PCR-BASED IDENTIFICATION, GENETIC DIVERSITY AND PATHOGENICITY OF *CYLINDROCLADIUM PARASITICUM* IN THE SOUTHEASTERN UNITED STATES

by

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Under the Direction of Ronald R. Walcott

ABSTRACT

In the last five years the intensity of Cylindrocladium black rot (CBR) of peanut has increased in Georgia and outbreaks have been observed in previously uninfested fields. One explanation for this trend might be changes in the population structure of the causal agent, *Cylindrocladium parasiticum*. To investigate this trend genetic analysis and pathogenicity studies were conducted using *C. parasiticum* isolates from the southeastern U.S. Genetic analysis revealed low levels of diversity within the population and while there were statistically significant differences in aggressiveness between isolates, these differences did not correlate to the geographical origin. Overall, the data suggest that recent trends of increasing CBR intensity in Georgia are not due to changes in population structure. Alternatively, it is likely that the trends can be explained by undetected inoculum accumulation in soil and local distribution of inoculum as fewer farmers produce peanuts on larger farms

INDEX WORDS: *Cylindrocladium parasiticum*, random amplified polymorphic DNA, microsatellite primed PCR, pathogenicity

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is thought to have originated in South America where it thrives in tropical and subtropical climates. Because the edible seeds of this annual legume start above ground but mature underground, peanuts are also known as groundpeas or groundnuts. Spanish explorers are believed to have spread peanut production through the Americas, Europe, Asia and Africa (http://www.peanutsusa.com). Before the U.S. Civil War, peanuts were thought to be food of the poor because the growing and harvesting practices were slow and difficult. By the end of the nineteenth century improvements in farming equipment contributed to an increase in peanut production in the U.S. (http://www.peanutsusa.com). The technological advancements in peanut production satisfied the increased demand for peanut oil, peanut butter, roasted and salted peanuts, and confections. In addition, George Washington Carver identified numerous manufactured nonfood uses for peanut and encouraged the use of peanut as a rotational crop for cotton production (http://www.peanutsusa.com).

Peanuts are an important component in human and animal diets in semiarid and tropical regions of the world (26). Peanuts contain 40-50% fat, 20-30% protein, 10-20% carbohydrate, 13 essential vitamins and minerals and are cholesterol free (26). According to the USDA's Foreign Agricultural Service, China is the largest producer of peanuts, with more than double the production of India, the world's second largest peanut producer (37). The U.S. is ranked third worldwide in peanut production followed by Nigeria, Indonesia, Senegal,

Myanmar, Zaire, Argentina and Chad (37). Furthermore, China is also the largest exporter of peanuts followed by the U.S. and Argentina (37). Peanut production for oil versus food varies greatly among countries. In most African countries peanuts are consumed for food, but in Asian countries peanuts are consumed as oil. However, food consumption is increasing, and in the U.S. peanuts are primarily consumed as snacks (37).

Georgia peanut farmers provide almost half of the U.S. peanut crop each year and peanuts are the state's official crop. Peanuts are grown in over 80 counties in Georgia on over 4,800 farms. The Georgia peanut industry employs more than 50,000 people and is worth $2.0 billion (http://www.gapeanuts.com/index.asp). In 2005, the value of Georgia's peanut crop was > $423 million and it ranked 5th in value of agricultural commodities produced in the state (6).

As with any agricultural commodity, Georgia peanut farmers have to contend with many obstacles in order to maintain their production and revenue. Environmental factors such as drought can be a problem for peanut producers, but plant diseases are more threatening. Tomato spotted wilt is the most economically important peanut disease in Georgia, even with the use of resistant peanut cultivars. Other diseases that affect Georgia peanut production include early and late leaf spot, rust, stem rot, Rhizoctonia limb rot and Cylindrocladium black rot (CBR).

In recent years, CBR cost Georgia farmers up to $13.75 million in control costs and yield losses (47). In 2005, approximately 1% of the total peanut

acreage was treated with metam-sodium to control CBR at $50/A (27). However, this control measure is often not economically feasible. Also the fungicides labeled for CBR suppression, Folicur (Bayer) and Abound (Syngenta),do not provide adequate control (2).

Since CBR is a soil borne disease, efforts are made to avoid planting peanuts in previously infested fields. Yet in recent years CBR has been observed in fields not previously planted to peanut and in fields in which the disease had not previously been observed (personal communication, Dr. Brenneman, UGA, Tifton, GA). The primary source of inoculum for CBR is microsclerotia overwintering in soil and in plant debris, and it was reported that microsclerotia could be dispersed by movement of farm equipment, hay, and root tissue (35,38).

By 1985, there were strong indications that the pathogen was seed-borne and seed transmission of *Cylindrocladium parasiticum*, the causal agent of CBR, was confirmed in 2001 (19,36). The dissemination of *C. parasiticum* via infested seeds may explain the sudden appearance of the disease in new fields. However, the viability of microsclerotia on stored seed declines during storage, with the greatest reduction occurring within the first month of storage (35). CBR was also shown to spread from colonized seeds to healthy seedlings and it was suggested that peanut genotype and inoculum density influenced seed transmission (9,11). While it is now clear that infected seed can transmit CBR, the epidemiological significance of seed borne inoculum is still unknown. However, surveys suggested that approximately 25% of commercial lots in

Georgia were infested in 2001 (45).

Since seed can be infested and transmit CBR, it is possible that seed introduce non-indigenous strains of *C. parasiticum*. In Virginia, the incidence of CBR in metam-sodium treated fields raised the awareness of seed borne inoculum (3). Recently, CBR was reported in a previously uninfested field in Texas (46). This observation supports the hypothesis that infected/infested seeds may spread *C. parasiticum*, since the seeds used to plant the field in Texas were produced in the southeastern U.S.

The purpose of this study was to develop a polymerase chain reaction (PCR)-based assay for the rapid diagnosis and confirmation of *C. parasiticum.* A second objective was to assess the genetic diversity of the pathogen and investigate variations in aggressiveness amongst isolates from different geographical regions in the southeastern U.S.

CYLINDROCLADIUM PARASITICUM

HISTORY

In 1965, Bell reported a new fungal disease of peanut in southwest Georgia (4). Signs of this disease included numerous red to orange perithecia on the stems, and symptoms included black lesions on peanut pods and roots. The fungus was identified as a member of the genus *Calonectria* and the name *Calonectria crotalariae* was proposed. The fungus was subsequently be reclassified as *Cylindrocladium parasiticum* (anamorph) and *Calonectria ilicicola* (teleomorph) (10). Between 1968 and 1970, *C. parasiticum* was observed in South Carolina, North Carolina and Virginia, and by 1975, the disease was

reported in Florida. Subsequently the disease was observed in peanut producing regions of Australia, Japan, China and India (12,14,22,34,35). Additionally, *C. parasiticum* infection of soybeans (*Glycine max*) was observed in Japan in 1968 (21).

Since its first observation, *C. parasiticum* has been a continuous problem in the major peanut producing areas of the U.S. In 2001, a new concern was raised when seed transmission of *C. parasiticum* was confirmed (36). Dissemination by seeds was investigated in 1970, when *Cylindrocladium* was recovered from discolored peanut seeds produced in Puerto Rico (13). Unfortunately, attempts to recover the pathogen from seed failed, and this prevented the confirmation of seed infection (13). In 1985 infected seeds were implicated as a vector for long distance dissemination when CBR appeared in previously unaffected areas in Australia (19). Researchers noted that drying seeds induced dormancy but did not kill the fungus (19). Recently, *C. parasiticum* was observed in a previously uninfested field in Texas (46). The importance of this incident is that the seeds planted in this field were obtained from the southeastern U.S., once again highlighting the potential of seeds as primary inoculum source. This incident also highlighted the need for more stringent standards for excluding infested seeds during peanut sorting.

HOST RANGE AND DISTRIBUTION

Cylindrocladium black rot has been observed in all peanut-producing states in the U.S. Generally it infects legumes such as peanuts and soybeans; however, it has also been reported on patridgepea (*Cassia fasciculate* Michx),

sicklepod (*C. obtusifolia L.*), Florida beggar weed (*Desmodium tortuosum*), and blueberry (*Vaccinium ashei* and *V. corymbosum*) (7,30,31). *C. parasiticum* has been recovered from tea (*Camellia sinensis*), yellow poplar (*Liriodendron tulipifera*), sweetgum (*Liquidambar styraciflua*), eucalyptus (*Eucalyptus* spp.) and other hardwood seedlings, rhododendron (*Rhododendron* sp.) and papaya (*Carica papaya*) (9). Worldwide distribution of *C. parasiticum* includes the southeastern U.S. and Hawaii, United Kingdom, Australia, Japan, India, Sri Lanka, Malaysia, Taiwan, Indonesia, China, Korea, Kenya, Ecuador, Colombia, Brazil (9).

ETIOLOGY

C. parasiticum was renamed in 1993 after analysis of morphological characteristics and whole protein profiles for a range of *Cylindrocladium* species (10). The new names given were *Cylindrocladium parasiticum* (anamorph) and *Calonectria ilicicola* (teleomorph). A phylogenetic study of the genus *Cylindrocladium* showed that *C. parasiticum*, *C. floridanum* and *C. curvisporum* belonged to the same clade; however, these taxa are linked only by their unique sphaeropedunculate vesicle morphology (44).

The morphology of *C. parasiticum* and *Ca. crotalariae* has been extensively characterized (9). The fungus is homothallic and produces orange to red perithecia. Asci are clavate, long-stalked, and eight-spored and while ascospores are primarily single-septate, they can have three cells. Macroconidiophores of the fungus are septate, hyaline and terminate in a sphaeropedunculate vesicle. The fungus grows well on potato dextrose agar

(PDA) at an optimal temperature of 25°C. Colonies of the fungus are salmon buff in color and chlamydospores are produced readily on PDA. The fungus does not grow at temperatures below 5°C or above 35°C. There is slight to no sporulation on aerial mycelium.

SYMPTOMS AND SIGNS

CBR symptoms on peanuts include chlorosis and wilting on the main axis followed by premature death of the plant. Hypocotyls and taproots of diseased plants become necrotic and the entire root system can appear blackened. CBR develops, most rapidly under cooler conditions and when soil moisture is near field capacity (3,33). Perithecia appear just above the soil line on diseased stems, and infected pegs and pods can be dark brown to black with slightly sunken lesions. Testae of peanut seeds may have reddish-brown speckles due to the presence of microsclerotia (36).

EPIDEMIOLOGY

Microsclerotia in the soil serve as primary inoculum for CBR epidemics (38). Microsclerotia are unaffected during mild winters, but when temperatures fall below 5°C for 4 to 5 weeks their viability decreases. Spatial distribution of microsclerotia in the soil is clustered or clumped (41). Rotating peanuts with soybeans is not recommended, since soybeans can be an alternative host for the pathogen and can increase microsclerotia populations in the soil (40). Inoculum levels as low as 10 microsclerotia/g of soil have been reported to initiate epidemics (33).

Histological response studies conducted by Harris & Beute, described

microsclerotia germinating and producing numerous slender hyphae that penetrate taproots intercellularly (17). Sites of fungal penetration included emerging branch roots, cortex phellem or phellogen, nodules and breaks in the periderm of the taproot. Secondary branch roots do not have secondary growth or periderm and this makes them ideal infection courts for the fungus.

Short distance dispersal of microsclerotia has been attributed to birds, movement of farm equipment, hay and roots (35). However, the role of ascospores and conidia in CBR epidemiology remain unresolved (32). Conidia occur rarely in nature and ascospores rapidly desiccate and lose their viability (19).

The mechanisms of long distance microsclerotia dispersal are not clear. In 1970, a *Cylindrocladium* species was isolated from cured peanut seeds produced in Puerto Rico leading to the hypothesis of seed infection (13). In 1985 long distance dissemination by seeds was postulated and it was also suggested that curing seeds might induce dormancy but not kill the pathogen (19). Randall-Schadel et al. confirmed seed transmission of *C. parasiticum* in 2001 (36) by visually examining seeds from commercial seed shelling plants in North Carolina. Seed were plated on a semi-selective media to recover the fungus and samples from the same lots were planted under field conditions and evaluated for CBR incidence under standard peanut cultivation practices. It was concluded that CBR-infested seeds could be a source of primary inoculum to initiate disease epidemics. This initial inoculum could spread to a 10 m focus by the subsequent year if the field was replanted with peanuts, thereby establishing local inoculum

sources and increasing the potential for crop losses (36).

In 2005 reports of *C. parasiticum* in previously uninfested peanut fields in Texas were confirmed (46). The importance of this outbreak is that the seeds planted in this field were obtained from the southeastern U.S. This incident is an example of the threat that *C. parasiticum* infested seeds may pose to new peanut production fields.

DISEASE MANAGEMENT

Currently, a combination of tactics is recommended to manage CBR. Most importantly, where available, resistant peanut cultivars should be planted. Well drained fields should be used for peanut production and to prevent soil movement, farm equipment should be thoroughly cleaned prior to field-to-field transport. Delaying planting and bedding the fields also reduce CBR by facilitating increases in soil temperature (39,40). Peanuts should not be rotated with soybeans since both crops are hosts of *C. parasiticum* and such rotations would lead to increased microsclerotia density in the soil (40). Pre-plant fumigation with metam-sodium is recommended where economically feasible. Additionally, attempts have been made to identify peanut cultivars with resistance to CBR under field and greenhouse settings. Results indicate that Spanish-type peanuts were least susceptible, Valencia-type peanuts were most susceptible and Virginia-type peanuts showed intermediate resistance (8). The existence of *C. parasiticum* races have been strongly suggested but no data are available to support this hypothesis (15). Difficulties determining races arise because variations in the environmental conditions drastically influence CBR development

(16).

MOLECULAR BASED ASSAYS AND POPULATION GENETICS

Polymerase chain reaction

Several species of the genus *Cylindrocladium*, including *C. parasiticum*, are important plant pathogens of numerous crops worldwide. Conventionally, morphological and cultural characteristics and pathogenicity assays are used to confirm the identity of suspected *C. parasiticum* isolates. These tests require expertise and are time consuming. However, since the development of the polymerase chain reaction (PCR), identification of fungi can be performed within a day. PCR is the enzyme-driven *in vitro* amplification of nucleic acids in which a specific sequence of DNA can amplify target DNA (23). PCR can be divided into three steps that include denaturation, annealing and extension. Denaturation of double stranded DNA occurs by briefly heating sample at 90-95°C. This is followed by annealing in which specific oligonucleotide primers hybridize to their complementary sequences on the template DNA strand. This normally takes place at 40-60°C. In the final step, Taq DNA polymerase adds nucleotides to the 3' end of each primer, which extends the new strand to a full length complement of the template. Extension normally occurs at temperatures ranging from 70-75°C. Successive cycles of denaturation, annealing and extension allow the template DNA to be doubled each cycle. The final amount of target DNA amplified is approximately 2^n, where n is the number of PCR cycles employed (23). PCR specificity is mainly determined by primer sequence and amplification conditions.

PCR has the advantage of rapidly and objectively confirming the identity of fungal isolates based on unique DNA target sequences. This technique can be more reliable than visual identification, since it does not require the user to be familiar with fungal morphology. Additionally, growth conditions can alter the morphological characteristics of a fungus, increasing the difficulty of visual identification. For PCR *C. parasiticum* DNA can be extracted from isolates growing on agar plates, from soil or from seeds.

Population Genetics

Management of soilborne diseases is a constant challenge for growers, who routinely use integrated pest management approaches. One major obstacle in disease management is the limited understanding of genetic structure of pathogen populations. Genetic structure is defined as the amount and distribution of genetic variation within and among populations (29). Since the advent of molecular based techniques, plant pathologists have gained knowledge about the genetic structure of many pathogen populations (29). Information gathered from such studies can be used to develop effective disease management strategies (29). However, assessing the genetic structure of fungi that exist mainly in the soil has proven to be difficult. This has been due to the fact that, distribution of fungal propagules in soil is difficult to quantify and visualize.

Only recently, a study of the population structure of *C. parasiticum* was performed using a limited population of *C. parasiticum* isolates from peanut in Georgia (49). Based on microsatellite-primed PCR and amplified fragment

length polymorphism patterns it was concluded that the *C. parasiticum* population was clonal (49). However, it has been suggested that unique strains may arise when anastomosis of the hyphae occurs followed by karyogamy and parasexual recombination of genes (20).

Random amplified polymorphic DNA

PCR based techniques can be used to generate DNA fingerprints that can be used for population structure analysis. One such technique is random amplified polymorphic DNA (RAPD) that utilizes short oligonucleotides (usually 10 bases long) of arbitrarily selected DNA sequences under low stringency conditions to amplify random segments of genomic DNA (5). This relatively simple approach yields polymorphisms and in instances, may detect single nucleotide changes in genomic DNA (18). Genetic mapping using RAPD markers has several advantages over other methods including 1) a universal set of primers can be used for genomic analysis in a wide variety of species and 2) no preliminary genetic information is required.

RAPD has been used to successfully distinguish the homothallic fungus *C. floridanum* from other heterothallic *Cylindrocladium* species (48). Additionally, two major RAPD-profiles were observed amongst *C. floridanum* isolates. RAPD makers have been used extensively to assess genetic diversity, genome mapping and molecular diagnostics of many fungal species (44).

Microsatellite primed PCR

Microsatellites are described as loci where short sequences of DNA are often repeated in tandem arrays (42). In particular, microsatellite primers that

allow PCR amplification of intergenic regions from closely related phylogenetic groups can be derived from highly conserved sequences, e.g., rRNA and tRNA genes (28). Microsatellite primers derived from tRNA regions have multiple advantages including 1) the tRNA region is conserved among species and allows a PCR product to be amplified from almost any member of a genus and 2) while the tRNA genes are highly conserved, the lengths of tRNA intergenic spacers can vary greatly, from 2 bp to 208 bp, depending on the organism (28). Such tRNA derived microsatellite primers have been successfully used for intraspecific populations studies of several plant pathogenic fungi (24,25,43,49,50).

PATHOGENICITY STUDIES

C. parasiticum virulence studies have been conducted to investigate the effects of inoculum density, pathogen response to resistant host selection pressure, pathogen response to environmental factors, and the effects of rotations and isolate origin disease severity (1,25,43). These have been conducted primarily to develop CBR-resistant peanut cultivars. These studies indicate that resistance is an effective way to manage CBR. Currently, no studies have been conducted to assess differences in virulence amongst *C. parasiticum* isolates from different geographical origins. The information gathered from these studies might further improve the efficiency and effectiveness of resistance breeding.

Research Objectives

The overall objective of this research was to elucidate the genetic population structure of *C. parasiticum* in the southeastern U.S. Data collected in

such a study may aid the understanding of the epidemiology of CBR. For example, the genetic population structure may indicate the patterns of movement of *C. parasiticum* and provide clues as to the role of seeds in dissemination. The specific objectives were to 1) develop a PCR-based assay to rapidly detect and diagnose *C. parasiticum*; 2) assess the genetic diversity among *C. parasiticum* populations using molecular based techniques and 3) determine the relative aggressiveness of genetically distinct *C. parasiticum* isolates from different geographical locations throughout the southeastern US.

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CHAPTER 2

DEVELOPMENT OF A RAPID PCR-BASED DIAGNOSTIC ASSAY FOR THE IDENTIFICATION OF *CYLINDROCLADIUM PARASITICUM*[1]

[1]Castro, A.C., Brenneman, T., and Walcott, R. R. 2006. To be submitted to Plant Disease.

ABSTRACT

Castro, A.C., Brenneman, T., and Walcott, R. R. 2006. Development of a rapid PCR based diagnostic assay for the identification of *Cylindrocladium parasiticum*. To be submitted to Plant Disease.

Cylindrocladium black rot (CBR), caused by *Cylindrocladium parasiticum* is a serious and potentially devastating disease of peanuts that was first observed in Georgia, USA in 1965. The fungus produces characteristic reddish brown perithecia that are useful for diagnosis and confirmation; however other fungi including *Nectria* spp. and *Neocosmospora* sp. also produce red perithecia on peanuts. To improve the speed and accuracy of diagnosing CBR, a PCR-based assay was developed. Specific oligonucleotide primers based on the β-tubulin gene were designed and evaluated for their ability to detect *C. parasiticum* isolates. The PCR primers, CBR1X/2X, were highly specific and did not yield the expected 560-bp amplicon with template DNA from the non-target *Cylindrocladium* spp. or other commonly occurring fungi tested. In contrast, the expected amplicon was produced with 92.6% (100/108) of the *C. parasiticum* isolates screened. These results suggest that CBR1X/2X is highly sensitive and can be useful for rapid confirmation of *C. parasiticum*.

Introduction

Cylindrocladium parasiticum, the causal agent of Cylindrocladium black rot (CBR) of peanut (*Arachis hypogaea* L.) was first reported in Georgia, USA in 1965 (3). By the 1970s, CBR was observed in all major peanut producing areas of the U.S, China, Japan, India and Australia (6,7,13,14,18,19). The disease continues to be a potential threat to peanut production and in 2005, it was responsible for $4.2 million in damages and $0.4 million in control costs in Georgia alone (16).

C. parasiticum survives as soilborne microsclerotia that serve as primary inoculum for CBR epidemics (21). Small-scale dispersal of microsclerotia has been attributed to birds, movement of farm equipment, hay and roots (19). Additionally, the fungus infects and survives on peanut seed coat as brown hyphae and microsclerotia (Figure 1) (20). Seed transmission of *C. parasiticum* was confirmed in 2001 and it has been suggested that seed may serve as vectors for long distance dissemination of the fungus (20). A recent CBR outbreak in Texas provides support for the role of seeds in *C. parasiticum* dissemination (27). This is particularly compelling since the pathogen was not found in that region previously, and the seeds for the Texas field were produced in the southeastern U.S. This outbreak emphasizes the need for more stringent phytosanitary standards, the implementation of which will require rapid sensitive and accurate detection and diagnostic assays.

Foliar symptoms of CBR include chlorosis and wilting of the main axis of the plant followed by premature plant death (Figure 2A). At advanced stages of

disease development abundant reddish-brown perithecia are produced on stems just above the soil line (Figure 2B). The appearance of perithecia is useful to identify and distinguish the pathogen from other soilborne diseases. However, diagnosis and confirmation of *C. parasiticum* in the field is confounded by the fact that perithecia often are not present, and in some peanut producing regions, related fungi including *Nectria* spp. and *Neocosmospora* sp. also produce red perithecia (14). In order to confirm *C. parasiticum*, field observations must be complemented with laboratory assays and routinely, surface sterilized peanut seeds and/or tissue are screened by plating on potato dextrose agar or V8-agar. After incubation at 25°C for 7 – 10 days, the fungal colonies are visually examined for characteristic morphological traits including: burnt orange mycelia, yellow fluffy aerial mycelia and production of perithecia. In addition to the time required, accurate confirmation of *C. parasiticum* requires training and experience in basic mycology, in order to recognize the conidiophores and vesicle morphology that are unique to this fungus. Finally, with regards to seedborne inoculum, it is generally difficult to detect the pathogen by agar plating once seeds have been stored (9). Hence, a technique that could improve the efficiency and objectivity of CBR identification would not only be important for rapid and accurate diagnosis, but also an effective tool for epidemiological studies. One such tool is the polymerase chain reaction (PCR) (22)

PCR is the enzyme mediated, in vitro amplification of specific DNA sequences (22). Because it is can be rapid, sensitive and highly specific, PCR-based assays have been developed for a wide array of plant pathogenic

organisms including fungi, bacteria and viruses (5,15,17). The specificity of PCR is conferred by short oligonucleotides (primers) (20 – 30 base pairs) that are designed to flank unique sequences of the template DNA. After denaturation of template DNA, primers hybridize to unique template DNA followed by the extension of specific sequences. Finally, there is the amplification of millions of copies of the target (amplicon). After PCR, gel electrophoresis can be used to separate the PCR products to verify the size of the amplicon.

As compared to culture media and visual observation of morphological characteristics, DNA extraction and PCR can be completed within one day, which makes it highly suitable for diagnosis and detection of *C. parasiticum*. To date there have been no reports of attempts to develop a PCR-based diagnostic assay for *C. parasiticum*. Hence, the objective of this study is to design species-specific primers to be used in a PCR assay for the detection and diagnosis of *C. parasiticum*.

Materials and Methods

Fungal isolates. One hundred and eight *C. parasiticum* isolates and six other *Cylindrocladium* species were used for this study (Table 1). The *C. parasiticum* isolates were collected from fields in Georgia and Alabama or submitted by contributors from Florida, North Carolina and Virginia. The isolates were collected from peanut, soybean, beggar weed, and sickle pod and represent populations of *C. parasiticum* in different peanut growing regions in the southeastern U.S. *Cylindrocladium* sp. isolates were routinely grown on potato dextrose agar (PDA) (Difco, Beckton Dickinson, Sparks, MD) at 25°C with

alternating 12 h intervals of darkness and fluorescent light. Isolates were maintained at 25°C on PDA for short term storage. For longer storage 1 cm^2 agar plugs were taken from the edges of 7 day old PDA colonies and transferred into sterile 20 ml glass vials half filled with sterile water. Vials were wrapped with Parafilm (Pechiney Plastic Packaging, Chicago, IL) and stored at 25°C. As needed, isolates were recovered by plating agar plugs onto PDA.

DNA extraction. Routinely, DNA was extracted from single conidium cultures of each isolate. Single spore cultures were produced by incubating each isolate on carnation leaf agar (2 cm^2 pieces of sterile carnation leaves placed on the surfaces of 1.5% water agar plates) (CLA) to induce sporulation (4). Isolates were incubated for 7 d at 25°C with 12 h cycles of darkness and fluorescent light and using a sterile needle, individual conidia were picked from carnation leaf pieces and transferred to PDA. After 5 days growth on PDA a sterile needle was used to transfer mycelial strands of each isolate into a sterile 1.5 ml microcentrifuge tube containing 1 ml of potato dextrose broth (PDB) (Difco, Beckton Dickinson). Tubes were incubated with continuous agitation on a bench top shaker (Innova, New Brunswick Scientific, Edison, NJ) for 72 h at 23-25°C. After incubation, mycelial tissue was harvested by centrifugation at 13,000 rpm for 3 min. and the PDB was decanted. The mycelial pellet was resuspended in 1 ml fungal DNA extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% w/v SDS) and transferred into a 2 ml polypropylene, screw-cap tube (BioSpec Products Inc., Bartlesville, OK), half-filled with 0.5 mm silica glass beads (BioSpec Products Inc.). Using a Mini-Bead beater apparatus (BioSpec

Products Inc.) cells of the mycelial tissue for each isolate were disrupted by vigorous agitation for 3 min. Approximately 500 µl of crude fungal cell extract was pipetted into a sterile 1.5 ml microcentrifuge tube, and DNA was purified by phenol/ethanol extraction (1). Purified DNA was quantified using a NanoDrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE) and stored at –20°C until used.

Design of *C. parasiticum*-specific PCR primers. *C. parasiticum*-specific primers were designed based on a comparison of the highly conserved β-tubulin gene sequences of several *Cylindrocladium* species (23). The β-tubulin gene sequence was used previously to phylogenetically analyze *Calonectria* (*Cylindrocladium*) species (23). β-tubulin gene sequences for 10 *Cylindrocladium* species were retrieved from the National Institutes of Health National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) (Table 2). Sequences were aligned using the BioNumerics sequence analysis software (Applied Maths, St-Martens, Belgium) and regions of the gene sequence that were unique to *C. parasiticum* were visually identified. Using available primer design software (www.idtdna.com), *C. parasiticum*-specific oligonucleotides CBR 1X (5'-CTG GGG ATT CAC TAA CAT TG-3') and CBR 2X (5'-TCG AGG GAC ATA CTT GTT GC-3') were designed to amplify a 560-bp fragment. The primers were synthesized at the University of Georgia Molecular Genetics Instrumentation Facility, Athens, GA.

Evaluation of the specificity of PCR with CBR1X/2X. To determine the specificity of the CBR1X/2X primer set for *C. parasiticum*, PCR was conducted

on DNA extracted from 108 *C. parasiticum* isolates (Table 1) as well as a range of other related and unrelated fungal species including *Fusarium sp*., *Bipolaris maydis*, *Collectotrichum sp.*, *Alternaria sp.*, *Sordaria sp*., *Rhizoctonia sp.*, and *Sclerotium rolfsii*. PCR was routinely conducted in 25 µl reaction volumes using a commercial PCR kit (2.5 units Taq DNA, 10mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$, 200µM dATP, dCTP, dGTP and dTTP, and stabilizers, including BSA) (PuReTaq Ready-to-Go PCR, Amersham Biosciences, Piscataway, NJ, USA). To each Ready-to-Go lyophilized PCR reagent bead, 22 µl of sterile high performance liquid chromatography water, 25 µM of each primer and ~2 ng of purified fungal DNA were added. The optimal PCR thermal profile conditions were empirically determined using an Eppendorf Mastercycler Gradient programmable thermal cycler (Eppendorf, Hamburg, Germany). The optimized PCR thermal profile included an initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Amplification was completed with a final cycle of 95°C for 30 sec, 55°C for 30 s and 72°C for 5 min. Ten microliters of PCR product were loaded on horizontal 1% agarose gel, separated by electrophoresis at 90 V for 60 min in 1X Tris-acetate EDTA (TAE) buffer (1X TAE: 40mMTris base, 11.4 ml of acetic acid glacial, 2mM EDTA disodium salt) and stained with ethidium bromide. Subsequently, PCR products were visualized under an ultraviolet light and gel images were digitally captured using a Stratagene Eagle Eye camera (Stratagene, La Jolla, GA).

Sensitivity of the *C. parasiticum* PCR assay. To determine the

detection sensitivity of the assay, PCR was conducted on ten-fold serial dilutions *C. parasiticum* ATCC 26110 DNA ranging from 10 ng/µl to 0.0001 ng/µl. Concentrations of purified *C. parasiticum* DNA were quantified using the Nanodrop spectrophotometer (Nanodrop Technologies, Inc) and serial dilutions were made in sterile water. All reactions were conducted in 25 µl as described previously. For comparison, PCR was also conducted on the dilution series using primer set ITS1 (5'-TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC 3') that targets the fungal internal transcribed spacer region. This experiment was repeated 4 times.

Results

Design of *C. parasiticum* specific PCR primers. Alignment and visual analysis of the β-tubulin gene sequences for ten *Cylindrocladium* species yielded sufficient sequence dissimilarities to facilitate the design of a *C. parasiticum* specific primer. The unique sequences were used to develop CBR 1X and CBR 2X, which directed the amplification of a 560-bp fragment with *C. parasiticum* template DNA.

Specificity of CBR1X/2X. As expected, CBR 1X/ 2X did not direct the amplification of the expected 560-bp amplicon with template DNA from *Fusarium sp*., *Bipolaris maydis*, *Collectotrichum sp.*, *Alternaria sp.*, *Sordaria sp*., *Rhizoctonia sp.*, nor *Sclerotium rolfsii.* Additionally, the expected amplicon was not generated with the related *Cylindrocladium* species evaluated (Fig. 3). Of the 108 *C. parasiticum* isolates 92.60% (100/108) yielded the expected 560-bp amplicon with CBR1X/2X and the PCR conditions described. The *C. parasiticum*

isolates whose DNA was not amplified with the species specific primers were CBR 01-94, CBR 01-201, CBR 01-77, CBR 01-163, CBR 01-165, CBR 01-10, CBR 01-17 and CBR 01-6 (soybean). Attempts to amplify DNA from these eight isolates with universal primers ITS1/ITS4 were successful, suggesting that the DNA was PCR quality and perhaps, CBR1X/2X was incapable of detecting a subpopulation of *C. parasiticum* isolates.

Detection sensitivity of CBR 1X/CBR 2X. Using a DNA serial dilution that ranged from 10 ng to 0.0001 ng/µl, the ITS1/ITS4 primer set demonstrated a consistent detection threshold of 0.01 ng/µl (4/4 attempts). In contrast, the CBR1X/2X primer set demonstrated a detection threshold of 1ng DNA /µl (4/4 attempts).

Discussion

CBR continues to threaten peanut production in the southeastern U.S. and more recently the disease has been observed in peanut fields in Texas (27). This observation suggests that the fungus has effective strategies for long distance dissemination. One possible vector for such long distance spread is peanut seed, and there is evidence that the fungus is both seedborne and seed transmitted (12,20). If this is the case, one effective strategy to limit the dissemination of the pathogen is by seed health testing to detect and exclude contaminated seedlots. To date, detection and diagnosis of *C. parasiticum* has been via the use of agar media and by visual recognition of unique signs associated with the fungus in the field. While *C. parasiticum* produces characteristic perithecia on infected tissues, the ability of several non-target fungi

including *Nectria* spp. and *Neocosmospora* sp. to produce similar signs necessitates laboratory-based testing of isolates for confirmation (14) . Additionally, even though characteristic microsclerotia might be present on infested/infected seeds, the ability to recover the fungus from seeds declines with time (9). Hence, more rapid and reliable assays are required to enhance the detection and diagnosis of *C. parasiticum* in seed, soil and plant tissues. As the role of seedborne inoculum becomes clear, a PCR-based assay might be necessary to enforce quarantine restrictions and limited the long distance dissemination of the pathogen.

PCR is highly suitable for detection of *C. parasiticum* because it is rapid, sensitive, specific, reliable and easy to conduct. PCR-based detection and diagnostic assays have been developed for a wide array of plant pathogens including fungi and the technique is becoming commonplace in many state and federal diagnostic laboratories (10,25). To our knowledge, this work represents the first attempt to develop a PCR based diagnostic assay for *C. parasiticum*.

An essential component of any diagnostic PCR assay is the oligonucleotide primers. These short single stranded DNA sequences direct the amplification of DNA fragment that are unique to the target organism and hence, confer specificity. Many techniques have been used to generate specific oligonucleotide primers including RAPD, AFLP, single nucleotide polymorphisms and the examination of DNA sequence variations in conserved and hypervariable genes. The latter approach has been widely used for fungal pathogens and has employed genes such as the ribosomal RNA and internal transcribed spacer

regions (10,25). Another gene that has been utilized is the β-tubulin gene (24). The β-tubulin gene is critical for microtubule function and cell division and the β-tubulin protein is also a target site of benzimidazoles fungicides (2). The β-tubulin gene sequence has been found to be conserved in many fungi (2,8,23,24). Schoch et al. used this gene to successfully assess the phylogeny of *Calonectria* (*Cylindrocladium* spp.) (23). Hence, it has many of the properties that would make it suitable for primer design. Other investigators have similarly used the β-tubulin gene sequence to design diagnostic PCR primers (8,24). Based on these facts the *C. parasiticum* β-tubulin gene was selected to develop a specific PCR primer for *C. parasiticum*.

By comparing the gene sequence for ten *Cylindrocladium* species, the primer pair CBR1X/2X was designed for *C. parasiticum*. CBR1X/2X did not amplify DNA from any of the other *Cylindrocladium* sp. nor the other genera tested. On the other hand, it produced the expected 560-bp amplicon from 92.6% of the *C. parasiticum* isolates tested indicating a high level of specificity. Unfortunately, the primers failed to amplify DNA from eight *C. parasiticum* isolates. Attempts to verify the identity of these isolates with microsatellite primed PCR proved that they were *C. parasiticum* (see Chapter 3).

One other disappointing aspect of the CBR1X/2X-based PCR assay was the low level of sensitivity observed (1ng/µl), which was 100 fold less than with ITS1/ITS4. One possible explanation for this observation is that there is a single copy of the β-tubulin gene in most fungal species, including *Cylindrocladium* sp. (23,24). This might explain the differences in sensitivity observed between the

primers ITS1/ITS4 (target multi-copy loci) and CBR1X/CBR2X.

Despite this limitation CBR1X/CBR2X primers could be used for the rapid diagnosis of most *C. parasiticum* isolates in soil or seeds. To implement this tool DNA could be rapidly extracted from soil or seeds using a commercially available kit and used as template for PCR. This would significantly enhance the efficiency and reliability of *C. parasiticum* diagnosis. To enhance the sensitivity of the assay, modification such as nested PCR, and magnetic capture hybridization (11) could be considered. Magnetic capture hybridization relies on single stranded nucleic acid probes anchored to polystyrene magnetic beads to sequester and concentrate target DNA prior to PCR. This approach has been used successfully to enhance the detection of *Botrytis allii* in onion seeds (26).

It is clear that further work is needed to optimize the PCR assay to ensure that all *C. parasiticum* isolates can be detected. This work should include the optimization of PCR run condition and further exploration of the β-tubulin gene for developing more appropriate primers. Despite its shortcomings, this work represents the first attempt to develop a PCR-based assay for *C. parasiticum*, and through seed testing this protocol may be useful for limiting the spread of CBR.

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Table 1. Different fungal isolates used for PCR assay using CBR1/CBR 2X primers

Fungus	Storage Code	Year received	Origin	Host	Source	PCR reaction with CBR1X/2X
Cylindrocladium parasiticum	CBR 84, 85, 91	2002	Suwannee Co., FL	peanut	T. Kucharek[1]	+
	CBR 782-783	2002	Levy Co., FL	peanut	T. Kucharek	+
	CBR 780-781	2002	Decatur Co., GA	peanut	T. Kucharek	+
	CBR 93-4	2002	Screven Co., GA	beggar weed	T. Brenneman[2]	+
	CBR 93-8	2002	Worth Co., GA	peanut	T. Brenneman	+
	CBR 94-4	2002	Burke Co., GA	beggar weed	T. Brenneman	+
	CBR 94-6	2002	Burke Co., GA	sicklepod	T. Brenneman	+
	CBR 94-8	2002	Worth Co., GA	peanut	T. Brenneman	+
	CBR 97-3	2002	Miller Co., GA	peanut	T. Brenneman	+
	CBR 97-8	2002	Emmanuel Co., GA	unknown	T. Brenneman	+
	CBR 97-11	2002	Georgia	partridge pea	T. Brenneman	+
	CBR 99-6	2002	Georgia	unknown	T. Brenneman	+
	CBR 202-2, 3, 4, 5, 6	2002	Tift Co., GA	peanut	A. Castro	+
	CBR 202-11, 12, 13, 14	2002	Sumter Co., GA	peanut	A. Castro	+
	CBR 202-15, 16, 17, 18	2002	Burke Co., GA	peanut	A. Castro	+
	CBR 202-21, 22, 23, 24	2002	Henry Co., AL	peanut	A. Castro	+
	CBR 00-25	2000	unknown	unknown	Seed Pathology Lab[3]	+
	CBR 00-27	2000	unknown	unknown	Seed Pathology Lab	+
	CBR 01-6	2001	unknown	soybean	A. Castro	-
	CBR 01-10	2002	Baker Co., GA	partridge pea	T. Brenneman	-
	CBR 01-14	2002	Oglethorpe Co., GA	peanut stem	T. Brenneman	+

Table 1.cont.

Fungus	Storage Code	Year received	Origin	Host	Source	PCR reaction with CBR1X/2X
Cylindrocladium parasiticum	CBR 01-17	2002	Burke Co., GA	peanut shell	T. Brenneman	-
	CBR 01-18, 21,36	2002	Burke Co., GA	peanut shell	T. Brenneman	+
	CBR 01-20, 56	2002	Burke Co., GA	peanut kernel	T. Brenneman	+
	CBR 01-61	2002	Miller Co., GA	peanut kernel	T. Brenneman	+
	CBR 01-64	2002	Miller Co., GA	peanut shell	T. Brenneman	+
	CBR 01-65	2002	Miller Co., GA	peanut kernel	T. Brenneman	+
	CBR 01-71, 79, 102, 116	2002	Calhoun Co., GA	peanut kernel	T. Brenneman	+
	CBR 01-77	2002	Calhoun Co., GA	peanut kernel	T. Brenneman	-
	CBR 01-94	2002	Calhoun Co., GA	peanut kernel	T. Brenneman	-
	CBR 01-73, 86, 89, 93, 95, 104	2002	Calhoun Co., GA	peanut shell	T. Brenneman	+
	CBR 01-120, 122, 125, 130, 137, 139, 141, 144, 152, 160, 161, 166, 175, 179, 180, 200, 203, 206, 218, 220, 221, 224, 225, 227, 230, 231, 233, 234, 236, 238, 239, 254, 274, 278, 287	2002	Burke Co., GA	peanut	T. Brenneman	+

Table 1. cont.

Fungus	Storage Code	Year received	Origin	Host	Source	PCR reaction with CBR1X/2X
Cylindrocladium parasiticum	CBR 01-163	2002	Burke Co., GA	peanut	T. Brenneman	-
	CBR 01-165	2002	Burke Co., GA	peanut	T. Brenneman	-
	CBR 01-201	2002	Burke Co., GA	peanut	T. Brenneman	-
	Purvis 26	2003	Martin Co., NC	peanut	B. Shew[4]	+
	Jones 5	2003	Martin Co., NC	peanut	B. Shew	+
	Jones 20	2003	Martin Co., NC	peanut	B. Shew	+
	111-4	2003	unknown	unknown	B. Shew	+
	205	2003	Florida	peanut	B. Shew	+
	CPRC 02	2003	Virginia	peanut	P. Phipps[5]	+
	26110	2003	North Carolina	peanut	ATCC[6]	+
C. floridanum	18882	2003	Florida	peach roots	ATCC	-
C. avesiculatum	6825	2003	Florida	unknown	S. Rizvi[7]	-
C. angustatum	P99 0454	2003	Florida	unknown	S. Rizvi	-
C. angustatum	P99 1321	2003	Florida	unknown	S. Rizvi	-
C. gordoniae	P02 6389	2003	Florida	unknown	S. Rizvi	-
C. rumohrae	P94 3976	2003	Florida	unknown	S. Rizvi	-
C. spathiphylli	0576	2003	Florida	unknown	S. Rizvi	-
Alternaria solani		2000	unknown	unknown	Seed Pathology Lab	-
Collectoctrichum sp.		2000	unknown	unknown	Seed Pathology Lab	-
Bipolaris maydis		2000	unknown	unknown	Seed Pathology Lab	-
Fusarium sp.		2000	unknown	unknown	Seed Pathology Lab	-
Rhizoctonia sp.		2000	unknown	unknown	Seed Pathology Lab	-
Sordaria sp.		2000	unknown	unknown	Seed Pathology Lab	-
Sclerotium rolfsii		2000	unknown	unknown	Seed Pathology Lab	-

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Table 3. Accession numbers for *Cylindrocladium* species β-tubulin gene sequences used for oligonucleotide development

Fungus	Gene Bank no.
C. angustatum	AF207543
C. avesiculatum	AF333392
C. candelabrum	AF210857
C. curvisporum	AF333394
C. floridanum	AF320193
C. gracile	AF232850
C. macroconidiale	AF232855
C. parasiticum	AF333411
C. pteridis	AF232860
C. scoparium	AF210872

Fig. 1. Peanut seed infested with *C. parasiticum*. Microsclerotia on the testa give the peanut its cinnamon brown speckled color.

Fig. 2. A) Wilted and prematurely dead peanut plants infected with *Cylindrocladium parasiticum*. B) Perithecia above soil line on 14 week old peanut cv. Georgia Green.

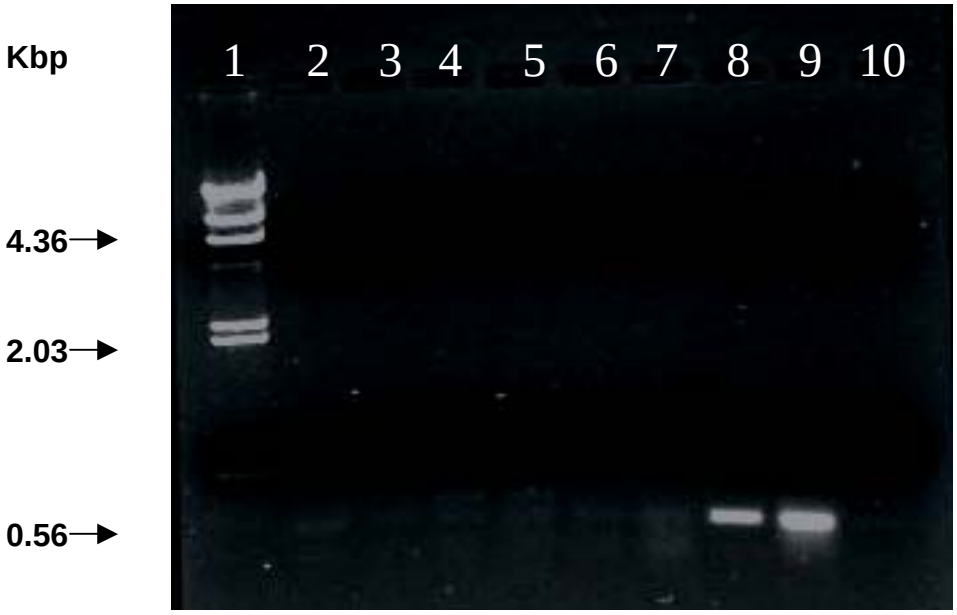

Fig. 3. Agarose gel depicting the specificity of polymerase chain reaction using primers CBR 1X and CBR 2X. Lanes 1-10 contain Lambda marker (Promega, Woodshollow, WI), *C. angustatum* P99 0454, *C. angustatum* P99 1321, *C. avesiculatum* 6825, *C. gordoniae* P02 6389, *C. rumohrae* P94 3976, *C. spathiphylli* 0576, *C. parasiticum* 95-4, *C. parasiticum* 96-1, and water, respectively.

CHAPTER 3

INTRASPECIFIC VARIATION AND PATHOGENIC ANALYSIS OF *CYLINDROCLADIUM PARASITICUM* ISOLATES FROM THE SOUTHEASTERN UNITED STATES[1]

[1]Castro, A. C., Brenneman T., Glenn, A., and Walcott, R. R. 2006. To be submitted to Plant Disease.

ABSTRACT

Castro, A. C., Brenneman T., Glenn, A., and Walcott, R. R. 2006. Intraspecific variation and pathogenic analysis of *Cylindrocladium parasiticum* isolates from the southeastern United States. To be submitted to Plant Disease.

To account for recent increases in disease severity and incidence in Georgia, the genetic diversity of *Cylindrocladium parasiticum* populations in the southeastern U.S. was investigated. Random amplified polymorphic DNA (RAPD) and microsatellite primed-polymerase chain reaction (MP-PCR) assays were conducted on a population of 108 *C. parasiticum* isolates collected from Georgia, Virginia, Florida, Alabama and North Carolina. While RAPD analysis indicated that the population was clonal, MP-PCR polymorphisms revealed three clusters of isolates. Cluster I contained 84.3 % (91/108) of the isolates while clusters II and III contained 14 and 3 isolates, respectively. The clusters did not correlate to the geographical origin of the isolates. To further investigate differences amongst *C. parasiticum* populations, the relative aggressiveness of 17 isolates representing different geographic regions were compared on peanut seedlings under greenhouse conditions. Statistically significant differences in aggressiveness were observed ($P < 0.001$) with CBR 323 from Florida being the most aggressive. However, there was no obvious correlation between isolate aggressiveness and geographical origin. These data do not indicate that recent introduction of aggressive, non-indigenous strains account for increases in CBR intensity in Georgia.

Introduction

Cylindrocladium parasiticum is the causal organism of Cylindrocladium black rot (CBR) of peanuts (*Arachis hypogaea* L.). This disease was first reported in Georgia in 1965, but since then it has been observed in all major peanut producing states in the southeastern U.S., Australia, Japan, China and India (2,7,9,14,19,20). Despite being first observed in Georgia, CBR was a more significant threat to peanut production in Virginia and North Carolina. It was assumed that this was due to the types of peanuts grown in these regions and the cooler weather conditions (8,9). However, within the last 5 years, the incidence and severity of CBR on peanuts in Georgia have been increasing (personal communication, T. Brenneman, The University of Georgia, Tifton, GA). Most notably, in 2001, peanut yield losses in Georgia due to CBR were estimated at $13.75 million (29). This increase in CBR in Georgia prompted a search for possible explanations that could lead to improved management strategies.

C. parasiticum is a fungus whose primary sources of inoculum include mycelia and microsclerotia that overwinter in the soil and plant debris (22). While conidia and ascopores are produced by this fungus, the epidemiological significance of these propagules is unclear because they are short-lived and desiccate readily (21). On the other hand, microsclerotia can be dispersed by birds and by movement of farm equipment, hay, root tissues and soil (22).

Seeds represent another possible vector for long distance dissemination of *C. parasiticum* but the significance of seed infection and seed transmission has long been disputed (13). In 2001, Randall-Schadell et al., demonstrated

transmission of *C. parasiticum* with chemically treated and untreated seeds (20). The long distance movement and introduction of aggressive, non-indigenous isolates on seeds provides one possible explanation for the recent CBR trends of increased incidence and severity in Georgia. To further support this hypothesis, CBR was first reported in a seed production field in Texas in 2005 (28). Since seeds produced in the southeastern U.S. were used to plant this field, it is possible that these seeds were the source of primary inoculum. However, the 2005 season was wetter and cooler than average and this might also provide a possible explanation for the disease outbreak. Nevertheless, there is still a need to explore possible explanations for the trends of increasing CBR in Georgia.

Management of CBR has traditionally involved an integrated approach that includes planting resistant peanut cultivars in well-drained fields, delayed planting and bedding to increase soil temperature at planting, crop rotation with non-legumes and pre-plant applications of metam sodium (23,24). However, there is still a need to improve CBR management. Such improvements could be developed based on a better understanding of the epidemiology and population structure of regional populations of *C. parasiticum*. In particular, knowledge of the genetic diversity of *C. parasiticum* could improve the efficiency of peanut resistance breeding and selection programs. While it has been strongly suggested that races of the pathogen exist, no definitive research has been conducted to test this hypothesis (24). Difficulties in determining races arise because variations in the environment drastically influence CBR development (10). However, assessing the genetic diversity of *C. parasiticum* might provide a

clue as to the existence sub-populations or biological races. Recently, using amplified fragment length polymorphisms, Wright et al. found that a *C. parasiticum* population consisting of 40 isolates from peanut in Georgia was clonal (30). In the event that this population size was limited and not fully representative of populations in the southeastern U.S., the objective of this work was to assess the genetic diversity of a larger population of isolates (108 isolates) recovered from GA, AL, VA, FL and NC. A second objective was to investigate differences in aggressiveness between a subpopulation of 17 isolates recovered from GA, AL, FL, NC and VA.

Materials and Methods

Fungal isolates. One hundred and eight *C. parasiticum* isolates and seven other *Cylindrocladium species* were used for this study (Tables 1-3). Isolates were collected or submitted by contributors from GA, AL, FL, VA and NC. Isolates were recovered from a range of hosts including peanut, soybean, sicklepod, beggar weed, and partridgepea. Single conidium isolates of *C. parasiticum* and other *Cylindrocladium* sp. were obtained by culturing on carnation leaf agar (4 cm^2 pieces of sterile carnation leaves placed on 1.5% water agar) to induce sporulation (6). Isolates were incubated at 25°C for 7 days with 12 h cycles of darkness and fluorescent light. Individual conidia produced on the carnation leaves by each isolate were transferred to potato dextrose agar (PDA) (Difco, Beckton Dickinson, Sparks, MD, USA). *Cylindrocladium* sp. isolates were routinely cultured on PDA at room temperature and for long term storage, 3 to 5 1 cm^2 plugs from the edges of 5 day old PDA cultures were

maintained in glass vials containing 10 ml of sterile water. Vials were wrapped with Parafilm (Pecheney Plastic Packaging, Chicago, IL) and stored at 23–25°C.

DNA extraction. To extract DNA from each isolate a sterile needle was used to transfer mycelia from a five-day-old culture to a sterile 1.5 ml microcentrifuge tube with 1 ml of potato dextrose broth (PDB) (Difco, Beckton Dickinson). Tubes were incubated with agitation on an Innova bench top shaker (New Brunswick Scientific, Edison, NJ) for 72 h at 23-25°C. To harvest mycelia, tubes were centrifuged at 13,000 rpm for 3 min. and the PDB was decanted with a micropipettor. The mycelial pellet was then resuspended in 1 ml of fungal extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.55% w/v SDS) and transferred to a 2 ml polypropylene, screw-cap tube (BioSpec Products Inc., Bartlesville, OK, USA), half filled with 0.5 mm silica glass beads (BioSpec Products Inc.). The sample was placed in a BeadBeater apparatus (BioSpec Products Inc.) and vigorously agitated (5,000 rpm) for 3 min. to physically disrupt fungal cells. Approximately 500 µl of crude fungal cell extract was pipetted into a sterile 1.5 ml microcentrifuge tube, and purified by phenol/ethanol extraction as described by Ausubel et al. (1).

Random amplified polymorphic DNA (RAPD). Purified genomic DNA from 50 *C. parasiticum* isolates, seven *Cylindrocladium* sp. and seven other plant pathogenic fungi were selected for this analysis (Tables 1-2). Primers OPE 2, OPE 3, OPE 4, OPE 14 and OPE 20 were previously used for RAPD characterization of *C. floridanum* (27) and after evaluating several primers singly

and in combinations (data not shown), the combination of OPE 14 and OPE 20 was selected for this analysis. Template DNA from each isolate was amplified with OPE14 (5'TGC GGC TGA G3') and OPE20 (5'AAC GGA GAC C3'). PCR was conducted using PuReTaq Ready-to-Go PCR mastermix (Amersham Biosciences, Piscataway, NJ) and each reaction was conducted in a 25 µl reaction volume comprised of 22 µl of sterile high performance liquid chromatography-grade water, 0.25 µM of each primer and ~2 ng of purified template DNA. RAPD amplification was carried out in an Eppendorf Mastercycler Gradient programmable thermal cycler (Eppendorf, Hamburg, Germany), with a thermal profile that included an initial denaturation at 96°C for 2 min, followed by 40 cycles of denaturation at 92°C for 30 s, annealing at 37°C for 30 s, and extension at 72°C for 1 min. Ten microliters of PCR product were separated by electrophoresis at 150 V/cm for 180 min on a 2% agarose gel and subsequently stained with ethidium bromide. RAPD profiles were visualized by exposure of the gels to ultraviolet light and digital images were captured with a StraTrategene EagleEye (Stratagene, La Jolla, GA).

Microsatellite-primed PCR. The tRNA derived primer T3B (5'-AGG TCG CGG GTT CGA ATC C- 3') was used (17). MP-PCR was conducted on DNA from 108 *C. parasiticum* isolates (Table 3) and on *C. floridanum* and *C. avesiculatum* as representative outliers. *C. parasiticum* isolate ATCC 26110 was used as a standard to ensure MP-PCR reproducibility. The DNA from this isolate was amplified independently 13 times.

To reduce run-to-run variability of MP-PCR results the PuReTaq Ready-

to-Go PCR mastermix (Amersham Biosciences) was employed and PCR was conducted as previously described with thermal run conditions that included an initial denaturation step for 3 min at 95°C, 40 cycles denaturation at 94°C for 1 min, annealing of primer at 50°C for 1 min, and extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on a 1.5% agarose gel, at 150 V for 360 min in 1x TAE. Gels were stained with ethidium bromide and images were captured under ultraviolet light as previously described.

Data analysis. Using the MP-PCR generated DNA profiles a distance matrix was generated using Dice's coefficient of similarity and a dendrogram was constructed by the unweighted pairwise group with arithmetic mean algorithm (UPGMA)(5). A dendrogram indicating isolate relatedness was generated using BioNumerics software (Applied Maths, St-Martens-Latem, Belgium).

Pathogenicity study. Seventeen *C. parasiticum* isolates that represented different geographic regions in the southeastern U.S. (Table 4) were chosen to evaluate differences in pathogen aggressiveness. To prepare inoculum, each isolate was cultured on glucose yeast extract agar (30 g-0.2 g/liter) amended with tyrosine (80 mg/liter) after autoclaving (12). Glucose yeast extract agar was selected because *C. parasiticum* produces large numbers of microsclerotia in 6-8 days (12). To harvest microsclerotia, cultures were comminuted for 2 min. in a blender (Waring Products, Inc, Torrington, CT) and passed through sieves with 246 and 240 µm pore sizes (W.S. Tyler Company, Mentor, OH) (12). Microsclerotia in the 240 µm sieve (W.S. Tyler Company) were

separated from mycelial fragments by forcefully running water through the sieve for 1 min. (12). Microsclerotia were resuspended in water and counted using a dissecting microscope. Ten milliliters of a microsclerotia suspension (1000 microsclerotia/ml) were used to inoculate one kilogram of Pro-Mix BX growing medium (Premier Horticulture LTD., Dorval, Canada). The steam pasteurized Pro-Mix BX (Premier Horticulture LTD.) growing medium was placed into a plastic bag and inoculum was evenly distributed by hand mixing. Inoculated growth medium was used to fill 20 conetainers (Stuewe and Sons, Inc., Corvallis, OR) per isolate. Soil treated with water served as a negative control. One peanut of cv. Georgia Green treated with Vitavax (Bayer CropScience, Research Triangle Park, NC) was planted into each conetainer (Stuewe and Sons, Inc.). The conetainers (Stuewe and Sons, Inc.) were incubated under greenhouse conditions (23°C-29°C) in a complete randomized block arrangement. Plants were watered daily to promote disease development.

Four to six weeks after planting, seedling germination was evaluated and after 10 – 14 weeks disease severity was evaluated after removing soil from roots. A CBR damage scale of 0 (no visible damage) to 5 (complete decay) (11) (Fig. 1) was employed to evaluate aggressiveness of the isolates and CBR incidence was also recorded. Subsequently, plants were allowed to air-dry on greenhouse benches for 48 h before plant dry weight of whole peanut plant was measured.

Statistical analysis. The pathogenicity experiments were analyzed as a split-plot, with replications as main plots and *C. parasiticum* isolate as subplots.

The experiment was conducted three times and analysis of variance (ANOVA) was used to determine the significance of fungal isolates on plant weight, CBR incidence and CBR severity. Statistical analyses were done with SAS statistical package (SAS Institute Inc., Cary, NC).

Results

Random amplified polymorphic DNA and microsatellite-primed PCR. Using the RAPD primers OPE 14 and OPE 20 twelve reproducible markers were generated with DNA from 50 *C. parasiticum* isolates. All 50 isolates yielded the identical DNA fingerprinting profile, indicating that the population was clonal (Figure 2). Using the same primers, a range of distinct RAPD profiles were generated for the other fungal isolates (*Cylindrocladium* spp. and others). These RAPD profiles revealed polymorphisms between *Cylindrocladium* spp. and between *C. parasiticum* and other *Cylindrocladium* spp. (Figure 3).

The fingerprints generated using MP-PCR primer T3B revealed 8 to 22 reproducible markers. The fingerprint generated for isolate *C. parasiticum* ATCC 26110, was consistently repeated on 13 different runs. A dendrogram containing the 108 isolates and the two outliers revealed that *C. floridanum* 18882 and *C. avesiculatum* 6825 were 48% similar to all of the *C. parasiticum* isolates and did not group within the *C. parasiticum* fingerprints. Overall the *C. parasiticum* isolates were 52% similar to each other (Figure 4). Fingerprints could be grouped into three major clusters. Cluster I comprised 91 *C. parasiticum* isolates that were 56% similar to each other within this cluster. Within cluster I subgroups were grouped in by their degree of similarity ranges at 60%-70%, 70%-80% and

>80% similar, subgroups were labeled Ia-Ig (Figure 4). The subgroups were divided as follows based on their similarity within cluster I: Ia, 1/91 isolates with 63% similarity, Ib, 1/91 isolates with 65% similarity, Ic, 4/91 isolates with 74% similarity, Id, 2/91 isolates with 73% similarity, Ie, 71/91 isolates with 81% similarity, If, 4/91 isolates with 83% similarity, and Ig, 7/91 isolates with 83% similarity (Figure 4). The origin of the isolates was mixed within the subgroups, in particular subgroup Ic had isolates from VA, GA, AL, NC, and FL. Additionally, *C. parasiticum* ATCC 26110 used as the reference isolate in this study was grouped within subgroup Ic.

Clusters II and III were 63% similar and 62% similar respectively (Figure 4). There were 14 isolates in cluster II, 13/14 isolates where from Georgia and 1/14 isolates was from Alabama. In cluster III there were three isolates and two were from North Carolina (111-4 and Edmondson 41) and the third was from Florida (205). The DNA profiles observed for cluster III were the most dissimilar within the *C. parasiticum* fingerprints (Figure 4).

Pathogenicity study. All peanut plants exposed to a *C. parasiticum* isolate became diseased in all three experiments. As expected the control plants generally showed little or no disease symptoms. During experiment three, two control peanuts plants displayed root discoloration; however, perithecia were not observed on these plants and it was suspected that this discoloration was not due to CBR. There was statistical significant difference between experiments, however, there was no significant block (replication) effect (Tables 4 and 5) and the data from the three experiments were pooled and analyzed using PROC

general linear models (GLM) of SAS. The mean weight and severity varied significantly depending on *C. parasiticum* isolate ($P < 0.001$) (Tables 4 and 5). The highest mean peanut weight was 4.37 g for treatment 202-15 GA (Fig. 5). However, the mean weight for plants inoculated with isolate 202-115 GA was not significantly different to the mean weights of plants inoculated with water, 84 FL, 205 FL, Purvis 26 NC, 202-28 GA, 93-08 GA, 202-13 GA, CPRC 02 VA, 202-24 AL, Elliot 40 NC, ATCC 26110 NC, 01-254 GA, 111-4 NC, 783 FL, and 202-1 GA (Fig. 5). The weight for plants inoculated with 323 FL was significantly different than that for plants inoculated with 84 FL, 202-15 GA, and 202-21 AL (Fig. 5). The lowest mean plant weight, 2.95 g was observed for plants inoculated with 202-21 AL and this treatment was significantly different to all other isolates (Fig. 5).

Analysis of variance indicated that the effect of *C. parasiticum* isolate on disease severity was statistically significant ($P < 0.0001$) (Table 5). Overall the most aggressive isolate was 323 Fl with a rating of 3.17. This isolate was significantly more aggressive that 205 FL, Purvis26 NC and 84 FL. However, differences in aggressiveness between 323 FL and the other 13 isolates were not statistically significant (Fig. 6). Overall, the least aggressive isolates were 205 FL, Purvis 26 NC and 84 FL (Fig. 6). When linear regression was used to determine the relationship between disease severity and dry weight a significant negative relationship was found ($y = 6.15 – 1.09\ x$; $r^2 = 0.847$, $P = < 0.0001$) (Fig. 7). Additionally, 15/17 *C. parasiticum* isolates used for the pathogenicity study belonged to cluster I. The remaining 2 isolates 205 Fl and 111-4 were in

cluster III.

Discussion

The notable increase in CBR incidence and severity in Georgia over the past 5 years has lead to much speculation about the epidemiology of this disease. In particular, it has been suggested that changes in spatial spread and disease severity might have been due to the introduction new and more aggressive isolates from outside the state. The confirmation of seeds as a possible long distance vector for the pathogens provided anecdotal evidence for this claim. However, concrete support for the idea that populations in different regions of the southeastern U.S. are genetically and pathogenically different could come only from an analysis of the population structure of the fungus.

To date, there has been one study conducted to investigate the genetic diversity of *C. parasiticum* populations (30). This study involved 40 isolates from peanut in Georgia and based on AFLP analysis, the authors concluded that the population was clonal (30). Results of the current study, which involved RAPD analysis of a larger population of isolates from a wider geographical range (GA, AL, Fl, NC and VA), supported the conclusions drawn by Wright et al. (30). AFLP is considered to be better than RAPD analysis for measuring genetic diversity because it has greater discriminating power (distinguishing up to one nucleotide) (18). Additionally, RAPD analysis generally has low levels of repeatability. Despite this, RAPD analysis of 50 *C. parasiticum* isolates using primers OPE14/OPE20 yielded identical DNA profiles with high levels of repeatability. On the other hand, the same RAPD primers allowed the

discrimination of other *Cylindrocladium* sp. These data are consistent with the conclusion that *C. parasiticum* populations in the southeastern U.S. are clonal. As such, the data generated by this technique do not support the idea that recent trends in CBR severity and incidence are due to novel populations being introduced from outside the state.

Because of concerns that the OPE14/OPE20 primer set was not suitable for determining intraspecific variation amongst *C. parasiticum* isolates, a microsatellite primed PCR approach was taken. Microsatellites are described as loci where short sequences of DNA are repeated in tandem arrays (25). In particular, microsatellite primers that allow PCR amplification of intergenic regions from phylogenetically related groups can be derived from highly conserved sequences, e.g., rRNA and tRNA genes (17). Primers derived from the tRNA region have multiple advantages including 1) the tRNA region is conserved and will allow a PCR product to be amplified from almost any member of a genus; and 2) while the tRNA genes are highly conserved, the lengths of tRNA intergenic spacers can vary from 2 bp to 208 bp, depending on the organism (17). Such tRNA derived microsatellite primers have been successfully used for population studies of several plant pathogenic fungi (15,16,25,26,30,31). MP-PCR targets single sequence tandem repeats that are dispersed throughout genomes of fungi and as such, it has advantages over RAPD analysis including increased reliability and higher discriminating power. For this study, the microsatellite primer T3B was used to generate DNA fingerprints for *C. parasiticum* (17). This primer was previously used to compare isolates of

Botryosphaeria dothidea in pistachio orchards in California (15). Contrary to the results observed with RAPD analysis, MP-PCR revealed some polymorphisms amongst a population of 108 *C. parasiticum* isolates. UPGMA analysis of the DNA fingerprint profiles revealed three clusters. The majority of isolates (84%) fell into cluster I and approximately 90% of the members of this cluster were >80% or more similar. The remaining two clusters were 62 and 63% similar. Despite the variability revealed by MP-PCR analysis, it is still likely that the population of *C. parasiticum* has a low level of genetic diversity. Possible explanations for the differences in genetic diversity observed amongst *C. parasiticum* isolates with RAPD and MP-PCR analysis include 1) that T3B targets species or sub-species specific polymorphic intergenic regions and 2) small differences in the intergenic regions can be detected with specifically designed microsatellite primers.

Similar to this study, Wright et al (2006) also employed MP-PCR to compare a sub-population of *C. parasiticum* isolates. In this case specific microsatellite primers were designed and the authors evaluated17 *C. parasiticum* isolates. Wright et al. observed 45 alleles for 17 isolates; however, they concluded that based on both MP-PCR and AFLP the *C. parasiticum* population was clonal (30)

To further explore the possibility of differences in aggressiveness amongst *C. parasiticum* isolates from different geographical regions in the southeastern U.S., greenhouse pathogenicity assays were conducted. All of the isolates tested caused some level of disease on peanut cv. Georgia Green;

however, the isolates did not differ in their effect on plant weight. Additionally, while 323 FL was the most aggressive isolate, its aggressiveness was not statistically different from 13 other isolates. In general, these data indicate a general lack of differences in aggressiveness between the isolates from around the southeastern US. However, there is significant correlation ($P < 0.0001$) observed between disease severity and peanut dry weight. As isolate aggressiveness increases peanut weight decreases. In light of the fact that RAPD and AFLP (Wright et al. 2006) analysis indicated clonality amongst *C. parasiticum* populations and MP-PCR analysis (this study) showed limited genetic variation, the observation of limited differences in aggressiveness was not surprising. It is suspected that the small differences in aggressiveness observed might be due to variation in environmental conditions in the greenhouse. In general however, the data from the greenhouse pathogenicity study did not support the idea that recent trends of increases CBR intensity in Georgia are due to the introduction of genetically distinct strains with higher levels of aggressiveness.

Based on the data generated in this and previous studies there appears to be low genetic diversity amongst *C. parasiticum* isolates collected from different southeastern states. Because of the observed genetic homogeneity the *C. parasiticum* population, these data do not support the hypothesis that new and aggressive isolates have been recently introduced into Georgia. Additionally, they neither support nor reject the role of seeds as long distance vectors of *C. parasiticum*. Since it now seems unlikely that the trends of increasing CBR

intensity in Georgia are due to increased genetic diversity of the pathogen, other explanations must be sought. Alternative explanations include gradual increases in inoculum density in field soils that go unnoticed until inoculum thresholds are reached. Additionally, the occurrence of weather conditions that favor disease development, combined with increased inoculum might account for increased disease outbreaks. In particular, increased soil moisture during the early stages of plant development might account for low levels of soilborne inoculum leading to severe disease outbreaks. Interestingly, in 2001, the year with the most severe CBR outbreaks in Georgia, environmental conditions were cooler and wetter than in previous years (29). Both inoculum accumulation over time and favorable weather conditions have previously been suggested as possible reasons for increased CBR intensity (3,4).

While limited genetic diversity of *C. parasiticum* populations in the southeastern U.S. was unexpected, this information suggests that the prospects for managing this disease through cultivar resistance are great. A limited range of isolates can be used to screen peanut lines for effective CBR resistance genes and once identified, it is likely that resistance will be durable. The epidemiological significance of seeds as long distance vectors for *C. parasiticum* remains to be determined. However, if seeds are important vectors, they may still be potential threats for introducing genetic variants of *C. parasiticum* from different peanut producing regions of the world. Thus increased vigilance through seed heath testing is needed ensure the future profitability of the Georgia peanut industry.

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Table 1. Fungal isolates used for RAPD analysis with primers OPE4/OPE20

Storage Code	Year received	Origin	Host	Source
CBR 84, 85, 91	2002	Suwannee Co., FL	peanut	T. Kucharek[1]
CBR 782-783	2002	Levy Co., FL	peanut	T. Kucharek
CBR 780-781	2002	Decatur Co., GA	peanut	T. Kucharek
CBR 934	2002	Screven Co., GA	beggar weed	T. Brenneman[2]
CBR 942, 948	2002	Burke Co., GA	peanut	T. Brenneman
CBR 951	2002	Mitchell Co., GA	unknown	T. Brenneman
CBR 962	2002	Miller Co., GA	peanut	T. Brenneman
CBR 984	2002	Decatur Co., GA	peanut	T. Brenneman
CBR 986	2002	Macon Co., GA	peanut	T. Brenneman
CBR 987	2002	Screven Co., GA	unknown	T. Brenneman
CBR 9711	2002	Georgia	partridge pea	T. Brenneman
CBR 9811	2002	Georgia	unknown	T. Brenneman
CBR VA	2001	Virginia	unknown	P. Phipps[3]
CBR VA 3	2001	Virginia	unknown	P. Phipps
CBR VA 4	2001	Virginia	unknown	P. Phipps
CBR 202-2, 3, 4, 5, 6	2002	Tift Co., GA	peanut	A. Castro
CBR 202-11, 12, 13, 14	2002	Sumter Co., GA	peanut	A. Castro
CBR 202-15, 16, 17, 18	2002	Burke Co., GA	peanut	A. Castro
CBR 202-21, 22, 23, 24	2002	Henry Co., AL	peanut	A. Castro
CBR 01-4, 5, 6	2001	unknown	soybean	A. Castro
CBR 01-3, 8, 18	2002	Baker Co., GA	peanut	T. Brenneman
CBR 01-1, 66, 69	2002	Worth Co., GA	peanut	T. Brenneman
CBR 01-62,63,64	2002	unknown	unknown	T. Brenneman

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[2] Tim Brenneman, Department of Plant Pathology, University of Georgia, Tifton, GA

[5] Pat Phipps, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech, Suffolk, VA

Table 2. Fungal isolates used for random amplified polymorphic DNA amplification with primers OPE 14 and OPE 20

Fungus	Storage Code	Year received	Origin	Host	Source
C. floridanum	18882	2003	Florida	peach roots	ATCC[1]
C. avesiculatum	6825	2003	unknown	Florida	S. Rizvi[2]
C. angustatum	P99 0454	2003	unknown	Florida	S. Rizvi
C. angustatum	P99 1321	2003	unknown	Florida	S. Rizvi
C. gordoniae	P02 6389	2003	unknown	Florida	S. Rizvi
C. rumohrae	P94 3976	2003	unknown	Florida	S. Rizvi
C. spathiphylli	0576	2003	unknown	Florida	S. Rizvi
Alternaria solani		2000	unknown	unknown	Seed Pathology Lab[3]
Collectoctrichum sp.		2000	unknown	unknown	Seed Pathology Lab
Bipolaris maydis		2000	unknown	unknown	Seed Pathology Lab
Fusarium sp.		2000	unknown	unknown	Seed Pathology Lab
Rhizoctonia sp.		2000	unknown	unknown	Seed Pathology Lab
Sordaria sp.		2000	unknown	unknown	Seed Pathology Lab
Sclerotium rolfsii		2000	unknown	unknown	Seed Pathology Lab

[1] American Type Culture Collection (ATCC), Manassas, VA

[2] Shabbir Rizvi, Florida Department of Agriculture, Gainesville, FL

[3] Ron Walcott, Department of Plant Pathology, University of Georgia, Athens, GA

Table 3. *Cylindrocladium* sp. Used for microsatellite primed-PCR analysis.

Fungus	Storage Code	Year received	Origin	Host	Source
Cylindrocladium parasiticum	CBR 84, 85, 91	2002	Suwannee Co., FL	peanut	T. Kucharek[1]
	CBR 782-783	2002	Levy Co., FL	peanut	T. Kucharek
	CBR 780-781	2002	Decatur Co., GA	peanut	T. Kucharek
	CBR 93-4	2002	Screven Co., GA	beggar weed	T. Brenneman[2]
	CBR 93-8	2002	Worth Co., GA	peanut	T. Brenneman
	CBR 94-4	2002	Burke Co., GA	beggar weed	T. Brenneman
	CBR 94-6	2002	Burke Co., GA	sicklepod	T. Brenneman
	CBR 94-8	2002	Worth Co., GA	peanut	T. Brenneman
	CBR 97-3	2002	Miller Co., GA	peanut	T. Brenneman
	CBR 97-8	2002	Emmanuel Co., GA	unknown	T. Brenneman
	CBR 97-11	2002	Georgia	partridge pea	T. Brenneman
	CBR 99-6	2002	Georgia	unknown	T. Brenneman
	CBR 202-2, 3, 4, 5, 6	2002	Tift Co., GA	peanut	A. Castro
	CBR 202-11, 12, 13, 14	2002	Sumter Co., GA	peanut	A. Castro
	CBR 202-15, 16, 17, 18	2002	Burke Co., GA	peanut	A. Castro
	CBR 202-21, 22, 23, 24	2002	Henry Co., AL	peanut	A. Castro
	CBR 00-25	2000	unknown	unknown	Seed Pathology Lab[3]
	CBR 00-27	2000	unknown	unknown	Seed Pathology Lab
	CBR 01-6	2001	unknown	soybean	A. Castro
	CBR 01-10	2002	Baker Co., GA	partridge pea	T. Brenneman
	CBR 01-14	2002	Oglethorpe Co., GA	peanut stem	T. Brenneman
	CBR 01-18,21,36	2002	Burke Co., GA	peanut shell	T. Brenneman
	CBR 01-17	2002	Burke Co., GA	peanut shell	T. Brenneman

Table 3. cont.

Fungus	Storage Code	Year received	Origin	Host	Source
Cylindrocladium parasiticum	CBR 01-20, 56	2002	Burke Co., GA	peanut kernel	T. Brenneman
	CBR 01-61	2002	Miller Co., GA	peanut kernel	T. Brenneman
	CBR 01-64	2002	Miller Co., GA	peanut shell	T. Brenneman
	CBR 01-65	2002	Miller Co., GA	peanut kernel	T. Brenneman
	CBR 01-71, 79, 102, 116	2002	Calhoun Co., GA	peanut kernel	T. Brenneman
	CBR 01-77	2002	Calhoun Co., GA	peanut kernel	T. Brenneman
	CBR 01-94	2002	Calhoun Co., GA	peanut kernel	T. Brenneman
	CBR 01-73, 86, 89, 93, 95, 104	2002	Calhoun Co., GA	peanut shell	T. Brenneman
	CBR 01-120, 122, 125, 130, 137, 139, 141, 144, 152, 160, 161, 166, 175, 179, 180, 200, 203, 206, 218, 220, 221, 224, 225, 227, 230, 231, 233, 234, 236, 238, 239, 254, 274, 278, 287	2002	Burke Co., GA	peanut	T. Brenneman
	CBR 01-163	2002	Burke Co., GA	peanut	T. Brenneman
	CBR 01-165	2002	Burke Co., GA	peanut	T. Brenneman
	CBR 01-201	2002	Burke Co., GA	peanut	T. Brenneman

Table 3. cont.

Fungus	Storage Code	Year received	Origin	Host	Source
Cylindrocladium parasiticum	CBR 01-201	2002	Burke Co., GA	peanut	T. Brenneman
	CBR 01-201	2002	Burke Co., GA	peanut	T. Brenneman
	Purvis 26	2003	Martin Co., NC	peanut	B. Shew[4]
	Jones 5	2003	Martin Co., NC	peanut	B. Shew
	Jones 20	2003	Martin Co., NC	peanut	B. Shew
	323	2003	Levy Co., FL	peanut	B. Shew
	763	2003	unknown	unknown	B. Shew
	Bateman 30	2003	unknown	unknown	B. Shew
	607	2003	unknown	unknown	B. Shew
	Elliot 40	2003	unknown	unknown	B. Shew
	111-4	2003	unknown	unknown	B. Shew
	205	2003	Florida	peanut	B. Shew
	CPRC 02	2003	Virginia	Peanut	P. Phipps[5]
	26110	2003	North Carolina	Peanut	ATCC[6]
C. floridanum	*18882*	2003	Florida	Peach roots	ATCC
C. avesiculatum	6825	2003	Unknown	Florida	S. Rizvi[7]

[1] Tom Kucharek, Department of Plant Pathology, University of Florida, Gainesville, FL.
[2] Tim Brenneman, Department of Plant Pathology, University of Georgia, Tifton, GA
[3] Ron Walcott, Department of Plant Pathology, University of Georgia, Athens, GA
[4] Barbara Shew, Department of Plant Pathology, North Carolina State University, Raleigh, NC
[5] Pat Phipps, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech, Suffolk, VA
[6] American Type Culture Collection (ATCC), Manassas, VA
[7] Shabbir Rizvi, Florida Department of Agriculture, Gainesville, FL

Table 4. *Cylindrocladium parasiticum* isolates used in the pathogenicity study

C. parasiticum **isolate**	***Host***	**Origin**	**Source**
202-21 AL	Peanut	Henry Co., Al	A. Castro
202-13 GA	Peanut	Sumter Co., GA	A. Castro
202-15 GA	Peanut	Burke Co., GA	A. Castro
84 FL	Peanut	Suwannee Co., FL	T. Kucharek[1]
ATCC 26110	Peanut	North Carolina	ATCC[2]
202-28 GA	Peanut	Burke Co., GA	A. Castro
93-08 GA	Peanut	Worth Co., GA	T. Brenneman[3]
Purvis 26 NC	Peanut	Martin Co., NC	B. Shew[4]
CPRC 02 VA	Peanut	Virginia	P. Phipps[5]
202-1 GA	Peanut	Tift Co., GA	A. Castro
202-24 AL	Peanut	Henry Co., AL	A. Castro
Elliot 40 NC	Unknown	North Carolina	B. Shew
01-254	Peanut	Burke Co., GA	T. Brenneman
323 Fl	Peanut	Levy Co., FL	B. Shew
111-4	Unknown	North Carolina	B. Shew
783 Fl	Peanut	Levy Co, FL	T. Kucharek
205 FL	Peanut	Florida	B. Shew

[1] Tom Kucharek, Department of Plant Pathology, University of Florida, Gainesville, FL.
[2] American Type Culture Collection (ATCC), Manassas, VA
[3] Tim Brenneman, Department of Plant Pathology, University of Georgia, Tifton, GA
[4] Barbara Shew, Department of Plant Pathology, North Carolina State University, Raleigh, NC
[5] Pat Phipps, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech, Suffolk, VA

Table 4. Results of a split-plot analysis of variance to determine the effects of different *Cylindrocladium parasiticum* isolates on the mean weight (grams per plant) of peanut cv. Georgia Green in greenhouse experiments

Source	df	Mean Square	*F*	*P>F*
Experimental run	2	180.13	109.18	0.0003
Replication (main plot)	2	1.41	0.85	0.4916
Main plot error	4	1.65	--------	--------
C. parasiticum isolate (subplot)	17	1.92	3.22	0.0001
Rep x *C. parasiticum* isolate	34	0.45	0.76	0.8217
Sub-plot error	101	0.59	--------	--------

Table 5. Results of a split-plot analysis of variance to determine the effects of different *Cylindrocladium parasiticum* isolates on the mean severity of CBR on greenhouse grown peanuts cv. Georgia Green

Source	df	Mean Square	*F*	*P>F*
Experimental run	2	16.05	17.38	0.0106
Replications (main plot)	2	0.66	0.71	0.5445
Main plot error	4	0.92	--------	--------
C. parasiticum isolate (subplot)	17	5.05	8.27	<.0001
Rep x *C. parasiticum* isolate	34	0.41	0.66	0.9123
Sub-plot error	101	0.61	--------	--------

Fig. 1. Symptom rating scale for Cylindrocladium black rot of peanuts. Severity rating increases as stem discoloration, wilting increases and plant death is observed.

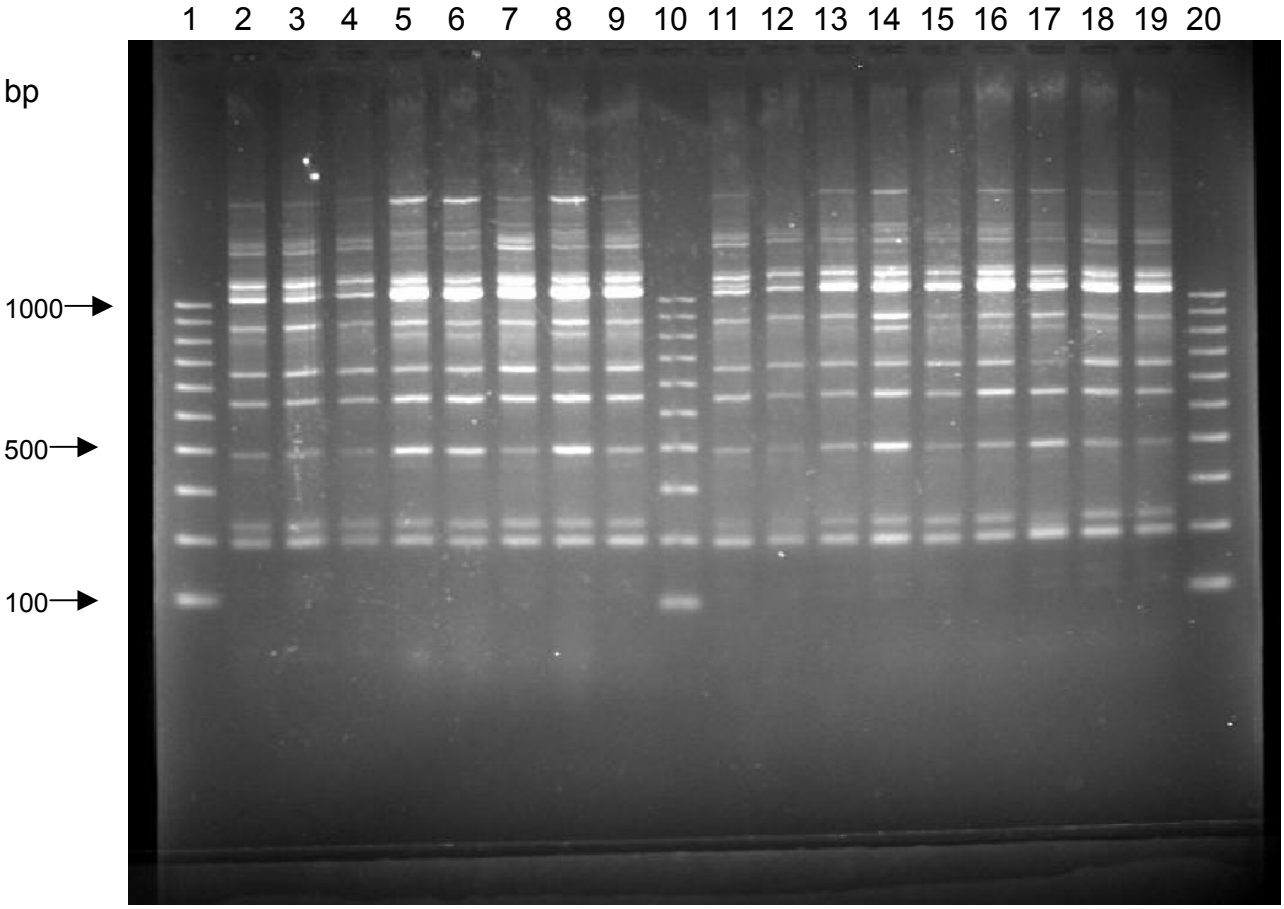

Fig. 2. Agarose gel depicting RAPD fingerprints for different *Cylindrocladium parasiticum* isolates using primers OPE 14 and OPE 20. Lanes 1-20 contained: Sigma low ladder marker (Sigma Aldrich, St. Louis MO), CBR 84, CBR 85, CBR 91, CBR 782, CBR 783, CBR 780, CBR 781, CBR 98-4, low ladder marker, CBR 93-4, CBR 98-7, CBR VA, CBR VA 3, CBR VA 4, CBR VA 5, CBR 9711, CBR 9811, CBR 95-1, and low ladder marker, respectively

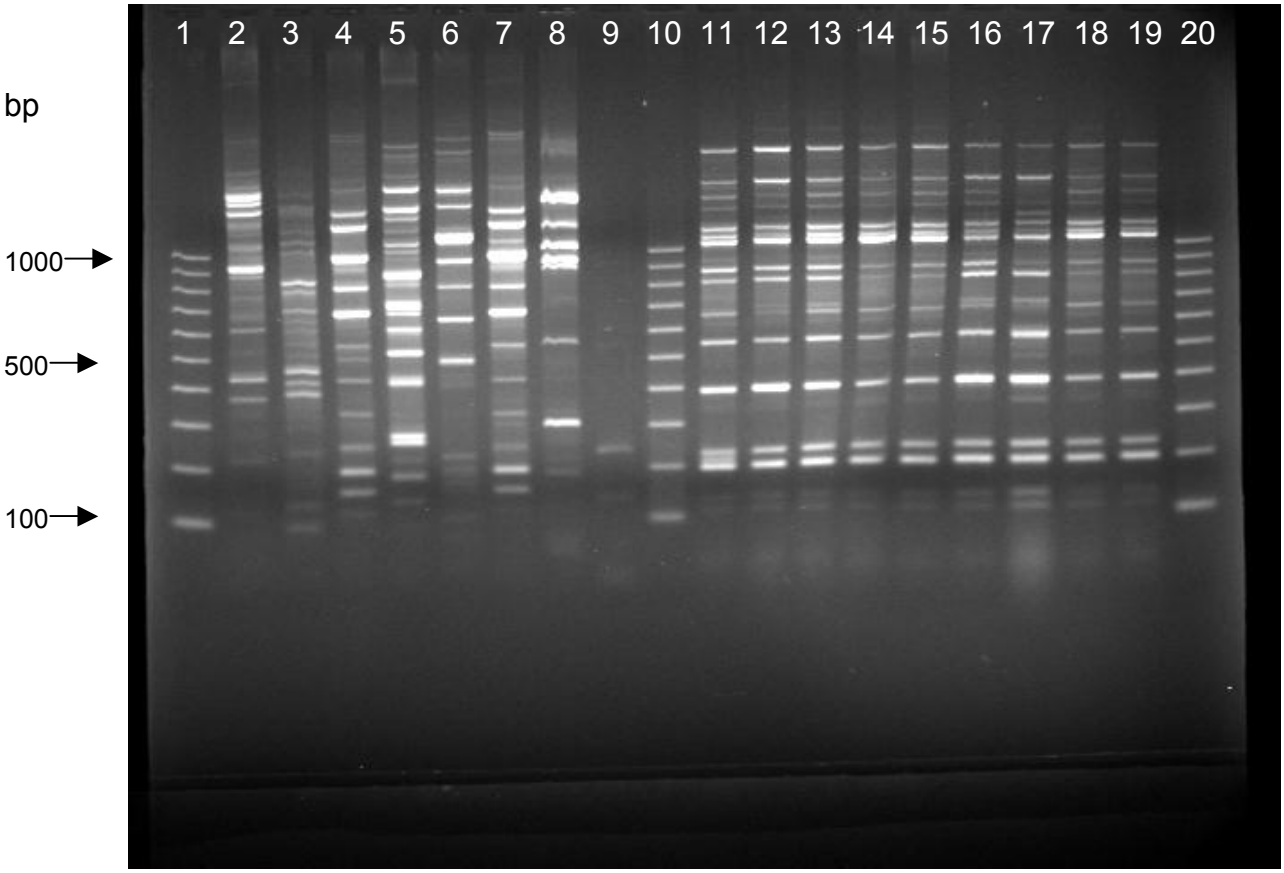

Fig. 3. Agarose gel depicting RAPD fingerprints for *Cylindrocladium parasiticum* and different fungal isolates using primers OPE 14 and OPE 20. The contents of each lane are as follows: lanes 1-20: Sigma low ladder marker (Sigma Aldrich, St. Louis MO), *Alternaria solani*, *Collectoctrichum* sp, *Bipolaris maydis*, *Fusarium* sp., *Rhizoctonia* sp., *Sordaria* sp., *Sclerotium rolfsii*, water, low ladder marker, CBR 84, CBR 782, CBR 780, CBR 202-3, CBR VA 3, CBR 01-4, CBR 202-15, CBR 202-21, CBR 202-22, and low ladder marker, respectively

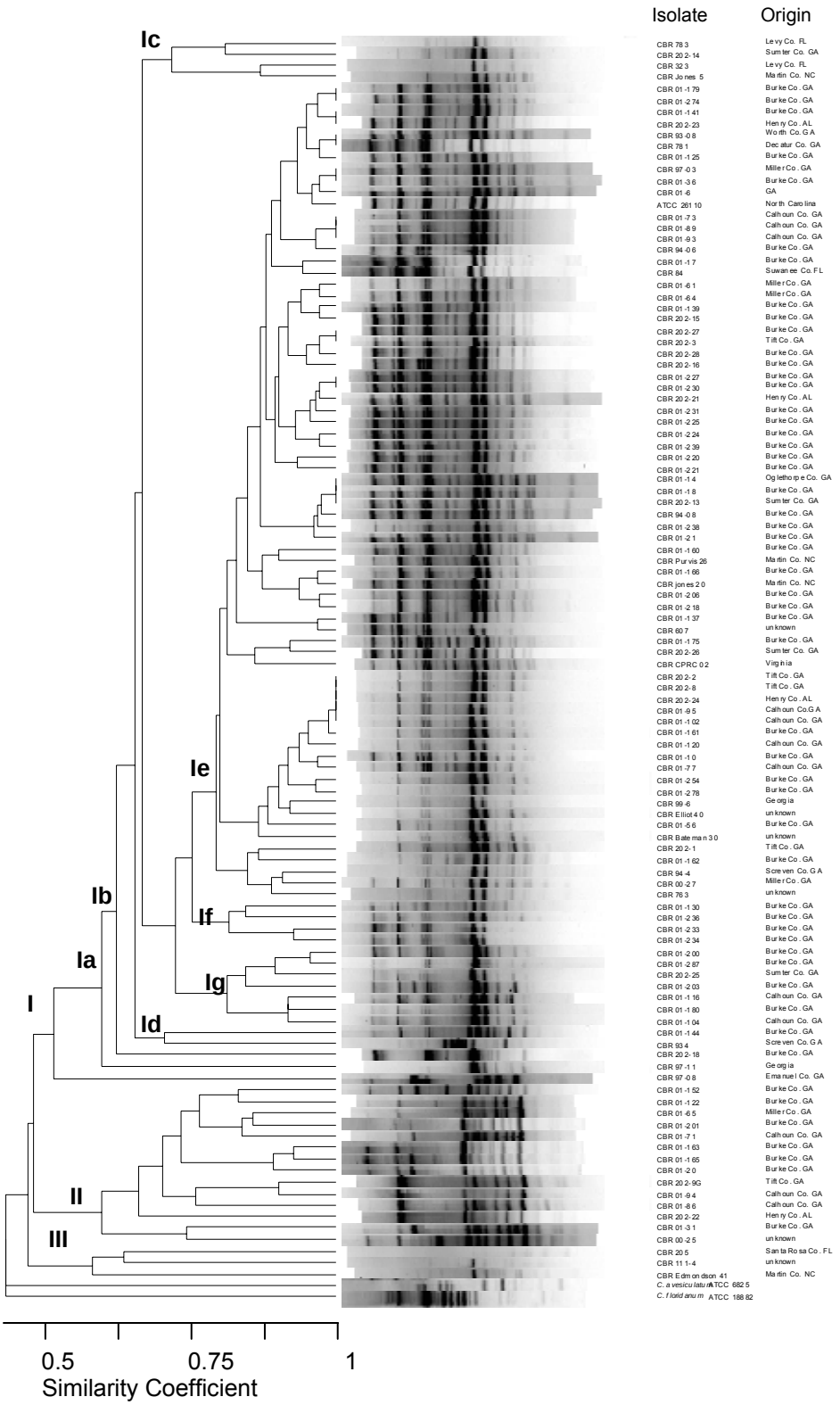

Fig. 4. Dendrogram indicating the relationships between isolates of *Cylindrocladium parasiticum, C. floridanum* and *C. avesiculatum* recovered from the southeastern U.S. DNA profiles were generated using microsatellite primed PCR and analyzed using Dice's coefficient. The dendrogram was constructed using the unweighted pair group method with arithmetic averages algorithm.

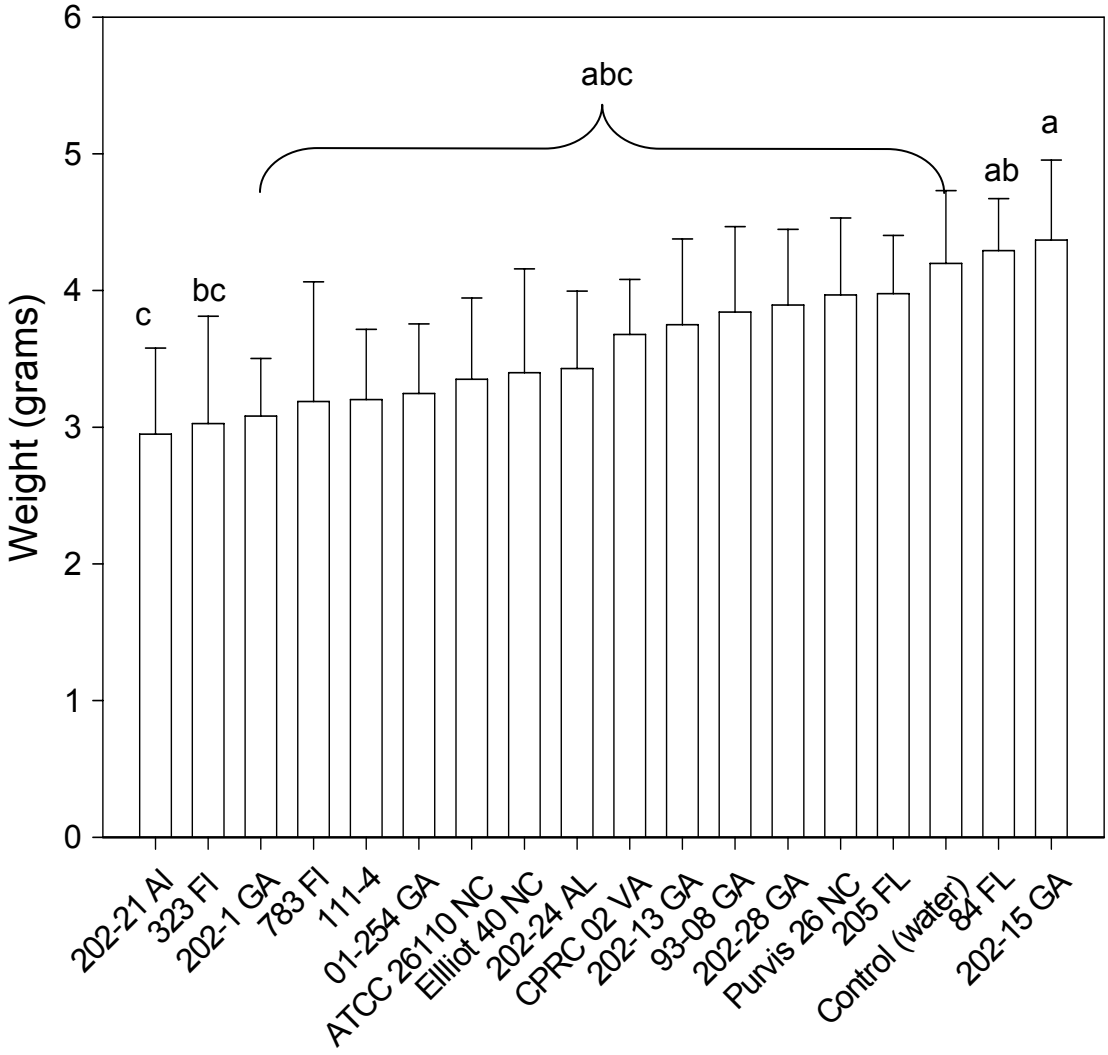

Fig. 5. Mean dry weight of peanut plants cv. Georgia Green 10-14 weeks after inoculation with different *Cylindrocladium parasiticum* isolates. Bars indicate the means of three experiments each comprised of 20 plants per isolate and the lines indicate the standard errors of the means. Isolates with different letters were significantly different according to Tukey's studentized test.

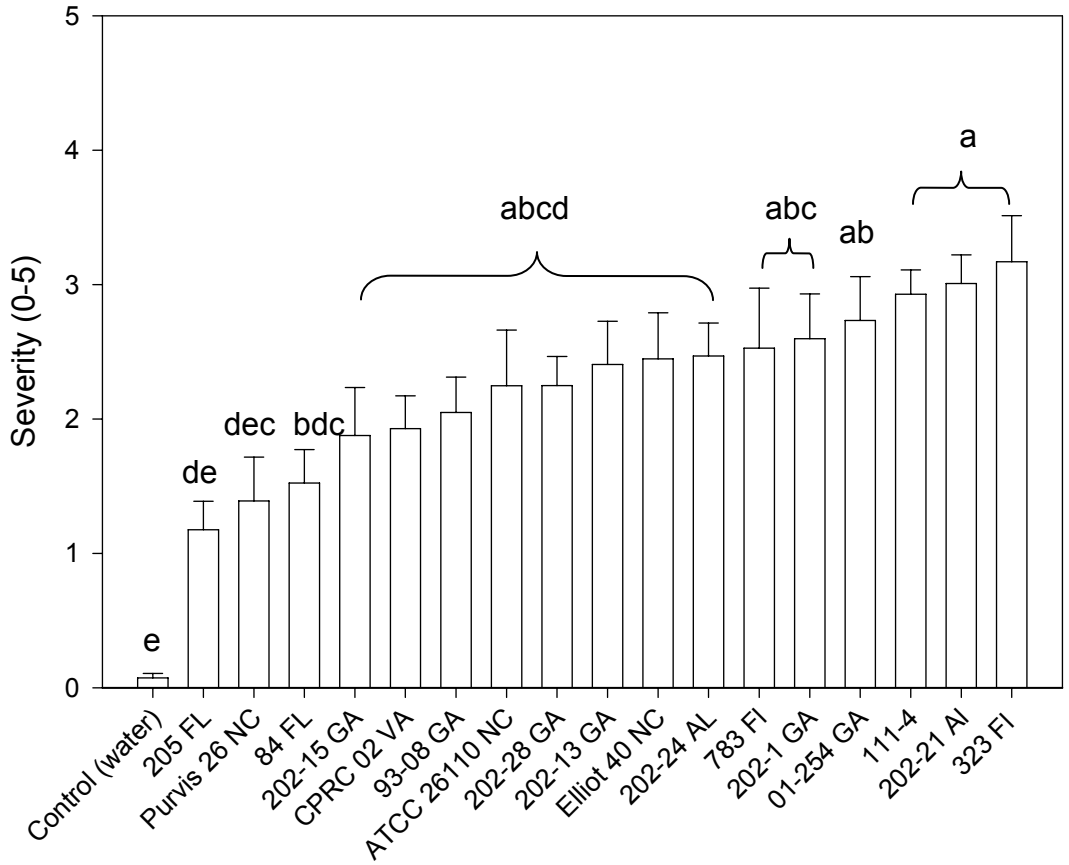

Fig. 6. Mean severity Cylindrocladium black rot observed on peanut plants 10-14 weeks after being inoculate with different *Cylindrocladium parasiticum* isolates. Bars indicate means of 3 experiments each consisting of 20 individual plants. Bars represent the standard error of the means. Bars with different lettering are significantly different according to Tukey's studentized test

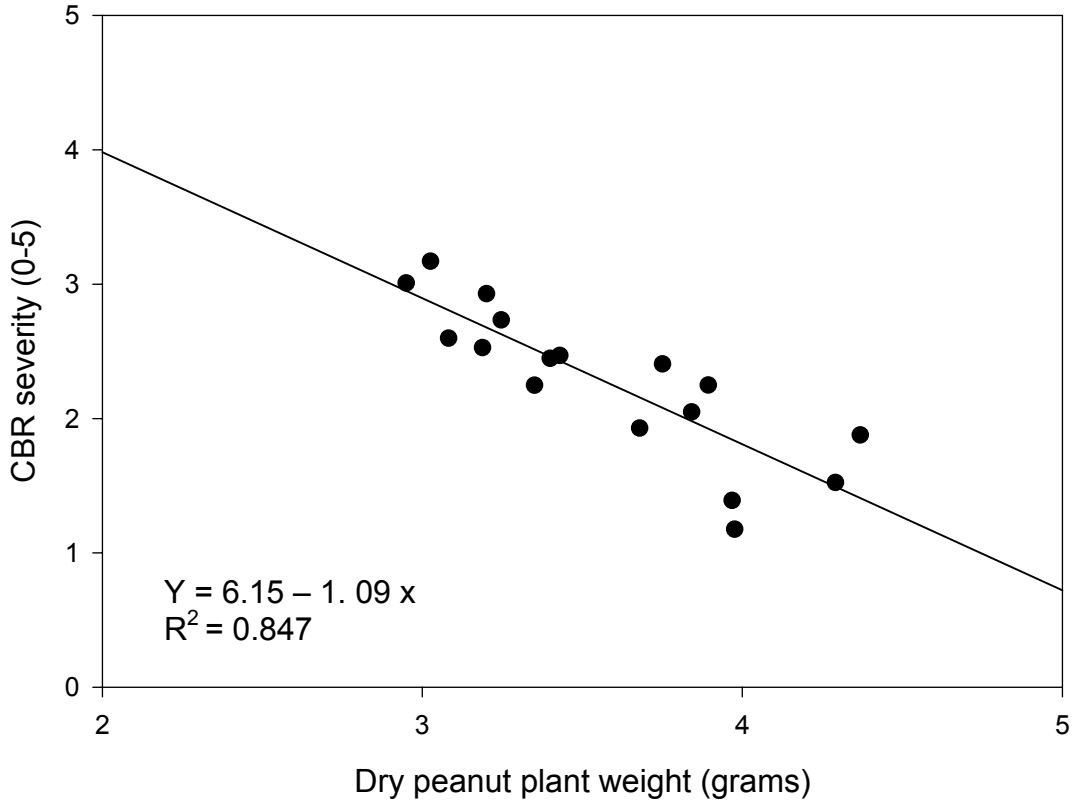

Fig. 7. Relationship between dry peanut plant weight and CBR disease severity with 17 isolates of *C.parasiticum*

CHAPTER 4

CONCLUSIONS

Conclusions

Cylindrocladium black rot (CBR) of peanut (*Arachis hypogaea* L.) was first reported in Georgia in 1965 and it continues to threaten the Georgia peanut industry (1). In 2001 CBR was responsible for over $13.75 million in control and yield losses in Georgia (4). Disease management is often aided by rapid and accurate diagnosis. However, at present, the tools used to diagnose *C. parasiticum* include visual recognition of unique fungal morphological traits. This is time consuming and requires trained experts to ensure accurate diagnosis. Due to recent increases in CBR intensity in Georgia and the 2005 outbreak in Texas, the need to determine the role of seeds in CBR epidemiology is critical (3). Seed transmission of CBR was confirmed in 2001, however the role of seeds as long distance dissemination vectors has not been fully explored (2).

For this study 108 isolates from the major peanut producing areas in GA, AL, FL, VA, and NC were collected. These isolates came from a range of hosts including soybean, peanut, partridge pea, beggar weed and sickle pod. Species-specific PCR primers based on the β-tubulin gene (CBR1X/2X) proved to be highly specific, yielding a 560-bp amplicon with DNA from *C. parasiticum* but none of the other fungi tested. However, 7.8% of the *C. parasiticum* isolates failed to yield the expected amplicon with the CBR1X/2X primer set. Additionally, the sensitivity of the PCR assay was 1 ng/µl of template DNA that may limit its applicability as a detection assay. Nevertheless, CBR1X/2X represent a first attempt to develop a species-specific PCR assay for *C. parasiticum*, and in their present form, they can be used to enhance the efficiency and accuracy of *C.*

parasiticum diagnosis. In the future, through the optimization of PCR run conditions or modification of primer sequences, a more suitable primer set could be developed.

To further investigate the trend of increasing CBR intensity in Georgia, RAPD and microsatellite-primed PCR analysis was used to assess the genetic diversity of 108 *C. parasiticum* isolates in the southeastern U.S. A previous study on a subpopulation of 40 *C. parasiticum* isolates from peanut in Georgia concluded that the population was clonal (5). In the current study, a similar conclusion was drawn based on RAPD analysis of population of 50 *C. parasiticum* isolates. However, based on microsatellite primed-PCR, some polymorphisms were observed, suggesting a limited degree of genetic diversity. Interestingly, clusters established based on MP-PCR analysis did not correlate to geographical origin.

Further evidence of a homogeneous *C. parasiticum* population of in the southeastern U.S. was provided from greenhouse studies to compare the relative aggressiveness of 17 isolates. In general, little differences in the relative aggressiveness of the strains were observed and there was no evidence that the differences in aggressiveness correlated to geographical origin.

Based on data from this study, it is unlikely that recent changes in genetic diversity account for recent trends in CBR intensity in Georgia. It is more likely that the recent trends are due to a gradual accumulation of soilborne inoculum and in weather conditions that favor CBR development. While unexpected, the limited diversity of *C. parasiticum* indicates that resistance should be an effective

and durable strategy for managing CBR in the future. Nevertheless, the threat of introduction of genetic variants of *C. parasiticum* on infested seeds must be considered and addressed. Hence a successful integrated disease management program for CBR should incorporate exclusion of inoculum, resistant cultivars and chemical and cultural practices to reduce soilborne inoculum. Development and implementation of this multi-pronged strategy should limit CBR losses and ensure the future profitability of the Georgia peanut industry.

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