suggestive of recombination. To detect recombination events not reflected in the tree topology, a combination of methods that use substitution patterns and site incompatibility to infer the presence of conflicting signal were employed. After removing indels from the aligned multilocus data set, sequences were collapsed into unique haplotypes using SNAP Map and SITES version 1.1 (29) launched through SNAP Workbench (51). Since the majority of the ingroup taxa in our analyses are likely to be recently diverged, all sites violating the infinite sites model, which assumes that the possibility of multiple mutations at a single nucleotide site can be ignored due to extremely low mutation rates, were eliminated. The resultant haplotype data set was used to identify the presence of reticulating relationships inappropriately represented in an acyclic tree topology; such relationships would be generated by evolutionary processes acting at the population level such as hybridization between lineages or recombination between genes, or homoplastic events such as convergent, parallel, or reverse mutations. We used the split decomposition network method, a transformation-based approach that uses distance parsimony to partition data sets into "splits" of sequences (8). The splits are combined successively, with any incompatible, contradictory groupings introducing a loop into the network to indicate the conflict. We employed the computer program SplitsTree version 4.0 b14 (35) to visualize reticulation networks in the data set, with branch support estimated by performing 10,000 bootstrap pseudo-replicates. In the split-graph network, reticulating taxa will be connected by more than one branch, with each connection representing alternative solutions; if the data are perfectly phylogenetic, a bifurcating tree topology will result.

To explore further the boundary between hierarchal and non-hierarchal, tokogenetic relationships in our sample, a site compatibility matrix was generated from the haplotype data set using the SNAP Clade and SNAP Matrix functions of SNAP Workbench. The compatibility matrix was used to identify the presence of compatibility/incompatibility among the haplotypes, with any

resultant incompatible sites removed from the data set. The data set was also evaluated for the signature of recombination events by using Hudson's four gamete test (32) executed in DnaSP (53); any sites identified as potentially recombinant were also excluded from the matrix. The resultant haplotype data set, with any potentially confounding recombinant or homoplastic sites removed, was used to perform MP analysis in PAUP* to visualize those portions of the tree topology uniquely characterized by hierarchal, species-level relationships.

Morphological analysis. To determine if the fungi described in the present study as *C. cereale* conform to the morphological description established by Selby and Manns (54), we inspected syn-type specimens from the Kansas State Herbarium, presently curated by the New York Botanical Garden Steere Herbarium (NYBG) (NYBG specimen numbers 305598 [type] and 276683 on *Bromus secalinus*; 305599 on *Avena sativa* [type]; 305595 on *Arrhenatherum elatius* [type]; 305596 [type] and 276680 on *Secale cereale*; 305597 [type], 276684 and 276681 on *Triticum vulgare*; 276686 on *Dactylis glomerata*; 276687 on *Phleum pratense*; 276685 on *Agrostis alba*; and unnumbered samples from *Agrostis alba*, *Arrhenatherum elatius*, and *Poa pratensis*). Morphological examination was also performed on *Colletotrichum* specimens isolated from pooid grasses in our collection (KS-20BIG from *Bromus inermis*; ONT-00128, NY-16, CT-18, MA-20, MA-21, MA-24, CT-25, and CT-27 from *Agrostis stolonifera*; NJ-6722, NJ-6795, CA-1715, NJ-4990, NH-23, NBR-13, RI-9, CT-8, MA-11, PA-50114, PA-WH3, PA-50623, PA-50231, PA-5005, PA-50111, PA-50183, PA-4410, PA-V1, and PA-50002 from *Poa annua*; KS-20DGU and KS-20DGY from *D. glomerata*; NJ-CA1 from *Calamagrostis acutifolia*; and KS-20EVM from *Elymus virginicanus*), with cultures grown on PDA under constant light at 26°C for 7 to 10 days. Microscopic observations and measurements were made with an Olympus CX40 microscope with bright field or phase contrast illumination. At least 50 conidia from each isolate were measured; dimensions of other fungal structures are given as the range of at least 20 measurements where possible. Photomicrographs were obtained using an Olympus BX41 microscope equipped with a MagnaFire Digital Firewire camera and software (Optronics, Goleta, CA).

RESULTS

Data set characteristics and analysis. Because ribosomal DNA genes are maintained at high copy numbers in eukaryotic genomes, nonorthologous copies may potentially be present (46), violating the expectation of homology required to reconstruct phylogenetic relationships accurately. In the present study, two lines of evidence support the presence of only orthologous rDNA sequences: nucleotide base calling from the ITS rDNA chromatographs generated by sequencing directly from the PCR products was clean and unambiguous, and all three gene trees were concordant at main branch points and tip clades where phylogenetic signal is adequate to resolve such relationships.

Among members of the *C. graminicola*/*C. cereale*/*C. sublineolum* ingroup, few insertion-deletion events were observed in the nucleotide sequence alignments, and none of the gaps introduced rendered the ingroup problematic; ambiguous alignment was restricted to outgroup comparisons. The combined molecular data set comprised a total of 1,229 nucleotides, with 130 indels coded as standard characters (Table 2). Much of the coded indel data were autapomorphic only with respect to the outgroup taxa and were otherwise parsimony-informative between ingroup taxa; 8 indels of 2 to 4 bp in length within introns were characteristic of particular monophyletic groupings recovered by the ingroup phylogeny.

Given the number of variable characters in the data sets, all three genes appeared to be potentially informative. A preliminary analysis of a 650-bp region of the *TUB-2* gene from the taxa NJ-

6340, PA-50005, NJ-6491, MO-1001178, and NY-15182 showed only one variable character; therefore, it was not included in the phylogenetic reconstruction (data not shown). In particular, the 536-bp *SOD2* sequence consists of 41% parsimony-informative characters within the ingroup taxa (ITS = 12%; HMG = 12%; combined = 25%). Individual gene genealogies constructed using strict consensus trees under parsimony showed visually concordant topologies, although the HMG and ITS sequences were unable to recover some of the groups reconstructed by the more informative *SOD2* and the combined data set due to their lower levels of phylogenetic signal. This relative lack of phylogenetic resolution from the *C. graminicola* ITS sequence is consistent with our previously published results (19) and a study conducted by Hsiang and Goodwin (31); similar reports have been made in other *Colletotrichum* species (7). The lower resolution exhibited by the ITS gene tree may also result to some degree from the extremely large number of equally parsimonious tree topologies recovered during the heuristic searches: 44,034 MP trees were inferred from the ITS sequence data (HMG = 4; *SOD2* = 72).

To assess whether the ITS, HMG, and *SOD2* sequences generate tree topologies that are congruent, we visually compared the individual gene genealogies for evidence of contradictory phylogenetic relationships. Although the incongruence length difference test (ILD; implemented as the partition length homogeneity test in PAUP) is still used in many phylogenetic studies to assess potential conflict among data sets, several properties of this test are known to generate misleading results, particularly when among-site rate variation is present (9,21,23,24,57). Because this is the case in our study, the ILD is probably not a reliable method to determine congruence of data sets stringently. We therefore relied on visual inspection to evaluate congruence between the individual gene genealogies. We found only three isolates with inconsistent placement between the gene trees: MA-6722 from *Poa annua* grouped with *C. cereale* clade A for the *SOD2* and combined trees, but with *C. cereale* clade B for the ITS and HMG; NJ-8467 from *Poa annua* grouped with *C. cereale* clade A for the ITS, HMG, and combined trees, but with *C. cereale* clade B for the *SOD2* tree; and NY-15182 from *Z. mays* grouped with *C. graminicola* for the HMG, *SOD2*, and combined trees, but with the *C. cereale* lineage for the ITS tree. Aside from these three individuals, the three gene genealogies all recovered monophyletic groups of *C. sublineolum*, *C. graminicola*, and *C. cereale* taxa,

were topologically congruent with one another at these main clades, and were also in agreement with population subdivisions recovered through an analysis of three transposable element restriction fragment length polymorphism (RFLP) patterns (J. Crouch, B. B. Clarke, and B. I. Hillman, *unpublished data*). The individual data sets were therefore combined and used for multilocus analysis.

Analysis of the three gene sequences using the AIC in the computer program Modeltest determined that the evolutionary signature of each sequence was best modeled separately for the Bayesian analyses (Table 3). Although the genes ultimately reconstruct the same relationships between the ingroup taxa, a distinct set of parameters uniquely characterizes the evolutionary processes experienced by each gene. In particular, all three gene sequences showed different rates of variation among different nucleotide sites (among-site rate variation). To correct for the presence of among-site rate variation in the data set, which can cause the number of unobserved, multiple substitutions to be underestimated, the α shape parameter from the gamma distribution was incorporated into the models for each gene (which were established as priors in the Bayesian ML analysis) with α inversely proportional to the amount of among-site rate heterogeneity (when rates are equal, α = infinity). Among-site rate heterogeneity in the *SOD2* sequence was considerable (α = 0.8737); heterogeneity present in the other genes was also estimated to be high (ITS, α = 0.9962; HMG, α = 1.8284).

In the Bayesian likelihood analyses, one run plateaued at 60,000 generations, the second after 119,000 iterations, and the third reached stationarity at 53,000 (ln likelihood values: run 1: -8785.664 to -8699.69, average -8733.853; run 2: -8826.63 to -8740.83, average -8740.83; and run 3: -8794.062 to -8658.552, average -8697.4453). All three runs converged on the same topology, so all generations sampled from the posterior distribution were combined (83,539 trees) to produce a 75% consensus tree (Fig. 1) representing our hypothesis of descent for these taxa. The consensus tree had an ln likelihood score of -8,746.140; only clades with relatively strong support (>75% support for nodes from the posterior distribution) were retained.

Phylogenetic relationships. All phylogenetic analyses, separate and combined, using either the complete data set, the data set reduced to unique haplotypes, or the haplotype data set reduced to strictly compatible sites (83 total sites) resolved three distinct,

TABLE 2. Characteristics of the genomic regions used in this investigation

Gene	Exon regions	Intron regions	Nucleotide characters, excluding indels	Coded characters (indels)	Invariable nucleotide characters ^a	Variable, parsimony uninformative nucleotide characters ^a	Parsimony informative nucleotide characters ^a	MP tree length	Number of equally parsimonious trees
ITS	1	2	482	49	358	68	56 (12%)	357	44,034
HMG	2	1	211	13	170	14	26 (12%)	139	4
SOD	3	2	536	38	504	0	221 (41%)	518	72
Combined data set	6	5	1,229	130	1,032	82	303 (25%)	1,058	8,813

^a Parsimony informative characterization of data set considers ingroup taxa only, outgroups are excluded.

TABLE 3. Best fit evolutionary models used in Bayesian analysis as determined by the Akaike information criterion (AIC) in Modeltest

<i>ITS1/5.8S/ITS2</i> 482 nucleotide characters	HMG (<i>MAT1-2</i>) 211 nucleotide characters	<i>SOD2</i> 536 nucleotide characters
SYM+G A→C 1.0161 A→G 1.2515 A→T 1.0096 C→G 1.7520 C→T 2.7189 G→T 1.000 ... Invariable = 0 Gamma α = 0.9962	HKY+G Frequency A = 0.305 Frequency C = 0.269 Frequency G = 0.224 Frequency T = 0.201 ... Ti/Tv ratio = 1.2350 Invariable = 0 Gamma α = 1.8284	HKY+G Frequency A = 0.2352 Frequency C = 0.2575 Frequency G = 0.3088 Frequency T = 0.1986 ... Ti/Tv ratio = 1.5889 Invariable = 0 Gamma α = 0.8737

KEY TO HOST PLANT ORIGIN

- ◻ *Agrostis canina*
- ⊕ *Agrostis stolonifera*
- ♥ *Bromus inermis*
- ◁ *Calamagrostis acutifolia*
- ◇ *Dactylis glomerata*
- ◁ *Elymus canadensis*
- ◻ *Elymus virginicanus*
- ◻ *Lolium perenne*
- ◻ *Poa annua*
- ◁ *Poa pratensis*
- *Sorghum bicolor*
- ◁ *Zea mays*

Fig. 1. Seventy-five percent majority rule Bayesian maximum likelihood phylogenetic tree constructed from the 83,539 trees sampled from the posterior distribution representing the well-supported hypothesis of descent recovered in the multilocus sequence analysis. The scale bar indicates divergence, measured in nucleotide changes per site. All groups illustrated by this tree are supported by posterior probabilities of at least 0.75. Posterior probabilities supporting the topology are shown above the nodes where space permits; elsewhere on the tree two asterisks were supported by posterior probabilities of >99%; branches with one asterisk were supported by posterior probabilities of >95%. Maximum parsimony analysis also recovered this same basic phylogeny; bootstrap values from 1,000 pseudoreplicates are shown below the nodes. Symbols next to taxa indicate host plant origin; the key to host plant origin is to the left of the phylogeny.

well-supported groups: (i) a monophyletic group comprising isolates from Pooideae grasses (clades A and B, Fig. 1), (ii) a group comprising isolates from sorghum (*C. sublineolum*, clade C), and (iii) a group comprising isolates from maize (clade D). In the *SOD2* and the multilocus tree, a further subdivision was observed: the *C. cereale* clade subdivided into unique lineages (clades A and B). *C. cereale* clade B further diverged into two smaller groupings. These groups were supported by both bootstrap values and posterior probabilities greater than 95%. The phylogenetic trees recovered under both parsimony and Bayesian likelihood optimality criteria are in agreement at all of the main clades. Figure 1 shows the 75% consensus tree constructed from the 83,539 trees sampled from the Bayesian posterior distribution; both the posterior probabilities and bootstrap support values are plotted at the nodes (the MP tree is not illustrated separately due to topological congruence with the ML phylogeny; groups not supported by at least a value of 0.75 by both bootstrap and posterior probabilities were collapsed). The results of the molecular phylogenetic analysis reconstructs a species history characterized by the presence of morphologically similar sister species: *C. graminicola*, which is pathogenic to corn, and a group of *C. cereale* lineages found on a wide range of pooid grasses, including cultivated C3 turfgrasses.

A much less well-supported association between *C. graminicola* and *C. sublineolum* was consistently recovered in all of the phylogenetic analyses, with these two species always forming a monophyletic group, separate from the *C. cereale* group. This association between the cereal-derived lineages was not, however, entirely well supported, with a posterior probability of only 0.63 in the Bayesian consensus tree. Analysis of the haplotype data set

using the split decomposition network reconstruction method (Fig. 2) also estimates a close relationship between *C. graminicola* and *C. sublineolum*, but confidence in a recent common ancestry for these taxa was extremely low (bootstrap = 56).

Reticulation and incompatibility in the data set. A total of 57 unique multilocus haplotypes were identified from the ingroup sample, with 611 nucleotides distinctively establishing the haplotypes after the removal of introns and any positions violating the infinite sites model. The split-decomposition splits-graph used to visualize the presence of reticulate evolution in the haplotype data set (Figs. 2 and 3) recovered tree-like relationships between the main species clades also estimated by the phylogenetic investigation: *C. sublineolum* from sorghum, *C. graminicola* from corn, and the *C. cereale* species group from the subfamily Pooideae grasses. Within the clades, on the intraspecific level, a reticulating network of relationships clearly emerged: a single loop was present among the *C. graminicola* haplotypes (five haplotypes, 23 individuals), a single loop connected the *C. sublineolum* haplotypes (three haplotypes, four individuals), and more complex patterns were found individually among the *C. cereale* lineages (Fig. 3). The two main *C. cereale* phylogenetic clades A and B were inferred in the splits-graph, with reticulate lineages occurring only below the level of these groups. *C. cereale* clade A consisted of 27 unique haplotypes from 59 individuals, all radiating from haplotype 5 (H5), which, given its central position in the splits-graph, is likely the ancestral haplotype for the *C. cereale* lineages. This interpretation is further supported by the observation that H5 contained the largest number of *C. cereale* isolates (22), and was widely distributed throughout the entire range of our sample. The majority of the haplotypes were tightly interrelated in clade A,

Fig. 2. Evolutionary relationships between the sampled taxa, inferred using split-decomposition network analysis as described in the text.

Fig. 3. An enlarged view of the splits-graph topology (Fig. 2), illustrating *Colletotrichum cereale* clades A and B. The small filled circles represent the positions of inferred intermediate haplotypes.

with only a few divergent lineages. Based upon its position within the splits-graph, clade A haplotype 47 also appeared to be a central lineage among the group, and served as the connecting point between *C. cereale* clades A and B. Haplotype 46, represented by *C. cereale* isolate NJ-8467 from *Poa annua*, was positioned directly between clades A and B, which is consistent with the observed conflict between gene genealogies for this isolate (ITS, HMG, and multilocus = clade A; *SOD2* = clade B). Collectively, these data suggest that this haplotype is a hybrid between the two *C. cereale* lineages. Haplotype 3, consisting of *C. cereale* isolate MA-6722, while similarly characterized by conflicting gene trees in the phylogenetic analyses (*SOD2* and multilocus tree = clade A; ITS and HMG = clade B), appears in the splits-graph to be a highly divergent taxon, but uniquely a member of the clade A lineage.

The haplotypes of *C. cereale* clade B, although numerically less prevalent in our sample, were nevertheless a much more genetically diverse assemblage, with 22 haplotypes resulting from 27 isolates interwoven into a complex pattern of cycles. While the phylogenetic analyses support the split of clade B into two distinct lineages, the splits-graph was unable to recover the subdivision, suggesting that these taxa are all of the same species.

Analysis of the haplotype data set by means of the compatibility matrix and Hudson's four gamete test identified the majority of characters as being incompatible; these characters were subsequently removed from the data set. The resultant 83 character data set was used to further investigate species-level boundaries through MP phylogenetic analysis. This reduced MP phylogeny (data not shown) confirmed the splits-graph topology: *C. sublineolum* from sorghum, *C. graminicola* from corn, and *C. cereale* from the Pooideae grasses are each individual species; however, the species level divergence of *C. cereale* clades A and B found in all other analyses was not recovered.

Taxonomy. Based upon the unique patterns of fixed nucleotide differences in DNA sequence data at the *ITS1/5.8S/ITS2*, *MAT1-2*, and *SOD2* nuclear loci, it is evident that *Colletotrichum* isolated from the pooid grasses examined in this study form a species taxon distinct from *C. graminicola*, which is limited to the fungus pathogenic to maize host plants. Based upon these nucleotide data, we formally propose to resurrect and emend the species *C. cereale* and to emend the species descriptions of *C. graminicola* and *C. sublineolum*.

Colletotrichum cereale Manns, Ohio Agric. Exp. Stn. Bull. 203:207 (1909).

Teleomorph. Unknown.

Emended description. Inhabits grasses of the subfamily Pooideae; pathogenic and sometimes particularly aggressive on *Poa annua*, *Poa pratensis*, *P. sapina*, *Agrostis stolonifera*, *Agrostis canina*, and *Lolium perenne*. *C. cereale* is uniquely described by the following fixed nucleotide characters (positions are relative to the nucleotide sequence alignment of the epitype specimens): *ITS1/5.8S/ITS2* positions 15 (C), 39 (T), 56 (C), 98 (C), 104 (C), 117 (G), 145 (C), 165 (A), 178 (T), 409 (A), 517 (T), 522 (C); *MAT1-2* positions 2 (A), 54 (C), 79 (A/T), 87 (G), 97 (A), 101 (C), 109 (A), 123 (T), 129 (G), 131 (A), 154 (C), 163 (C), 172 (A), 199 (A); and *SOD2* positions 35 (C/T), 45 (A), 47 (C), 53 (A), 58 (A), 61 (G), 70 (C), 73 (A), 77 (T), 88 (C), 119 (G), 127 (C), 133 (C), 136 (G), 142 (C), 151 (C), 169 (A), 175 (C), 205 (C), 211 (C), 226 (G), 244 (A), 247 (C), 259 (C), 262 (C), 268 (C), 280 (T), 292 (G), 332 (G), 355 (C), 373 (G/A), 376 (C), 382 (C), 386 (G), 394 (T), 403 (C), 451 (C), 454 (C), 455 (G), 500 (T), 501 (G), 512 (C), 518 (T), 519 (T), 520 (G), 521 (G), 522 (A), 525 (G), 534 (A), 537 (C), 541 (C), 545 (T), 546 (T), 547 (C), 551 (C), 558 (T), and 571 (G).

Colonies on PDA under constant illumination highly variable; usually form dark mat of tight setae masses across agar surface;

commonly heavy conidia accumulation gives orange cast to brown/black culture. Some cultures exhibit thin layer of hyphae along agar surface, copious amounts of conidia give orange appearance. Some cultures possess fluffy aerial mycelium growing over setae, producing gray appearance; generally as colonies age mycelia overtakes entire culture surface. Hyphae septate, normally hyaline, sometimes dark brown when present at the base of setae, 1.0 to 6.5 μm , often guttulate. Conidia falcate or fusiform, apices acute, individually hyaline but appear salmon-orange en mass, may be mono-, bi-, or up to seven-guttulate or oil drops may be absent from the cytoplasm, measuring 6.0 to 33.8 μm $\times$ 2.2 to 6.3 μm with an average of 23.3 μm $\times$ 3.4 μm . Germinating conidia

Fig. 4. *Colletotrichum cereale* morphological structures (Bar = 25 μm). **A**, Heavily melanized setae emerging from an acervulus on the leaf of *Bromus secalinus* from the lectotype specimen (NYBG-305598); **B**, setae and conidia, grown on potato dextrose agar from epitype strain KS-20BIG; and **C**, germinating conidia, with the formation of germ tubes generally (but not always) leading to the formation of heavily melanized appressoria.

form single or infrequently two hyaline germ tubes that terminate in dark brown/black appressoria, sometimes appressoria absent; germ tubes separated from appressoria by septa (Fig. 4), occasionally appressoria forms directly from conidia. Appressoria rounded and smooth or irregular or lobate or multi-lobate, measuring 8.5 to 11.6 $\mu\text{m} \times 6.5$ to 10.2 μm . Setae develop from dark brown, tight masses of hyphae (Fig. 4); in culture dense, rounded masses of setae form, (Fig. 4) sometimes partly or completely covered in thick deposition of conidia. Individual brown-black setae separated from hyphae by septa. Setae base swollen or not swollen, irregularly septate with up to 7 septa, measuring 32 to 120 $\mu\text{m} \times 6$ to 8 μm at base, tapering at tip.

Type specimen. Examination of herbarium syntype specimens confirmed the presence of acervuli on the grass hosts, in association with black-brown setae that are characteristic of *Colletotrichum*. Very few conidia were observed; those that were present were somewhat shriveled and without cytoplasmic contents, falcate in shape, measuring 20 to 25 $\mu\text{m} \times 3 \mu\text{m}$. Morphological comparison of the syntypes of *C. cereale* and the published descriptions and illustrations against our recent samples obtained from pooid grasses confirmed that the *Colletotrichum* isolated from pooid grasses in the present study are within the range of morphological variation consistent with the *C. cereale* species description. Although we attempted to extract DNA from the herbarium material, we were not successful, as the fungal tissue was in close association with the plant tissue, and relatively little fungal tissue was present. Attempts to revive the specimens on PDA were also unsuccessful; therefore comparisons of contemporary *Colletotrichum* strains with syntype specimens are necessarily confined to morphological estimations. Four of the New York Botanical Garden specimens were catalogued in the herbarium database as type specimens (although the published description never makes such a specification): 305598 on *Bromus secalinus*, 305599 on *Avena sativa*, 305595 on *Arrhenatherum elatius*, 305596 on *Secale cereale*, and 305597 on *Triticum vulgare*. A holotype was not designated; we therefore establish 305598 (*Bromus secalinus*) as the lectotype for the species, and 305599, 305595, 305596, and 305597 are specified as paratypes. To facilitate species interpretation using the molecular characters described in this study, we designate KS-20BIG, NJ-6795, PA-5062-3, and NJ-4990 as epitypes; cultures of the epitype strains have been deposited in the American Type Culture Collection (ATCC), Manassas, VA, and the Centraalbureau voor Schimmelfcultures (CVS), Utrecht, the Netherlands.

Comments. *C. cereale* also possesses several fixed autapomorphic characters that serve to uniquely distinguish the species from *C. graminicola* (positions are relative to the nucleotide sequence alignment of the epitype specimens): *ITS1/5.8S/ITS2* positions 54 (no base), 69-72 (no bases), 100 (C), 111 (A), 115 (G), 121 (no base), 158 (no base), 404-406 (no bases), 525 (no bases); *MAT1-2* positions 115 (no base), 120 (G); and *SOD2* positions 18-19 (AA), 25-27 (no bases), 29 (T), 56 (A), 60 (no base), 78 (no base), 82 (no base), 85 (T), 503 (no base), 508 (T), 562 (A), and 567 (no base).

In general, *C. cereale* is morphologically very similar to *C. graminicola*. Two continuous morphological characters (conidia length and hyphopodium area) have recently been suggested to differentiate approximately between *C. graminicola* and *C. cereale* (12,25); however, the variability and overlap of range in these types of quantitative characters precludes diagnosis of these taxa uniquely and consistently on this basis (61). Conidia in these two species are sometimes different in length, with the conidia of *C. graminicola* typically, but not always, larger than those from *C. cereale* (6,12,25,54, our data); overall, *C. cereale* conidia grown on PDA measured 6.0 to 33.8 μm , while *C. graminicola* conidia ranged from 13.0 to 44.0 μm . Hyphopodium (mycelial appressoria) area is also notably different in the two species (12,25), with the hyphopodium area of *C. graminicola*, on average,

significantly larger than those of *C. cereale*, but this continuous character also is present in overlapping ranges (*C. cereale* 63.8 to 315.6 μm^2 and *C. graminicola* 136.7 to 1,027 μm^2) (12,25,61).

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= *Colletotrichum graminicola* f. sp. *zae* Messaien, Lafon & Malot, *Ann. Epiphyt., ser. C.*, 10:454 (1959).

Teleomorph. *Glomerella graminicola* Politis, *Mycologia* 67:56-72 (1975).

Emended description. Parasitic on *Zea mays*. *C. graminicola* is uniquely distinguished by the following synapomorphic fixed nucleotide characters (positions are relative to the nucleotide sequence alignment of the epitype specimens): *ITS1/5.8S/ITS2* positions 15 (T), 39 (C), 56 (T), 92 (G), 98 (C), 99 (A), 104 (T), 117 (C), 145 (T), 165 (G), 178 (C), 409 (T), 517 (C), 522 (T); *MAT1-2* positions 2 (T), 54 (T), 79 (G), 87 (A), 97 (G), 101 (T), 109 (G), 123 (C), 129 (A), 131 (C), 154 (T), 163 (T), 172 (G), 199 (G); and *SOD2* positions 35 (A), 45 (G), 53 (T), 58 (T), 61 (A), 70 (G), 73 (G), 77 (C), 88 (T), 119 (A), 127 (T), 133 (T), 136 (A), 142 (G), 151 (T), 169 (G), 175 (A), 205 (T), 211 (A), 226 (T), 244 (T), 247 (T), 259 (T), 262 (G), 268 (A), 292 (C), 332 (A), 255 (T), 373 (T), 376 (T), 382 (T), 386 (A), 394 (C), 403 (T), 451 (T), 454 (T), 455 (A), 500 (C), 501 (A), 512 (T), 518 (A), 519 (A), 520 (A), 521 (A), 522 (G), 525 (A), 534 (G), 537 (A), 541 (T), 545 (A), 546 (A), 547 (T), 551 (T), 558 (C), and 571 (A).

Epitype establishment. The lectotype established for this species was examined by Sutton (58) from *Z. mays* (IMI83255). To facilitate species interpretation using the molecular characters described in this study, we designate MO-100178 from *Z. mays* (also known as *C. graminicola* isolate M1.001) as an epitype; a culture of this specimen has been deposited in the ATCC and the CVS.

Comments. The reader is referred to any of several excellent morphological studies of *C. graminicola* sensu stricto Sutton that have been published (10,25,58-62). In general, *C. graminicola* is morphologically quite similar to *C. cereale* except for the two continuous quantitative conidial and hyphopodial characters noted above, but the overlapping range of these morphological characters precludes their usage for consistent and unique diagnosis of these species.

C. graminicola also possesses several fixed autapomorphic characters that can serve to uniquely distinguish the species from *C. cereale* (positions are relative to the nucleotide sequence alignment of the epitype specimens): *ITS1/5.8S/ITS2* positions 54 (T), 69-72 (TCCG), 100 (no base), 111 (no base), 115 (no base), 121 (G), 158 (A), 404-406 (GTA), 525 (C); *MAT1-2* positions 115 (C), 120 (no base); and *SOD2* positions 18-19 (no bases), 25-27 (AAC), 29 (no base), 47 (no base), 56 (no base), 60 (T), 78 (C), 82 (C), 85 (no base), 500 (C), 508 (no base), 562 (no base), and 567 (C).

Colletotrichum sublineolum Henn. *apud* Kabat & Bub. *Fungi imp. exs.* 186 (1905) (as “*sublineola*”).

= *Colletotrichum graminicola* f. sp. *sorghi* Messaien, Lafon & Malot, *Ann. Epiphyt., ser. C.*, 10:456 (1959).

= *Colletotrichum graminicola* var. *zonatum* Rajasab & Ramal., *Curr. Sci.* 50(1):34 (1981).

Teleomorph. Unknown.

Emended description. Parasitic on *Sorghum bicolor* and *Sorghum halapense*. *C. sublineolum* is uniquely distinguished by the following synapomorphic fixed nucleotide characters (positions are relative to the nucleotide sequence alignment of the epitype specimens): *ITS1/5.8S/ITS2* positions 39 (T), 67 (T), 93 (C), 98 (T), 99 (C), 105 (C), 112 (C), 131 (G) 132 (A), 133 (G), 135 (A), 136 (T), 137 (A), 139 (G), 142 (A), 143 (A), 147 (T), 178 (T), 417 (T), 419 (A), 420 (C); *MAT1-2* positions 2 (A), 54 (C), 66 (T), 77 (A) 79 (A) 87 (G), 88 (A), 95 (G), 106 (G), 130 (A), 145 (A), 154 (C), 163 (C), 181 (T), 202 (T); and *SOD2* positions 40 (C), 42 (T), 44 (T), 48 (T), 88 (C), 115 (A), 125 (G), 142 (C), 148 (A), 151 (C), 166 (T), 175 (C), 184 (T), 187 (A), 205 (C), 211 (C), 226 (G), 244 (A), 268 (C), 332 (G), 355 (C), 367 (A), 373 (G), 376 (C), 382 (C), 386 (C), 403 (C), 408 (T), 455 (G), 457 (C), 460 (A), 495 (A), 498 (G), 504 (G), 505 (G), 509 (T), 512 (G), 518 (G), 519 (G), 524 (A), 530 (G), 534 (A), 537 (T), 538 (C), 541 (G), and 563 (A).

Epitype establishment. To facilitate species interpretation using the molecular characters described in this study, we designate S3.001 from *Sorghum bicolor* as an epitype; a culture of this specimen has been deposited in the ATCC and the CVS.

Comments. *C. sublineolum* also possesses several fixed autapomorphic characters that serve to uniquely distinguish the species from *C. graminicola* (positions are relative to the nucleotide sequence alignment of the epitype specimens): *ITS1/5.8S/ITS2* positions 25 (T), 69-72 (no bases), 100 (C), 101-102 (TC), 107 (no bases), 111 (A), 113-115 (GGG), 119-120 (CG), 138 (A), 414-416 (no bases), 514 (T), 522 (no base); *MAT1-2* positions 105 (C), 116 (no base), 120 (G); and *SOD2* positions 35 (no base), 37 (no base), 39 (A), 47 (T), 50 (no base), 52 (no base), 503 (no base), 508 (G), and 544-546 (GGA).

We have not listed a teleomorph state for the species; in a review of the species taxonomy, Sutton suggests that the teleomorph may be *Glomerella cingulata* var. *sorghicola* Saccas (62).

It is worth emphasizing that although *C. graminicola* is still frequently employed to describe *Colletotrichum* from sorghum, Sutton's 1980 emendment of the species description rendered such application of the name illegitimate. *C. sublineolum* is the validly established taxon describing *Colletotrichum* associated with sorghum; this distinction has been repeatedly confirmed through morphological examinations (10,12,25,59-61), host range and pathogenicity testing (6,12,20,36,40), and molecular analyses (12,19,25,31,45,52,55,56,67,68, this study).

DISCUSSION

The purpose of this study was to explore the evolutionary history of the *C. graminicola* lineages that cause anthracnose disease in turfgrasses, in particular, we sought to identify the point at which interspecific boundaries have been erected between taxa, defining the extreme limits of gene flow and population-level processes.

Species boundaries. The phylogenetic analyses performed in this study generated an extremely well-supported hypothesis of evolutionary descent for the taxa currently recognized as *C. graminicola* sensu lato G.W. Wilson and conclusively established that there are two species within this circumscription. Although we employed several methods to identify reticulate evolution among the taxa, the evidence is overwhelmingly in favor of a long-standing biological isolation between these two distinct, monophyletic groups.

In light of the pattern of fixed nucleotide differences and the unique host plant associations observed between lineages of *Colletotrichum* from corn and from those isolates obtained from the C3 grasses in the subfamily Pooideae, the species level phylogenetic divergence present in *C. graminicola* sensu lato G.W. Wilson is unambiguous. We therefore propose to resurrect the species *C. cereale* to describe *Colletotrichum* from pooid grasses,

and *C. graminicola* (Ces.) G.W. Wilson sensu stricto Sutton 1980 is reserved for the fungus pathogenic to corn, with each species uniquely characterized by their molecular identities at the *ITS1/5.8S/ITS2*, *MAT1-2*, and *SOD2* loci and their host range. These physiological host range limitations are well documented in the literature (2,6,12,20,36,38,40,41,43,44,72,73) and correspond with the emended phylogenetic species described in this paper. Since *C. cereale* likely has a much more extensive host range as a pathogen than that which is explicitly described in this manuscript, we emphasize that it is the pattern of fixed differences on the molecular level and the association with grasses of the Pooideae that serve uniquely to distinguish this species, rather than strict pathogenicity criteria. This is an important distinction: *C. cereale* transcends its ability to induce anthracnose disease symptoms in a given host plant, since the species is capable of inhabiting many pooid grasses without inducing disease in the host.

Even in our most conservative analyses, where only the most reliable, unconflicted nucleotide characters are considered, these species fully meet the criteria of the genealogical concordance phylogenetic species concept (64), which is capable of efficiently and objectively marking species boundaries in asexually reproducing organisms such as *Colletotrichum* that defy characterization based upon morphological or reproductive criteria. The specific designation of these groups is dictated by taxonomic priority: *C. graminicola*, as *Dictadium graminicolum*, was first described by Cesati (15) in 1852 from the stems of corn and barnyard grass (*Echinochloa crus-galli*); the first formal description of *Colletotrichum* from a pooid grass did not occur until 1909 (54) when Selby and Manns first described *C. cereale*. Most of the taxonomic uncertainty surrounding the circumscription of *C. graminicola* completely by-passed members of the genus found in association with pooid grasses and instead concentrated on those strains responsible for economically important levels of disease in the host plant: corn, sorghum, and sugarcane. Even Sutton, the recognized authority in *Colletotrichum* systematics, has evaluated only two pooid strains as *C. graminicola* (from *Avena sativa*) using conidial measurements. Herbaria material inspected from *Bromus* spp., *Calamagrostis epigeios*, *C. villosa*, *C. neglecta*, *Lolium perenne*, and *Poa annua* were accepted by Sutton to be *C. dematium* since a distinction between these taxa could not be made based on morphological structures examined (58). The study of Selby and Manns that served to erect *C. cereale* was not cited or discussed in any of Sutton's publications (58-62).

Because our current research goals are focused on the population dynamics of *Colletotrichum* from pooid grass hosts rather than conducting an expansive taxonomic survey of the genus, we have not sampled *Colletotrichum* from the other C4 grasses evaluated by Wilson (*Panicum* spp., *Echinochloa crus-galli*), leaving the species status of these members of the genus *Colletotrichum* still undefined; Sutton's 1980 treatment of *C. graminicola* precludes the legitimate use of the name to describe these taxa. In a review of the taxonomy of the genus *Colletotrichum* in 1992 (62), Sutton introduced the idea that a still unnamed species closely related to *C. falcatum* occurs on grass hosts other than sugarcane, basing his suggestion upon earlier morphological studies of samples from several "miscellaneous" C4 grass genera of the Panicoideae including *Andropogon*, *Digitaria*, *Echinochloa*, *Eleusine*, *Eragrostis*, *Miscanthus*, *Panicum*, and *Rottboellia* (58). Molecular data presented in two recent phylogenetic analyses (12,25) corroborate Sutton's hypothesis, providing additional evidence that a new species will need to be erected to describe properly the *Colletotrichum* associated with *Echinochloa* spp., since these taxa do not conform phylogenetically to any of the species now described from graminaceous host plants.

One particular question that remains unresolved is the relationship between *C. graminicola* and *C. sublineolum*: have they recently diverged from a common ancestor? This is an intriguing

premise that continually arises in all of the evolutionary inferences, albeit with minimal support. For several years these two species, along with *C. falcatum* and *C. caudatum*, were considered to be varietal forms of a single species (5,43,44), and the unique status of *C. sublineolum* is still rejected by many researchers of sorghum anthracnose (e.g., 69). Is this association between species real, or is it an artifact of ancestral polymorphisms? To fully consider this possibility, a substantially larger portion of the genome should be sampled, with an extended sampling from these taxa along with their sister species from other C4 grass hosts.

Unresolved, potentially species-level divergences. We envision that *C. cereale* sensu lato Crouch, Clarke, and Hillman may eventually need to be further subdivided—minimally into two species, and potentially into as many as four distinct species based solely upon this limited sampling. We regard the description of *C. cereale* to be a species group rather than a single homogeneous species, but the data from the present study are inadequate for the purpose of formally proposing subdivision of the group. Both ML and MP phylogenetic inferences and the splits-graph strongly support the divergence of *C. cereale* clades A and B, but the phylogeny of the data set reduced of all potentially incompatible sites does not recover these lineages as independent entities. While this discrepancy is likely due to the elimination of a great many of the informative characters from the analysis rather than continued gene flow, we cannot at this time confidently suggest the establishment of two unique species based upon these data alone, since the eliminated characters may have yielded erroneous species phylogenies. It is not surprising that a great deal of potentially misleading data can be found when considering a group of organisms so close to the population level, as any number of processes can result in inconsistent, misleading conclusions due to incomplete lineage sorting, recombination, and hybridization. We expect that the application of several analytical techniques—vegetative compatibility, host range analysis, pathogenicity, and virulence—will be required before these unique groups can be defined in a substantive, biologically meaningful manner. This work merely represents the first step in an ongoing process; nevertheless, it does provide a vital framework for future experimental work.

The evolutionary history of *C. cereale*. In any phylogenetic analysis, the assumption that only a single phylogeny underlies the evolution of the population sampled is violated by the presence of recombination. Recombination is a truly creative force in organismal biology, but has the unfortunate side effect of completely confounding the accurate estimation of phylogenies when present (14,48,49 for reviews). This is particularly true in sexual species and, as our data will attest, in putatively asexual entities like those found in the genus *Colletotrichum*. It is evident that although *C. cereale* may for the most part propagate in an asexual fashion, recombination between taxa has occurred, as independently estimated by the split-decomposition network and compatibility matrix analysis; results from transposon RFLP fingerprinting assays also confirm the existence of mosaic genotypes in the species group (J. Crouch, B. B. Clarke, and B. I. Hillman, unpublished data). Although the three individual gene genealogies considered in this study were topologically congruent, suggesting a clonal, nonrecombining species history, overall our data provide evidence for the presence of recombination that is not reflected in the tree topologies. Many experts in the field of evolutionary biology recommend using a combination of assays to detect recombination in order to maximize the possibility of identifying recombination with a minimum of false positives (50 for review). In our research, three separate analyses detected the potential for recombination between *C. cereale* clades A and B; we believe these data are sufficient to recommend further analysis before establishing these clades as phylogenetic species when they may in fact only represent divergent populations.

Regardless of whether one accepts the clades of *C. cereale* as individual species or simply highly divergent populations, there are undeniably two unique lineages of this fungus causing disease in turfgrasses maintained as golf course greens. The clade A isolates are geographically widespread and were isolated from numerous turfgrass and noncultivated C3 pooid grass species; they are the numerically dominant form of *C. cereale* in the sample discussed in this manuscript and in our entire culture collection. To date, over 200 additional specimens of *C. cereale* in our culture collection have been identified as part of clade A through the application of a PCR-based screening protocol using lineage-specific transposable element primers, in some instances supported by sequence analysis. Also included in *C. cereale* clade A are several isolates from wheat plants from the Midwest United States, as inferred from *SOD2* and *ITS* sequences (J. Crouch, B. B. Clarke, and B. I. Hillman, unpublished data). In contrast, the clade B lineage is quite rarely encountered and appears to have a much more restricted host range and geographic distribution. Additionally, there are several hints of an association between host plant derivation and the *C. cereale* clade B lineage: the phylogenetic tree topology in particular shows an almost perfect division between *Agrostis stolonifera* and *Poa annua* isolates within this lineage. Whether the peculiarities of clade B with respect to distribution and host range are based on real phenomena or have arisen due to sampling bias will require further study.

One of the most important conclusions generated by these analyses is the identification of the very common and widely distributed H5 ancestral haplotype in the *C. cereale* clade A population. The proposition that all North American *C. cereale* lineages are ultimately derived from the H5 haplotype, regardless of their host-plant derivation, could have very important implications for the development of effective disease control strategies.

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