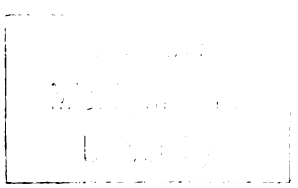

COMPARISON OF *COLLETOTRICHUM* SPP. CAUSING CROWN ROTTING ANTHRACNOSE TO *COLLETOTRICHUM GRAMINICOLA* AND *COLLETOTRICHUM SUBLINEOLUM* ISOLATES USING ISOZYME MARKERS

By

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ABSTRACT

COMPARISON OF *COLLETOTRICHUM* SPP. CAUSING CROWN ROTTING ANTHRACNOSE TO *COLLETOTRICHUM GRAMINICOLA* AND *COLLETOTRICHUM SUBLINEOLUM* ISOLATES USING ISOZYME MARKERS

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Crown rotting anthracnose (CRA) caused by *Colletotrichum graminicola*, is a serious problem on annual bluegrass (*Poa annua*) and creeping bentgrass (*Agrostis palustris*) under high maintenance conditions. The objectives for this study were: 1) determine the genetic relatedness of isolates of *C. graminicola* from annual bluegrass and creeping bentgrass, and 2) compare the genetic relatedness of isolates from annual bluegrass and creeping bentgrass to isolates from two economically important hosts, corn (*Zea mays*) and sorghum (*Sorghum* spp.) using isozyme genetic markers. Our results show that isolates from annual bluegrass and creeping bentgrass are related at the sub-species level, and that isolates from the turfgrass hosts are more distantly related to the crop hosts, corn and sorghum. These results are also consistent with a sympatric speciation system where genetic isolation occurs because of niche specialization. These results question the use of this pathogen as a biological control agent for annual bluegrass.

To my wife, Laura, whose undying love and support gives me the strength to complete a project this large, and to my parents, Jean and Joe, who taught me that if I put my mind to it, I can do anything.

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Literature Review

Annual bluegrass (*Poa annua* L.) is one of the five most widely disseminated weeds in the world (1) and is often troublesome in golf course turfs because no control measures exist to selectively eliminate it from a mixed stand of grasses (2). However, due to *P. annua*'s cosmopolitan nature and the lack of effective controls, it is tolerated and managed as a turfgrass on golf courses. There has been controversy over the life cycle of *P. annua* since 1965 when Timm (3) separated *P. annua* into two distinct subgroups: an erect annual subgroup, and a prostrate biennial or perennial subgroup. Johnson, *et al.* (4) assert that *Poa annua*, as a species, is almost anything but annual. Others report that "an endless array of genotypes can exist within the same golf green" (5). The divergent reports in the literature suggest a very high level of genetic diversity in *P. annua* populations. Because all of these studies have been based on morphological and physical characteristics that may be plastic over time, the true life cycle of *P. annua* is still in question.

Poa annua's growth requirements as a turfgrass were first characterized in 1937 (6). Its prolific seedhead production and susceptibility to fungal pathogens during periods of high temperature and humidity were also described (6). This susceptibility is the primary obstacle to *P.annua*'s acceptance as a desirable turfgrass, and suggests this turfgrass might be amenable to biological control. Diseases that cause widespread damage on a single undesirable species in a

mixed stand of plants are often investigated as potential biological controls because of their putative ability to selectively eliminate the undesirable species. In the case of *P. annua*, crown rotting anthracnose (CRA), caused by *Colletotrichum graminicola* (Ces.) G.W. Wils., has the potential to be a biological control.

One reason for the lack of interest in biological control of grass species is the fear that organisms used for biological control could severely damage important non-target crop species. Therefore, a good biological control agent is characterized by its host specificity. The literature presents a conflicting account of the host specificity of *C. graminicola*. This pathogen infects many grass species including, maize (*Zea mays* L.), sorghum (*Sorghum* spp.), creeping bentgrass (*Agrostis palustris* Huds.), and annual bluegrass (*P. annua*). Different species of turfgrass from several geographic locations in Canada were inoculated with an isolate of *C. graminicola* from *P. annua* and only the *Poa* species was infected (16). Another study with 381 isolates of *C. graminicola* from six hosts (primarily *Sorghum* spp., and sugarcane) revealed that isolates only infected the host from which they were isolated (17). An evaluation of *C. graminicola* as a possible biological control for johnsongrass (*Sorghum halepense* (L.) Pers.) determined that isolates from johnsongrass were severely damaging to all *Sorghum* spp. tested, but that other graminaceous hosts were not damaged (18). These reports each demonstrate that host specificity may exist in the *C. graminicola* pathosystem. However, Jamil and Nicholson (19) showed that, depending on a plants physiological condition, isolates from other

hosts can be as damaging as isolates from the same host. This account shows that host specificity may be affected by external factors such as plant health.

CRA was first reported in 1954 in Great Britain, and symptoms occurring on infected turfgrass were described as "measly" and "piebald" (7). The disease manifests itself as small patches of chlorotic, dying turf that may increase in diameter to 15 cm or more. Initially, older leaves are discolored, but eventually the central leaf becomes discolored and plant death usually follows (8). The detached crowns of infected plants appear black and rotted, and numerous acervuli are apparent upon close examination.

CRA is often associated with stressful growth conditions such as low mowing heights, reduced N fertility, and wet soils. A possible explanation for this association is that the onset of pathogenesis is triggered by stress-induced signals from the plant. *C. graminicola* belongs to a group of *Colletotrichum* species that exhibit hemibiotrophic disease cycles (9). Pathogens in this group penetrate the host without triggering the plant defense mechanisms and remain latent until the plant is stressed (9). Ethylene production by the plant has been reported as a potential signal for pathogen invasion in this group of hemibiotrophs (10,11). Ethylene production is triggered by wounding and other mechanical stresses (12). The production of ethylene in older tissues has also been shown to cause leaf senescence (14). In the 1980's, Vargas suggested that the death of *P. annua* was related to several factors described as HAS (Helminthosporium, Anthracnose, Senescence) decline (15). Coleman and Hodges (12) found that when leaves of Kentucky bluegrass (*Poa pratensis*) were

wounded, ethylene production increased, and spores of *Bipolaris sorokiniana* germinated and produced penetration structures on glass cover slides when the spores were exposed to the wounded leaves. Maize plants can also be predisposed to stalk rot as a result of wounding stress (13). Such predisposition to infection by wounding or other stresses is hypothesized to play a similar role in *P. annua*.

The current taxonomy of *Colletotrichum* spp. occurring on grass hosts is not well defined. Isolates that occur on graminaceous hosts are commonly assigned to *C. graminicola*. The characterization of *Colletotrichum* spp. using molecular techniques has greatly facilitated the delineation of many species in this genus and these techniques have become the method of choice to identify species that are difficult to distinguish morphologically (20,21,22,23,24,25). Isozyme analysis, Randomly Amplified Polymorphic DNAs (RAPDs), Restriction Fragment Length Polymorphisms (RFLPs) and DNA sequence analysis have been used to examine genetic diversity in the *C. graminicola* pathosystem (20,21,22,23,24,25,26). Molecular techniques are particularly useful in this genus because the morphological characteristics used for identification (e.g. conidia, perithecia, ascus, and ascospore sizes; setae length; and appressorium diameter) have been demonstrated to be variable. Thus, without the use of molecular techniques, meaningful identifications are extremely difficult to obtain (22,23,24).

The relationships between isolates of *C. graminicola* from different grass hosts have been examined in maize (*Zea mays*) and sorghum (*Sorghum bicolor*)

(22,23). Taxonomically, these two hosts are very closely related, both belonging to the family Andropogoneae, subfamily Panicoideae (27). Therefore, isolates of *C. graminicola* infecting these hosts could also be closely related. However, Hugenin found large differences in zymograms between isolates from maize, sorghum, and sugarcane, but because the isozymes used were highly variable, it was impossible to make inferences about the taxonomy of these isolates (23). In another study, isolates from maize and sorghum from various geographic regions were differentiated using RAPDs, and the profiles were separated according to geography (21). The authors concluded that *C. graminicola* exhibits a high level of genetic variation (21). Recently, Vaillancourt and Hanau conducted a comprehensive study demonstrating the genetic separation of isolates from maize and sorghum (22). Data generated from crossing studies, RAPDs, and RFLPs of mtDNA demonstrated that isolates from the two hosts could not interbreed, and RAPDs showed only 45% similarity between isolates from different host origins (22). These data support the reclassification of isolates of *C. graminicola* from sorghum (*Sorghum bicolor*) to a new species (22). Sherriff, *et al.* confirmed these results using the ribosomal DNA internal transcribed spacer region (ITS-1) sequence and suggested *C. sublineolum* as the name for isolates from sorghum (28). This is significant because isolates from corn and sorghum have been shown to be distinct genetic lineages that are reproductively isolated. Therefore, isolates from corn and sorghum cannot share genetic information such as genes for pathogenicity on other hosts. Isolates from maize

and sorghum can now be considered sibling species that are defined as being morphologically nearly identical, but reproductively completely isolated (29).

A single isolate from *P. annua* was recently included in a study of *Colletotrichum* species relationships using ITS-1 sequencing. The ITS-1 sequence from the *P. annua* isolate was nearly identical to sequences found in isolates from *Sorghum* (20). This suggests that isolates from *P. annua* may be accommodated under *C. sublineolum*. Backman (24) found significant variation between isolates from *A. palustris* and *P. annua* using RAPD markers. Cluster and principal coordinate analyses grouped isolates from the same host together (24). Browning, Rowley, Zeng, Chandlee, and Jackson (30) also studied morphological and RAPD variation present in isolates of *C. graminicola* from *P. annua* and *A. palustris*. They found that morphological comparisons were useful in distinguishing between isolates from the two hosts. They also found that RAPD fingerprints from isolates from *A. palustris* and *P. annua* were very different from those generated by the isolates from *Z. mays* and *S. bicolor*. Because of the sampling limitations of these studies, determining what the actual relationship is between isolates from the amenity turfgrasses and isolates from corn and sorghum is difficult to ascertain.

The relationship of isolates from amenity turfgrasses to isolates from maize and sorghum has not been a subject of much study. Because the turfgrass hosts *P. annua* and *A. palustris* are in a different plant family than the crop hosts, and the two turfgrasses are in different subfamilies from each other, is reasonable to hypothesize that isolates from these hosts would demonstrate

some specialization (27). Understanding the relationships between isolates from maize, sorghum, creeping bentgrass, and annual bluegrass would allow us develop information about the reservoirs of inoculum, gene flow, genetic drift, and taxonomy.

Isozymes are often used as an initial tool to examine the variation present in an unstudied system. Isozyme analysis has been used to study variation in other species of *Colletotrichum* (23,25,26). As with any technique, isozyme analysis has advantages and disadvantages. The advantages of this technique include: low cost, minimal use of toxic chemicals, rapid results, true genetic loci, codominant markers, easy preparation, and the ability to screen for a large number of markers in a single run (30,31). Since only 1/3 of the mutations in isozymes result in a change of charge that would result in a change in migration distance, isozyme analysis represents a conservative estimate of variation present within genomes (30). The disadvantages include: the need for sufficient quantities of fungal tissue, a limited number of loci, and the possibility of cryptic, post-translational modifications of functional enzymes (30,31). Overall, isozyme data provide a broad view of the variation present in a system upon which further studies using DNA-based technologies can be built.

Since most golf course managers consider *P. annua* a weed, a pathogen that could selectively eliminate *P. annua* from the turf stand would be very useful. The literature presents an inconsistent view as to whether biological control is possible. One step in assessing the feasibility of using *C. graminicola* as a biological control organism is to examine the genetic relationships of the

pathogen from *P. annua* to the closely related and well-studied *Colletotrichum* pathogens in maize and sorghum and to isolates originating on the primary non-target host, *A. palustris*. Unless the isolates from *P. annua* can be demonstrated to be genetically distinct from other hosts, then the possibility of using *C. graminicola* as a biological control agent is at best a risky proposition.

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Chapter 1

Genetic comparison of crown rotting anthracnose isolates from amenity turfgrass hosts to isolates from corn (*Zea mays*) and sorghum (*Sorghum* spp.) using isozyme analysis

Introduction

Crown rotting anthracnose (CRA) caused by *Colletotrichum graminicola* is a serious disease of *Poa annua* L. CRA and other fungal diseases severely diminish *P. annua*'s ability to survive; this high susceptibility to disease presents a major obstacle to developing this grass as a desirable species (2). As one of the five most widely disseminated weeds in the world (1), *P. annua* is often troublesome in golf course turfs because no effective control measures exist at this time to selectively eliminate it from the turf stand (2). For this reason, *P. annua* is tolerated and managed as a turfgrass on golf courses. However, a disease that selectively infects *P. annua* in a mixed stand has the potential to be used as a biological control. Biological control of *P. annua* has been attempted with several pathogens, but as yet no disease has provided economically effective control. One of the problems that golf course superintendents face when their golf course is infected with CRA is the unpredictable ball roll caused by the small voids of bare earth left as a result of the disease. These voids also negatively affect the appearance of the playing surface. Because aesthetics are critically important to pleasing golfers, CRA can cause significant problems for golf course operators. There are basically two competing viewpoints on how to

deal with *P. annua* encroachment. The first group views *P. annua* as good turfgrass that tolerates low mowing, and should be managed as a desirable turfgrass. In fact, some in this school would even argue for improving the wild types of this species through directed breeding. The second group views *P. annua* as a weed that should be eradicated. No matter which viewpoint one subscribes to, diseases like CRA need to be understood either to better manage *P. annua*, or to take advantage of one of its weaknesses.

CRA was first reported in 1954 in Great Britain (3). The symptoms occurring on an infected turfgrass area were described as "measly" and "piebald" (3). The disease begins as small, diffuse patches of chlorotic, dying turf that may increase in diameter to 15 cm or more. Initially, older leaves are discolored, but eventually the central leaf becomes discolored, and plant death usually follows (2,3,4). The base of a detached crown from an infected plant appears black and rotted. Close inspection of the rotted tissue reveals numerous acervuli.

The causal agent of CRA is presently identified as *Colletotrichum graminicola*. Traditionally, all *Colletotrichum* isolates that infect graminaceous hosts are identified as *C. graminicola* by default. This convention is due to the assumption that if an isolate of *Colletotrichum* is morphologically similar to the type isolate from corn (*Zea mays* L.), then it is identified as *C. graminicola*. However, recent research has shown that multiple species of *Colletotrichum* infect graminaceous hosts (5,6). Vaillancourt and Hanau used data generated from mtDNA RFLPs, RAPDs, and crossing studies to demonstrate that *Colletotrichum* isolates from maize and sorghum were probably different species

(5). Data from RAPD fingerprinting showed only 45% similarity between isolates from different host origins (5). These data supported the reclassification of isolates originating on sorghum from *C. graminicola* to a new species (5). Sherriff, *et al.* confirmed these results using the ribosomal DNA internal transcribed spacer region (ITS-1) sequence and suggested placing those isolates from sorghum into a new species, *C. sublineolum* (6). Currently, there is a lack of information about the species composition of *Colletotrichum* occurring on amenity turfgrasses.

Taxonomic identification of isolates within this group is important for practical reasons. The principal reason is to facilitate regulatory control of the development of pathogens as biological control agents, which must be regulated to reduce the possibility of destroying economically important crops due to cross infection. Inaccurate identifications result in regulations that may prevent useful organisms from becoming safe biological control agents. Once a correct identification has been obtained, additional information (e.g., host specificity, host range, and morphological measurements) can be correctly referenced to the appropriate species and informed regulatory decisions can be made. Therefore, information about the genetic background of isolates as it relates to taxonomy is a crucial part of evaluating the potential organisms have as biological control agents.

In the case of *Colletotrichum* spp., using only morphological characteristics for identification is unreliable as evidenced by the recent identification of the new species, *C. sublineolum* (5,6). Therefore, molecular

genetic techniques should become the method of choice to identify species within *Colletotrichum*, and unless these techniques are used, meaningful identifications of isolates that infect graminaceous hosts will remain extremely difficult to obtain.

One of the first steps in examining potential taxonomic variation in fungi is isozyme research. Isozyme analysis provides a broad view of variation that may be present, and the relative amount of genetic information that is generated compared to other techniques makes isozyme analysis an efficient and cost-effective first step. The lack of meaningful data regarding the amount of genetic variation in isolates of *Colletotrichum* from *Agrostis palustris* Huds. (creeping bentgrass) and *P. annua* indicates the need to determine the amount of genetic variation present. Isozyme analysis has been used to study variation in other species of *Colletotrichum* (7,8,9). Bonde *et al.* used isozymes to examine *Colletotrichum spp.* infecting strawberries (7). They found that for the 5 species tested, all the isolates supported the current species designation with the exception of *C. acutatum* and *C. gloeosporioides* that were more closely related than the species level (7). Their findings were that isozymes provide an effective method for distinguishing the *Colletotrichum* species tested (7).

Our objective for this study was to examine and compare the interspecific and intraspecific variation present in isolates of *C. graminicola* originating on *P. annua*, *A. palustris*, *Z. mays*, and *Sorghum* spp. using isozyme techniques. Data resulting from this study of *Colletotrichum* species on turfgrasses could be used as a basis for further research using DNA-based techniques to examine species-

level relationships and help determine if host specificity exists in isolates that infect graminaceous hosts. Ultimately, data from this study will begin to assess the potential of using CRA as a biological control for *P. annua*.

Materials and Methods

Isolate Collection

Isolates of *C. graminicola* were collected from several different geographic locations (Table 1). Isolates were collected from the following hosts: *P. annua* (25), *A. palustris* (21), *Z. mays* (10), and *Sorghum spp* (4). The isolate collection from the turfgrass hosts was developed by collecting isolates from areas that I visited, and also from Dr. Noel Jackson at the University of Rhode Island, Dr. Randy Kane at the Chicago District Golf Association/University of Illinois and Dr. Peter Landschoot at Pennsylvania State University. The collection of isolates from corn and sorghum were from the collections of Dr. Ralph Nicholson at Purdue University, Dr. Gary Bergstrom at Cornell University, Dr. Don White at the University of Illinois and Dr. David TeBeest at the University of Arkansas. Isolates were single spored and stored as a spore suspension in 20% glycerol solution at -80°C. Several isolates were also stored as potato dextrose agar (PDA) (Difco, Detroit, MI) plugs in 1 mL sterile heavy mineral oil. Isolates were maintained in storage for up to two years until they were needed for electrophoresis.

Culture Preparation

Isolates were removed from storage and prepared for electrophoresis by seeding 15 mL V8 juice broth (200 mL low Na V8, 800 mL distilled H_2O, 6 g

$CaCO_3$) with 200 µl of spore suspension and incubating them in a 25 cm^2 tissue culture flask (Corning, Corning, NY) under cool-white fluorescent lighting for a 24 hr photoperiod for four days.

Extraction and Electrophoresis

The techniques concerning the procedures for isozyme analysis were modified as described below from Quiros (10). Proteins were extracted immediately prior to electrophoresis. The contents of the tissue culture flask were harvested and a small portion of mycelium (~2-3 cm^2) was excised and blotted dry. The mycelium was placed in a sample well of a Plexiglas grinding tray that was placed on ice to prevent protein degradation. Cold 2% glutathione extraction buffer (120µl) was added to each sample to further prevent protein degradation. The mycelium was macerated with a blunt Plexiglas rod to release the proteins into the extraction buffer. Extracted proteins from one sample were absorbed into four filter paper wicks (Whatman 3mm Chr, Maidstone, England) measuring approximately 3 x 8 mm. Both a pH 8.3 system, and a pH 5.7 system were used to detect enzymes present. Horizontal starch gels (12% w/v) were prepared using the appropriate gel buffers for each system (Table 2). The gel origin was sliced perpendicular to the direction of the current, and two wicks from each sample were placed vertically at the origin of the gel. The gels were maintained at 4°C, placed in trays containing the appropriate running buffer (Table 2), and electrical current was applied across the gel at the appropriate amperage for each pH system (Table 2). The protein front was allowed to progress for about 40 minutes before the wicks were removed from the origin. The gel was then covered with

transparency film followed by a glass plate on top of which ice bags were placed to cool the gel and prevent protein denaturation. Amperage (Table 2) was monitored closely throughout each 3 hr run to ensure consistent migration of the protein front.

Table 1- Electrophoretic phenotypes (EP) of crown rotting anthracnose isolates collected from various hosts and locations.

Isolate ID	Host	Region[a]	EP	Geographic Origin
IW 94-1	*P. annua*	M	1	Detroit, MI
DB 95-1	*P. annua*	M	1	Detroit, MI
WH 95-1	*P. annua*	M	1	Lansing, MI
CCL 95-1	*P. annua*	M	3	Lansing, MI
CL 95-1	*P. annua*	M	4	Missouri
WGC 95-1	*P. annua*	M	4	Weirton, WV
CW 95-1	*P. annua*	M	2	Vienna, OH
LS 95-1	*P. annua*	---	1	Chicago, IL
KWIL 95-1	*P. annua*	M	1	Chicago, IL
EV 95-1	*P. annua*	M	1	Chicago, IL
BA 95-1	*P. annua*	---	3	Bel Aire, CA
WF 95-1	*P. annua*	A	2	Wakefield, RI
CAM	*P. annua*	A	2	State College, PA
QR 95-1	*P. annua*	A	1	Scarsdale, NY
RK 96-1	*P. annua*	M	2	Detroit, MI
SUN 1	*P. annua*	A	1	Johnston, PA
OAK 2	*P. annua*	A	2	Pittsburgh, PA
OAK 3	*P. annua*	A	2	Pittsburgh, PA
VAL 1	*P. annua*	A	2	State College, PA
MCC 95-1	*P. annua*	A	2	Madison, CT
AP 95-1	*P. annua*	A	2	Cranston, RI
GR 95-1	*P. annua*	M	2	Grand Rapids, MI
IV 95-1a	*P. annua*	M	2	Toledo, OH
IWIL 95-1	*P. annua*	M	2	Chicago, IL
SPC 95-1	*P. annua*	A	2	Sands Point, NY
CH 95-2	*A. palustris*	A	5	Southbridge, MA
SS 95-2	*A. palustris*	A	5	Ridgefield, CT
BOB 95-2	*A. palustris*	A	6	Brewster, NY
AV 95-2	*A. palustris*	A	6	Weston, CT
FCC 95-2	*A. palustris*	A	7	Farmington, CT
GW 95-2	*A. palustris*	A	6	Greenwich, CT
PA 95-2	*A. palustris*	A	6	Bellingham, MA
SCC 95-2	*A. palustris*	A	6	Stonington, MA
VAL 2	*A. palustris*	A	6	State College, PA
WFCC 95-2	*A. palustris*	A	6	Wethersfield, CT
ACC 95-2	*A. palustris*	A	8	Plymouth, MA
AT 96-2	*A. palustris*	---	3	Atlanta, GA
CCN 95-2	*A. palustris*	---	3	Pinehurst, NC

Table 1 (cont'd)

Isolate ID	Host	Region[a]	EP	Geographic Origin
FF 96-2	*A. palustris*	---	3	Frankfort, KY
GM 95-2b	*A. palustris*	---	3	Missouri
ICC 95-2	*A. palustris*	A	3	Ipswich, MA
KCC 95-2	*A. palustris*	A	3	Yarmouthport, MA
KEN-1	*A. palustris*	---	3	Lexington, KY
MA 95-2	*A. palustris*	---	8	Kennesaw, GA
PCC 95-2	*A. palustris*	A	3	Litchfield, NH
PQ 95-4	*A. tenuis*	A	3	Bristol, ME
UNK 95-2	*A. palustris*	---	9	Chicago, IL
COR 2	*Z. mays*	---	10	Rock Springs, PA
Cg 151	*Z. mays*	---	10	New York
COR 3	*Z. mays*	---	14	New York
Cg ASK 88	*Z. mays*	---	12	Unknown[b]
Cg M6	*Z. mays*	---	12	Missouri
Cg 46	*Z. mays*	---	11	Unknown[b]
Cg M9 Mo	*Z. mays*	---	13	Missouri
Mo 940 D	*Z. mays*	---	12	Indiana
Cg 17	*Z. mays*	---	12	Unknown[b]
Cg 122	*Z. mays*	---	12	Indiana
Cg 1042 (2-3-9)	*S. halepense*	---	15	Stuttgart, AR
Cg 1034	*S. halepense*	---	16	Devil's Den, AR
Cg 1031	*S. halepense*	---	18	College Station, TX
FRM 1993 236I	*S. bicolor*	---	17	Griffin, GA

[a] Isolate region assignment for geographic data analysis (where M=midwest, A=atlantic

[b] Unknown locations from Ohio to Nebraska

Table 2- Enzymes and buffer systems used for the detection of variation in collected *C. graminicola* isolates.

Enzyme[a]	Abbreviation	EC No.	Activity[b]	Electro-morphs[c]	Buffer System[d]
Aspartate aminotransferase	AAT	2.6.1.1	A, B	4	1
Isocitric dehydrogenase	IDH	1.1.1.42	A	1	2
Malate dehydrogenase	MDH	1.1.1.37	A,B	3	2
Phosphoglucose isomerase	PGI	5.3.1.9	A,B	4	2
Phosphoglucomutase	PGM	5.4.2.2	A,B	2	1
6-Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	A	1	2
Triosephosphate isomerase	TPI	5.3.1.1	A,B	3	1

[a] All stain recipes from (11)

[b] Activity visualized in isolates from: A= amenity turfgrasses, B= crop plants

[c] Number of different activity bands visualized in each enzyme studied

[d] Buffer systems used (11): 1= Lithium borate/ Tris-citrate, pH 8.3, 55 mA

2= Histidine-citrate, pH 5.7, 35 mA

Visualization of enzyme activity

Following electrophoresis, gels were sliced into four approximately 1 mm thick slabs and each slab was placed in a separate enzyme stain. Enzyme activity was assayed using common histochemical stains that produce an insoluble dye where enzyme activity is present. The staining protocols were modified from Vallejos (11) by optimizing the intensity of the staining through adjustments to the substrate amount in the stain. To ensure the repeatability of the zymograms, each isolate was run on two replicate gels and an isolate with a defined banding pattern was used as an internal reference standard for each gel. A total of 16 enzyme systems were assayed for activity and resolution in two buffer systems. Seven enzyme systems were resolved in isolates originating from A. *palustris* and *P. annua*, while only five enzyme systems were resolved in isolates from *Z. mays* and *Sorghum spp.* (Table 2). The nine enzyme systems remaining that were not repeatedly resolved were not investigated further.

Genic Nomenclature

Alleles found in the different enzyme systems (Table 2) were numbered sequentially from the anodal end of the gel using one number for locus and a second number for allele (abbreviations with all capital letters refer to enzymes, and those with the first letter capitalized refer to the locus coding for the particular enzyme). For example, the first locus, third allele of the enzyme PGI would be abbreviated as, Pgi13.

Analysis of Data

Data generated from each gel run were recorded in a matrix identifying the presence (1) or absence (0) of a particular band. Allele frequencies were calculated from the presence/absence matrices and subdivided based on host origin or geographic location (Table 6). Nei's unbiased genetic distance (corrected for small sample sizes) (12) was calculated for each group of isolates, and is defined as follows:

Let p_i and q_i be the frequencies of the ith allele in populations X and Y, respectfully, and x_i and y_i are the corresponding allele frequencies from the sampled populations.

$D = -\ln[G_{xy}/\sqrt{G_x G_y}]$, where G_x, G_y, and G_{xy} are the means of Σp_i^2, Σq_i^2, and $\Sigma p_i q_i$ over all loci in the genome. Replacing the population gene identities with the sampled population gene identities J_x, J_y, and $J_x J_y$ which are the means of Σx_i^2, Σy_i^2, and $\Sigma x_i y_i$ over r loci studied. However, with small sample sizes the measure of genetic distance is biased. Therefore, Nei's genetic distance (12) can be corrected for sample size as follows:

Let n_x and n_y be the number of individuals sampled from populations X and Y, respectively. Substituting an unbiased estimator for each frequency value, yields an unbiased estimate of genetic distance. Namely,

$\hat{D} = -\ln[\hat{G}_x\hat{G}_y / \sqrt{\hat{G}_x\hat{G}_y}]$ where the caret (^) denotes an unbiased estimator for the frequency means.

The estimators are defined such that,

$\hat{G}_x$ and $\hat{G}_y$ are the averages where, $\frac{2n_xJ_x - 1}{2n_x - 1}$, and $\frac{2n_yJ_y - 1}{2n_y - 1}$ over *r* loci studied, respectively, and $G_{xy} = J_{xy}$. Nei's distance essentially is calculating the probability that an allele came from one of two sampled populations such that a large distance (> 1.000) indicates that one of the sampled populations is probably similar to the other population. However, a small distance measure (< 0.300) indicates that one of the populations is not as closely related to the original population. The small distances could be potential areas where speciation may be occurring. Areas where the distance is between the two extreme values (0.3< X < 1.0), typically indicates the possibility of sub-species organization levels.

Results

Seven enzymes produced distinct and repeatable zymograms in isolates from *A. palustris* and *P. annua*. Only five enzymes produced repeatable zymograms in isolates from *Z. mays* or *Sorghum* spp. The enzymes 6-PGDH (E.C. number 1.1.1.44) and IDH (E.C. number 1.1.1.42) were dropped from all analyses because they were monomorphic in the *A. palustris* and *P. annua* groups of isolates, but were not resolvable in the *Z. mays* or *Sorghum spp.* isolate groups. Therefore, only the five enzymes that were resolved in all isolates

were used for analysis.

Sixteen putative allelles were resolved with these 5 enzymes. Eighteen unique electrophoretic phenotypes (EPs) were observed in the 61 isolates sampled. All isolates produced single banded phenotypes consistent with a haploid genetic condition in all enzyme systems tested. Crossing studies were not possible with these isolates because the teleomorph of *C. graminicola* has not been formed using isolates from amenity turfgrasses.

Nei's corrected genetic distance between isolates from *P. annua* and *A. palustris* was consistent with a relationship at the sub-species level (Table 4A). Isolates from *P. annua* were more closely related to isolates from *Sorghum spp.* than to isolates from *Z. mays* (Table 4A). In contrast, isolates from *A. palustris* were more closely related to isolates from *Z. mays* than to isolates from *Sorghum spp.*, and the distances between each of these groups of isolates are consistent with species level relationships (Table 4A).

Table 3- Nei's corrected genetic distance (12) by host origin (A) and geographic location (B).

A)

Host	*P. annua*	*A. palustris*	*Z. mays*
A. palustris	0.1870		
Z. mays	0.5683	0.9133	
Sorghum	0.4371	1.1540	1.6080

B)

Location	Atlantic *P. annua*
Midwest *P. annua*	0.1165
Atlantic *A. palustris*	0.3242

Table 4- Allele frequencies for isozyme loci tested, subdivided by host origin and geographic location

A) Allele frequency by host origin

	Isozyme Loci															
Host Origin	Pgi11	Pgi12	Pgi13	Pgi14	Mdh11	Mdh12	Mdh13	Pgm11	Pgm12	Got11	Got12	Got13	Got14	Tpi11	Tpi12	Tpi13
P. annua	0.6800	0.0000	0.3200	0.0000	0.6800	0.0000	0.3200	0.6800	0.3200	0.4000	0.0800	0.0000	0.5200	1.0000	0.0000	0.0000
A. palustris	0.5455	0.0000	0.0000	0.4545	0.5000	0.0000	0.5000	0.9091	0.0909	0.4091	0.5909	0.0000	0.0000	1.0000	0.0000	0.0000
Z. mays	0.0000	0.5000	0.5000	0.0000	0.0000	0.9000	0.1000	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.1000	0.6000	0.3000
Sorghum	0.2500	0.5000	0.2500	0.0000	0.0000	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	0.2500	0.7500	0.7500	0.2500	0.0000

B) Allele frequency by geographic location

	Isozyme Loci															
Location	Pgi11	Pgi12	Pgi13	Pgi14	Mdh11	Mdh12	Mdh13	Pgm11	Pgm12	Got11	Got12	Got13	Got14	Tpi11	Tpi12	Tpi13
MW[a] *P. annua*	0.6000	0.0000	0.4000	0.0000	0.6000	0.0000	0.4000	0.6000	0.4000	0.5333	0.1333	0.0000	0.3333	1.0000	0.0000	0.0000
Atl[a] *P. annua*	0.8000	0.0000	0.2000	0.0000	0.8000	0.0000	0.2000	0.8000	0.2000	0.2000	0.0000	0.0000	0.8000	1.0000	0.0000	0.0000
Atl[a] *A. palustris*	0.3300	0.0000	0.0000	0.6700	0.3300	0.0000	0.6700	0.9333	0.0667	0.5333	0.4667	0.0000	0.0000	1.0000	0.0000	0.0000

[a]MW= Midwest and Atl.= Atlantic

The small distance value between isolates from *P. annua* in the Atlantic region and those from the midwest region (Table 1) was inconsistent with expected distances resulting from geographic specialization (Table 4B). Distances for eastern seaboard isolates of *P. annua* and *A. palustris* (Table 1) were consistent with isolates that were specialized based on host type (Table 4B). Therefore, host specialization appears to be a primary source of diversity in these two groups of isolates.

Since the sample sizes for isolates from *Z. mays* and *Sorghum* spp. were small, meaningful conclusions regarding the relationship of these two groups are difficult. However, distances of isolates from these two hosts were consistent with the known species-level relationship that these two groups of fungi have with each other (Table 4A).

Four EPs contained multiple members that all originated on a single host species. EP-2 had eight members that were geographically diverse, but all members originated on *P. annua* (Table 1). EP-3 had thirteen members that also were geographically diverse and of *P. annua* origin (Table 1). EP-6 had two members from NY and CT, and both isolates were from *A. palustris* (Table 1). EP-7 had seven members from three states that were all from *A. palustris* (Table 1). Two EPs contained members from both *P. annua* and *A. palustris*. EP-5 had one member each from the two hosts (Table 1). EP-4 had ten members from *A. palustris* and two members from *P. annua*. Isolates in EP-4 were also geographically diverse thus reducing the significance of geographic specialization

(Table 1). *Zea mays* isolates were made up of five EPs, and they were not observed in any isolates from any other host sampled (Table 1). Likewise, *Sorghum* isolates were all unique from each other in their EP as well as to all other isolates EPs (Table 1).

Discussion

This is the first report to compare variation in *Colletotrichum* spp. from amenity turfgrass hosts to the variation observed in *Colletotrichum* spp. from corn and sorghum at the genetic level. Based on these data, two general conclusions arise: host specialization, not geography, appears to be the primary factor affecting the pattern of variation in the isolates tested, and morphological characteristics are of limited value when examining isolates of *Colletotrichum* from graminaceous hosts. A limitation of this study was the small sample size of isolates. Because of the small sample size, it is difficult to draw conclusions regarding the processes involved in the divergence of these fungi. However, these data present a baseline to which future data can be compared. Both of the conclusions that are supported by the data illustrate why understanding taxonomy is an important part of assessing the potential of a biological control agent.

There was a significant amount of variation observed in screened isolates as 30% of them produced unique EPs. Our results are consistent with reports that also show a large amount of genetic diversity in other species of *Colletotrichum* (5,6,7,9,14). The diversity found in this genus contrasts with the lack of diversity often found in other genera when using isozymes (8). The

diversity in our study is similar to that observed in the Bonde, *et al.* (7) study of *Colletotrichum* spp. on strawberry, and indicates that there is enough variation present to use isozymes to study diversity in populations of *Colletotrichum* infecting graminaceous hosts (Figure 1).

Isolates from *P. annua* were geographically distributed evenly from the midwest to the Atlantic seaboard, and this provided the opportunity to test the possibility of geographic specialization. If geographic specialization existed in the isolates tested, then the expected Nei's distance would be large (i.e. >1.000). However, Nei's corrected distance (12) revealed a very close relationship between isolates from the midwest and those from the seaboard (0.1165), and this distance was the smallest of all the comparisons made by either host type or geography.

The possibility of host specialization was tested by comparing isolates from *P. annua* and *A. palustris* from the Atlantic seaboard. If host type specialization was present, then the observed distance value would be large (i.e. >1.000). Although observed distances were consistent with a sub-species relationship, this distance was larger than the distance observed between isolates from these two hosts for the entire collection, indicative of host type specialization.

The distances between isolates from *A. palustris* and the isolates from the crop hosts indicated a strong divergence consistent with a species level relationship. However, the same comparison between isolates from *P. annua*

and the crop hosts indicated a relationship below the species level. One possible reason for this inconsistency is that *P. annua* is an ubiquitous weed found in agricultural and turf areas while *A. palustris* is usually only found in turf areas. These inconsistent distances could reflect a possible geographic association between isolates from *P. annua* and those from the two crop hosts that could not be tested in our study. This is important if *C. graminicola* is to be used as a biological control for *P. annua*.

Morphological differences have often been used to study speciation processes in fungi (15). Isolates of *Colletotrichum* that infect corn have been shown to be closely related, but distinct species from those isolates that infect sorghum. This raises the possibility of other such species relationships within *Colletotrichum* spp. that infect graminaceous hosts. Numerous reports have shown that morphology is of limited value when studying closely related species because the character measurements often overlap making determinations difficult, if not impossible (5,9,13). Traditionally, when samples of isolates lack morphological differences, they are classified as a single species. Morphological comparisons between selected isolates in this study showed no differences between their measurements and those published previously (Horvath, *unpublished data*, 5,9,13).

Sympatric speciation is a possible theoretical framework for the differences observed both in this study and other published studies using graminaceous infecting isolates of *Colletotrichum* (15). With sympatric speciation, an organism diverges rapidly from its basal population, and eventually

becomes different enough to be reproductively isolated from the original basal population (15). One of the major ways an organism can diverge from its basal population is by exploiting a new niche (15). Niche divergence would explain the observed differences in this group of *Colletotrichum* spp. Sympatric speciation has been the subject of much study in the apple fruit fly species group *Rhagoletis pomonella* (16,17,18). Larvae of *Rhagoletis* from apple and hawthorn were analyzed using isozyme loci (17). The allele frequencies of two of the six loci tested in the different populations varied significantly between host types for all three years tested. In a sympatrically speciating population other influences like host preference, mate selection, and survivorship can result in genetic barriers to gene flow. Collectively, these barriers restrict gene flow in two isolated groups such that these two groups sympatrically diverge into two sibling species with alternative host preferences (16,17,18). In the *Colletotrichcum* system, isolates from corn could have taken advantage of the presence of sorghum nearby and those isolates that could exploit this new niche eventually isolated themselves so that now isolates from sorghum belong to a new species, *C. sublineolum*. Isolates infecting the crop hosts could also capitalize on the presence of an ubiquitous weed, *P. annua*, as another possible under-utilized niche. Like isolates from corn and sorghum, isolates that infect the amenity turfgrass hosts are also spatially associated, so there is the possibility of these isolates also diverging into other isolated niches. This model would account for the variation observed in this study and other studies with the grass infecting *Colletotrichum* spp. Future work in this area would involve sampling local populations and using

DNA based techniques to determine how these groups of pathogens are evolving.

Understanding the genetic relationships a potential biological control agent has is an important part of assessing the biological control potential for the organism of interest. Based on the data from this study, isolates from *P. annua* share a large amount of genetic information with isolates from *A. palustris*. This close relationship would seem to limit the potential use of *Colletotrichum* isolates from *P. annua* as biological control agents because of the potential risk of cross infection. Isolates from *P. annua* are currently identified as *C. graminicola* as are all isolates from *Z. mays*, and *A. palustris*. However, our data indicate that isolates from the amenity turfgrass hosts are more closely related to each other than they are to isolates from corn. Isolates from sorghum are currently identified as *C. sublineolum*, and have been shown to be reproductively isolated from these other groups identified as *C. graminicola* (5,6). Isolates from *P. annua* were more closely related to the isolates from sorghum than to isolates from corn. The significance of properly assigning taxonomic names is that since isolates are currently named based on morphological characteristics, those that have potential as biological control agents may not be investigated because of a perceived threat to a major crop. Therefore, for isolates to be properly assigned a correct taxonomic name, molecular genetic techniques should become the primary method by which closely related isolates in *Colletotrichum* are identified and classified.

The data from this study illustrate that the genetic relationships of

pathogens can be tremendously important in assessing the potential any pathogen might have as a biological control agent. In this case, isolates from *P. annua* are so closely related to isolates from *A. palustris* that this relationship significantly decreases the potential of using CRA as a safe and effective biological control against *P. annua*. The risk of non-target exposure to a potentially damaging pathogen is significant given our current understanding of this pathogen.

Future work on this disease must include a study of host specificity, population studies using DNA-based techniques, and taxonomic studies to determine the species relationships in grass infecting *Colletotrichums*.

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APPENDICES

APPENDIX A

EPILOGUE

These few pages at the end of this document are intended to highlight the current state of the field in this area of research and bring attention to techniques and methods that were attempted during the course of this project that are not mentioned in the formal text because they did not work out. I do this with the hope that those reading this epilogue use the information contained within to avoid some of the same trouble spots I encountered.

This project initially set out to study whether crown rotting anthracnose (CRA) could effectively kill annual bluegrass (*Poa annua* L.) from within a healthy stand of creeping bentgrass. The idea of using a fungus to selectively eliminate one undesirable plant from a stand of desirable plants is attractive especially since, in this case, *P. annua* currently can not be controlled through any other means. In order to study the efficacy of the pathogen, inoculation experiments must be done. The problem with this pathogen is that it is a stress-related disease that does not seem to be virulent against healthy, vigorous plants like those usually maintained in the greenhouse. The inoculation methods that were attempted included: sand/cornmeal around the base of the plant, wounding with carborundum powder, wounding with needles, and infested soil. None of these methods worked. There were several possible reasons for the lack of infection. The first is that the plants were not stressed enough to allow the pathogen to

gain entry and begin destroying tissue. This is probably not the likely because the wounding methods did result in several plants being infected, but the infection rate was very low. Another possible reason could be that the pathogen was not able to colonize where it was inoculated and so could not establish high enough inoculum levels to result in a successful infection rate. Again, this is unlikely because with several of these methods an excess of inoculum was used to avoid this very problem. The most likely problem with the methods that were attempted is that a combination of the above factors coupled with the a lack of control over environmental parameters caused the lack of success. One method did work sufficiently to make it a small-scale inoculation technique useful for working with this pathogen. This method involved injecting a spore suspension directly into the crown region of the plant and then placing the injected plants into a high humidity environment for 48-96 hrs. This method did result in successful infections that were repeatable. This success however, was too late in my project to be of use. Future work involving inoculations can use this information as a starting point to develop a protocol that would allow large scale inoculations to be used for screening plants for resistance to CRA and to screen isolates for the possibility of altered virulence levels in populations.

Research often raises more questions than it answers. Colleagues often comment that this principle is the sign of a good project. My research also raised more questions than it raised. The two basic conclusions were; host type, not geography was responsible for the variation observed in the isozyme patterns, and that morphological characteristics are of limited value when trying to identify

isolates of *Colletotrichum*.

The questions that were raised by this my research include:

- Is speciation occurring in this genus of *Colletotrichum*?
- What are the evolutionary forces driving any observed speciation events?
- What organizational levels exist in these populations of *C. graminicola*? Geographical? Host Type? Species? Environmental?
- Is there any association between certain ecotypes of *P. annua* and different EP types in the pathogen?

This is not a comprehensive list, but these questions could provide a much improved understanding of the processes involved in this important group of pathogens. The continuing use of molecular techniques to help study these questions will only serve to make arriving at an answer to these questions easier.

It is my hope that these short words would help the reader understand some of the thought processes involved in deciding on a project. I also hope that this document is used to continue study on this diverse group of pathogens that infect grasses, and I hope that ultimately someone benefits from the information that I developed.

APPENDIX B

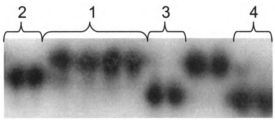
Table 5- Unique electrophoretic phenotypes (EP) of *Colletotrichum* isolates sampled

		Isozyme Loci															
EP #	No. of Isolates	PGI 11	PGI 12	PGI 13	PGI 14	MDH 11	MDH 12	MDH 13	PGM 11	PGM 12	GOT 11	GOT 12	GOT 13	GOT 14	TPI 11	TPI 12	TPI 13
EP-1	8	0	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0
EP-2	13	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0
EP-3	12	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0
EP-4	2	1	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0
EP-5	2	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0
EP-6	7	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0
EP-7	1	0	0	0	1	0	0	1	0	1	1	0	0	0	1	0	0
EP-8	1	1	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0
EP-9	1	1	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0
EP-10	1	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0
EP-11	2	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1
EP-12	5	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	0
EP-13	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0
EP-14	1	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	1
EP-15	1	0	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0
EP-16	1	0	1	0	0	0	0	1	0	1	0	0	0	1	1	0	0
EP-17	1	0	0	1	0	0	0	1	0	1	0	0	0	1	1	0	0
EP-18	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0

APPENDIX C

Figure 1- Figure showing the different alleles for the enzymes PGI (**A**), PGM (**B**), and GOT (**C**) (NOTE: each set of two bands is from a single isolate).

A) PGI- 4 alleles



B) PGM- 2 alleles

C) GOT- 3 alleles