USING MOLECULAR ANALYSIS TO INVESTIGATE PHYLOGENETIC RELATIONSHIPS IN TWO TROPICAL PATHOSYSTEMS: WITCHES' BROOM OF CACAO, CAUSED BY *MONILIOPHTHORA PERNICIOSA*, AND MANGO ANTHRACNOSE, CAUSED BY *COLLETOTRICHUM* SPP.

By

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To David, for the endless love and support

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The increasing availability of DNA sequence data has enabled rapid advances in molecular systematics. This is especially true for the Fungi, where systematics and taxonomy relied previously on largely artificial, morphologically based systems. Many of the advances in fungal systematics have been made with phytopathogens. Characterizing the relationships of lineages among phytopathogenic genera provides insight into not only basic systematic information, but can also address applied questions on host specificity, life strategy and pathogenicity. With DNA sequence-based phylogenetic analyses, two broad topics were investigated: 1) relationships among biotypes of *Moniliophthora perniciosa*, causal agent of witches' broom of cacao, and 2) host and tissue specificity of *Colletotrichum gloeosporioides* sensu lato on mango.

Sequences from the ITS1-5.8S-ITS2 ribosomal DNA region (ITS), intergenic spacer (IGS) region, and RNA polymerase large subunit (RPB1) regions were analyzed for 36 accessions of *M. perniciosa* from all reported biotypes of the pathogen. Maximum parsimony and maximum likelihood analyses resolved three major clades within the species: a clade that contained all isolates from *Theobroma* spp. and most isolates from *Solanum* spp.; a clade that contained most

isolates from malpigheaceous hosts; and a clade that contained three isolates from *S. cernum*, a bignoniaceous liana, and an unknown host. Analysis of morphological characters did not reveal striking differences among the clades. The molecular findings indicate that *M. perniciosa* likely evolved from a saprophytic ancestor and that pathogenicity may have evolved with a switch from a heterothallic to homothallic lifestyle. Host jumps have resulted in distinct lineages within the pathogen.

Sequences from the ITS, mating type (MAT) 2 gene, and a cloned region from a randomly amplified DNA fragment were analyzed to examine 58 accessions of *C. gloeosporioides* sensu lato that represented all anthracnose-affected organs of mango, as well as avocado, banana, carambola and guava. Phylogenies from maximum parsimony and maximum likelihood analyses revealed a mango-specific clade that comprised all blossom blight and leaf anthracnose agents, and some fruit anthracnose agents. Other mango fruit anthracnose and peduncle isolates resolved in two general clades that also contained isolates from other fruit hosts (avocado, guava, carambola, banana). The pathogenicity of representive isolates supported the phylogenetic findings, in that the mango-specific clade isolates cause blossom blight, leaf anthracnose, and fruit anthracnose, and the general clade isolates caused only fruit anthracnose. There were no differences among clades with respect to conidium size and shape, and hyphopodium size. Hyphopodia produced by isolates from the mango-specific clade were clavate and smooth, while isolates from other clades produced irregular, lobed hyphopodia.

CHAPTER 1
INTRODUCTION

Current State of Fungal Systematics

The Kingdom Fungi includes at least 74,000 described species, with estimates of the total number of species from one to nine million (Hawksworth 1991, 2001). Fungi possess diverse life strategies and inhabit diverse habitats (Hawksworth 2001). One of the major groups of fungi is the plant pathogens, which cause the majority of plant diseases and reduce yields of food crops worldwide by an estimated 10% (Oerke and Dehne 2004).

A major challenge is the correct identification and characterization of the species that are responsible for these diseases. Although fungal taxa should be classified on both phylogenetic and biological bases, morphological criteria have played a more significant role historically. In the last 20 years, multilocus DNA sequence-based phylogenies have been used to build a tree of life for the Fungi that would not have been possible with morphological data alone (Lutzoni et al. 2004, James et al. 2006a). The group of scientists involved in the Assembling the Fungal Tree of Life program (AFTOL, http://aftol.org/) produced a kingdom-wide phylogeny based on ribosomal DNA data (Lutzoni et al. 2004), and more recently published the Deep Hypha issue of *Mycologia* (2006, v. 89, issue 6), which contains the most comprehensive analyses to date of all major groups of Fungi. Especially important were the studies of the Chytridiomycota and Zygomycota, which elucidated the relationships of taxa within these phyla and addressed their phylogenetic placement in the Kingdom Fungi (O'Donnell et al. 2001, Tanabe et al. 2004, James et al. 2006a,b, White et al. 2006). With gene sequence data, mycologists can now decipher relationships at every level of fungal systematics, and coordinated projects like AFTOL are paving the way towards a less artificial fungal classification system that is based on phylogenetic relationships.

Classification of Different Phyla of Fungi

Recent molecular kingdom-wide studies supported the recognition of two of the four traditional fungal phyla, the Basidiomycota and Ascomycota (Lutzoni et al. 2004, James et al. 2006a). The Chytridiomycota and Zygomycota are polyphyletic and resolved into several distinct, early-diverging lineages within the Fungi (O'Donnell et al. 2001, Tanabe et al. 2004, White et al. 2006, James et al. 2006a). Two new phyla have been recognized, the Blastocladiomycota (formerly classified as the Blastocladiales in the Chytridiomycota) (James et al. 2006b) and the Glomeromycota (the arbuscular mycorrhizal fungi, formerly in the Zygomycota) (Schüssler et al. 2001). The AFTOL project has helped clarify relationships within each phylum at the subphylal, ordinal, or familial level. The relationship among fungal phyla according to phylogenies presented in the Deep Hypha issue is shown in Figure 1-1.

James et al. (2006b) described a core Chytridiomycota clade containing the majority of previously defined chytrid taxa with the following exceptions: the Blastocladiomycota; two *Rozella* species, which appeared to represent the oldest known lineage in the true Fungi; and *Olpidium,* which was nested in a zygomycete lineage. The paraphyly of the Zygomycota was detailed by White et al. (2006). Nine zygomycete orders formed at least three paraphyletic lineages between the Chytridiomycota and the Glomeromycota+Ascomycota+ Basidiomycota clade. Although the Glomeromycota is hypothesized to be sister to the Ascomycota+Basidiomycota clade, protein coding-data do not always support this hypothesis (Redecker and Raab, 2006).

The sister phyla Ascomycota and Basidiomycota are the most recently evolved lineages in the Fungi (Lutzoni et al. 2004, James et al. 2006a), and have a larger number of recognized taxa and a larger database of DNA sequences than the other phyla. Within the Ascomycota, there are three traditionally recognized subphyla: the Taphrinomycotina, the Saccharomycotina, and the

Pezizomycotina. The relationships among Taphrinomycotina taxa were only partially resolved by Sugiyama et al. (2006), who suggested that additional taxon sampling is needed for further resolution. The Pezizomycotina analysis utilized one of the most comprehensive data sets, which included five loci and 191 taxa (Spatafora et al. 2006). The study supported the monophyly of the Pezizomycotina and the Saccharomycotina, and resolved most of the classes in the Pezizomycotina. As had been suggested in prior molecular phylogenies (Lutzoni et al. 2004), apothecia were indicated as the most ancient ascomal state.

The Basidiomycota are also classified in three subphyla: the Agaricomycotina, Ustilagomycotina, and Pucciniomycotina. Recent phylogenetic analyses (Aime et al. 2006, Hibbett 2006, Matheny et al. 2007) suggest that the Pucciniomycotina are basal to the Ustilaginomycotina and Agaricomycotina. This relationship is supported by the simple septal pore structure and cell wall sugars present in the Pucciniomycotina compared to the latter subphyla (Lutzoni et al. 2004, Prillinger et al. 2002).

Although much progress has been made in resolving deeper nodes in the tree, the "leaves" of the Fungal Tree of Life are still largely nonresolved (relationships below the familial level). This is where plant pathology has contributed most to fungal systematics. Molecular data have resolved relationships for groups of important pathogens, such as several *Fusarium* species complexes (O'Donnell et al. 2000, O'Donnell et al. 2004) and *Ophiostoma* (de Meyer et al. 2008, Roets et al. 2009). The extent to which these and other data may contribute to a species concept for the Fungi is discussed below.

Species Characterization in Fungi

Species defined by morphology

Fungal classification has traditionally been based mainly on morphology. These classifications often did not reflect evolutionary relationships among fungal taxa. There are

significant problems when morphological data are used in phylogenetic analyses of fungi, most importantly the scarcity of homologous characters that can be used across taxa. For example, yeasts in the order Saccharomycetales are quite divergent based on sequence data, despite having almost no morphological differences (Suh et al. 2006).

Morphological classifications above the ordinal level have often conflicted due to the use of different sets of morphological characters and the scarcity of characters that are present among all taxa. Thus, relationships among groups are generally not resolved above the ordinal level, especially in the Ascomycota (Alexopolous et al. 1996). At the genus or species level, morphological characters used for classification often lack consistency. Due to the indeterminate nature of fungal growth and development, characters such as the shapes and sizes of spores, reproductive structures, and production of sterile hyphae often differ with a specimen's age or under different environmental conditions (Ekpo 1978, Sutton 1992, Andrews 1995, Spiers et al. 2000).

An additional challenge for fungal systematics has been the placement of asexual taxa. Many fungi have asexual (anamorph) and sexual (teleomorph) stages with dissimilar morphologies. The respective stages have often been classified separately, leading to an overestimation of species. In contrast, only the anamorph is known for other fungi. In the past, anamorphic fungi formed a separate taxon called Fungi Imperfecti or Deuteromycota, an artificial phylum that had no hypothesized relationship with other fungal groups (Gams 1995).

DNA sequence data have established relationships between asexual and sexual taxa, and have enabled phylogenetic classification of difficult taxa and new understanding of fungal diversity and evolution (Taylor 1995). However, despite these clarifications anamorph and teleomorph species names will probably not be merged in the near future, due to their long

history, opposition to change and the confusion that would likely ensue in mycology and plant pathology should this occur.

Given the scarcity of useful morphological characters in fungi, cryptic speciation, wherein phylogenetically distinct species cannot be distinguished morphologically, is widespread (Hawksworth 2001, Kohn 2005). These are likely lineages that have not been isolated long enough for notable morphological differences to develop (Kohn 2005), or in which a morphological character has evolved convergently (i.e. chlamydospores in *Fusarium* or setose acervuli in *Colletotrichum*; Sutton 1992, O'Donnell et al. 1998b). These lineages are especially common in asexual, clonal species. Although some cryptic species can be recognized as distinct vegetative compatibility groups or mating populations, the widespread availability of DNA sequence data has allowed cryptic species to be more easily detected and characterized (O'Donnell et al. 2000, Hawksworth 2001, Baker et al. 2003, O'Donnell et al. 2004). Molecular phylogenies also suggest that some morphological characters used to describe major lineages in Fungi are homoplasious and need to be revisited (e.g. ascocarp and ascus development in the Pezizomycotina; Spatafora 1995, Lutzoni et al. 2004, Spatafora et al. 2006).

What role can morphology play in fungal systematics? Undoubtedly, many morphological characters represent important evolutionary changes that are still useful in classifying lineages of fungi. Likewise, there is evidence that ultrastructural characters may be a future source of informative data (Celio et al. 2006), and may be able to answer phylogenetic questions that molecular data has left unclear. For example, Lutzoni et al. (2004) suggested a (Pucciniomycotina, (Ustilaginomycotina, Agaricomycotina)) relationship among the basidiomycete subphyla based on septal morphology before molecular phylogenies supported the same relationship (Aime et al. 2006, Hibbett 2006, Matheny et al. 2007). Unfortunately, the

scarcity of these characters ensures that only small data sets are possible and, since most morphological characters are not represented across broad taxonomic groups, they cannot be used to discern relationships among distantly related groups. In addition, morphological data usually cannot be used to accurately predict phylogenetic relationships due to limited understanding of the evolution of these characters.

Molecular data can help reconstruct the evolution of morphological traits and determine whether characters are inherited via common descent or convergent evolution (McLaughlin et al. 1995). Although molecular data will likely continue to be the major future contributor to fungal systematic analysis, morphology cannot be ignored if we are to understand organismal and functional evolutionary changes. By using molecular data as a guide to more complete phylogenies, we can understand the evolution of the diverse forms of the Fungi.

Biological species concept

The biological species concept describes species as "groups of interbreeding populations that are reproductively isolated from other such groups" (Mayr 2000). Reproductive isolation can result from physiological, genetic, ecological, and behavioral factors. In fungi, interfertility is governed by mating-type loci that code proteins for all aspects of mating (Kronstad and Staben 1997). Applying the traditional biological species concept to fungi is problematic due to the complexity of these mating systems and the widespread phenomenon of sexual sterility.

There are two principal mating strategies in fungi, heterothallism and homothallism. In heterothallic fungi, individuals are self-sterile and gamete nuclei must come from different mating types for karyogamy and meiosis to occur (Alexopolous et al. 1996, Kronstad and Staben 1997). Ascomycetes generally exhibit bipolar heterothallism, where mating types are determined by idiomorphs at a single locus (Kronstad and Staben 1997). An example of bipolar heterothallism is exhibited by the yeast *Saccharomyces cereviseae*, where mating type is

determined by the MAT locus that usually has two idiomorphs, a and α. The MAT locus must be heterozygous (i.e. MATa and MATα individuals must fuse) in order for meiosis to take place (Moore 1998). Basidiomycetes generally exhibit a more complex tetrapolar mating system that involves two loci, with many combinations of alleles (Kronstad and Staben 1997). In *Coprinus cinereus*, there are two mating type loci, A and B, which are located on different chromosomes (Moore 1998). Subloci exist at each locus, and many alleles exist for each sublocus. Compatible individuals must differ at both loci, and because there are several alleles for each locus, many compatible combinations exist for successful fusion and meiosis.

To divide groups of morphologically similar fungi into mating groups or biological species, non-identified strains are crossed with fertile tester strains in the laboratory. In the *Giberrella fujikuroi* species complex (GFSC) nine mating populations (A-H) have been identified, each of which contains morphologically similar *Fusarium* anamorphs that exhibit varying levels of host specificity (Leslie 1995, Britz et al. 1999, O'Donnell et al. 1998a). Mating tests have also identified cryptic, biological species in *Neurospora*, where *N. crassa*, *N. intermedia*, *N. sitophila*, *N. discreta* and *N. tetrasperma* have been described (Turner et al. 2001).

Although mating tests can be used to detect biological species, several factors limit their utility. In mating tests, isolates may fail to cross with standard testers because they belong to a non-characterized mating population, have lost the ability to undergo sexual reproduction, or due to poor fertility (Leslie 1991). Thus, highly fertile mating testers must be developed to increase mating probabilities. In addition, it may be difficult to interpret results of *in vitro* mating tests. For example, Guerber et al. (2003) used a seven point scale to rate results from crosses among strains of *Colletotrichum acutatum*, which ranged from the production of no structures to

perithecia that produced viable ascospores. In contrast, Leslie (1995) considered crosses in the GFSC to be fertile when perithecia were formed and exuded asci. For both of these studies, empty perithecia were considered infertile, even though such a result might suggest that occasional outcrossing may occur in nature. Mating tests are also cumbersome and time consuming, and highly dependent on environmental factors (Guerber et al. 2003). Another drawback to the biological species concept is that it cannot be applied to asexual species. Phylogenetic data can help define new biological species by: a) indicating where genetic barriers exist; b) identifying which lineages should be crossed to check for mating compatibility, thereby reducing the numbers of crosses that are needed; and c) specifying in which mating population non-characterized isolates or new lineages may reside.

Phylogenetic species concept

Currently, the phylogenetic species concept predominates in fungal systematics. During the past 20 years it has relied increasingly on molecular data, and transitioned from the use of isozyme protein markers to an array of PCR-based molecular markers. Large sets of multilocus DNA sequence data are now most common and several genome-level analyses have been completed (Hu and Leger 2004, Delsuc et al. 2005, Wolfe 2006, Aguileta et al. 2008, Stuckenbrock et al. 2009). These technological advances have radically strengthened our ability to resolve relationships among evolutionary lineages, and enabled the identification and characterization of numerous cryptic lineages. As expected, close relationships between mating populations (biological species) and phylogenetic species have been demonstrated experimentally (O'Donnell et al. 1998a, Steenkamp et al. 2000). The new challenge is to recover accurate and meaningful phylogenies with DNA sequence data.

Phylogenetic resolution at different taxonomic levels depends on utilizing appropriate regions of DNA. Three are most common in the phylogenetic analysis of fungi: nuclear

ribosomal DNA (currently most common), nuclear protein coding DNA, and mitochondrial (mt) DNA; the utility and attributes of each is discussed below.

Nuclear ribosomal DNAs include the large subunit (26S, LSU), small subunit (18S, SSU), and the 5.8S genes, as well as non-coding regions such as the internal transcribed spacer (ITS1 and ITS2) and the intergenic spacer (IGS) regions (Bridge et al. 2005). The most widely used loci are the ITS1 and ITS2 regions that flank the 5.8 subunit, and the majority of phylogenetic studies published involving fungi include ITS data (Hillis and Dixon 1991, Nilsson et al. 2008). The wide use of ribosomal DNAs in systematic studies in Fungi is due to high copy number that facilitates PCR amplification, the availability of universal primers, and the resolution they provide at different taxonomic levels (Álvarez and Wendel 2003, Bridge 2002). Whereas the ITS and IGS regions are generally less conserved and can be used to resolve species and some subspecific taxa, the SSU and LSU are more conserved and appropriate for studies at the super-generic level. When the ITS is not sufficiently polymorphic to identify cryptic species (de Meyer et al. 2008, Crouch et al. 2009a), regions with stronger signals must be identified to resolve such lineages.

A large variety of the second type of locus, nuclear protein-coding genes, has been used in the past. Some of the most common include the elongation factor 1 alpha (EF-1α), beta-tubulin (β-tubulin), RNA polymerase large subunit (RPB2), and the mating (MAT) loci (O'Donnell et al. 1998, Poggeler 1999, O'Donnell et al. 2000, Reeb et al. 2004, Du et al. 2005, Matheny et al. 2007). The resolution that these loci provide depends on the group that is studied. For example, in *Colletotrichum* (Du et al. 2005), *Fusarium* (O'Donnell et al. 2004), *Ascochyta* (Barve et al. 2003), *Cochliobolus* (Turgeon 1998), and *Neurospora* and *Sordaria* (Poggeler 1999), intraspecific variability was low in the high mobility group (HMG) region of the MAT1-2

mating locus, but interspecific variability was high, compared to the ITS. In *Fusarium*, for which the ITS regions cannot be used, EF-1α is informative (Geiser et al. 2004). And in studies of *Ophiostoma*/*Sporothrix* taxa, the β-tubulin gene has been used (De Meyer et al. 2008, Roets et al. 2009). While these genes provide valuable data for multilocus phylogenies, they are usually not as conserved as the ribosomal subunits. Therefore, specific primers may be needed for the studied taxa. Also, since large data sets, such as those that are available for the ITS, LSU and SSU ribosomal regions, may not exist for many nuclear loci, comparisons of these data with other studies or taxonomic groups may not be possible.

Mitochondrial DNA has also been employed in phylogenetic studies in fungi. It is usually inherited uniparentally and, thus, subject to low levels of recombination (Xu 2005). It also evolves faster than nuclear DNA and may be more useful at lower taxonomic levels than the above loci (Waugh 2007). Nearly all fungal mitochondrial genomes characterized to date contain genes that code for cellular respiration chain subunits, including *cox* and *nad* genes and ATP-synthetase subunits (*atp* genes) (Paquin et al. 1997). These gene sequences are often employed in phylogenetic analysis at the species level, especially the cytochrome *c* oxidase 1 gene (*COX1*) (Waugh 2007, Vialle et al. 2009). Mitochondrial genes have been proposed as alternatives to ITS for barcoding in basidiomycetes (Vialle et al. 2009) and *Penicillium* (Seifert et al. 2007).

Caution should be used when incorporating mtDNA data in fungal phylogenetic studies. The wide range in gene/genome size due to intergenic regions and introns (Gray 1998) that occurs among and within species could make amplification of a gene region among taxa unreliable. For example, in a study of 23 *Chrysomixa* taxa, only 29% of the *COX1* sequences were successfully amplified (Vialle et al. 2009). In addition, high rates of genome rearrangement have been observed in some fungi (Gray 1989), which could complicate alignment of mtDNA

datasets among taxa, especially at higher taxonomic levels. Although it is assumed that mtDNA is inherited uniparentally with a low rate of recombination, biparental inheritance and genome recombination have been reported (May and Taylor 1988, Barroso and Laberére 1997, de la Bastide and Horgen 2003, Xu 2005). Lastly, the preferential inheritance of one mitochondrial genome over another has been demonstrated (Xu et al. 2000, de la Bastide and Horgen 2003). Edwards (2009) discussed the effect of natural selection on phylogeny reconstruction, and concluded that although many types of selection can be addressed by analytic methods and should not affect tree topologies, balancing selection and selection-based convergence of amino-acid substitutions could result in the identification of monophyletic lineages among evolutionarily discrete clades. Since some nuclear loci share this problem, consideration must be given to the poor understanding of the selective forces that act on the loci that are used in phylogenetic analyses and the impact that this may have on the resultant phylogenies. Testing for congruence among trees from several DNA regions can help address this problem.

Since different evolutionary factors affect different classes of DNA, employing data from at least two classes may provide more robust phylogenetic assessments (Taylor et al. 2000, O'Donnell et al. 1998b). Depending on the level of taxonomic resolution that is desired, each DNA region has advantages and disadvantages. For example, highly conserved loci do not resolve closely related species but may be useful at the generic or family level. When such studies are begun, preliminary analyses of several loci in a few accessions are advisable as they can help focus more comprehensive, subsequent work.

Evolutionary species concept

Whereas species that are defined by the morphological, biological and phylogenetic species concepts do not always overlap, and in some cases directly disagree, the evolutionary species concept utilizes these concepts to identify a species as a "single lineage of ancestral

descendant populations of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley 1978). The strength of the evolutionary species concept lies in this combination of morphological, biological and phylogenetic evidence to identify divergent evolutionary lineages.

Taylor et al. (2000) differentiated between the theoretical (the evolutionary species concept) and the operational (morphological, biological, and phylogenetic) nature of these species concepts, and suggested that the operational concepts could be used to fulfill the theoretical. They recommended using a phylogenetic approach to define species that fit Wiley's (1978) evolutionary species concept, and cited several studies with morphological data that underestimated lineage number, presumably because lineage divergence occurred before morphological differences developed. Wiley (1978) recognized the limited ability to recognize evolutionary species that existed at that time, and stated that "real evolutionary lineages exist in nature outside man's ability to perceive these lineages."

The ability to detect cryptic species has improved dramatically since Wiley's (1978) publication, but the challenge now is to identify the levels at which satisfactory and useful lineage resolution occurs. This is where the operational species concepts can play a role. Progress towards defining real evolutionary species would be made with holistic considerations of biological (mating compatibility, ecology, pathology, etc.), phylogenetic and other types of data. In the meantime, broad-based evaluations are preferable to a reliance on a single or limited sets of characters for species delineations.

The Role of Plant Pathology in Fungal Systematics

The correct identification and characterization of lineages within phytopathogenic genera has both practical and academic applications, and has relied increasingly on molecular phylogenetics. Molecular data can be especially useful to identify cryptic species (Kohn 2005,

Couch et al. 2005, de Meyer et al. 2008, Crouch et al. 2009b), which in turn enhances our understanding of the complexity of and interactions among plant pathogenic populations. For example, DNA sequence data can address questions on disease ecology and epidemiology, host specificity, pathogen nutrition and life strategies, and the evolution of pathogenicity.

Since human-derived selection pressure can be substantial in agroecosystems, it is important to be able to predict these effects on pathogen evolution (Burdon and Thrall 2008). To determine these effects, pathogen evolution is assessed over time. The evolution of resistance genes in natural host populations, fungicide sensitivity in agricultural pathogen populations, and host specificity in natural and agricultural settings are some of the processes that can be better understood by tracking with molecular phylogenetic analyses the evolution of genotypes that are associated with these phenotypes.

Understanding host specificity in pathogenic species and populations is necessary to understand pathogen evolution and ultimately devise effective disease management strategies. Many phytopathogenic fungi that have broad host ranges (e.g. *Colletotrichum* spp*., Fusarium* spp*., Rhizoctonia solani*, *Alternaria alternata* and *Botrytis cinerea*) are now known to be comprised of cryptic species that are themselves often host-specific (Salazar et al. 2000, Steenkamp et al. 2002, O'Donnell et al. 2004, Fournier et al. 2005, Peever et al. 2005, Peres et al. 2008, Crouch et al. 2009b). Recognizing and characterizing these lineages can help understand the epidemiology of a disease, measure infection of hosts and nonhosts, and regulate pathogens that may or may not be morphologically distinguishable. National and international quarantines for these pathogens will depend increasingly on rapid and reliable molecular tools for identification.

To investigate host specificity and coevolution in *Magnaporthe oryzae*, Couch et al. (2005) used phylogenetic analysis combined with construction of haplotype networks that used sequence data from 10 loci. They were able to track host shifts in the pathogen to rice and other grassy weeds that were associated with rice cultivation. They combined the molecular data with pathogencity tests to associate host specificity with haplotype lineages.

Another study investigated host specificity in the *Colletotrichum graminicola* species complex. Crouch et al. (2006) used sequence data from the MAT1-2 HMG-box locus, the ITS region, and a superoxide dismutase gene to demonstrate that the closely related species *C. graminicola*, *C. sublineolum*, and two divergent lineages of *C. cereale* all formed well-supported, host specific lineages. Further study of the lineages in *C. cereale* with the same three loci and the MAT1-flanking apurinic DNA-lyase gene enabled the identification of two lineages associated with C_3 and C_4 hosts, respectively, and the resolution of several new host-specific cryptic species (Crouch et al. 2009b).

Phylogenetic analysis can also be used to investigate whether fungal symbionts are latent pathogens or saprotrophs. Promputtha et al. (2007) used a phylogenetic approach to evaluate relationships between endophytic and saprotrophic strains of *Colletotrichum*, *Fusarium*, and *Phomopsis*. Although endophytic strains often fell in the same clade as saprotrophic and pathogenic strains, they did not test the abilities of endophytes to cause disease or colonize senescent tissue. As discussed above, the value of phylogenetic studies can be enhanced considerably with corresponding biological information for a fungus.

The evolution of pathogenicity has been inferred using molecular phylogenetics. Studies involving the origin of several pathogenic *Fusarium oxysporum* forma speciales are the best example of this application. Baayen et al. (2000) constructed phylogenies of several formae

speciales that cause wilt disease and bulb rot (f.sp. *asparagi, dianthi, gladioli, lilii, opuntiarum, spinaciae,* and *tulipae*). They indicated that several of the formae speciales were polyphyletic and each appear to have evolved from at least two different lineages. Similar studies have found multiple evolutionary origins for other formae speciales: two races of f. sp. *lactucae* from lettuce in Arizona appear to have evolved separately (Mbofung et al. 2007); five distinct lineages of the Panama disease pathogen of banana, f. sp. *cubense* exist from different geographic origins (O'Donnell et al. 1998b); and two formae specialis affecting cucurbits, f. sp. *radicis-cucumerinum* and f. sp. *cucumerinum* form separate lineages (Vakalounakis and Fragkiadakis 1999). Recent genomic studies suggest that pathogenicity evolves in *Fusarium* through horizontal gene transfer of pathogenicity factors, particularly on the supernumerary chromosomes of the genome (Coleman et al. 2009, Michielse and Rep 2009).

Another example of the use of phylogenetic analysis to track the evolution of pathogenicity is in *Pyrenophora tritici-repentis*, which causes tan spot of wheat. It has been shown that the fungus gained its pathogenicity through the horizontal gene transfer of a peptide toxin-encoding gene, *ToxA*, from another wheat pathogen *Stagonospora nodorum* (Friesen et al. 2006). This hypothesis was supported through analysis of the *ToxA* gene sequences in isolates of both species, where *Stagonospora nodorum* displayed more diversity in the gene (11 haplotypes) than *P. trici-repentis* (1 haplotype), coupled with the fact that the gene was required in both species for disease development.

Phylogenetic analysis of sequence data can be used to track the geographic spread of pathogens. A four-gene phylogenetic analysis of a global collection of *Heterobasidium annosum* indicated that the pathogen originated in Eurasia (where a total of six lineages were found) and then spread to North America (two lineages) (Linzer et al. 2008). They also identified a likely

human-derived introduction into a region of Italy, based on the resolution of isolates from Italy in the North American clade.

The studies described above illustrate how molecular phylogenetic analysis can be used to answer specific questions about phytopathogenic fungi. As DNA sequencing becomes easier and more affordable, and more loci are developed for studies at the species and intraspecific level, additional applications will be found for these types of data. As we enter the era of phylogenomics, these possibilities grow.

The overall objective of this dissertation research was to use DNA sequence data and phylogenetic analyses to address question of host and tissue specificity on two important tropical plant pathogens. The pathogens chosen cause witches' broom of cacao, *Moniliophthora perniciosa*, and anthracnose of tropical fruit crops, *Colletotrichum* spp. In the first study, phylogenies of *M. perniciosa* were used to test the validity of biotype designations that are currently used for the species. These phylogenies were used to determine if *M. perniciosa* should be considered a single species or a species complex, and to hypothesize on whether different lineages and biotypes might affect other host taxa. The second study focused on the *C. gloeosporioides* as a mango pathogen in south Florida. Phylogenetic and pathological data were used to determine: if a mango-specific population exists; whether this population should be considered a separate species; and if there are tissue-specific lineages within the mango population.

Witches' Broom of Cacao

Cacao, *Theobroma cacao*, is a neotropical crop with two reported centers of diversity (Bartley 2005). The primary center of diversity is in the vast Amazon region, extending in the north from southern Colombia and Venezuela to eastern Peru and Bolivia in the south, and from east of the Andes to northern Brazil in the west. Due to pre-Columbian cultivation of cacao, a

secondary area of diversity exists in the Caribbean region, including Mexico, Central America and the Caribbean islands. The Mayans and Aztecs prized cacao as a foodstuff and currency (Coe and Coe 1996). The first European contact with cacao was by Colombus in 1503, and it quickly gained popularity in Spain and then the rest of Europe (Coe and Coe 1996). Today chocolate is one of the most beloved foodstuffs in the world.

Significant cacao production occurs in tropical Africa, Asia, and America. According to World Cocoa Foundation statistics (2008, http://www.worldcocoafoundation.org/info-center/statistics.asp), the 2006/2007 crop was almost 3.5 million tons of cacao beans. Most cacao was produced in Central and South America before the 20th century, but West Africa has now become the world leader (Gray 2001). West Africa produces about 70% of the world cacao crop, with Côte d'Ivoire being the largest producer by far. South America currently produces just above 10% of the global crop, largely due to losses and management costs that are associated with witches' broom and frosty pod diseases (Gray 2001).

The greatest limiting factor in cacao production is a "trilogy" of important diseases: black pod, witches' broom, and frosty pod (Fulton 1989, Evans 2006). Black pod, caused by *Phytopthora palmivora*, *P. megakarya*, *P. capsici*, and *P. citrophthora* (Appiah et al. 2004), is the most economically damaging disease globally (Taylor 1994, Bowers et al. 2001, Evans 2006). However, witches' broom (caused by *Moniliophthora perniciosa*) and frosty pod (*M. roreri*), have the potential to devastate the world cacao supply if they were to spread outside their current American range. The cacao industry was decimated in Ecuador after the appearance of witches' broom in the early 1900s (Evans 1981b). The potential for catastrophic damage from the disease is evident in Bahia, the largest cacao-producing region of Brazil. After witches' broom appeared in Uruçuca and Camacan in 1989 (Pereira et al. 2006), it spread rapidly

throughout the region and reduced production in Brazil from 378,000 tons in 1990 to less than 120,000 tons in 1999 (Gray 2001). Losses due to frosty pod are similarly dramatic (Evans et al. 1998). The disease spread from Ecuador and Colombia in the early 1900s south into Peru (Evans et al. 1998), and then recently into Central America and southern Mexico (Phillips-Mora et al. 2006a, b).

Moniliophthora roreri and *M. perniciosa* are closely related basidiomycetes in the Marasmiaceae and Agaricales (Aime and Philips-Mora 2005, Matheny et al. 2006). Both fungi: a) are indigenous to South America, b) affect *Theobroma cacao*, c) are hemibiotrophs, d) influence the hormonal balance of the host, e) share a mushroom odor, f) have the dolipore septum characteristic of basidiomycetes, g) produce incrusted red pigmented hyphae, and e) produce similar early symptoms in affected pods. Due to these similarities a close relationship between the two fungi was proposed (Evans et al. 1978, Evans 1981a, Evans et al. 2002), and has been confirmed with molecular data. Based on a five-locus molecular phylogeny, Aime and Phillips-Mora (2005) found that *M. roreri* and *M. perniciosa* were sibling species in the Marasmiaceae, and that *M. perniciosa* was more closely related to *M. roreri* than to other *Crinipellis* species. The pathogen was therefore transferred to the genus *Moniliophthora*.

Classification of *M. roreri* proved difficult, due to its exceptional biology. The fungus was first described as *Monilia roreri* (Ciferri and Parodi 1933), an anamorphic discomycete genus in the Ascomycota. This classification was based on the production of conidia in long chains. However, Evans et al. (1978) discovered that *M. roreri* produced conidia basipetally rather than acropetally, which is a characteristic of *Monilia*, and produced dolipore hyphal septa, a characeteristic of the Basidiomycota. They erected a new genus, *Moniliophthora*, to accommodate the pathogen, and described it as an anamorphic (asexual) basidiomycete. Evans et

al. (2002) reported that the supposed conidia of *M. roreri* were actually dikaryotic probasidia that have undergone the first but not the second meiotic division; thus, *M. roreri* appears to undergo sexual reproduction without forming basidiocarps. These meiospores are produced in white powdery masses on white, fleshy pseudostroma on the surface of affected fruit (Evans 1981a, 2006). Spores are dispersed by wind and rain and infect immature cacao pods, cause swelling and premature ripening of infected areas, and eventually produce abundant inoculum for new infections (up to 44 million spores per square centimeter) (Evans 1981a).

Moniliophthora perniciosa (= *Crinipellis perniciosa*) was originally described as *Marasmius pernicious* by Stahel (1915), and transferred to the genus *Crinipellis* (Singer 1942) due to the presence of long thick-walled pileal hairs. The name of the disease it causes, witches' broom, refers to the characteristic proliferation of swollen and twisted shoots that develop on cacao, which were first described by Went (1904) in Surinam. Cacao pods and flower cushions (woody meristematic tissue above leaf scars; de Almeida and Valle 2007) may also be affected, resulting in large yield losses. Only basidiospores, which are produced on basidiocarps on necrotic host tissue, are capable of infection.

Much progress has been made in understanding the biology of *M. perniciosa* and its interaction with the host. It is a hemibiotroph (Pegus 1972), with a biotrophic phase that consists of primary, monokaryotic mycelia that infect and colonize host tissue. As the host tissue necroses, the fungus switches to a saprotroph that produces secondary, dikaryotic mycelia. These changes have been observed in histological studies that show swollen, thick-walled, monokaryotic hyphae growing intercellularly in infected host tissue until brooms necrose; thereafter, the fungus switches to a dikaryotic, fine mycelium that colonizes the necrotic tissue (Evans 1980, Ceita et al. 2007).

It is assumed that *M. perniciosa* uses endogenous or host hormone production to induce broom formation. The formation of brooms is the result of cell enlargement rather than cell proliferation (Orchard et al. 1994) leading to less organized tissue. Indoleacetic acid (IAA) and salicylic acid (SA) levels are elevated in infected leaf tissue (Kilaru 2007), however, there is little evidence of the involvement of cytokinins (Orchard et al. 1994).

Infected cacao tissue undergoes programmed cell death as it necroses, which is accompanied by DNA degradation, calcium oxalate production, and increased concentrations of ascorbic acid and hydrogen peroxide (Ceita et al. 2007). *Moniliophthora perniciosa* resists oxidative stress and utilizes the breakdown products of host cells as nutrients as it transitions to its saprotrophic form (Ceita et al. 2007, Santos et al. 2008).

Recent work has also shed light on mechanisms of pathogenicity. Multiple biotypes of *M. perniciosa* produce degradative enzymes, including cellulase, protease, lipase, amylase, esterase, and peroxidase (Bastos 2005). Several necrosis and ethylene-inducing proteins (MpNEP1, MpNEP2, MpNEP3) have been identified in the fungus, which may play a role in disease development (Garcia et al. 2007), as well as cerato-platanin protein (MpCP1), which is similar to that produced by *Ceratocystis platani* (Zaparoli et al. 2009). The synergistic effect of MpNep2 and MpCP1 produced symptoms similar those caused naturally by *M. perniciosa* (Zaparoli et al. 2009).

Witches' broom is difficult to manage. Several fungicides reduced the incidence of pod rot, but were not able to protect vegetative shoots (Laker and Ram 1992). In addition, the high cost of frequent applications that are needed to protect growing pods has reduced the utility of fungicide application (Laker and Ram 1992). Pod removal decreases disease incidence (Soberanis et al. 1999), but is also labor intensive. Alternative measures are key to the future of

cacao production in the Americas. In this regard, biocontrol and disease resistance have received much recent attention.

Potential biological control agents utilize antibiosis, parasitism and competition, and include *Cladobotryum amazonense* (Bastos et al. 1981), *Streptomyces* (Macagnan et al. 2006, Macagnan et al. 2008), several *Trichoderma* spp. (Krauss and Soberanis 2002, Sanogo et al. 2002, Aneja et al. 2005, Bailey et al. 2006, Bailey et al. 2008), and even *Colletotrichum gloeosporioides* and *C. rosea* (Mejía et al. 2008). Despite the amount of work that has been devoted to this area (Coe et al. 2006, Bastos et al. 1981, Aneja et al. 2005, de Marco and Felix 2007, Bailey et al. 2008, Macagnan et al. 2008, Mejia et al. 2008), limited success has been observed in field trials (Aneja et al. 2005, Mejía et al. 2008).

The best hope for cacao in South and Central America lies with the development of cacao clones with durable resistance to witches' broom, frosty pod, and black pod. Breeding programs exist in many producing regions, and often focus on resistance to the most important local disease(s).

The first step in breeding resistance to witches' broom is to identify potential resistant parents in the wild and in collections. Bartley (2005) gives a detailed description of known *T. cacao* diversity worldwide, including the historical provenance of major groups of cacao clones. The International Cocoa Germplasm Database (ICGD, http://www.icgd.rdg.ac.uk/), based at the University of Reading, UK, is available online and gives detailed descriptions of most known clones, including compiled photographs, physiological characteristics, SSR profiles, and available disease resistance data. Several independent studies have been undertaken in Brazil to characterize genetic diversity of cacao collections with a focus on witches' broom resistance (Marita et al. 2001, Paim et al. 2006). Through breeding efforts, several progeny display useful

tolerance to witches' broom. Several resistance QTLs have been identified, with the ultimate goal of pyramiding resistance loci to produce clones with durable resistance (Queiroz et al. 2003, Brown et al. 2005, Faleiro et al. 2006, Paim et al. 2006, Santos et al. 2007).

The diversity of *M. perniciosa* has been studied, and molecular tools are yielding further insight into the origins and evolution of the pathogen. Basidiocarp variation was first described by Pegler (1978), who identified three varieties: a) var. *perniciosa*, with a crimson pileus in the center and fading to white at the margins, identified in Trinidad and Surinam; b) var. *ecuadorensis*, with a uniformly red pileus, identified in Ecuador; and c) var. *citriniceps* , with a yellow pileus and identified from a single broom in Ecuador. This varietal classification has given way to a host-based system of biotypes. Five biotypes have been described: a) C-biotype, which affects *Theobroma* spp. (including cacao) and *Herrania* spp. (Evans 1981b); b) S-biotype, which affects *Solanum* spp. (*Solanum lasiantherum*, *S. rugosum, S. cernum, S. lycocarpum* and *S. paniculatum*) (Bastos and Evans 1985, Pereira et al. 1997, Rincones et al. 2006); c) B-biotype, which affects *Bixa orelleana* (Bastos and Anderbrhan 1986); d) L-biotype, which includes presumed saprotrophs found on lianas in the Bignoniaceae and Malpighiaceae (Evans 1978, Pegler 1978, Griffith and Hedger 1994b); and e) H-biotype from *Heteropterys acutifolia* (Resende et al. 2000). Mating studies have suggested that the C- and S-biotypes are homothallic, but the L-biotype is heterothallic (Griffith and Hedger 1994a,b).

Molecular data have been used to investigate relationships between biotypes. RAPD and RFLP (restriction fragment length polymorphism) fingerprints support the hypothesis that the S, C, H, and L-biotypes represent distinct populations (Anderbrhan and Furtek 1994, de Arruda et al. 2003a,b, Rincones et al. 2006). However, there have been only two phylogenetic analyses for *M. perniciosa*. One, which included an analysis of the IGS region of 14 isolates in the C, S, and

H-biotypes, indicated that the biotypes formed well-supported clades (de Arruda et al. 2003a). Another analysis of the ITS region in a similar range of isolates, found that an H-biotype isolate resolved sister to a clade containing isolates from *Theobroma, Herrania* and *Solanum* (de Arruda et al. 2005). A multilocus phylogenetic analysis, which would facilitate a more complete picture of lineages within *M. perniciosa*, has not been conducted.

Colletotrichum-induced Anthracnoses: Plagues to Tropical Fruit

Taxonomic History of *Colletotrichum*

Corda erected the genus *Colletotrichum* in 1837 with the type species *C. lineola* (Corda 1831). Many genera have been placed in synonymy with *Colletotrichum*, including *Gloeosporium*. The two genera were previously distinguished by the presence of setae in the acervulus of *Colletotrichum*, but not *Gloeosporium*. Before the 1950s, species of *Colletotrichum* were erected based on host substrate, leading to a proliferation of species in the genus with little biological or phylogenetic basis. In his revision of the genus in 1957, von Arx reduced more than 750 species of *Colletotrichum, Glomerella, Gloeosporium,* and 40 other genera[1] to only 19 species of *Colletotrichum* (Table 1-1). This classification was based solely on morphology, and von Arx used type specimens (approx. 25% of the taxa that were reviewed), pure cultures, and descriptions to group taxa into species. Morphological characters that were used to distinguish species were conidium shape and size, the presence of sclerotia in culture, chlamydospore development, host specificity, and acervulus size. Most notable about this classification are *G. cingulata*/*C. gloeosporioides*, which von Arx synonymized with over 600 previously described species. He also distinguished several forms of *G. cingulata* with anamorphs that differ from *C.*

[1] *Amerosporium, Ascochyta, Chaetostroma, Colletostroma, Colletotrichopsis, Cryptosporium, Dicladium, Discella, Discula, Elisiella, Excipula, Exosporium, Fellneria, Fusarium, Fusisporium, Fusoma, Gloeosporidium, Gloeosporopsis, Hainesia, Hypodermium, Macrophoma, Monostichella, Myxosporium, Phellomyces, Phoma, Phyllosticta, Physalospora, Piggotia, Psilonia, Rhizoctonia, Sclerotium, Septogloeum, Septoria, Sphaeria, Sphaeropsis, Steirochaete, Trullula, Tubercularia, Vermicularia, Volutella*

gloeosporioides, including *C. lindemuthianum* from *Phaseolus* (bean), *C. orbiculare* from Cucurbitaceae (cucurbits), *C. musae* from *Musa* (banana), *C. malvarum* from *Gossypium* (cotton), *C. gnaphalii* from *Gnaphalium*, *C. psoraleae* from *Psoralea*, *C. helichrysi* from *Helichrysum*, *C. trifolii* from *Medicago* and *Trifolium* (leguminous forages), and *C. miyabeana* from *Salix*.

The next revision of *Colletotrichum* was by Sutton (1980), who described 22 species based on conidium shape and size, presence of sclerotia, appressorium shape and size, and host specificity (Table 1-2). *Colletotrichum acutatum* and *C. coccodes* were included as newly described species, and several species that were synonymized by von Arx were distinguished: *C. higginsianum* and '*C. coffeanum*' from *C. gloeosporioides*; *C. caudatum*, *C. falcatum*, and *C. sublineolum* from *C. graminicola*; and *C. capsici* and *C. truncatum* from *C. dematium*. A standardized description was not given for *C. gloeosporioides*, as Sutton considered such a description meaningless considering the species' wide variation. Due to intrataxon variation, *C. gloeosporioides, C. dematium, C. capsici,* and *Colletotrichum* spp. on graminaceous hosts were each considered species complexes.

Sutton (1992) provided a subsequent description of 33 widely accepted *Colletotrichum* species (Table 1-2). Several of the additional taxa were described by von Arx (1957) (*C. destructivum*, *C. fusarioides*, *C. gnaphalii*, *C. helichrysi*, *C. malvarum*, and *C. paludosum*), whereas others were new (*C. curvatum*, *C. fragariae*, *C. nigrum*, *C. nymphaeae*, *C. spinaciae*, and *C. truncatum*). Since Sutton's (1992) list was published, several new *Colletotrichum* species have been described[2].

[2] *Colletotrichum agaves*: **D.F. Farr & M.E. Palm**, in Farr, Aime, Rossman & Palm, *Mycol. Res.* **110**(12): 1401 (2006)

Currently there are 674 entries of *Colletotrichum* taxa in the Index Fungorum (http://www.indexfungorum.org/). *Colletotrichum boninense*, a species with straight conidia that was described by Moriwaki et al. (2003), is differentiated from *C. gloeosporioides* by having wider conidia, and by its phylogenetic placement outside the large *C. gloeosporioides* sensu lato clade. Since *C. boninense* was erected, it has been associated worldwide with hosts in *Passiflora*, *Dendrobium*, *Cattleya*, *Cucumis*, *Persea* and the Agavaceae and Proteaceae (Moriwaki et al.

Colletotrichum araliae: **Kamal & R.P. Singh**, *Indian Phytopath.* **33**(4): 594 (1981)

Colletotrichum boninense: *Mycoscience* **44**(1): 48 (2003)

Colletotrichum latium: Wang & Li, *Acta Mycol. Sin.* **6**(4): 212 (1987)

Colletotrichum lilii: **Boerema & Hamers**, *Neth. Jl Pl. Path.* **94**(suppl. 1): 12 (1988)

Colletotrichum kahawae: **J.M. Waller & Bridge** *Mycol. Res.* **97**(8): 993 (1993)

Colletotrichum lupini: **Nirenberg, Feiler & Hagedorn**, *Mycologia* **94**(2): 309 (2002)

Colletotrichum dracaenophilum: **D.F. Farr & M.E. Palm**, in Farr, Aime, Rossman & Palm, *Mycol. Res.* **110**(12): 1401 (2006)

Colletotrichum nupharicola: **D.A. Johnson, Carris & J.D. Rogers**, *Mycol. Res.* **101**(6): 647 (1997)

Colletotrichum sinuatisetiferum: **Matsush.** [as '*sinuatosetiferum*'], *Matsush. Mycol. Mem.* **9**: 6 (1996)

Colletotrichum phormii: **D.F. Farr & Rossman**, in Farr, Aime, Rossman & Palm, *Mycol. Res.* **110**(12): 1403 (2006)

Colletotrichum sansevieriae: **Nakamura, Ohzono, Iwai & Arai,** (2006) Index of Fungi 7: 929

Colletotrichum solidaginis: Chevassut & Pellicier, *Bull. Soc. mycol. Fr.* **117**(3): 197 (2002) [2001]

Colletotrichum neriicola: *Lidia* **5**(5): 149 (2001)

Colletotrichum suttonii: *Journal of the Hebei Academy of Sciences*(1): 66 (1992)

Colletotrichum taiwanense: *Mycol. Res.* **97**(12): 1525 (1993)

Colletotrichum xanthorrhoeae: *Mycol. Res.* **102**(3): 280 (1998)

Colletotrichum yunnanense: **Liu, Xie & Duan**, *Mycotaxon* **100**: 139 (2007)

2003, Lubbe et al. 2004, Farr et al. 2006, Avila-Quezada et al. 2007). Based on its morphology and phylogenetic placement outside *C. gloeosporioides*, Farr et al. (2006) re-established *C. agaves* for setose *Colletotrichum* taxa found on *Agave* and *Furcraea* (the species had been included in *C. gloeosporioides* by von Arx). Farr et al. (2006) also erected two new species to accommodate host specific taxa on the Agavaceae: *C. dracaenophilum* (on *Dracaena*) and *C. phormii* (on *Phormium*). Studies such as these that combine morphological examinations of new isolates and herbarium materials with robust multi-locus phylogenetic analyses will be key to meaningful classifications in the genus.

Sutton (1980) considered *C. gloeosporioides* to be a species complex, and wrote later that "no progress in the systematics and identification of isolates belonging to this complex is likely to be made based on morphology alone" (Sutton 1992). Wide ranges in *C. gloeosporioides* have been reported for colony color and growth, conidium dimensions, appressorium and hyphopodium shape and size, and host range. For example, Sutton (1980) reported that hyphopodia can be clavate, ovate to lobed, and that conidia can measure 6-20 x 4-12 µm, a far greater range than is recognized for other species, such as *C. acutatum* (conidia 8.5-16 x 2.5-4 µm) or *C. capsici* (conidia 18-23 x 3.5-4 µm).

The *C. gloeosporioides*/*G. cingulata* complex contains several species that have not been distinguished with molecular phylogenies: *C. musae*, cause of banana anthracnose; *C. kahawae*, cause of coffee berry disease; and *C. fragariae*, cause of crown rot of strawberry. Unfortunately, there are few informative sites in DNA regions that have been used to date that differentiate these taxa, and the relationships among the three species and their relationship to *C. gloeosporioides* sensu stricto is not clear (Sreenivasaprasad et al. 1993, Sreenivasaprasad et al. 1996, Munaut et al. 2002, Martinez-Culebras et al. 2003, Du et al. 2005).

As phylogenetic analyses with DNA sequence data become commonplace, the systematics of *C. gloeosporioides* and other species in this genus will recognize true evolutionary relationships in these fungi. Understanding the different diseases that are caused by these pathogens is not possible without recognizing the different causal agents. Recent work has shown that *C. gloeosporioides* is comprised of distinct genetic clades that often display different host specificities (Abang et al. 2002, 2005, Munaut et al. 2002, MacKenzie et al. 2007). A systematic review of the *C. gloeosporioides* species complex that uses both molecular and biological data would help clarify the evolutionary history of this important plant pathogen and enable more focused work on the epidemiology and management of the corresponding diseases.

Genetic diversity studies on *C. gloeosporioides* include comparisons of electrophoretic patterns of molecular markers (mostly random amplification of polymorphic DNAs, RAPDs), occasionally accompanied by construction of dendrograms based on UPGMA (unweighted pair group method with arithmetic mean) analysis. These studies include the characterization of: a) two populations from yam that differ in pathogenicity (Abang et al. 2002); b) populations on different *Stylosanthes* species in Mexico, Brazil and Colombia (Munaut et al. 2002, Weeds et al. 2003); c) distinct mango and avocado isolates in Australia (Giblin 2005); and d) *C. gloeosporioides* and *C. fragariae* on strawberry (Martinez-Culebras et al. 2002, Ureña-Padilla et al. 2002, Xiao et al. 2004, MacKenzie et al. 2007). Use of sequence data is rare, and phylogenetic analysis to determine taxonomic relationships among lineages in the species complex is lacking.

Colletotrichum gloeosporioides as the Cause of Mango Anthracnose

Almost every fruit and vegetable crop is affected by postharvest anthracnose caused by *Colletotrichum* spp. *Colletotrichum gloeosporioides* is most often associated with tropical hosts, and causes severe economic damage on a number of important fruit crops worldwide (Freeman

et al. 1998). On mango, the pathogen causes blossom blight, leaf anthracnose and fruit anthracnose. Three taxa have been associated with these diseases: *Colletotrichum gloeosporioides* (worldwide), *C. gloeosporioides* var. *minor* (Australia, Simmonds 1965), and *C. acutatum* (Australia, Fitzell 1979; Taiwan, Weng and Chuang 1995; and Homestead, Florida, Riveras-Vargas et al. 2006).

Several groups have studied the influence of relative humidity and temperature on conidium germination and appressorium formation (Dodd et al. 1991b, Estrada et al. 2000, Dinh et al. 2003). According to Dinh et al. (2003), conidia germinate 48 hr after inoculation onto mango peels, and appressoria form 30-96 hr after germination. High relative humidity of 95-100% is necessary for germination, and appressorium formation peaks at 25ºC (Dodd et al. 1991b, Estrada et al. 2000). Reservoirs of inoculum exist on fallen leaves and fruit, as well as necrotic peduncles that failed to set fruit in the previous season (Arauz 2000). Affected leaf flushes also serve as important sources of inoculum (Fitzell and Peak 1984, Dodd et al. 1991b). Newly emerged inflorescences and leaves are susceptible, resulting in blossom blight or leaf anthracnose. Conidia are most prevalent during rainy periods, and rain events are important for disease development (Fitzell and Peak 1984). The role of the *G. cingulata* teleomorph in the epidemiology of the disease is not clear. Fitzell and Peak (1984) were able to produce ascospores in culture, but they were never found in the field.

Although more than one cycle of infection can occur on leaves and blossoms, fruit anthracnose is monocyclic (Arauz 2000). Infections remain latent on fruit until they begin to ripen. Most work studying latent infection on fruit by *C. gloeosporioides* has been done on avocado (Prusky and Lichter 2007, 2008). After fruit set, conidia on the fruit surface germinate and form appressoria that penetrate the fruit cuticle and form a biotrophic latent infection.

Colonization of the fruit is inhibited by host defenses (such as the production of reactive oxygen species, and the presence of an antifungal diene) until fruit maturation (Beno-Moualem and Prusky 2000, Guetsky et al. 2005), when hydrolytic enzymes secreted by the fungus enable tissue maceration and saprotrophic colonization (Prusky and Lichter 2007, 2008). Guetsky et al. (2005) showed that laccase produced by *C. gloeosporioides* breaks down epicatechin, which enables the break down of the antifungal diene, activating the latent infection. Pectate lyase secreted by *C. gloeosporioides* and other species has been shown to be an important factor for host penetration by and pathogenicity on avocado (Yakoby et al. 2000a, 2001). The regulation of synthesis and secretion of this pectate lyase is dependent on alkalization of the host tissue by the pathogen (Yakoby et al. 2000b), and the fungus secretes ammonia to enable this pH change (Prusky et al. 2001, Kramer-Haimovich et al. 2006). The presence of sugars, such as fructose, glucose and sucrose, also play a role in enzyme secretion (Miyara et al. 2008).

Droby et al. (1986, 1987) have investigated latent infections of *Alternaria alternata* on mango and found that, like the *C. gloeosporioides*-avocado pathosystem, mango peels contain antifungal compounds (resorcinols) that limit colonization of the pathogen and symptom development until fruit maturation. To date, fifteen resorcinols have been identified in mango peels (Knödler et al. 2007), and a direct correlation has been demonstrated between the presence of resorcinols and anthracnose lesion size on mango fruits (Hassan et al. 2007).

Due to the latent infection exhibited by the pathogen, it has been suggested that *C. gloeosporioides* coevolved as a climacteric fruit pathogen (Flaishman and Kolattukudy 1994, Arauz 2000). The latent infection exhibited by the pathogen works to the advantage of both host and pathogen. By not causing disease until the fruit is mature, anthracnose allows the host to

reproduce and then facilitates rapid seed germination by decomposing the fruit flesh (Arauz 2000).

Anthracnose management in humid growing regions consists mainly of pre and postharvest fungicide treatment, and is especially necessary when fruit develops in humid, wet conditions (Arauz 2000). Frequent applications continue from shortly before bloom until shortly before harvest. A number of chemistries have shown varying levels of efficacy, including thiophanate-methyl, benomyl, copper, prochloraz, sulfur, ferbam, and azoxystrobin (McMillan 1984, Dodd et al. 1997, Ploetz and Prakash 1997, Mossler and Nesheim 2002, Sundravadana et al. 2006). The classes at highest risk for resistance are the benzimidazoles and thiophanates (benomyl and thiophanate-methyl) (Sanders et al. 2000), whereas inorganic compounds such as copper and sulfur carry little risk of resistance (Anonymous 2009). The use of chemistries such as prochloraz and thiabendazole has not been shown to lead to the development of resistance in *C. gloeosporioides* (Kuo 2001, Sanders et al. 2000), even though resistance has developed in other fungi (Dyer et al. 2000, Smilanick et al. 2003). When there is a risk of resistance developing in a given fungicide, its use should be: a) alternated with products with different modes of action; b) limited; and c) according to the manufacturer label (do not use reduced rates); recommendations for specific fungicides are reported by Brent and Hollomon (2007).

The use of weather-based models that predicted the likelihood of disease development reduced the numbers of sprays that were needed for effective anthracnose management in the Philippines (Dodd et al. 1991b, Estrada et al. 1996). And postharvest treatments are as important as field treatments to manage anthracnose on fruit. Immersion in hot water, especially when accompanied by fungicides, is very effective in lowering anthracnose on fruit (Muirhead 1976,

Thompson 1987, Dodd et al. 1991a, McGuire and Campbell 1993). However, no fungicides are currently labeled for postharvest application in the United States (Prusky et al. 2009).

Several studies have attempted to identify and develop biocontrol agents for anthracnose. Koomen and Jeffries (1993) identified 121 microorganisms that were antagonistic to *C. gloeosporioides*, and showed that a single strain of *Pseudomonas fluorescens* decreased anthracnose levels on fruit in the Philippines. Vivekananthan et al. (2004) found strains of *Bacillus subtilis*, *P. fluorescens,* and *Saccharomyces cerevisiae* that decreased anthracnose incidence on fruit when applied with a chitin formulation. Most recently, a formulated product containing *Rhodoturula minuta* was as effective as benomyl in decreasing anthracnose in 'Haden' and 'Kent' in the field (Patiño-Vera et al. 2005). In addition, Bugante and Lizada (1996) found that bagging immature fruit with brown paper bags decreased disease levels by 50%.

No complete resistance has been identified in mango, although partial resistance has been noted for some cultivars. Resistance has been quantified for several cultivars. Knight (1993) used a 1-9 scale, with 1 being the most susceptible, and 9 the most resistant. He reported that the cultivars 'Tommy Atkins', 'Keitt', 'Kensington', and 'Van Dyke' were the most resistant (ratings of 7 or higher), and cultivars 'Pope' and 'Alfonso' were the most susceptible (ratings of 2-3). Crane et al. (2003) described characteristics of 28 cultivars and gave them one of four resistance ratings: moderately resistant, moderately susceptible, susceptible, and very susceptible. Ten cultivars were moderately resistant, including 'Edward', 'Florigon', 'Keitt', 'Tommy Atkins', and 'Van Dyke', whereas 'Irwin' and 'Kent' were very susceptible. Dinh et al. (2003) investigated anthracnose resistance in Thai mango cultivars and found that 'Rad', 'Kaew', and 'Chok Anan' were more resistant than 'Nom Doc Mai' and 'Nan Klang Wang'.

Mango-specific populations of *C. gloeosporioides* have been identified in several studies. With RFLPs and isolates from four tropical fruit species, Hodson et al. (1993) showed that only isolates from mango were genetically similar. Other RAPD and RFLP studies corroborated the presence of a distinct mango population in worldwide collections from tropical fruits (Alakahoon et al. 1994, Hayden et al. 1994). Cross inoculation studies have shown that isolates of *C. gloeosporioides* usually cause symptoms on all fruit, regardless of the original host, but that lesion diameter is generally greater on the original host (Alakahoon et al. 1995, Sanders and Korsten 2003). Additional work is warranted to qualify and quantify the phylogeny and biology of *C. gloeosporioides* populations from mango and other hosts. Contemporary investigations of the tissue-specificity of mango populations are also needed. Although pectic zymograms (Gantotti and Davis 1993) and RAPDs (Davis 1999) demonstrated some correlation between isolate and host organ (leaf, inflorescence and fruit), modern data are needed to clarify the existence of these biotypes.

Table 1-1. von Arx classification of species of *Colletotrichum* (1957), including anamorph, teleomorph (when described), numbers of synonyms von Arx placed in each taxon, and host plant(s).

Species (anamorph)	Species (teliomorph)	# synonyms	Host
C. gloeosporioides	*G. cingulata*	600	Numerous
C. lindemuthianum	*G. cingulata*	3	*Phaseolus vulgaris*
C. orbiculare	*G. cingulata*	12	Cucurbits
C. musae	*G. cingulata*	2	*Musa*
C. malvarum	*G. cingulata*	5	Malvaceae
C. gnaphalii	*G. cingulata*	1	*Gnaphalium spicatum*
C. helichrysi	*G. cingulata*	1	*Helichrysum petiolatum*
C. trifolii	*G. cingulata*	1	*Medicago* and *Trifolium* (legume forage)
C. miyabeana	*G. cingulata*	1	*Salix*
C. crassipes	---	18	Numerous
C. lini	---	4	*Linum*
C. destructivum	---	2	Legumes
C. fuscum	---	3	*Digitalis*
C. fusarioides	---	3	*Asceplias*
C. phyllachoroides	---	2	*Artemisia*
C. paludosum	---	1	*Peltandra virginia*
C. atramentarium	---	15	Numerous
C. graminicola	*G. tucumanensis*	41	Graminaceae
C. dematium	---	89	Numerous

Table 1-2. Major revisions of *Colletotrichum* by von Arx (1957), Sutton (1980), Sutton (1992), and others.

von Arx (1957)		Sutton (1980)		Current[a]	
Anamorph	Teleomorph[b]	Anamorph	Teleomorph	Anamorph	Teleomorph
		C. acutatum	---	*C. acutatum*	*G. acutata*[c]
C. atramentarium	---				
		C. coccodes	---	*C. coccodes*	---
		C. corchori	---	*C. corchori*	---
C. crassipes	---	*C. crassipes*	---	*C. crassipes*	---
C. dematium	---	*C. dematium*	---	*C. dematium*	---
		C. capsici	---	*C. capsici*	---
		C. circinans	---	*C. circinans*	---
		C. curvatum	---	*C. curvatum*	---
				C. spinaciae	---
		C. trichellum	---	*C. trichellum*	---
		C. truncatum	---	*C. truncatum*	---
C. destructivum	---			*C. destructivum*	*G. glycines*
				C. dracaenophilum[d]	---
C. fusarioides	---			*C. fusarioides*	---
C. fuscum	---	*C. fuscum*	---	*C. fuscum*	---
C. gloeosporioides	*G. cingulata*	*C. gloeosporioides*	*G. cingulata*	*C. gloeosporioides*	*G. cingulata*
C. musae	*G. cingulata*	*C. higginsianum*		*C. agaves*[d]	---
		C. musae		*C. boninense*[e]	---
				C. fragariae	---
			---	*C. higginsianum*	---
				C. kahawae[f]	---
				C. musae	*G. musarum*
				C. nigrum	---
				C. nymphaeae	---
C. gnaphalii	*G. cingulata*			*C. gnaphalii*	---
C. graminicola	*G. tucumanensis*	*C. graminicola*	---	*C. graminicola*	*G. graminicola*
		C. caudatum	---	*C. caudatum*	---
		C. falcatum	*G. tucumanensis*	*C. falcatum*	*G. tucumanensis*

Table 1-2. Continued.

Anamorph	Teleomorph	Anamorph	Teleomorph	Anamorph	Teleomorph
		C. sublineolum	---	*C. sublineolum*	---
C. helichrysi	*G. cingulata*			*C. helichrysi*	---
C. lindemuthianum	*G. cingulata*	*C. lindemuthianum*	---	*C. lindemuthianum*	*G. lindemuthiana*
C. lini	---	*C. lini*	---	*C. linicola*	---
C. malvarum	*G. cingulata*			*C. malvarum*	---
C. miyabeana	*G. cingulata*				
C. orbiculare	*G. cingulata*	*C. orbiculare*	---	*C. orbiculare*	*G. lagenarium*
C. paludosum	---			*C. paludosum*	---
				C. phormii[d]	---
C. phyllachoroides	---	*C. phyllachoroides*	---	*C. phyllachoroides*	---
C. trifolii	*G. cingulata*				
				C. typhae	---

[a] Based on Sutton (1992) except where footnoted [b] Dashed line denotes no teleomorph described for the taxon [c] Guerber and Correll 2001 [d] Farr et al. 2006 [e] Moriwaki et al. 2003 [f] Waller et al. 1993

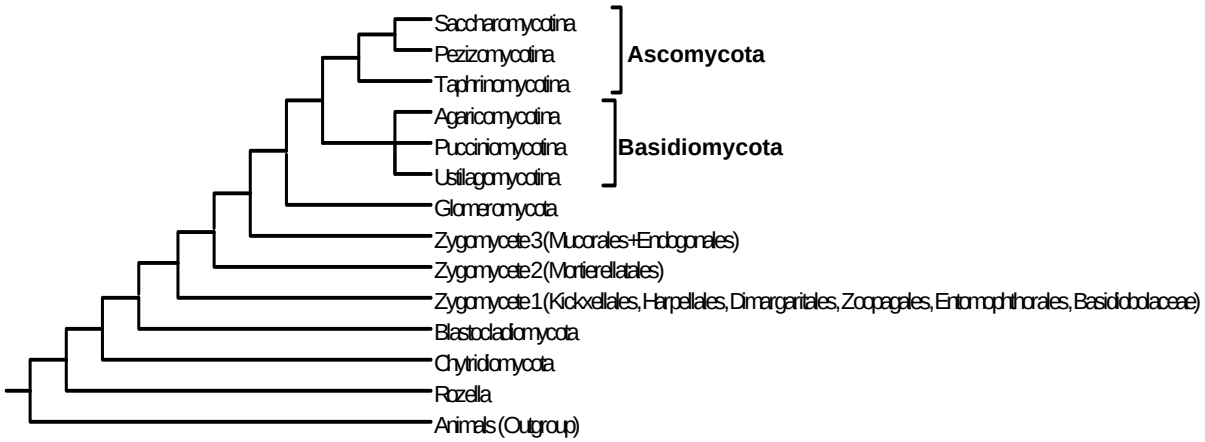

Figure 1-1. Proposed relationships among fungal phyla as reported in the AFTOL Deep Hypha issue of *Mycologia* (2006, v. 89, issue 6).

CHAPTER 2
INVESTIGATING THE RELATIONSHIPS AMONG BIOTYPES OF *MONILIOPHTHORA PERNICIOSA* USING PHYLOGENIES FROM MULTIPLE DNA REGIONS

Introduction

Cacao (*Theobroma cacao*) is an important tropical crop worldwide. It is native to the headwaters of the Amazon, with a secondary area of diversity in Mesoamerica due to extensive prehistoric cultivation (Bartley 2005). The Mayans and Aztecs used cacao not only as a popular beverage, but also as a form of currency. The Mayans held the crop in religious esteem (Coe and Coe 1996). After introduction to Europe in the 16th century its popularity grew, and chocolate has become one of the world's most loved foods. World production in 2006-2007 was estimated at 3.5 million tons, 70% of which occurs in West Africa (World Cocoa Foundation statistics, 2008, http://www.worldcocoafoundation.org/info-center/statistics.asp).

The greatest limiting factor in cacao production is disease. The three most important diseases are black pod, caused by several *Phytophthora* species (Appiah et al. 2004); witches' broom, caused by *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora; and frosty pod, caused by *M. roreri* (Cif.) H.C. Evans, Stalpers, Samson & Benny (Fulton 1989, Evans 2006). Black pod is the most economically damaging disease globally (Bowers et al. 2001, Evans 2006), but the two diseases caused by *Moniliophthora* spp. have the potential to devastate the world cacao supply if they spread to the largest production areas in Africa and Indonesia. The devastating effect of these two diseases is still being played out in South and Central America. After their appearance in Ecuador in the early 1900s, the cacao industry was decimated, and many plantations abandoned (Evans 1981b). The potential for witches' broom to cause catastrophic damage is evident from production numbers in the largest cacao-growing region of Brazil, Bahia. The disease initially appeared in Bahia in 1989 (Pereira et al. 2006), purportedly after the deliberate introduction by bioterrorists of inoculum from the state of Rondonia (Junior

2006). The rapid spread of witches' broom in Bahia resulted in a dramatic decrease in national production, from 378,000 tons in 1990 to less than 120,000 tons in 1999 (Gray 2001). Losses due to frosty pod are no less staggering (Evans et al. 1998). Frosty pod spread from Ecuador and Colombia in the early 1900s south into Peru (Evans et al. 1998), and north to Belize and southern Mexico (Phillips-Mora et al. 2006a,b, 2007), but is currently restricted to the west of the Andes, save for the Napo province of Ecuador (Evans 1981a, Phillips-Mora et al. 2007).

Moniliophthora roreri and *M. perniciosa* are basidiomycetes in the Marasmiaceae, a family in the Marasmioid clade in the Agaricales (Aime & Phillips-Mora 2005, Matheny et al. 2006). As early as the 1970s, similarities between these two pathogens were recognized (Evans et al. 1978, Evans 1981a, Evans et al. 2002). Both fungi are indigenous to South America, have similar host ranges, are hemibiotrophs, and influence the hormonal balance of their hosts. Due to these similarities, a close relationship between the two fungi was proposed and recent molecular data has confirmed this hypothesis. *Moniliophthora perniciosa* (= *Crinipellis perniciosa*) was originally described as *Marasmius pernicious* by Stahel (1915), and later transferred to the genus *Crinipellis* (Singer 1942) due to the presence of long, thick-walled pileal hairs. Aime and Phillips-Mora (2005) investigated the molecular phylogeny of *M. roreri* and *M. perniciosa* with DNA sequences from five loci. They found that they were sibling species within the Marasmiaceae, and that *M. perniciosa* was more closely related to *M. roreri* than to other *Crinipellis* species. Thus, it was transferred to the genus *Moniliophthora*.

The name witches' broom refers to the characteristic proliferation of swollen and twisted shoots that develop on affected cacao trees, symptoms that were first described by Went (1904) in Surinam. Cacao pods and flower cushions (woody meristematic tissue above leaf scars; de Almeida and Valle 2007) may also be affected, resulting in large yield losses. The sole inoculum

for infection is basidiospores that are released by basidiocarps that are produced on necrotic, infected tissue.

Singer's (1976) monograph of the Marasmiaceae indicated that *C. perniciosa* basidiocarps had a crimson red pileus that bleached to whitish with a crimson spot in the center, and was 5-15 mm in diameter. Pileocystidia were 80-150 x 4-12 μm, pseudoamyloid (i.e. dextrinoid) in Melzer's reagent, had a red thick wall, and were rounded-obtuse at the tip. Lamellae were white and distant (1-2 mm). Stipes were 5-10 x 0.4-0.7 mm and dark-brown red at the subbulbous base to white at the apex. Basidiospores were 7-11 x 4-5 μm, ellipsoid, hyaline and smooth, and basidia were 31 x 8 μm. Cheilocystidia were bottle-shaped and 35-50 x 9-14 μm.

Morphological and pathological diversity has been studied in *M. perniciosa*. Basidiocarp variation was first described by Pegler (1978), who reported three varieties: a) var. *perniciosa*, which was identified in Trinidad and Surinam and had a pileus that was crimson in the center and faded to white at the margins; b) var. *ecuadorensis*, identified in Ecuador and with a uniformly red pileus; and c) var. *citriniceps*, from a single broom in Ecuador and with a yellow pileus. In addition to morphologically based varietal classification, the pathogen has also been divided into biotypes based on host range. Five biotypes have been described; the: a) C-biotype affects species in the genera *Theobroma* (including cacao) and *Herrania* (Malvaceae) (Evans 1981); b) S-biotype affects *Solanum lasiantherum*, *S. rugosum, S. cernum, S. lycocarpum* and *S. paniculatum* (Solanaceae) (Bastos and Evans 1985, Pereira et al. 1997, Rincones et al. 2006); c) B-biotype affects *Bixa oreallana* (Bixaceae) (Bastos and Anderbrhan 1986); d) L-biotype includes presumed saprotrophs on lianas (Bignoniaceae) (Evans 1978, Pegler 1978, Griffith and Hedger 1994b); and e) H-biotype affects *Heteroptrys acutifolia* (Malpighiaceae) (Resende et al. 2000).

Moniliophthora perniciosa-type basidiocarps were first reported on lianas and other nonidentified hosts in 1949 along the Napo River in Ecuador (Desrosiers and von Buchwald 1949). Evans (1978) made the first collections of the L-biotype in Ecuador, and showed them to be nonpathogenic on cacao. The L-biotype is associated with dead or living liana vines, and due to limited symptom development on cacao (Evans 1978, Hedger et al. 1987, Purdy and Dickstein 1990), it is assumed that this biotype is either a non-pathogenic parasite or a non-host specific saprotroph (Hedger et al. 1987). Griffith and Hedger suggested that the L-biotype is host-specific to *Arrabidaea verricosa* (Bignoniaceae) (1994b), but basidiocarps have since been found on other liana species (R. Barreto, personal communication).

Moniliophthora perniciosa was first identified as a cause of witches' broom on the solanaceous hosts in Brazil, first on *Solanum rugosum* and *S. lasiantherum* by Bastos and Evans (1985), and later on *S. cernum* and *S. lycocarpum* by Pereira et al. (1997). S-biotype isolates have been shown to be non-pathogenic on cacao, but have caused broom symptoms and necrosis on tomato plants (*Lycopersicon esculentum*), and shortened internodes and galls on bell pepper (*Capsicum annum*) (Bastos and Evans 1985). The B-biotype was originally reported from *Bixa orelleana* (Bixaceae) (Bastos and Anderbhran 1986), but was shown to be genetically identical to C-biotype isolates, and has not been widely reported since. Therefore, its designation as a distinct biotype is questionable (Meinhardt et al. 2008b).

The most recently described biotype was found causing brooms on *Heteropterys acutifolia* (Malpighiaceae), and is reported to be pathogenic on cacao (Resende et al. 2000). Another malpighiaceous host has been reported, *Mascagnia* cf. *sepium*, and isolates from this host are also pathogenic on cacao (Bastos et al. 1998). de Arruda et al. (2005) compared the morphology of C-biotype (nine collections), S-biotype (one collection), and one collection from *H. acutifolia*.

They determined that the *H. acutifolia* collection produced slightly larger basidiospores (average 12 x 6.5 µm) and obpyriform, lageniform to mucronate cheilocystidia, compared with the other collections (10-11.5 x 5.5-6.5 µm basidiospores with sub-cylindrical, lageniform to obclavate cheilocystidia). Based on these morphological characters and the resolution of the *H. acutifolia* isolate outside of the other isolates in an ITS-based phylogeny, de Arruda et al. (2005) erected a new species name for the H-biotype, *Crinipellis brasiliensis* Arruda, G.F. Sepulveda, R.N.G. Mill., M.A. Ferreira & M.S. Felipe. A fuller systematic analysis with more isolates from the various biotypes is needed to resolve species level relationships in *M. perniciosa*.

Mating studies indicated that the C- and S-biotypes are homothallic, but that the L-biotype is heterothallic, with a complex bifactorial, multiple allele mating system (Griffith and Hedger 1994a,b). Whereas single spore, uninucleate cultures of isolates of the C-, B- and S- biotype quickly developed clamp connections and binucleate mycelia, isolates of the L-biotype persisted as uninucleate cultures without clamp connections (Griffith and Hedger 1994a).

Molecular data have been used in several studies to investigate relationships among biotypes. A RAPD study with eight isolates (including one S-, one B-, and six C-biotype isolates) suggested that an isolate of the S-biotype was genetically distinct from isolates of the B- and C-biotypes (Anderbrhan and Furtek 1994). More recently, RFLP analyses of rDNA and mtDNA were largely unable to distinguish the S- and C- biotypes, and found only one polymorphism for separating the H-biotype from the other biotypes (de Arruda et al. 2003a). When an almost identical set of isolates was analyzed with ERIC-PCR fingerprints, the C-, S-, and H-biotypes were distinguished (de Arruda et al. 2003b). Rincones et al. (2007) used microsatellite-primed PCR (mp-PCR) to investigate relationships between the C-, S- and L-

biotypes. Although genetic diversity was revealed within each biotype, the authors did not speculate on relationships among biotypes or specific lineages in the species.

Only two examples of phylogenetic analyses of *M. perniciosa* with DNA sequence data were found in the literature. de Arruda et al. (2003a) completed a parsimony analysis of the intergenic spacer (IGS) region of 14 *M. perniciosa* isolates, including 10 isolates from *T. cacao*, two isolates from *S. lycocarpum*, and two isolates from *Heteropterys acutifolia*. The three biotypes formed well-supported clades, one of which contained isolates from *S. lycocarpum* and *T. cacao*, and another that contained isolates from *Heteropterys acutifolia*. In a similar study using the ITS1-5.8S-ITS2 region, de Arruda et al. (2005) again found that an isolate from *H. acutifolia* was distantly related to isolates from *Theobroma*, *Herrania*, *Heteropterys nervosa,* and *Solanum*. All *M. perniciosa* isolates resolved as a sister group to *M. roreri* var. *gileri*, which was also supported in the multi-gene analyses of Aime & Phillips-Mora (2005). The isolate from *Heteropterys acutifolia* was sister to a largely unresolved clade that contained all other isolates of *M. perniciosa*.

A multiple regoion phylogenetic analysis comprised of isolates from all biotypes and hosts has yet to be completed, and is needed to resolve the conflicting evidence regarding relationships among biotypes and host-specific lineages. Clarification of these relationships would help to: estimate the threat that subspecific groups pose to the cacao crop; indicate whether *M. perniciosa*, as it is currently defined, should be considered a single species or species complex; and assess the utility of host plant associations to designate biotypes. In the present study, isolates of *M. perniciosa* from a range of hosts in Brazil, many of them newly reported for the pathogen, were analyzed with isolates from *T. cacao* and several South American countries. Three DNA regions were chosen for phylogenetic analyses: ITS, IGS, and the RNA polymerase

II large subunit (RPB1). In addition, macro- and microscopic morphological characters were examined in a subset of these isolates to determine if they reflected genetic relationships.

Materials and Methods

Isolate Selection

Isolates of *M. perniciosa* that were used in this study were collected previously by several individuals and are stored in the isolate collection of RCP in Homestead, FL (Table 2-1). All CPB isolates were collected from various states in Brazil by H. Evans and R. Barreto in March-June of 2006, and they represent the bulk of isolates collected from hosts other than *T. cacao*. Overall, 16 isolates of the C-biotype, 10 of the S-biotype, five of the L-biotype, one of the H-biotype, and three from other host plants were studied. Isolates of the C-biotype were from Bolivia, Brazil, Colombia, Ecuador, Venezuela, Trinidad, and Tobago; all other isolates were collected in Brazil. Isolate TCP7 was originally received as *M. perniciosa*, but phylogenetic analysis showed it to be consistently distinct, therefore it was considered as an additional *Moniliophthora* sp. isolate.

Three *Moniliophthora* taxa that are sister to *M. perniciosa* were included in analyses: *M. roreri* in the ITS, IGS and RPB1 analyses; *M. roreri* var. *gileri* in the ITS analysis; and *Moniliophthora* sp. MCA 2500, a grass endophyte, in ITS and RPB1 analyses. For the IGS analysis, *M. rorreri* and *M. roreri* var *gileri* were used as outgroup taxa. *Chaetocalathus liliputianus*, which is sister to *Moniliophthora* (Aime and Phillips-Mora 2005), was included as an outgroup taxon in the ITS and RPB1 analyses.

DNA Extraction and PCR of ITS, IGS, and RPB1 Regions

Isolates of *M. perniciosa* were grown at room temperature on PDA covered with a layer of sterile cellophane. After colonies had grown to at least 6 cm in diameter, mycelial mats were peeled from cellophane with sterile forceps, triple rinsed with sterile deionized water and dried

on sterile filter paper. DNA was extracted using a DNA genomic preparation protocol from the University of Wisconsin Biotechnology Center. Briefly, mycelia were ground in 500 µl Shorty DNA Extraction Buffer (0.2M Tris-HCl, pH 9.0, 0.4M LiCl, 25mM EDTA, 1% SDS) and incubated at 68°C for 10 minutes. Tissue was centrifuged at 14,000 rpm for 5 minutes, 400 µl of the supernatant was transferred to new tube and the DNA was precipitated with 400 µl 99% isopropanol. The tubes were centrifuged for 10 minutes at 14,000 rpm and the supernatant was decanted. The DNA pellets were air-dried for 5 minutes then resuspended in 400 µl TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) for 30 minutes at room temperature. 2µl DNA was used in PCR reactions.

DNA sequence data from three nuclear regions were used in phylogenetic analyses: two rDNA sequences (the ITS1-5.8S-ITS2 and the intergenic spacer, IGS), and a protein-coding gene, the RNA polymerase II large subunit, RPB1. In studies by de Arruda et al. (2003a, b), the IGS region was shown to have a stronger phylogenetic signal than ITS. Both ribosomal regions were included to provide two levels of resolution. Although it is generally more conserved than the ITS and IGS, the RPB1 locus was included as a protein-coding nuclear gene that is not linked to the ITS and IGS.

All PCR reactions were carried out in 50 µl reactions that contained 38.25 µl of sterile distilled, deionized water, 6.5 µl ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA), 1 µl 10 mM dNTP mix (New England Biolabs, Ipswich, MA), 0.25 µl Taq DNA polymerase (conc. 5,000 units/ml), 1 µl each of 15 µM primers and 2 µl DNA template. Standard cycling parameters with a 55 °C annealing temperature were used. The ITS region was amplified using the following primers: ITS1 (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The IGS rDNA region was amplified using primers CNL12 (Appel and Gordon 1995) and

O-1 (Duchesne and Anderson 1995). The RPB1 locus was amplified with primers RAS.RPB1-F2 and RAS.RPB1-R2 (Aime and Phillips-Mora 2005). Direct sequencing of the PCR products was performed at the University of Florida ICBR (Interdisciplinary Center for Biotechnology Research) Facility (Gainesville, FL) with the same primers used for amplification. For ambiguous bases, the base that agreed with the consensus sequence was retained. Because sequencing signal from direct sequencing was strong, cloning was not performed. For isolates with unexpected phylogenetic placements, sequences were confirmed by repeated DNA extraction, PCR reactions and direct sequencing.

Phylogenetic Analysis

Sequence alignments were done using CLUSTALX (Thompson et al. 1994) in Mega4 using default parameters, and adjusted manually. In the IGS data set, a single indel of 170bp present only in isolate TCP7 was excluded from all analyses. For the maximum parsimony analysis, two data sets were used: one which included indels but with indel sites treated as missing data, and a second in which indels were coded as separate characters following a coding system modified from Simmons and Ochoterena (2001). Indel sites were removed from the alignments, and indels were coded as unordered, numerical characters. Single site indels were coded as binary presence/absence characters. Contiguous indels that did not share 5' or 3' termini were considered as separate mutation events, and were coded as separate presence/absences characters. Contiguous indels that share a 5' or 3' terminus were coded as unordered multistate characters. Because no gaps in the data set were imbedded (one gap falls completely within another contiguous gap), step matrices (as outlined by Simmons and Ochoterena 2001) were not utilized. A microsatellite region in the IGS locus $(CAAA)_x$ was also coded as an unordered, multistate character reflecting the number of repeats of the microsatellite. Coded indels were excised from the data set, unless the nucleotide sequence among isolates not containing the

deletion contained informative sites, in which case the indel was left in the data set but gaps were coded as missing data. Alignments were submitted to TreeBASE (http://www.treebase.org)

Both maximum parsimony (MP, using PAUP* v.4.0b; Swofford 2000) and maximum likelihood (ML, using GARLI v.0.946) analyses were done on all data sets. Both data sets for each locus (indels treated as missing data or indels coded as separate characters) were used for MP analysis. Parsimony analysis was conducted using heuristic searches with 100 random addition replicates, with tree bisection reconnection (TBR) swapping, saving no more than two trees with tree scores greater than five per repetition. Consistency index (CI) and retention index (RI) values were calculated by PAUP*. To determine statistical support for groups in the phylogenies, the non-parametric bootstrap test (Felsenstein 1985) was performed using 1,000 repetitions and heuristic search criteria as described above except that 10 random addition replicates were used, saving no more than two trees with scores greater than five per repetition.

ML analysis was run using the data set with gaps treated as missing data only. Analysis was run on GARLI v.0.946, using default parameters and a randomly generated starting topology. Analysis was stopped after $5x10^6$ generations or a 0.01 decrease in ML score. Three independent runs were performed and resulting topologies compared in TreeView v1.6.6. If similar topologies were generated, bootstrap analysis was performed using the same parameters with 1,000 repetitions. Tree files were imported into MEGA 4.0 for visualization and editing of phylograms. For the ITS and RPB1 analyses, *C. liliputianus* was the outgroup; for the IGS analysis, *M. roreri* C21 and *M. roreri* Dis116a were outgroups.

Congruence of Molecular Data Sets and Combined Analysis

In order to determine if combining data from the three loci was appropriate, the level of congruence was first tested between pairs of the three data sets, testing either congruence between character sets or between topologies of phylogenetic trees (Seelanen et al. 1997,

Johnson and Soltis 1998). Overall incongruence between data sets was quantified using the Mickevich-Farris index (I_{MF}), which calculates a standardized value for the increase in the tree length of the most parsimonious tree from a combined analysis compared to the sum of the tree lengths of the two loci analyzed individually (Mickevich and Farris 1991). To determine the significance of the quantified incongruence, a concatenated data set was constructed with all three loci, and partitions were defined for each locus. The partition homogeneity test was implemented in PAUP* comparing all three loci, to determine the incongruence length difference (ILD) between loci pairs (Farris et al1994). *P*-values less than 0.05 contain significant incongruence between the data sets. The ILD test is known to be very sensitive (Yoder et al. 2001; Darlu and Lecointre 2002), so the decision as to whether to combine data sets was largely based on examination of topology and bootstrap support.

To assess topological congruence, an approach similar to that used by Seelanen et al. (1997) was followed. First, consensus trees for each of the three loci were compared empirically to determine if instances of topological incongruence were hard (isolates resolve in different well-supported clades in each topology), or soft (incongruence due to weak phylogenetic signal in one of the data sets). To determine the statistical significance of topological incongruence, we used 70% bootstrap trees from one region as a topological constraint in analysis of each additional region, and the Wilcoxon signed-rank (WSR) test to determine if the number of steps that were gained or lost in the resulting tree was significantly greater ($P<0.05$) than the original trees, an approach that was first described by Templeton (1983). In addition, strongly supported clades that were resolved in each single locus analysis were used as constraints for the other loci (Table 2-4). This approach allowed us to test the congruence of specific clades in each data set, enabling the identification of individual clades and/or accessions that cause incongruence

between data sets. This approach allowed congruence of specific clades to be tested in each data set and enabled the identification of individual clades and accessions that caused incongruence. This approach helps determine if data sets could be combined and provides information about evolutionary events that may have caused incongruence, such as cryptic outcrossing, hybridization, introgression, or horizontal gene transfer (Wendel and Doyle 1998).

Morphological Description

Basidiocarps of isolates CPB1, CPB2, CPB5, CPB9, CPB15 and TRDC74 were successfully produced using the bran media "cookie" method (Griffith and Hedger 1993, Niella et al. 1999). Preserved basidiocarps of CPB10 and CPB12 were provided by H. Evans. Fisherbrand 500ml polypropylene screw top jars were filled two-thirds full with bran media of 400 g vermiculite, 500 g wheat bran flakes cereal, 120 g $CaSO_4 \cdot 2H_2O$, 15 g $CaCO_3$ and 1200 ml de-ionized water and autoclaved for 1 h at 0, 24 and 72 h, then seeded with mycelial plugs of isolates. Once media was fully colonized, it was covered with a sterile casing of 200g mown turf, 50 g vermiculite and 50 g $CaCO_3$. After the casing was colonized, cookies were removed from plastic jars, cut into quarters to maximize surface area for basidiocarp production, and hung in a Plexiglass closet under a 12 hr light/dark cycle and and high humidity; cookies were misted daily with de-ionized water.

Basidiocarps were harvested 1-2 days after opening, and dried (50°C for approx. 20 min) for subsequent micromorphological characterization. For one to 10 basidiocarps of each isolate, the following macromorphological characteristics were recorded: pileus diameter and color; presence of lamellulae; lamellae spacing and color; and stipe color, length, and diameter at base and tip. The following microscopic characters were examined for two basidiocarps per isolate: pileocystidium pigmentation, shape and dimensions (15 cystidia per fruiting body); basidium

size and number of sterigmata (15 basidia per fruiting body); cheilocystidium shape and dimensions (15 cystidia per fruiting body); and basidiospore shape and dimensions (30 spores per fruiting body). In addition, tissue was stained with Melzer's reagent and pseudoamyloid reactions were recorded. Mean length, width, and length/width ratios of basidiospores were separated using Fisher's LSD test (PROC GLM in SAS v. 0.1.3; SAS Institute Inc., Cary, NC).

Results

Phylogenetic Analysis

Coding indels as separate unordered characters increased resolution and bootstrap support for the majority of nodes in the ITS analysis, and approximately half of the nodes in the IGS analyses. Thus, indels may be important in the evolutionary histories of these loci (Table 2-2). The indel characters in the RPB1 locus only marginally improved statistical support for nodes, and most separated the outgroup taxon *C. liliputianus* from the *Moniliophthora* isolates.

The ITS dataset contained 40 accessions, including one of *M. roreri*, two of *M. roreri* var. *gileri* and two of *Moniliophthora* sp. isolates. *Chaetocalathus liliputianus* was used as an outgroup. There were 678 total characters (35 indel characters), of which 122 were parsimony-informative. Within isolates of *M. perniciosa*, there were 22 parsimony informative characters (PIC). Maximum parsimony (MP) analysis resulted in 18 most parsimonious trees (MPT), with a tree length of 391 (CI=0.903, RI=0.893) (Figure 2-1). The maximum likelihood (ML) tree had a ln score of -2472.65, and largely similar topology to the 50% consensus tree from parsimony analysis, except that clade 2 was not completely resolved in ML (Figure 2-2). Both ML and MP analyses resolved isolates of *M. perniciosa* as a highly supported sister clade to *M. roreri.* Isolates of *M. perniciosa* were resolved into three main clades in both trees (Figures 2-1 and 2-2). In the MP phylogeny, Clade 1 resolved into two subclades. Subclade 1a contained all isolates from *T. cacao* and isolate 73-62, collected from an unidentified solanaceous weed (the species

was not identified). Subclade 1b contained most isolates from solanaceous hosts, and also included a broom isolate from a liana, CPB17, and a C-biotype isolate, CPB2, from *T. grandiflorum*. Subclades 1a and 1b were resolved in the 50% parsimony consensus tree, but did not have high bootstrap support. Examination of character state changes in the analysis revealed that only three and one synapomorphies supported the resolution of the 1a and 1b subclades, respectively. Clade 1 is sister to Clade 2 (59% in MP), which contains three isolates, CPB5 (*S. cernum*), TCP8 (unknown host), and CPB8 (bignoniaceous liana). It had 55% bootstrap support in the MP analysis, but isolates CPB5 and TCP8 were sister to each other with higher bootstrap support in both MP and ML analyses (60% and 62%, respectively). Clade 3 contained two liana isolates, CPB7 and CPB10, and three isolates from malpighiaceous shrubs and trees, CPB6, CPB9, and CPB12. It was highly supported in both analyses (90% MP, 88% ML), and was supported by 17 synapomorphies.

The IGS dataset included 38 accessions of *M. perniciosa*, and *M. roreri* C21 and *M. roreri* var. *gileri* Dis116e were used as outgroup taxa. There were 861 total characters (41 indel characters) and 300 PIC, 68 within isolates of *M. perniciosa*. MP analysis resulted in 200 MPT, with a tree length of 475 (CI=0.931, RI=0.934) (Figure 2-3). The ML tree had a ln score of -3023.85, and similar topology to the 50% consensus tree from MP analysis, except for the placement of Clade 2 (Figure 2-4). The resulting phylogenies resolved with high support the three main clades: Clade 1 contained all C-biotype and S-biotype isolates, without subclade resolution; Clade 2 contained CPB5, CPB8, and TCP8; and Clade 3 contained CPB6, CPB10, and CPB12 (Figures 2-3 and 2-4). The placement of Clade 2 differed from that in the ITS phylogeny, which placed it sister to Clade 1. Isolates CPB7 and CPB9 resolved in Clade 1 in the IGS phylogeny rather than Clade 3, where they were placed in the ITS analysis. Within Clade 1,

they were part of a large polytomy that contained all other Clade 1 isolates. This was an example of hard incongruence between the two loci (Seelanen et al. 1997). Support for Clade 1 was high (95% MP), and the group shared only two synapomorphies. Support for Clade 2 (isolates CPB5, CPB8, and TCP8) was high in both ML (92%) and MP (83%) analyses and the three isolates shared six synapomorphies. Clade 3 contained only CPB6, CPB12, and CPB10, and was highly supported (100% MP, 99% ML).

The RPB1 analysis included 36 accessions of *M. perniciosa*, and single accessions of *M. roreri* (C21), *M. roreri* var. *gileri* (Dis116a), and *Moniliophthora* sp. (MCA 2500). *Chaetocalathus liliputianus* was used as an outgroup. There were 747 total characters, and 58 PIC. Within *M. perniciosa*, there were only 12 PIC. This locus is largely conserved, hence the weak phylogenetic signal it gave among closely related taxa. MP analysis resulted in 200 MPT with a tree length of 230 (CI=0.948, RI=0.911) (Figure 2-5). The ML tree had a ln score of -2124.22, and a topology that was largely similar to the 50% consensus tree from parsimony analysis (Figure 2-6). The ML tree showed less resolution than the MP phylogeny, with no support for resolving Clade 2. There was less resolution in the RPB1 phylogeny compared to the other loci, likely due to the low number of parsimony informative sites. Subclades 1a (one synapomorphy) and 1b (two synapomorphies, and contained most S-biotype isolates and CPB17) had 64% MP and 87% MP, and 84% ML bootstrap support, respectively. Isolate CPB7 (which resolved into Clade 3 in the ITS phylogeny, and Clade 1 in the IGS phylogeny), was sister to subclade 1b, with moderate to high support (87% MP, 79% ML). The other incongruent taxon, CPB9 (Clade 3 in ITS, Clade 1 in IGS), resolved in Clade 3 in the RPB1 phylogeny. CPB8 (Clade 2 in ITS and IGS phylogenies) resolved in Clade 3, with 87% MP and 82% ML bootstrap support.

Congruence Tests and Combined Data Analysis

Partition homogeneity analysis in PAUP revealed significant incongruence (P=0.002) among all data set comparisons (Table 2-3). To identify the source of incongruence, we tested several topological constraints on each data set, with WSR tests to estimate the significance of incongruence of alternate topologies (Table 2-4). In general, enforcing RPB1 clades onto the ITS and IGS data sets resulted in significant incongruence (P<0.05), with the exception of RPB1 subclade 1b. Alternatively, enforcing ITS and IGS-supported clades onto the RPB1 data set resulted in less significant incongruence. This is likely due to fewer parsimony informative characters in the RPB1 data set; thus, part of the resulting phylogenetic signal may be due to chance. Clade 2 (CPB5, TCP8, and CPB8) was completely congruent in the IGS and ITS data set, and led to a four-step increase in the RPB1 phylogeny (P=0.0455). Clade 3 (CPB6, CPB10, and CPB12) was congruent in all data sets. The addition of CPB7 and CPB9 (which were resolved in Clade 3 in the ITS set, but not in the IGS set) made the clade significantly incongruent in the IGS set (P=0.0010), but not in the RPB1 set (0 length increase). Based on the results of the topological incongruence analyses, it was concluded that most of the incongruence between data sets was due to the placement of CPB7 and CPB9, as well as the low phylogenetic signal in the RPB1 set. Other instances of incongruence occurred in the placement of isolates in subclades 1a and 1b, and again could be due to a weak phylogenetic signal, as the resolution of these two clades was only supported by one or two synapomorphies in each data set. Because significant incongruence existed among all data sets, data were not combined for phylogenetic analysis.

Morphological Characterization

Basidiocarps were produced for four isolates from Clade 1 (CPB1, TRDC74, CPB2 and CPB15), one from Clade 2 (CPB5), and isolate CPB9. In addition, preserved basidiocarps of two Clade 3 isolates (CPB10 and CPB12) from original host material were examined.

The examined characters largely agreed with Singer's description of the species (1972) (Tables 2-5 and 2-6). Pileus diameter generally ranged from 7-25 µm, with larger pilei produced by CPB5 (Clade 2), which ranged 11-32 µm. Pileus color was crimson, and faded to crimson at the umbo to cream at the margins as basidiocarps aged, except in alcohol-preserved basidiocarps of CPB12, in which pigment had leached to result in a uniform cream color.

Isolates from Clade 3 (CPB10, CPB12) and CPB5 had significantly larger basidiospores (mean size of 10.7x6.0 µm, 10.9x5.8 µm, and 10.5x5.4 µm, respectively) than isolates from other clades (generally 9-9.5x5-5.3 µm) (Table 2-7). Isolate CPB9 had smaller basidiospores, 8.4x4.7 µm. Cheilocystidial shape, which de Arruda et al. (2005) indicated distinguished the H-biotype, was variable, even between basidiocarps of the same isolate. CPB5 and isolates in Clade 1 tended to have numerous lageniform (bottle-shaped) cheilocystidia in older basidiocarps (collected on 2nd day after opening) (Figure 2-7). Among isolates in Clade 3 (CPB10, CPB12) and CPB9, cheilocystidia were rare and had an obclavate to pyriform shape.

Pileocystidium pigmentation was cytoplasmic and often produced as extracellular incrustations. Pileocystidia were produced in a trichodermal layer, and were mostly clavate to cylindrical, but sometimes mucronate (Figure 2-7) and clamped. CPB5 had distinct pileocystidia, in that the first cell was swollen clavate, with a largely swollen second cell (Figure 2-7). Pigment was concentrated in the tip of the apical cell. Pileocystidia of all isolates were generally shorter than those reported by Singer (1976).

Discussion

The multilocus phylogenetic analysis presented here is the most complete analysis for *M. perniciosa* to date. It included isolates from four of the five reported biotypes; the fifth, B-biotype is thought to be conspecific with the C-biotype (Meinhardt et al. 2008).

Although Hedger et al. (1987) suggested that isolates of the L-biotype were saprotrophs or nonpathogenic endophytes, their isolates were from *Arrabidaea verrucosa* (Bignoniaceae). Interestingly, H. Evans and R. Barreto (unpublished) have collected isolates from brooms on malpighiaceous lianas in Brazil, and one of these isolates, CPB17, was examined in the present work. Thus, there may be pathogenic lineages of *M. perniciosa* on lianas in the Malpighiaceae, but not the Bignoniaceae. More work is needed to distinguish saprophytic and pathogenic populations of *M. perniciosa* that reside on tropical vines.

Morphological differences were slight among the isolates that were examined, and do not appear to distinguish subspecific groups. However, subspecific lineages were resolved in phylogenetic analyses.

Clade 1 contained most isolates, including all from *Theobroma* and *Solanum* hosts, as well as several from lianaisolates, including CPB17, which was recovered from a broom. Isolates from *T. cacao* all had similar sequences, and resolved into a single subclade (1a) with low bootstrap support (40-60%) in the ITS and RPB1 analyses. Isolates from solanaceous hosts (subclade 1b) were resolved as a sister to subclade 1a in the ITS and RPB1 analyses, with low to high support (49 and 87%, respectively). CPB2, CPB15, and 92-10-7 had equivocal resolution in Clade 1, in that they were alternately placed in subclade 1a or 1b in the single locus phylogenies. Subclades 1a and 1b may be distinct, but recently diverged lineages in *M. perniciosa*. This hypothesis is supported by low bootstrap support and the limited number of synapomorphies that resolved the *Solanum* and *Theobroma* subclades.

Isolate TCP7, *Moniliophthora* sp., was distantly related to isolates of *M. perniciosa*, although it was more closely related to *M. perniciosia* than the frosty pod pathogen, *M. roreri*. TCP7 was collected from a liana in 2001 in Brazil. Based on the phylogenetic evidence presented in this study, it could be considered a separate species, but fruiting bodies need to be examined for a complete taxomonic assessment.

Isolate CPB5 from *S. cernum*, CPB8 from a bignoniaceous liana, and TCP8 from an unknown host resolved into Clade 2 in the ITS and IGS analyses, but not in the RPB1 phylogeny. In the RPB1 analysis, CPB8 fell into Clade 3, and CPB5 into subclade 1a. Hard incongruence in the RPB1 phylogenies may be due to insufficient sampling, but the jumping of so many isolates between Clades 1 and 2 may also indicate that the genetic isolation between these lineages is not absolute, and that outcrossing occurs between lineages. Further taxon sampling may increase the collection of isolates in this clade, which could provide more information about its ecology.

Clade 3 contained malpighiaceous isolates CPB6, CPB10, and CPB12, as well as CPB7 and CPB9 for at least one locus. It was well resolved as sister to Clades 1 and/or 2 in all analyses, and could be considered a separate taxon. There was little morphological difference between isolates of Clade 3 and Clades 1 and 2, but isolates CPB10 and CPB12 had slightly, but significantly larger basidiospores, and slightly differently shaped cheilocystidia. Our observation of obclavate to pyriform cheilocystidia produced by malpigheaceous isolates contradicts that of de Arruda et al. (2005), who indicated that the H-biotype (*H. acutifolia*) produced lageniform cheilocystidia However, at least one study has shown that cheilocystidia can be morphologically variable, even within a single pileus and depending on basidiocarp age, humidity, and other environmental factors (Aime 2001).

The phylogenetic analyses presented here largely agree with previous studies comparing populations of different biotypes using molecular markers, in that the S- and C-biotypes were genetically distinct (de Arruda et al. 2003a,b, Rincones et al. 2007). Assuming that the isolates that were included in this study were correctly labeled with respect to host, the revealed phylogenetic lineages are pathogens of *Theobroma*, *Solanum*, or malpighiaceous hosts with a few exceptions: a liana isolate (CPB17) and *Theobroma* isolate (CPB2) fell in the *Solanum* clade (1b), and a *Solanum* isolate (73-62) fell in the *Theobroma* clade (1a). Although the C- and S-biotypes appear to define biologically meaningful subspecific populations of the fungus, the present results indicate that the L- and H- biotypes as they are currently defined are not valid. Biotypes of *M. perniciosa* should be redefined once the host ranges of these populations are better understood.

The host specificity of the different biotypes is of practical importance to cacao growers. As cacao cultivation expands in Brazil, there is concern about introducing the crop where *M. perniciosa* is found on solanaceous hosts. Previous pathogenicity tests indicated that host specificity exists among biotypes, in that cross inoculations of the L- or S-biotypes onto *T. cacao* yielded no symptoms or only slight swelling of inoculated nodes (Evans 1978, Evans and Bastos 1985). The present phylogenetic analyses indicate that these biotypes may be host specific, but that specificity may not be absolute. The extent to which phylogenetically distinct isolates of *M. perniciosa* would cause disease on cacao needs to be better understood. In addition, virtually nothing is known of the potential for host range expansion to occur in this fungus via gene flow. Although a homomictic, homothallic breeding strategy prevails in *M. perniciosa*, incongruent phylogenies for several isolates suggests that hybridization or outcrossing may have occurred among the lineages.

Moniliophthora is a relatively new genus that was erected in 1978 to accommodate a single species, *M. roreri* (Evans et al. 1978). It remained a monospecific genus until 2005, when *Crinipellis perniciosa* was transferred to *Moniliophthora* (Aime and Phillips-Mora 2005). While both species include cacao pathogens and are hemibiotrophs, their respective biologies exhibit striking differences. *Moniliophthora* was erected to accommodate an anamorphic basidiomycete, which does not produce basidiocarps. Evans et al. (2002) recently suggested that the "conidia" of *M. roreri* are actually meiospores. Aime and Phillips-Mora (2005) conjectured that other species of *Moniliophthora* exist as nondescribed biotrophs and that many species of *Crinipellis* currently placed in Section Iopodinae are likely congeners with *Moniliophthora.* Based on molecular phylogenies, it appears that at least seven other species of *Moniliophthora* are now known: a grass endophyte, isolate MCA2500 (Aime and Phillips-Mora 2005), used in this study; a liana-associated species, isolate TCP7, used in the present study; *C. eggersii* (Aime unpubl.); and four species (three transferred from *Crinipellis* to *Moniliophthora* and one new species) from Southeast Asia (Kerekes and Desjardin 2009). It is possible that isolate TCP7 is a previously described *Crinipellis* species in section Iopodinae, but basidiocarps need to be obtained and studied for this isolate to determine its affinity with other species. Because the lineages that were resolved in the present study may have outcrossed and lack distinctive morphological differences, they should remain subspecific groups of *M. perniciosa*.

The S- and C- biotypes are homothallic and the L-biotype heterothallic. It has been proposed that the divergence of lineages within the species was accompanied by a switch from a heterothallic to homothallic mating strategy (Griffith and Hedger 1994a,b). Homothallism can be advantageous for plant pathogens. With no enforced outcrossing, populations can become highly

specialized to a given environment or lifestyle. There is also no need for the presence of compatible individuals to complete a life cycle.

The classic hypothesis of the evolution of mating strategies is the evolution from homothallism to heterothallism (Whitehouse, 1949). Phylogenetic analysis of several *Aspergillus* species supported a single switch from homothallism to heterothallism (Geiser et al. 1998), and examination of mating type gene sequences in several species suggest that the switch was caused by the loss of mating genes (Galagan et al. 2005).

However, other homothallic fungi appear to be derived from heterothallic taxa. Homothallic species of *Neurospora* often contain both mating type loci, but their function is not known (Kronstad and Staben 1997). These loci may be artifacts from a heterothallic ancestor. The occurrence of homothallic *Neurospora* species at the geographic limits of the genus also suggests that homothallism may have evolved where opposite mating types were not present. Phylogenies of homothallic and heterothallic *Neurospora* and *Sordaria* species support a single switch from heterothallism to homothallism in both genera (Poggeler et al. 1999). A shift from heterothallism to homothallism has also been proposed for *Cochliobolus* (Yun et al. 1999) and *Fusarium* (O'Donnell et al. 2004). In *Cochliobolus*, there is evidence that homothallism evolved not once, but several times (Turgeon et al. 1998). A similar situation could be proposed for *M. perniciosa*, where pathogenicity and homothallism evolved to enable the survival of these specialized populations. L-biotype isolates from Ecuador that were reported to be heterothallic and saprotrophs or endophytes were not included in this study. It is possible that they represent another species of *Moniliophthora* that differs from the pathogenic *M. perniciosa*.

It has been speculated that both *M. perniciosa* and *M. roreri* evolved from a common forest endophyte (Evans 1981, Griffith et al. 1994, Evans et al. 2002), and that *M. perniciosa* co-

evolved with *Theobroma* and *Herrania* on the eastern side of the Andes (Pound 1943, Baker and Holliday 1957). However, Evans (1981) and then Griffith et al. (1994) proposed an alternative hypothesis to coevolution with cacao, suggesting that the homothallic C- and S- biotypes evolved from the heterothallic L-biotype in separate divergence events (Griffith et al. 1994). They studied the mating systems of the L-, S-, and C- biotypes and determined the L-biotype to be more diverse than the other biotypes, based on banding patterns of mtDNA and isozyme studies. They also noted that the S-biotype profiles were more similar to the L-biotype than the C-biotype. They also speculated that additional biotypes would be found that would represent older divergences from a heterothallic endophytic ancestor.

Recent evidence, including the phylogenies constructed in this study, seems to support an evolutionary scenario similar to that proposed by Griffith et al. (1994). Recent collections of *M. perniciosa* in the Brazilian Amazon have uncovered new host species in the Malpighiaceae, which in the present analyses fall in Clade 3 with bignoniaceous liana isolate ("L-biotype") CPB7. Thus, there is less phylogenetic support for distinct H- and L-biotypes than for a malpighiaceous clade that affects that family and contains bignoniaceous saprobes.

The present phylogenies also support the suggestion by Hedger et al. (1994) that the *Theobroma* and solanaceous lineages diverged more recently compared to other clades. Lineages that diverged earlier have mostly malpigheaceous and bignoniaceous hosts. Interpretation of these phylogenies suggests that the homothallic, highly specialized pathogen could have evolved from a heterothallic, saprotrophic or endophytic ancestor, and subsequent host jumps resulted in the lineages presently seen in *M. perniciosa*. *Moniliophthora perniciosa* as a pathogen on cacao therefore likely represents not a pathosystem involving coevolution, but a series of host jumps to plants in several different families. As more host species are identified in the Amazon, and more

Moniliophthora taxa are described and added to phylogenetic studies, the evolution of both *M. perniciosa* and *M. roreri* should become clearer.

Table 2-1. Description of isolates used in the study.

Isolate	Collector/Donator	Host origin	Collection tissue	Geographic origin	Biotype	Genbank Accession number		
						ITS	IGS	RPB1
4.	M. Shaw	*Theobroma cacao*	Broom	Colombia	C	GQ919116	GQ919081	GQ919152
7.	M. Shaw	*T. cacao*	Broom	Venezuela	C	GQ919117	GQ919080	GQ919153
17.	M. Shaw	*T. cacao*	Broom	Colombia	C	GQ919118	GQ919082	GQ919156
28.	M. Shaw	*T. cacao*	Broom	Colombia	C	GQ919119	GQ919083	GQ919154
31.	M. Shaw	*T. cacao*	Broom	Trinidad	C	GQ919120	GQ919084	GQ919155
46PR	H. Purdy	*T. cacao*	Broom	Ecuador	C	GQ919137	GQ919085	GQ919157
73-31	M&M/Penn State	*T. cacao*	Broom	Brazil	C	GQ919121	GQ919094	GQ919158
73-62	DIFIP/Penn State	Weed, Solanaceae	Broom	Brazil	S	GQ919122	GQ919108	GQ919159
92-10-7	ACRI/PennState	Weed, Solanaceae	Broom	Brazil	S	GQ919123	GQ919096	GQ919160
CPB1	H. Evans/ R. Barreto	*T. cacao*	Broom	Brazil	C	GQ919141	GQ919092	GQ919161
CPB2	H. Evans/ R. Barreto	*T. grandiflorum*	Broom	Brazil	C	GQ919124	GQ919101	GQ919162
CPB3	H. Evans/ R. Barreto	*Solanum lycocarpum*	Fruit	Brazil	S	GQ919125	GQ919102	GQ919163
CPB4	H. Evans/ R. Barreto	*S. lycocarpum*	Broom	Brazil	S	GQ919126	GQ919103	GQ919164
CPB5	H. Evans/ R. Barreto	*S. cernum*	Broom	Brazil	S	GQ919127	GQ919104	GQ919165
CPB6	H. Evans/ R. Barreto	Tree, Malpighiaceae	Broom	Brazil	?	GQ919147	GQ919113	---
CPB7	H. Evans/ R. Barreto	Liana, Bignoniaceae	Hanging litter	Brazil	L	GQ919146	GQ919106	GQ919166
CPB8	H. Evans/ R. Barreto	Liana, Bignoniaceae	Hanging litter	Brazil	L	GQ919128	GQ919111	GQ919167
CPB9	H. Evans/ R. Barreto	Tree, Malpighiaceae	Broom	Brazil	?	GQ919148	GQ919100	GQ919168
CPB10	H. Evans/ R. Barreto	Liana,	Basidiospore	Brazil	L	GQ919149	GQ919114	GQ919169
CPB12	H. Evans/ R. Barreto	*Heteropterys*	Broom	Brazil	L	GQ919150	GQ919112	GQ919171
CPB14	H. Evans/ R. Barreto	*S. lycocarpum*	Broom	Brazil	S	GQ919129	GQ919104	GQ919172

Table 2-1. Continued.

Isolate	Collector/Donator	Host origin	Collection tissue	Geographic origin	Biotype	Genbank		
						ITS	IGS	RPB1
CPB17	H. Evans/ R. Barreto	Liana	Broom	Brazil	L	GQ919131	GQ919097	GQ919173
CPB20	H. Evans/ R. Barreto	*S. grandiflorum*	Broom	Brazil	S	GQ919132	GQ919098	GQ919174
CPB21	H. Evans/ R. Barreto	*S. lycocarpum*	Broom	Brazil	S	GQ919133	GQ919099	GQ919175
CPB22	H. Evans/ R. Barreto	*S. lycocarpum*	Broom	Brazil	S	GQ919134	GQ919105	GQ919176
TBOC3-2	L. Johnson	*T. cacao*	Broom	Tobago	C	GQ919145	GQ919091	GQ919177
TCP3-2	R. Schnell	*T. cacao*	Broom	Bolivia	C	GQ919139	GQ919090	GQ919178
TCP8	A. Pommella	Unknown plant	---	Brazil	?	GQ919136	GQ919109	GQ919179
TCP24-1	unknown	*S. lycocarpum*	Broom	Brazil	S	GQ919135	GQ919107	GQ919181
TCP33-1	R. Ploetz	*T. cacao*	Broom	Brazil	C	GQ919138	GQ919088	GQ919180
TCP44-5	R. Ploetz	*T. cacao*	Broom	Brazil	C	GQ919140	GQ919093	GQ919182
TCP90	C. Suarez-Capello	*T. cacao*	Broom	Ecuador	C	GQ919142	GQ919089	GQ919183
TRDC15	L. Johnson	*T. cacao*	Broom	Trinidad	C	GQ919144	GQ919086	GQ919184
TRDC74	L. Johnson	*T. cacao*	Broom	Trinidad	C	GQ919143	GQ919087	GQ919185
Moniliophthora sp. (isolate	A. Pommella	Liana	Hanging litter	Brazil?	L	GQ919151	GQ919115	GQ919186
M. roreri C21	H. Evans	*T. cacao*	---	Costa Rica	N/A	AY916746	GU183377	AY91747
M. roreri var. *gileri* DIS116e	---	*T. gileri*	---	Ecuador	N/A	GU183375	GU183376	GU183378
M. roreri var. *gileri* IMI 389649	H. Evans	*T. gileri*	---	Ecuador	N/A	AY230255	---	---

Table 2-1. Continued.

Isolate	Collector/Donator	Host origin	Collection tissue	Geographic origin	Biotype	Genbank		
						ITS	IGS	RPB1
Moniliophthora sp. MCA2500	C. Aime	*Bouteloua eriopoda*	---	New Mexico	N/A	AY916754	---	AY916755
Chaetocalathus liliputianus (Outgroup)	not known	not known	---	---	N/A	AY571032	---	AY916683

Table 2-2. Maximum parsimony statistics for the ITS, IGS, and RPB1 datasets.

Locus	# accessions	# total characters	# indel characters	# PIC[a]	# nodes with increased support[b]	Mean increase in support[c]
ITS	40	678	35	122 (18.0)	10 (83)	3.9
IGS	36	861	41	300 (34.8)	3 (43)	2.7
RPB1	39	747	---	58 (7.8)	3 (27)	-3.7

[a] Number of parsimony informative characters, with percent of total characters in parentheses. [b] Number of nodes that display increased bootstrap support after indel characters were included, with percent of total nodes with >50% BS support in parentheses. [c] Mean percent increase of bootstrap support across all nodes with >50% BS

Table 2-3. Statistics from phylogenetic analyses

	ITS	IGS	RPB1	ITS+IGS	RPB1+ITS	RPB1+IGS
# characters	678	861	747	---	---	---
# PIC[a]	122	300	45	---	---	---
# PIC in *M. perniciosa* isolates[b]	22	68	13	---	---	---
# trees	18	200	200	---	---	---
# steps	391	475	230	---	---	---
CI	0.903	0.931	0.948	---	---	---
RI	0.893	0.934	0.911	---	---	---
ILD *P*-value [c]	---	---	---	0.002	0.002	0.002
I_{MF} [d]	---	---	---	0.038	0.251	0.341
Clade	Statistical support[e]					
1	59/-	95/-	63/-	---	---	---
1a	41/-	---	64/-	---	---	---
1b	49/50	---	87/84	---	---	---
2	55/62[f]	83/92	-/-	---	---	---
3	96/88	100/99	87/82	---	---	---

[a] # parsimony informative characters. [b] Excluding *M. roreri*, *M. roreri* var. *gileri*, *Moniliophthora* sp. MCA2500, and *C. liliputianus* [c] Incongruence length difference (ILD) [d] Mickevich-Farris index (I_{MF}). [e] Based on 1000 boostrap replications (MP/ML) [f] ML analysis supported the grouping of TCP8 and CPB5 only

Table 2-4. Topological congruence tests for single region data sets.

Data set with constraints	Tree length	Gain	Loss	Net	$P=$[a]
1. ITS	391				
a. IGS strict consensus tree	402	12	1	11	0.0023
b. IGS 70% bootstrap tree	399	8	0	8	0.0078
c. IGS clade 1	403	13	1	12	0.0013
d. IGS clade 2 (CPB5, CPB8, TCP8)	391	0	0	0	---[b]
e. IGS clade 3 (CPB6, CPB10, CPB12)	391	0	0	0	---
f. RPB1 strict consensus tree	495	104	0	104	<0.0001
g. RPB1 70% bootstrap tree	397	7	0	7	0.0384
h. RPB1 clade 1+2	411	20	0	20	0.0004
i. RPB1 clade 1a (CPB1, CPB2, CPB5, CPB15, TBOC3-2, TCP33-1, TCP44-5, TCP90, TRDC15, TRDC74, 4., 7., 17., 28., 31., 46PR, 73-31, 73-62)	398	7	0	7	0.0384
j. RPB1 clade 1b (CPB3, CPB4, CPB14, CPB17, CPB20, CPB21, CPB22, TCP24-1, 92-10-7)	392	1	0	1	---
k. RPB1 clade 3 (CPB9, CPB8, CPB12)	391	0	0	0	---
2. IGS	475				
a. ITS strict consensus tree	510	36	1	35	<0.0001
bITS 70% bootstrap tree	500	25	0	25	<0.0001
c. ITS clade 1	478	5	2	3	0.3340
d. ITS clade 1b (CPB3, CPB4, CPB14, CPB15, CPB17, CPB20, CPB21, CPB22, TCP24-1)	475	0	0	0	---
e. ITS clade 2(CPB5, CPB8, TCP8)	475	0	0	0	---
f. ITS clade 3 (CPB6, CPB7, CPB9, CPB10, CPB12)	489	16	2	14	0.0010
g. RPB1 strict consensus tree	571	96	0	96	<0.0001
h. RPB1 70% bootstrap tree	495	23	3	20	0.0020
i. RPB1 clade 1+2	524	56	5	61	<0.0001
j. RPB1 clade 1a	504	35	6	29	<0.0001
k. RPB1 clade 1b	476	2	1	1	0.5637
l. RPB1 clade 3	527	53	1	52	<0.0001
3. RPB1	230	---			
a. IGS strict consensus tree	238	8	0	8	0.0384
b. IGS 70% bootstrap tree	238	8	0	8	0.0384
c. IGS clade 1	235	5	0	5	0.0253
d. IGS clade 2	234	4	0	4	0.0455
e. IGS clade 3	230	0	0	0	---
f. ITS strict consensus tree	237	7	0	7	0.0196
g. ITS 70% bootstrap tree	235	5	0	5	0.0339
h. ITS clade 1	233	3	0	3	0.0833
i. ITS clade 1b	230	0	0	0	---
h. ITS clade 2	234	4	0	4	0.0455

Table 2-4. Continued.

Data set with constraints	Tree length	Gain	Loss	Net	*P*=
i. ITS clade 3	230	0	0	0	---

[a] *P*-values are based on the Wilcoxon signed-rank test of the number of steps gained or lost by using the corresponding topological constraints. $P<0.05$ indicate the topology is significantly less parsimonious than the trees from the data set being tested. [b] WSR cannot be used for topologies with 0 or 1 step changes.

Table 2-5. Macroscopic morphological characters of select accessions of *Moniliophthora perniciosa*.

Pileus characteristics							
Accession	Host	Shape	Diameter[a]	Color	Margin	Surface	Flesh
CPB1	*Theobroma cacao*	Conic turning extremely convex, broadly umbulate	7 (13) 20	Rose center turning to cream with white margins	Margins inrolled, becoming planar to uprolled; entire becoming undulating or eroded	Dull and dry, wrinkled	Cream colored, yellows after harvest
TRDC74	*T. cacao*	Conic, broadly umbellate	7 (9) 16	Crimson center fading to creamy-yellow margins	Margins inrolled	Dull and dry, wrinkled	Creamy-yellow colored, y
CPB2	*T. grandiflora*	Conic to catenulate, becoming slightly uplifted/convex, broadly umbellate	4 (9) 22	Crimson center fading to cream margins	Margins inrolled, becoming planar to uprolled; entire becoming undulating or eroded	Dull and dry, wrinkled	Cream colored, yellows after harvest
CPB 15	*S. lycocarpum*	Conic turning extremely convex, broadly umbellate	7 (16) 25	Pale crimson center to light rose or cream margin	Margins inrolled, becoming planar to uprolled; entire becoming undulating or eroded	Dull and dry, wrinkled	Cream colored, yellows after harvest
CPB5	*S. cernum*	Conic to catenulate, sometimes uplifted, broadly umbellate	11 (18) 32	Uniform rose color becoming rose in center fading to cream at margins	Margins inrolled, becoming planar to uprolled; entire becoming undulating or eroded	Dull and dry, wrinkled	Cream colored, yellows after harvest
CPB9	Malpighiaceae	Conic to catenulate, becoming slightly uplifted/convex; broadly umbellate	12 (14) 22	Pale crimson center to light rose or cream margin	Margins inrolled, becoming planar to uprolled; entire becoming undulating or eroded	Dull and dry, wrinkled	Cream colored, yellows after harvest
CPB12	*Heteropterys acutifolia*	Conic to catenulate, , broadly umbellate	7 (9) 10	Pigment present, but altered from preservation	Margins inroled becoming planar; entire becoming undulating or eroded	n/a	Cream colored
CPB10	Liana, Malpighiaceae	Conic to catenulate, , broadly umbellate	6 (8) 10	Pigment bleached from preservation	Margins inroled becoming planar; entire becoming undulating or eroded	n/a	Cream colored

Table 2-5. Continued

Stipe characteristics									
Accession	Host	Width			Length[a]	Color	Shape	Flesh	Surface
		Base	Center	Apex					
CPB1	*Theobroma. cacao*	0.5 -3.0	…	0.5 - 2	5 (8) 10	Dark crimson base to cream at apex	Round, slightly swollen base and apex	Hollow	striated
TRDC74	*T. cacao*	1.0-2.0	0.5-1.0	0.5-1.0	7 (9) 10	Dark crimson base to cream at apex	Round, slightly swollen base and apex	Hollow	striated
CPB2	*T. grandiflora*	0.9	0.5	0.5	5 (8) 12	Dark crimson base to cream at apex	Round, slightly swollen base and apex	Hollow	striated
CPB 15	*S. lycocarpum*	1.0-2.0	1.0-2.0	1.0-2.0	5 (7) 8	Dark crimson to rose at apex	Equal	Hollow	striated
CPB5	*Solanum. cernum*	1.0-1.5	.	1.5-4.0	6 (11) 14	Rose to crimson base to cream-pink at apex	Tapered from apex to base	Hollow	striated
CPB9	Malpighiaceae	0.5-1.8	0.5-1.8	0.5-1.8	10 (13) 17	Dark crimson base to cream at apex	Equal	Hollow	striated
CPB12	*H. acutifolia*	1.0	1.0	1.0	3 (3) 4	Crimson base to cream at apex	Equal	Hollow	striated
CPB10	Liana, Malpighiaceae	1.0	1.0	1.0	7 (only one stipe intact)	Crimson base to ream at apex	Equal	Hollow	striated

Lamellae characteristics								
Accession	Host	Length[a]	Color	Attachment	Spacing	Margin	Face	Lamellulae
CPB1	*Theobroma. cacao*	2 (3) 5	Cream	Adenate	Distant, 2 (4) 6	Smooth to undulating	Powdery	Present, 0-3
TRDC74	*T. cacao*	3 (3) 4	Creamy-yellow	Adenate	Distant, 1 (2) 4	Smooth to undulating	Powdery	Present, 0-1
CPB2	*T. grandiflora*	.	Cream	Adenate	Distant	Smooth to undulating	Powdery	Present, 0-3
CPB 15	*S. lycocarpum*	3 (4) 4	Cream	Adenate	Distant, 2 (3) 4	undulating	Powdery	Present, 0-3
CPB5	*Solanum. cernum*	.	Cream	Adenate	Distant, 2 (4) 9	Smooth to undulating	Powdery	Present, 0-3
CPB9	Malpighiaceae	1 (2) 3	Cream	Adenate	Distant, 1 (3) 4	Smooth to undulating	Powdery	Present, 0-3
CPB12	*H. acutifolia*	n/a	Cream	Adenate	Distant, 2 (3) 3	Smooth to undulating	n/a	Present, 0-1
CPB10	Liana, Malpighiaceae	n/a	Cream	Adenate	Semi-distant 1 (1) 2	Smooth to undulating	n/a	Present, 0-1

[a] Measurements are given as minimum (mean) maximum, in µm

Table 2-6. Cystidia, basidia, and basidiospore characters of select accessions of *Moniliophthora perniciosa* isolates.

Cystidia characteristics			Pileocystidia				Cheilocystidia			
Accession	Host		Shape	Pigment	Melzer's	Dimensions[a]	Shape	Pigment	Melzer's	Dimensions
CPB1	*T. cacao*	1[b]	clavate, trichoderm, clamps abundant	red pigment, cytoplasmic and incrusted, many hyaline	dextrinoid incrustations	30 (39) 52 x 7(7) 8	pyriform, swollen, clamped at base	hyaline	none	17 (25) 33 x 12(16) 25
		2	---[c]	---	---	28 (44) 60 x 5 (7) 8	pyriform, lageniform (bottle-shaped) to swollen elliptical	---	---	17(27) 40 x 10 (12) 22
TRDC74	*T. cacao*	1	clavate, elliptical to obclavate to mucorate; clamps abundant	elliptical cystidia darkly cytoplasmic ally pigmented; incrustations	dextrinoid incrustations	25 (40) 52 x 5 (9) 17	clavate to swollen; scarce	hyaline	none	20 (27) 38 x 7 (12) 17
		2	---	---	---	28 (39) 60 x 5 (9) 17	---	---	---	18 (30) 45 x 7 (10) 12
CPB2	*T. grandiflorum*	1	clavate, obclavate to mucorate	red pigment cytoplasmic, some hyaline, sometimes clamped; pigmented cystidia are thickwalled/ incrusted	dextrinoid incrustations ; 1 case of dextrinoid cytoplasmic	20 (36) 48 x 10 (8) 15	clavate	hyaline	none	17 (25) 30 x 8 (10) 12

Table 2-6. Continued.

Accession	Host		Shape	Pigment	Melzer's	Dimensions[a]	Shape	Pigment	Melzer's	Dimensions
		2	clavate, trichoderm, cylindrical to obclavate, mucorate; clamps abundant	red pigment cytoplasmic (thickwalled) and incrusted (thin walled)	dextrinoid cytoplasmic and incrustations	25 (54) 99 x 5 (6) 8	swollen elliptical to mucorate, lageniform	---	---	17 (23) 32 x 8 (12) 13
CPB 15	*S. lycocarpum*	1	clavate, trichoderm to obclavate to mucorate; clamps abundant	Cytoplasmic pigmentation	---	19 (42) 69 x 5 (7) 13	Pyriform, lageniform	Hyaline	None	22 (28) 34 x 9 (11) 14
CPB5	*S. cernum*	1	swollen clavate, often clamped, second cell swollen	red pigment often incrusted, sometimes cytoplasmic; often darker in tip of cell	dextrinoid in incrustations	18 (36) 57 x 7 (11) 15	clavate to obclavate to slightly mucorate, scarce	hyaline	none	23 (30) 42 x 8 (12) 17
		2	clavate, swollen, a few cylindrical; rarely mucorate; clamps abundant	pigment darker at tip of cell; cytoplasmic and incrusted	dextrinoid in incrustations	23 (40) 72 x 7 (9) 13	---	---	---	18 (26) 32 x 7 (10) 13

Table 2-6. Continued.

Accession	Host		Shape	Pigment	Melzer's	Dimensions	Shape	Pigment	Melzer's	Dimensions
CPB9	Malpighiaceae	1	clavate, elliptical to obclavate to mucorate; clamps abundant	elliptical cystidia darkly cytoplasmic ally pigmented; incrustations	dextrinoid cytoplasmic, incrustations	18 (38) 54 x 5 (8) 13	pyriform, napiform	hyaline	none	17 (23) 30 x 8 (10) 12
		2	clavate, elliptical to obclavate to mucorate; clamps abundant	elliptical cystidia darkly cytoplasmic ally pigmented; incrustations	---	28 (48) 93 x 5 (10) 12	pyriform, napiform, rarely mucronate	hyaline	none	23 (25) 29 x 6 (8) 9
CPB12	*Heteropterys acutifolia*	1	clavate, cylindrical, rarely obclavate	mostly hyaline, very few with light cytoplasmic pigment	---	40 (77) 112 x 6 (12) 19	swollen clavate to pyriform	hyaline	none	23 (28) 33 x 8 (10) 13
CPB10	Liana, Malpighiaceae	1	Clavate, trichoderm, rarely obclavate	Cytoplasmic pigment	---	43 (68) 99 x 5 (7) 12	Obclavate to pyriform, rare, subtle difference from basidia	hyaline	None	23 (27) 33 x 9 (11) 14
		2	Clavate, trichoderm, rarely obclavate	Cytoplasmic pigment	---	40 (78) 130 x 5 (8) 12	None observed	hyaline	None	---

Table 2-6. Continued.

Basidia characteristics			Basidia				Basidiospores			
Accession	Host		Shape	# cells	Dimensions	Color	Shape	Surface	Melzer's	Dimensions
CPB1	*T. cacao*	1	clavate	2-3	.	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	8 (9) 10 x 3 (5) 7
		2	---	4	17 (20) 27 x 7 (8) 10	hyaline	---	---	---	7 (9) 10 x 3 (5) 7
TRDC74	*T. cacao*	1	clavate	no sterigmata seen	---	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	8 (10) 12 x 5 (5) 7
		2	---	no sterigmata seen	18 (23) 25 x 5 (7) 8	hyaline	---	---	---	8 (9) 12 x 5 (5) 8
CPB2	*T. grandiflora*	1	clavate	mostly 4	20 (23) 27 x 5 (7) 8	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	8 (9) 12 x 5 (5) 7
		2	clavate	mostly 4	18 (20) 22 x 7	hyaline	---	---	---	8 (10) 12 x 3 (5) 5
CPB 15	*S. lycocarpum*	1	Clavate	no sterigmata seen	21 (24) 25 x 8 (8) 9	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	9 (10) 13 x 4 (5) 6
CPB5	*S. cernum*	1	clavate	4	17 (20) 27 x 7 (8) 10	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	8 (10) 12 x 5 (6) 7
		2	clavate	4	18 (24) 28x 7 (8) 8	hyaline	elliptical to amygdaliniform	thinwalled, smooth	none	8 (11) 12 x 5 (5) 7
CPB9	Maphigeaceae	1	clavate	No sterigmata seen	20 (23) 27	hyaline	elliptical to amygdaliniform	thinwalled, smooth	none	7 (9) 10 x 3 (5) 5
		2	clavate	2-4 celled	25 (28) 29	hyaline	elliptical to amygdaliniform	thinwalled, smooth	none	6 (8) 10 x 4 (5) 5
CPB12	*Heteropterys acutifolia*	1	clavate	2-4 celled	25 (27) 30 x 6 (7) 8	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	9 (11) 13 x 5 (6) 6

Table 2-6. Continued.

Accession	Host		Shape	# cells	Dimensions	Color	Shape	Surface	Melzer's	Dimensions
CPB12		2	clavate	no sterigmata seen	23 (24) 28 x 5 (7) 8	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	9 (11) 13 x 5 (6) 6
CPB10	Liana, Malpighiaceae	1	clavate	4-celled	25 (29) 30 x 6 (7) 8	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	10 (11) 13 x 6 (6) 8
		2	clavate	4-celled	no clear measures	hyaline	elliptical to amygdaliniform, apiculate	thinwalled, smooth	none	

[a] Measurements are given as minimum (mean) maximum, in µm
[b] Two basidiocarps were examined when available. When possible, basidiocarp 2 was older than basidiocarp 1
[c] Dashes denote identical description as basidiocarp 1

Table 2-7. Dimensions of basidiospores of select accessions of *Moniliophthora perniciosa*

		Spore dimensions (µm)		
Accession	Clade	Length	Width	Ratio
TRDC74	1a	9.6±0.2 b[a]	5.2±0.1 cd	1.9±0.0 b
CPB1	1a	8.9±0.1 c	5.0±0.1 d	1.8±0.0 b
CPB2	1	9.5±0.2 b	5.1±0.1 d	1.9±0.0 b
CPB15	1	9.9±0.2 b	5.9±0.1 b	1.9±0.0 b
CPB5	2	10.6±0.1 a	5.4±0.1 bc	2.0±0.0 b
CPB9	2/3	8.4±0.2 d	4.7±0.1 e	1.8±0.0 a
CPB10	3	10.7±0.1 a	6.0±0.1 a	1.8±0.0 b
CPB12	3	10.9±0.1 a	5.8±0.1 a	1.9±0.0 b

[a] Values within columns with the same letter are not significantly different based on Fisher's LSD, *P*=0.05

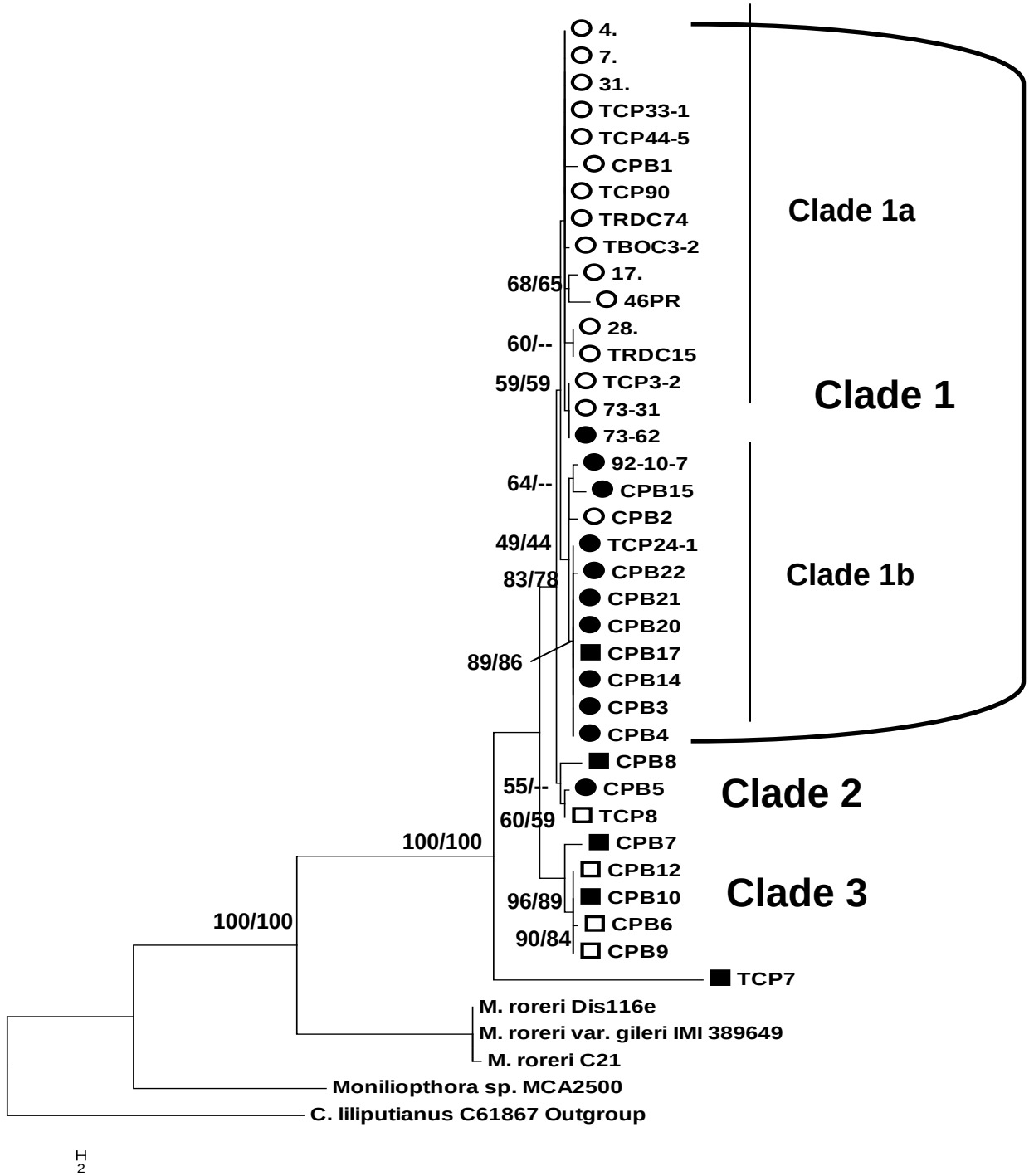

Figure 2-1. Phylogenetic relationships among 36 accessions of *Moniliophthora perniciosa* based on ITS sequence data. The phylogram represents one of 18 most parsimonious trees (391 steps, CI=0.903, RI=0.893). Support values are bootstrap values over 50% including indels coded as characters (before slash), and data set treating indels as missing data (second slash). Isolates are coded for biotype as follows (open circle=C, filled circle=S, open square=*Heteropterys* and other Malpighiaceae, closed square=L)

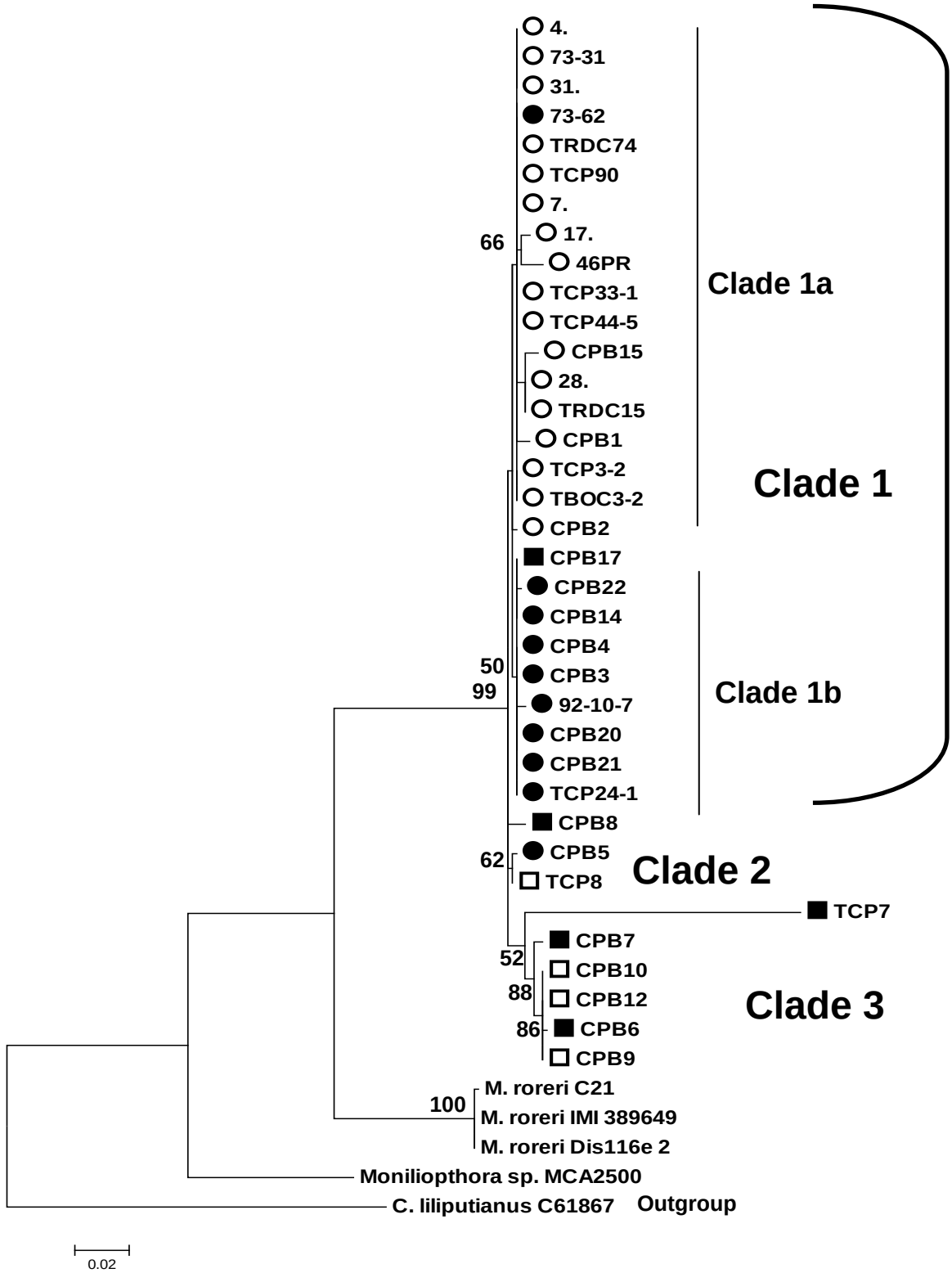

Figure 2-2. Phylogenetic relationships among accessions of *Moniliophthora perniciosa* resolved using ML analysis of ITS sequence data, ln score = -2472.65. Support values are bootstrap values over 50%. Isolates are coded for biotype as follows (open circle=C, filled circle=S, open square=*Heteropterys* and other Malpighiaceae, closed square=L)

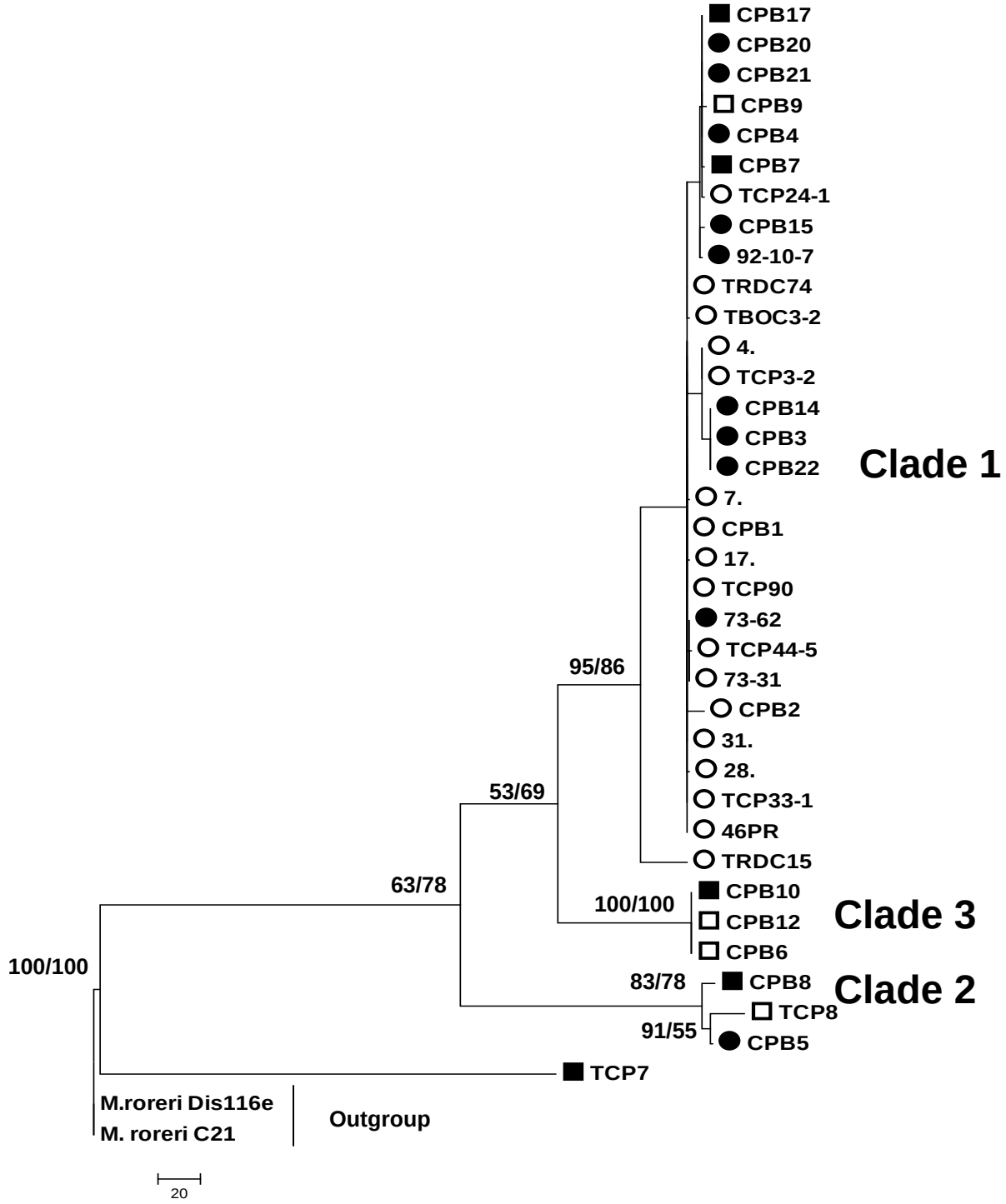

Figure 2-3. Phylogenetic relationships among 36 accessions of *Moniliophthora perniciosa* based on IGS sequence data. The phylogram represents one of 200 most parsimonious trees (475 steps, CI=0.931, RI=0.934). Support values are bootstrap values over 50% including indels coded as characters (before slash), and data set treating indels as missing data (second slash). Isolates are coded for biotype as follows (open circle=C, filled circle=S, open square=*Heteropterys* and other Malpighiaceae, closed square=L).

Figure 2-4. Phylogenetic relationships among accessions of *Moniliophthora perniciosa* \resolved using ML analysis of IGS sequence data, ln score = -3023.85. Support values are bootstrap values over 50%. Isolates are coded for biotype as follows (open circle=C, filled circle=S, open square=*Heteropterys* and other Malpighiaceae, closed square=L)

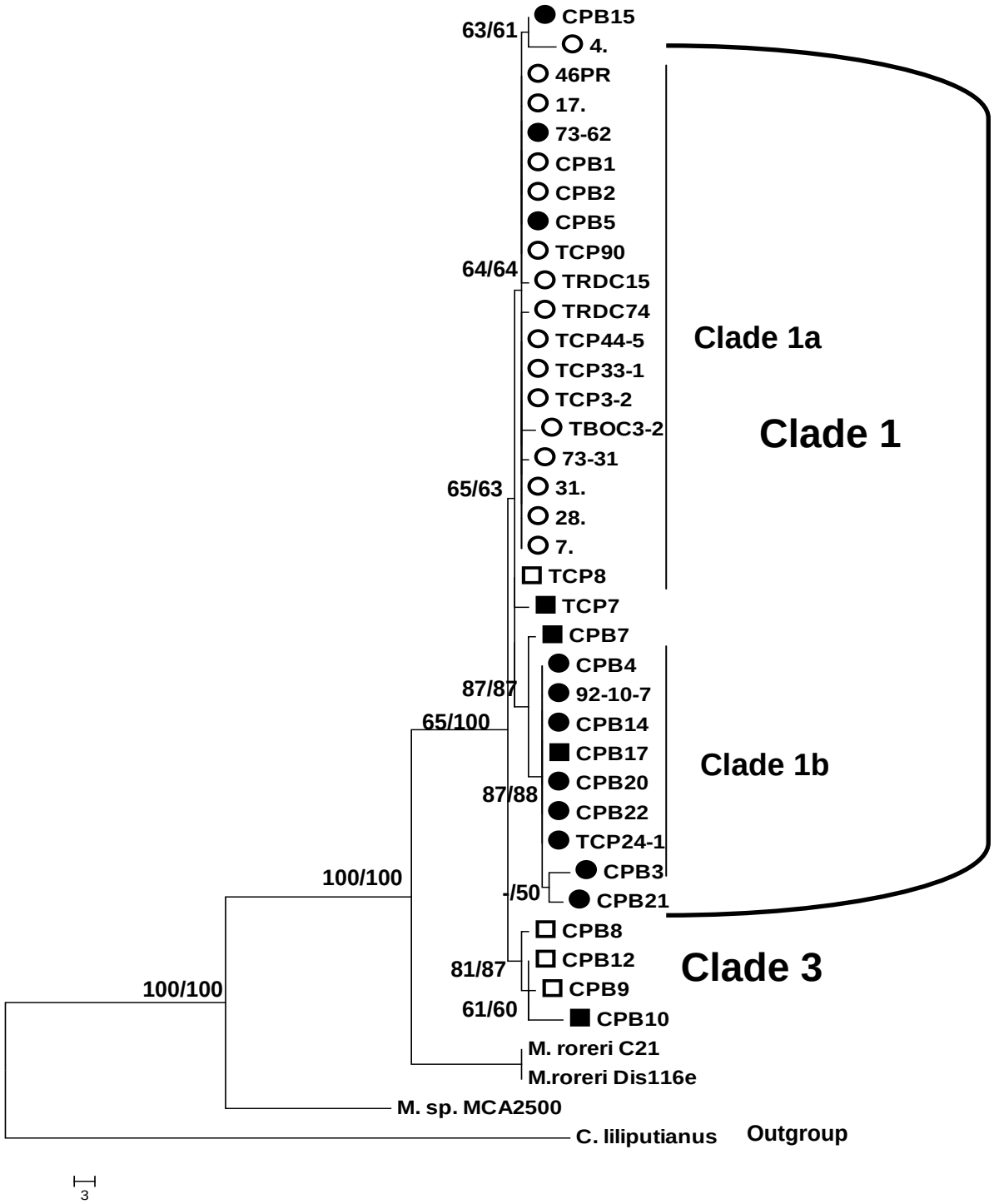

Figure 2-5. Phylogenetic relationships among 35 accessions of *Moniliophthora perniciosa* based on RPB1 sequence data. The phylogram represents one of 198 most parsimonious trees (230 steps, CI=0.948, RI=0.911). Support values are bootstrap values over 50% including indels coded as characters (before slash), and data set treating indels as missing data (second slash). Isolates are coded for biotype as follows (open circle=C, filled circle=S, open square=*Heteropterys* and other Malpighiaceae, closed square=L)

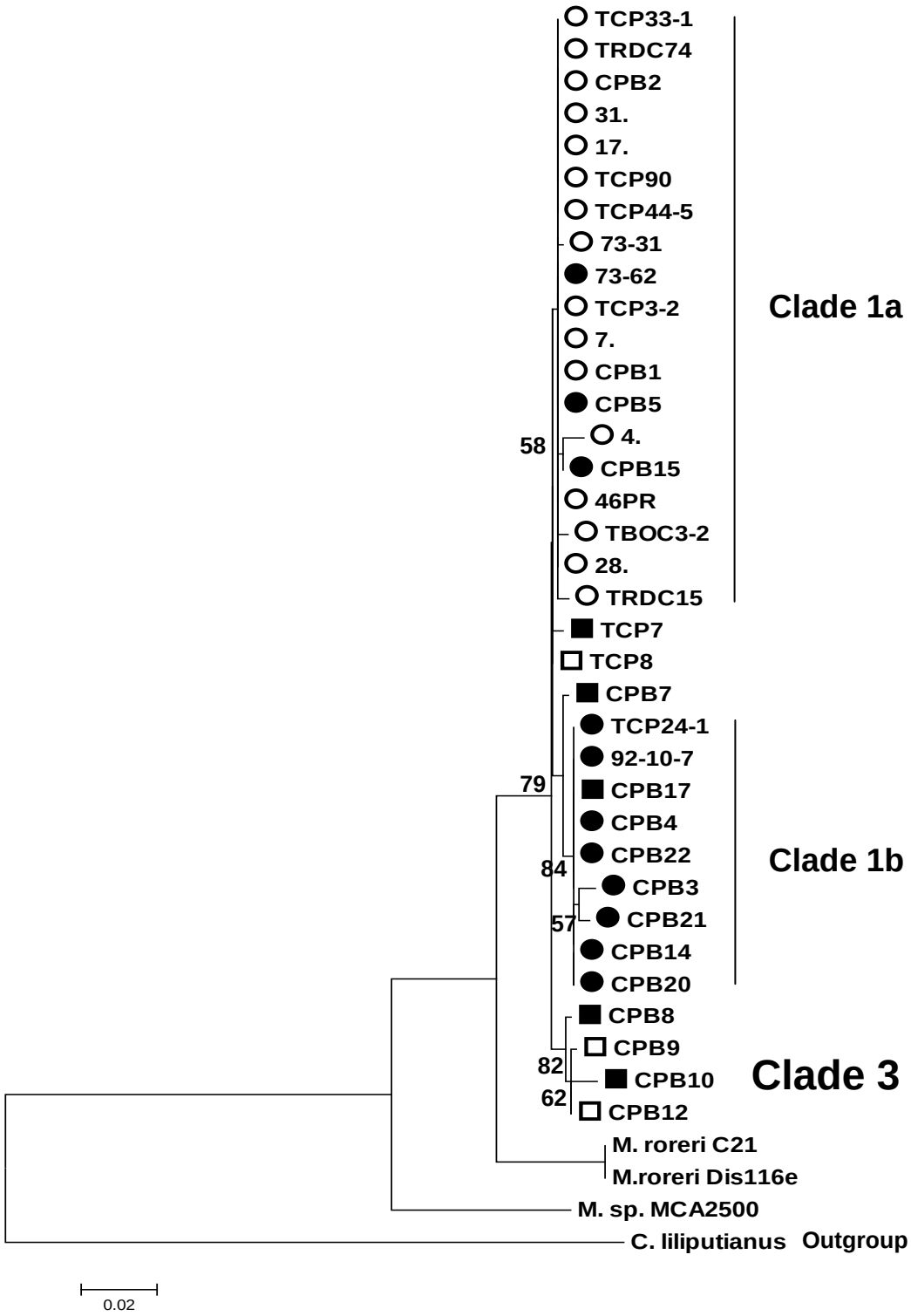

Figure 2-6. Phylogenetic relationships among accessions of *Moniliophthora perniciosa* resolved using ML analysis of RPB1 sequence data, ln score = -2124.22. Support values are bootstrap values over 50%. Isolates are coded for biotype as follows (open circle=C, filled circle=S, open square=*Heteropterys* and other Malpighiaceae, closed square=L)

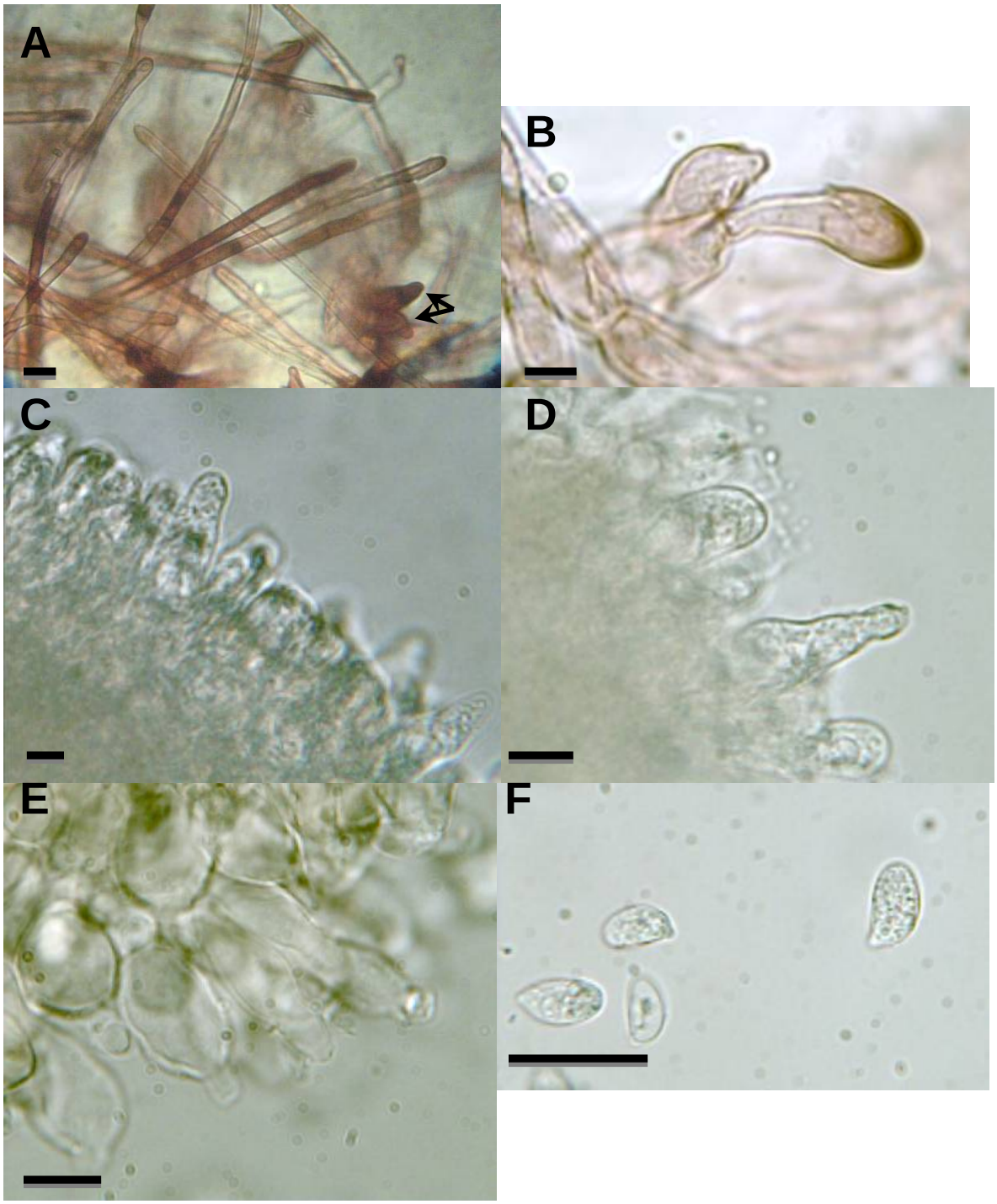

Figure 2-7. Microscopic characteristics of accessions of *Moniliophthora perniciosa*. A) Pileocystidia of isolate TRDC74 showing long, clavate-elliptical cystidia, as well as shorter mucorate cystidia (arrow); B) Obclavate pileocystidia of CPB5 with swollen second cell and dark pigmentation at tip; C) Obclavate to slightly pyriform cheilocystidia of CPB10; D) Swollen, pyriform cheilocystidia of CPB12; E) Lageniform (bottle-shaped) cheilocystidia of CPB15; F) Typical basidiospores (CPB10). Bars=10µm.

CHAPTER 3
USE OF PHYLOGENIES FROM MULTIPLE DNA REGIONS TO ASSESS THE DIVERGENCE OF LINEAGES OF *COLLETOTRICHUM GLOEOSPORIOIDES* SENSU LATO THAT ARE ASSOCIATED WITH MANGO ANTHRACNOSE IN SOUTH FLORIDA

Introduction

Mango (*Mangifera indica*) is an important fruit crop in the tropics and subtropics. The FAO (Food and Agricultural Organization) estimated world production of the fruit in 2007 at over 33 million tons (FAOSTAT 2009 statistics, http://faostat.fao.org/). Asian nations are the largest producers, including India, China and Thailand. Mexico is the largest producer in the Americas, with 2.05 million tons in 2007, and is also the world's largest exporter. Although production in Florida is marginal in comparison (2500 tons in 1997) (Mossler and Nesheim 2002), many commercially important cultivars were developed in the state, beginning with the release of 'Haden' in 1912, which served as a female parent for 'Tommy Atkins', 'Lippens', 'Zill', and several other cultivars (Knight and Schnell 1994). A high level of crop diversity exists in south Florida due to the array of germplasm that was introduced to the area (Schnell et al. 2006).

In humid growing regions, anthracnose is the most prevalent threat to mango production. Although anthracnose also affects leaves, it is most important on panicles, where it causes reduced fruit set, and on fruit, where postharvest losses can be severe if management measures are not implemented. Three taxa have been associated with these diseases: *Colletotrichum gloeosporioides* (worldwide), *C. gloeosporioides* var. *minor* (Australia, Simmonds 1965), and *C. acutatum* (Australia, Fitzell 1979; Taiwan, Weng and Chuang 1995; and Homestead, Florida, Riveras-Vargas et al. 2006).

The presence of a mango-specific population of *C. gloeosporioides* was suggested by several researchers in the 1990s. Hodson et al. (1993) showed with restriction fragment length

polymorphisms (RFLPs) of mitochondrial (mtDNA) and ribosomal (rDNA) DNA that isolates of *C. gloeosporioides* from mango were the only ones from four tropical fruit species that could be grouped by host. Further studies with RFLPs and random amplification of polymorphic DNAs (RAPDs) identified a distinct mango population when anthracnose isolates from tropical fruits in Sri Lanka and Australia were compared (Alakahoon et al. 1994, Hayden et al. 1994). Inoculation studies have shown that isolates of *C. gloeosporioides* usually cause symptoms on diverse fruit hosts, regardless of the original host, but that greater disease usually develops on the original host (Alakahoon et al. 1995, Sanders and Korsten 2003).

Prior work in south Florida investigated tissue specialization within populations of *C. gloeosporioides* on mango. Pectic zymograms of 63 isolates from leaves, inflorescences, and immature and mature fruit revealed 10 pectinase profiles that were somewhat related to host tissue (Gantotti and Davis 1993). For example, 75% of the immature fruit isolates had one profile, and 44% and 53% of the mature fruit and leaf isolates had another profile. A follow-up study with RAPDs and pathogenicity tests on fruits and inflorescences also suggested tissue specificity among isolates from mango (Davis 1999). One population tended to come from inflorescences and was less virulent on leaves and mature fruit than inflorescences. However, no phenetic or cluster analysess were performed with these data.

Since the 1990s, no subsequent work has been conducted to characterize mango-associated *C. gloeosporioides* in south Florida or elsewhere. Additional work is warranted to qualify and quantify phylogenetic and biological relationships for *C. gloeosporioides* populations from mango and other hosts, as are contemporary investigations of the tissue-specificity of mango-specific populations.

Colletotrichum gloeosporioides var. *minor* was first associated with mango anthracnose by Simmonds (1965), who distinguished it from *C. gloeosporioides* as having slightly narrower conidia (3.7 μm vs. 4.8 μm), despite the considerable overlap he observed in conidium width between the two taxa. Cox and Irwin (1988) later reported that conidium widths for *C. gloeosporioides* var. *minor* were 3.0-4.2 μm compared to 4.5-5.5 μm for *C. gloeosporioides*. Both taxa had unlobed or slightly lobed appressoria. Given these slight differences, *C. gloeosporioides* var. *minor* is no longer recognized.

Taxonomic classification within *Colletotrichum* was traditionally based on morphology or host specificity. The last thorough revision of the genus was completed by von Arx (1957), who sought to condense the hundreds of species that had been erected based largely on host origin into morphologically defined species. For fungi, the morphological species concept can be highly artificial, and morphology has repeatedly been shown to do a poor job of organizing phylogenetically or biologically relevant taxa, a generalization that holds true for *Colletotrichum* (Sutton 1992, Du et al. 2005). Cryptic species, in which phylogenetically distinct taxa cannot be separated by morphology, are common and especially problematic (Hawksworth 2001, Kohn 2005). Currently, more meaningful species that fit the evolutionary species concept (Wiley 1978, Taylor et al. 2001), and which incorporate phylogenetic analyses of molecular data, host specialization, and the traditional biological species concept (i.e. interfertility within species), are sought by mycologists and plant pathologists.

In von Arx's (1957) revision of *Colletotrichum*, approximately 600 previously described species were placed in synonymy with *C. gloeosporioides*, even though extensive morphological and host diversity were noted. *Colletotrichum gloeosporioides* sensu lato is currently considered a species complex that affects hundreds of plant hosts and contains several described species,

including *C. musae* (cause of banana anthracnose), *C. fragariae* (strawberry crown rot), and *C. kahawae* (coffee berry disease) (von Arx 1957, Sutton 1980, Sutton 1992). Although slight morphological differences have been reported for these host-specific species, phylogenetic analyses consistently place them in the *C. gloeosporioides* sensu lato clade (Sreenivasaprad et al. 1996a, Munaut et al. 2002, Martinez-Culebras et al. 2003, Du et al. 2005). The extent to which these taxa are separated phylogenetically and the emphasis given to host-specificity would determine whether they remain distinct species or are considered subspecific groups within the *C. gloeosporioides* species complex. Although the previously described mango biotype appears to be another example of a host-specific lineage within *C. gloeosporioides* senus lato, additional phylogenetic analysis is needed to determine if it represents a distinct taxon and, if so, the appropriate level of its classification.

The objective of this study was to determine whether mango-specific groups exist within *C. gloeosporioides* sensu lato. Phylogenetic analyses using DNA sequence data from several regions were used to characterize relationships among isolates of *C. gloeosporioides* from different mango organs, as well as other host species. Morphological and pathological data were assessed for isolates that represented the different phylogenetic taxa that were identified in the study. The case for a mango-specific taxon in *C. gloeosporioides* sensu lato is discussed, as are evolutionary patterns in *Colletotrichum*.

Materials and Methods

Collection and Storage of Isolates

A hierarchical sampling scheme was used to collect isolates. During June-July 2007, symptomatic mango leaves, peduncles, and mature fruit, as well as asymptomatic immature fruit and peduncles were collected in two groves at the University of Florida Tropical Research and Education Center (TREC), one of mixed cultivars and the other of cv. ‘Keitt’. During March-

August 2008, inflorescences, leaves, immature fruit and mature fruit from mango, as well as symptomatic fruit from *Persea americana* (avocado), *Musa* (banana), *Averrhoa carambola* (carambola), *Psidium guajava* (guava), and *Carica papaya* (papaya) were collected in plantings at TREC and elsewhere in Miami-Dade County.

Up to three symptomatic organs were chosen from each tree included in the study, from which up to three lesions were sampled, from which three acervuli were sampled, resulting in up to 27 subsamples per organ from each tree. Lesions were excised from tissues and surface disinfested (10 s 70% EtOH, 2 min bleach, rinsed in sterile H_2O) and placed in a moist chamber (plastic Petri plate with moist filter paper). After 2-3 days, conidia were collected from acervuli with a sterile needle and placed on half-strength PDA. Isolated colonies were streaked onto fresh PDA plates and single, germinated spores picked off and plated on PDA. Up to three asymptomatic, immature fruit were sampled from each tree included in the study. Three pieces of tissue, approximately 5x5 mm were excised and surface disinfested as described above. Tissues pieces were then placed in molten (ca. 50°C) PDA to ensure maximum surface area contact with tissue and media. *Colletotrichum* isolates growing out of tissue pieces were streaked onto fresh PDA, and single germinated spores were picked off and plated on PDA. All single-spore cultures were stored directly onto filter paper stored at 4ºC and in 10% glycerol stored at -80ºC. At least one isolate from each organ was stored for future analysis (Table 3-1).

DNA Extraction and PCR of the ITS, MAT1-2, and CGTT5 Regions

Molecular analyses were conducted with 51 isolates of *Colletotrichum* spp. that represented all mango organs and isolates from TREC and other locations (Table 3-2). Also included were three isolates from avocado, four from banana, three from carambola, three from guava, two from papaya, one from passionfruit and one from *Piper betle*. *Colletotrichum acutatum* and *C. magna* were used as outgroup taxa.

Isolates were grown for 3-5 days in PDB at room temperature (23-25ºC) on a shaker at ca. 80 rpm. Approximately 150 mg mycelia were harvested and triple rinsed with sterile deionized water and dried on sterile filter paper. DNA was extracted using a DNA genomic preparation protocol from the University of Wisconsin Biotechnology Center. Briefly, mycelia were ground in 500 µl Shorty DNA Extraction Buffer (0.2M Tris-HCl, pH 9.0, 0.4M LiCl, 25mM EDTA, 1% SDS) and incubated at 68°C for 10 minutes. Tissue was centrifuged at 14,000 rpm for 5 minutes, 400 µl of the supernatant was transferred to new tube and the DNA was precipitated with 400 µl 99% isopropanol. The tubes were centrifuged for 10 minutes at 14,000 rpm and the supernatant was decanted. The DNA pellets were air-dried for 5 minutes then resuspended in 400 µl TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) for 30 minutes at room temperature. 2µl DNA was used in PCR reactions.

Three nuclear regions were used in phylogenetic analyses: the widely used ITS1-5.8S-ITS2 (ITS); a gene that encodes the high mobility group-box (HMG) region of the MAT1-2 mating locus; and CGTT5, a locus that was cloned from *C. gloeosporioides* and exhibits homology to a hypothetical protein from *Gibberella zeae*, *Magnaporthe grisea*, and *Neurospora crassa.* The MAT1-2 locus has previously been shown to be more useful than ribosomal sequences or protein-coding gene introns in phylogenetic studies of closely related taxa at the subgeneric level (Turgeon, 1998; Du et al., 2005). The CGTT5 marker was developed by cloning a 895-bp band from isolate Cg49 of *C. gloeosporioides* that was generated with the $(TCC)_5$ RAPD primer (Appendix A); it contained a higher number of phylogenetically informative sites than the ITS and MAT1-2 regions (Table 3-3).

All PCR reactions were carried out in 50 µl reactions that contained: 38.25 µl of sterile distilled, deionized water; 6.5 µl ThermoPol Reaction Buffer (New England Biolabs, Ipswich,

MA); 1 µl 10 mM dNTP mix (New England Biolabs, Ipswich, MA); 0.25 µl Taq DNA polymerase (conc. 5,000 units/ml); 1 µl each of 15 µM primers; and 2 µl DNA template. Standard cycling parameters with a 55°C annealing temperature were used. The ITS region was amplified with the ITS1 (Gardes and Bruns 1993) and ITS4 (White et al. 1990) primers, the MAT locus was amplified with primers HMGgloF1 and HMGgloR1 (Du et al. 2005), and CGTT5 with primers pTT5F and pTT5R (Appendix A). Direct Sanger sequencing of both strands of the PCR products was performed at the University of Florida ICBR Facility with the above primers. For ambiguous bases, the base that agreed with the consensus sequence was retained. Because sequencing signal from direct sequencing was strong, cloning was not performed.

Phylogenetic Analyses

Sequences were aligned with CLUSTALX (Thompson et al. 1994) in Mega4 and default parameters, and alignments were adjusted manually. Both maximum parsimony (MP, using PAUP* v.4.0b; Swofford 2000) and maximum likelihood (ML, using GARLI v.0.946) analyses were conducted for all data sets, and gaps were treated as missing data. Parsimony analyses were conducted using heuristic searches with 100 random addition replicates, with tree bisection reconnection (TBR) swapping, saving no more than two trees with tree scores greater than five per repetition. Consistency (CI) and retention indices (RI) values were calculated with PAUP*. To determine statistical support for groups in the phylogenies, the non-parametric bootstrap test (Felsenstein 1985) was performed using 1,000 repetitions and heuristic search criteria as described above except that 10 random addition replicates were used, saving no more than two trees with scores greater than five per repetition.

ML analyse were with GARLI v.0.946, using default parameters and a randomly generated starting topology, and were stopped after $5x10^6$ generations or a 0.01 decrease in ML score.

Three independent ML runs were performed and resulting topologies were compared in TreeView v1.6.6. If similar topologies were generated, bootstrap analysis was performed using the same parameters with 1,000 repetitions. Tree files from analyses were imported into MEGA 4.0 for visualization and to edit phylograms. For the ITS and MAT1-2 analyses, *C. acutatum* and *C. magna* were included as outgroups. Because CGTT5 sequences for taxa outside of *C. gloeosporioides* sensu lato were not available, analysis of the CGTT5 data set was not rooted.

Congruence of Molecular Data Sets and Combined Analysis

In order to determine if combining data from the three loci was appropriate, levels of congruence were tested between pairs of the data sets and between topologies of phylogenetic trees (Seelanen et al. 1997, Johnson and Soltis 1998). Overall incongruence between data sets was quantified using the Mickevich-Farris index (I_{MF}) (Mickevich and Farris 1991) and the incongruence length difference (ILD) between loci (Farris 1994) using the partition homogeneity test in PAUP*. This test was run with 500 replicates of 10 random addition sequences, and $P < 0.05$ indicated significant incongruence between data sets. The ILD test is known to be very sensitive (Yoder et al. 2001; Darlu and Lecointre 2002), so the decision as to whether to combine data sets was largely based on examination of topology and bootstrap support.

To assess topological congruence, an approach similar to that used by Seelanen et al. (1997) was followed. First, consensus trees for each of the three regions were compared empirically to determine if instances of topological incongruence were "hard" (terminology of Seelanen et al. 1997 for isolates that resolve in different well-supported clades in different topologies), or "soft" (due to weak phylogenetic signal in one of the data sets). To determine the statistical significance of topological incongruence, topological constraints were applied to each DNA region corresponding to the 70% bootstrap consensus tree for the other two regions. The Wilcoxon signed-rank (WSR) test was applied to determine if the number of steps that were

gained or lost in the resulting tree was significantly different than the original trees (P=0.05, as described by Templeton 1983). In addition, strongly supported clades that were resolved in each single region analysis were used as single constraints for the other loci (Table 3-5). This approach allowed congruence of specific clades to be tested in each data set, and identified individual clades and taxa that caused incongruence between data sets. This approach helped determine if data sets should be combined, and provided phylogenetic information about evolutionary events that resulted in incongruence, such as cryptic outcrossing, hybridization, introgression and horizontal gene transfer (Wendel and Doyle 1998). Loci that were determined to be largely congruent were combined and analyzed using parsimony and maximum likelihood analysis as described above. For these analyses, accessions with missing sequences were pruned from the combined data set.

Morphology Studies

Thirteen isolates representing genetic clades that resolved in the phylogenetic analyses were characterized morphologically. The shapes and dimensions of conidia and hyphopodia are used to define *Colletotrichum* species and were shown recently to be phylogenetically useful (Du et al. 2005). Conidia were harvested from 7-day-old PDA cultures. Hyphopodia (vegetative appressoria) were produced by growing isolates over sterile glass coverslips at the edge of PDA colonies. The shapes and dimensions of conidia and hyphopodia were determined with a Leitz Laborlux 2 microscope and an ocular micrometer.

Pathogenicity Studies

For all pathogenicity tests, isolates were retrieved from -80ºC storage and grown on PDA for 7-10 days at room temperature (23-25ºC) and ambient light. Spores were harvested by flooding plates with sterile deionized water, scraping colonies, and straining suspensions through

a layer of sterile cheesecloth. Inoculum was adjusted to $5x10^5$ to $1x10^6$ conidia•ml^{-1} in sterile deionized water.

Field inoculations were carried out at TREC in two experimental groves: one of cv 'Keitt' and one of mixed cultivars. Trees were spaced 4 m apart in rows 7 m apart. 'Keitt' tree height was approximately 4 m; trees in the mixed cultivar block were approximately 6 m. Trees were chosen for experiments based on having sufficient numbers of organs at the appropriate developmental stage, and 4-5 trees were included in each experiment. At the end of each experiment, lesion margins were excised from leaves, surface disinfested, and plated on PDA to confirm presence of the pathogen.

Blossom blight

In 2009, mouse-ear to green-colored stage inflorescences (Schoeman et al. 1995) were inoculated in the field with 13 isolates; 10^6 conidia·ml^{-1} of each isolate was applied with a handheld manual spray bottle. Treatments (isolates and a water control) were replicated four (expt 3, cv 'Sensation') or five (expts 1 and 2, cv 'Keitt') times on single-inflorescence experimental units in a randomized complete block design (RCBD), where blocks were individual trees. Inflorescences were sprayed until runoff and then covered with plastic bags inside brown paper bags for 48 hrs. Severity ratings were taken six times for four weeks after inoculation, using a synoptic key (key 1.5, James 1971). Analysis of variance of the area under the disease progress curve (AUDPC) and y_{max} (highest disease severity) were performed for each experiment. Mean separations were performed using least significant differences (PROC GLM, SAS v.9.0, Cary, NC).

Leaf anthracnose

During June-July, 2009, two experiments were conducted on cv. 'Keitt' at TREC. Leaves on newly opened vegetative shoots were inoculated in the field as described for infloresences,

except that plastic bags were omitted due to high summer temperatures. Ten (expt 2) or 14 (expt 1) treatments (nine or 13 *C. gloeosporioides* isolates and a water control) were replicated four time in RCBDs, where buds and the subtending five leaves were experimental units and blocks were single trees.

Disease severity was measured after 4, 7, 10 and 14 days with a synoptic key (key 2.1.2, James 1971). Experiments were terminated after 14 days due to severe defoliation in some treatments. Analyses of variance of AUDPCs and y_{max}s were performed with PROC GLM in SAS. Least significant differences (PROC GLM, SAS) were used to separate means.

Fruit pathogenicity

Detached fruit assays. Imported, heat-treated fruit of cv. 'Tommy Atkins' were obtained from a local packinghouse (LimeCo, LLC, Naranja, FL). Treatments (seven *C. gloeosporioides* isolates and a noninoculated control) were replicated five times in a completely randomized design (CRD). Depending on fruit size, each fruit was treated at two or three points, each of which was wounded with a sterile needle and covered subsequently with 15µl droplets of sterile 0.3% water agar that did not contain (control) or contained 10^6 conidia·ml^{-1} of a given isolate (i.e., 15,000 conidia per inoculated site). Fruit were incubated in plastic sweater boxes on wire mesh over moistened paper towels at 25°C in the dark, and lesion diameters were measured in two directions at a right angle after 4, 5, 6, and 7 days. The experiment was conducted four times. Analyses of variance of AUDPCs and final lesion diameters were performed with PROC GLM in SAS, and least significant differences were used to separate means.

Attached fruit assays. In 2009, single experiments were conducted in the field on attached fruit of three cvs, 'Haden' (susceptible), 'Tommy Atkins' (moderately resistant) and 'Van Dyke' (moderately resistant). Immature fruit (ca 8 cm in dia) were surface disinfested (10 s 70% ethanol, 2 min 10% bleach, rinse with sterile H_2O) and dried before they were treated

individually with either one of seven *C. gloeosporioides* isolates or a water control; fruit were misted with a suspension of $5x10^5$ conidia·ml^{-1} sterile water or sterile water, and covered with brown paper bags. Bags were removed when fruit were harvested at maturity. Disease severity was rated four times over 10 days after harvest with a synoptic key (Corkidi et al. 2005). Single fruit were experimental units, treatments were replicated four times in RCBDs, and trees were blocks. Analyses of variance of AUDPCs and y_{max}s were performed with PROC GLM in SAS, and least significant differences were used to separate means.

Results

Phylogenetic Analyses

CGTT5 was more informative than either the ITS or MAT regions (Table 3-3). Overall, 61 PICs were found at the CGTT5 locus vs 27 and 39 for, respectively, ITS and MAT (Table 3-3). Moreover, all CGTT5 PICs were found in ingroup accessions (11.4% of all characters), whereas only 11 (2.0%) and 13 (6.0%) of the respective ITS and MAT PICs were found in the ingroups used in those analyses.

Most of the 65 accessions of *C. gloeosporioides* sensu lato in the ITS analyses were from mango, and *C. acutatum* and *C. magna* were used as outgroup taxa (Table 3-2). The data set contained 557 characters and MP analyses resulted in 11 trees with 112 steps (CI=0.938, RI=0.949). A MPT that agrees with the majority-rule consensus tree is shown in Figure 3-1, and the ML tree is shown in Figure 3-2 (ln score=-1071.9). In the ITS-based phylogeny, *C. musae* isolates were in a highly supported clade (93% MP, 98% ML) with equivocal placement either within (ML analysis) or sister to the rest of (MP analysis) the *C. gloeosporioides* isolates. Several clades were resolved with low to moderate bootstrap support. Clade 1 (40% MP) contained all mango isolates from blighted panicles and immature fruit, as well as most leaf isolates. Subclade 1a was well supported (86% MP, 61% ML) and contained four leaf isolates

and three immature fruit isolates from various groves. Clade 2 (75% MP) contained most isolates from mature fruit, peduncles and leaves, as well as single isolates from banana, guava and passionfruit. The remaining isolates of *C. gloeosporioides* did not resolve into a specific clade in the ingroup.

The MAT1-2 locus was analyzed in 58 accessions of *C. gloeosporioides* sensu lato, most of which were from mango (Table 3-2). *Colletotrichum acutatum* and *C. magna* were outgroup taxa. The data set included 216 characters. MP analysis resulted in 28 MPT with a tree score of 146 (CI=0.966, RI=0.981), one of which is shown in Figure 3-3. ML analysis resulted in the tree in Figure 3-4 (ln score=-1166.49). Clade 1 in the MAT1-2 analysis contained the same isolates as Clade 1 in the ITS phylogeny, and resolved in a larger clade (92% MP) that contained isolates of *C. musae* and isolate Gua 3. It was moderately supported in MP analysis (72% bootstrap), but the ITS subclades 1a and b were not resolved. All other isolates did not resolve into separate clades, but were part of the general ingroup clade. The organ sources for clades of mango isolates were similar in the MAT1-2 and ITS phylogenies.

The unrooted analysis of the CGTT5 region included 58 accessions of *C. gloeosporioides* sensu lato. The data set contained 535 characters, and MP analysis resulted in two MPT with a treelength of 73 (CI=0.959, RI=996) (Figure 3-5). ML analysis resulted in a tree of similar topology with a ln score of -1166.49 (Figure 3-6). Isolates of *C. musae* and Gua3 resolved with those in Clade 1 in the MAT phylogeny (100%MP, 99% ML). However, isolates from ITS Clade 2 also resolved with these isolates. Subclade 1a, similar to that in the ITS phylogeny, was resolved with CGTT5 data (82% MP, 79% ML), but it contained several different isolates; only isolates Cg34, Cg141, Cg164, and Cg165 were common to subclade 1a in both the ITS and CGTT5 phylogenies. CGTT5 data resolved two additional clades. Clade 2 had high MP support

(92%), and included a subset of eight isolates from the ITS Clade 2. Clade 3 contained the remaining isolates in the analysis (86% MP, 87% ML), except for two from carambola, Cm1 and Cm3.

In general, Clade 1 was resolved in all phylogenies and was well supported in the MAT1-2 and CGTT5+MAT analyses (Table 3-4). It was comprised of only mango isolates, including all from inflorescences and immature fruit, and 59 to 63% of the leaf isolates, depending on the region. However, a low percentage of isolates from mango fruit (27 to 33%) and peduncles (0 to 14%) resolved in this clade.

The remaining isolates of *C. gloeosporioides* resolved differently in the different analyses without clear location or host associations. The ITS Clade 2 was resolved with strong support within the larger general *C. gloeosporioides* clade, and contained 21 isolates, including seven from peduncles (78% of that total) and six from mature fruit (55%). In the MAT1-2 phylogeny, about one-half the isolates were not resolved, and included six of the mature fruit isolates (67%), all peduncle isolates, and isolates from other fruit hosts. Four of the peduncle isolates (57%) were resolved in the Clade 2 CGTT5 phylogeny. Less than half the isolates from any mango organ were found in any other clade/phylogeny.

Congruence Tests and Combined Analysis

Data-set-based congruence measures suggested that all sets were incongruent (Table 3-3). The MAT and ITS data were significantly heterogenous, but with less significance than the ITS and CGTT5 data sets. Using the 70% bootstrap trees as a limit, only the MAT1-2 and CGTT5 data sets did not display significant levels of incongruence or instances of hard incongruence (Table 3-5). Therefore, these two data sets were combined and analyzed.

Individual clades (MAT1-2 Clade 1, MAT1-2 Clade 1+*C. musae*+Gua3, ITS Clade 1-1a, ITS Clade 2, CGTT5 Clade 1-1a, CGTT5 Clade 2, and CGTT5 Clade 3) were also used as

constraints to determine where cases of hard incongruence were present between data sets (Table 3-5). With the ITS topology constrained on the MAT data set, only Clade 2 produced barely significant incongruence. ITS Clade 2 was significantly incongruent when constrained on the CGTT5 data set. Clade 1 and subclade 1a were congruent over every data set.

The CGTT5+MAT combined data set had 54 ingroup accessions with *C. acutatum* and *C. magna* as outgroup taxa. MP analysis resulted in 63 trees with a length of 224 (CI=0.91, RI=0.986), one of which is shown in Figure 3-7. The ML tree is shown in Figure 3-8 (ln score=-2075.2). Clade 1 and subclade 1a were highly supported (99/97% and 87/85% MP/ML, respectively), and the monophyly of Clade 1+*C. musae* +Gua3 was moderately supported (78% MP). Clades 2 and 3 were identical to those resolved in the CGTT5 phylogeny, and had moderate support (76% MP, 74% ML and 69% MP, 68% ML, respectively). A new clade, which contained carambola isolates Cm1 and Cm3, resolved with lower support (60% MP, 66% ML).

Morphology Studies

Conidia of the 13 isolates that were examined were typical of *C. gloeosporioides*: straight, cylindrical and sometimes tapered toward the base or constricted in the middle, and from10-20 x 3-6 µm, with an average of 13-15 x 4 µm (Table 3-6, Figure 3-9). Significant differences existed among isolates, but there was no relationship between conidium size and genetic clade (Table 3-7). Hyphopodia were irregular (lobed) or clavate-shaped and smooth (Table 3-6, Figure 3-10). With the exception of isolate Cg136, isolates from Clade 1 had smooth hyphopodia, whereas those from other clades had irregular hyphopodia. Hyphopodia ranged from 5-16 x 4-11 µm in size with an average of 8-10 x 6-9 µm, which was slightly shorter than that reported by Sutton (1992). Significant differences also existed in hyphopodia size, but without relationships between size and genetic clade (Table 3-7).

Pathogenicity studies

During blossom blight experiments, isolates had highly significant impacts on AUDPC and y_{max} (P<0.0001) (Table 3-8). Also, with the exception of Cg136, isolates in Clade 1 caused significantly greater disease than isolates in Clades 2 and 3. Less disease developed with Cg136 than for other Clade 1 isolates in Experiment 1 and 2, probably due to the limited numbers of conidia that this isolate produced during these experiments and the corresponding low volumes of inoculum and poor panicle coverage that resulted. Isolates in Clades 2 and 3 did not produce significant disease compared to the water control, and were considered nonpathogenic. In general, isolates in Clade 1 caused disease severities above 50% 28 days after inoculation (y_{max}), whereas isolates in Clades 2 and 3 caused severities less than 20% (Table 3-8). All pathogenic isolates produced similar blossom blight symptoms (Figure 3-11). Numerous small lesions coalesced, resulting in large areas of necrosis. Necrotic panicle tips curled downward to produce a shepherd's crook symptom. Florets often senesced, and when severities exceeded 75% the entire panicle usually senesced.

Leaf inoculation experiments produced similar results: isolates in Clade 1 caused greater disease than isolates in Clades 2 and 3, although these differences were not as pronounced as in the blossom experiments. In both experiments, block and treatment were significant (P<0.0001) (Table 3-9). Except for isolate Cg138 in Experiment 1, all Clade 1 isolates caused more severe leaf anthracnose than the water control. All other isolates of *C. gloeosporioides* were slightly to not pathogenic, and caused disease severities similar to those in the water control treatments. With the exceptions of isolates Cg134 and Cg129, isolates in Clade 1 caused severities greater than 20%, whereas severities for all other isolates were less than 10% (Figure 3-12).

For experiments with detached fruit, isolates generally had significant impacts on AUDPC and lesion diameter (Table 3-10). Although all isolates of *C. gloeosporioides* produced greater

disease than the water control, the significance of and rank orders for disease development were inconsistent for isolates in the different experiments (Figure 3-13). Average lesion diameters after 7 days were approximately 10-20 mm, but reached 30 mm.

Results from attached fruit inoculations generally agreed with resistance levels that have been reported for the tested cultivars (Crane et al. 2003), in that y_{max} values for the susceptible 'Haden' were higher (0.1-0.87) than for the moderately resistant 'Tommy Atkins' (0.1-0.34) and 'Van Dyke' (0.05-0.26) (Table 3-11). On 'Haden', isolate treatments were significant for AUDPC and y_{max} (respectively, P=0.0013 and 0.0018), but they were not significant for 'Tommy Atkins' and were significant on 'Van Dyke' for only AUDPC (P=0.0224). Isolate Cg141 (Clade 1) caused the greatest disease on all three cultivars, and isolates in Clade 1 produced greater disease than other isolates, although these differences were not always significant (Table 3-11). Although more disease developed on inoculated than on control fruit (Figure 3-13), these differences were usually not significant due to variation in these data.

Discussion

This study examined the occurrence and identification of a mango-specific taxon within *C. gloeosporioides* sensu lato. Alakahoon et al. (1994), Hayden et al. (1994) and Hodson et al. (1993) had suggested such a taxon, but relied on scant data obtained with old techniques. Likewise, tissue specificity had been previously indicated among mango isolates with isozyme and RAPD profiles (Davis 1999, Gantotti and Davis 1993). Considering the importance of these research topics, a modern and comprehensive re-examination of previous work was warranted. The current study used both phylogenetic and biological data to investigate the presence of distinct evolutionary species associated with mango anthracnose. The ecological and epidemiological dynamics of the three clades that were resolved in this work is discussed below.

With ITS, MAT1-2, and CGTT5 sequence data, a mango-specific biotype (Clade 1) was resolved, and was well supported in both the MAT1-2 and CGTT5+MAT phylogenies. Clade 1 contained only mango isolates, and isolates from mango inflorescences and asymptomatic immature fruit were found in no other clade. Isolates from ripe fruit and young leaves of mango were found in Clade 1, but were also found in Clades 2 and 3; isolates from necrotic peduncles were restricted to the latter clades. Isolates outside Clade 1 could be considered generalists as they came from mango and several other hosts. The prevalence of isolates in Clades 2 and 3 on mature mango fruit and peduncles suggests that host-specific factors were no longer present when they were recovered from these tissues. Unlike isolates in Clade 1, these isolates did not consistently resolve across all data sets, and hard incongruence was found among the clades that did resolve (Clade 2 between the ITS and CGTT5 data sets). While these incongruencies could be due to lineage sorting among recently diverged lineages, they could also indicate that recombination has occurred among these strains.

The placement of *C. musae* relative to the other *C. gloeosporioides* clades was equivocal in this study. In the ITS MP phylogeny, *C. musae* isolates formed a strongly supported clade sister to the *C. gloeosporioides* clade, but in the other phylogenies it resolved within *C. gloeosporioides.* Since the placement of *C. musae* within the larger, more general *C. gloeosporioides* clade differed by locus, it is difficult to speculate on its relationship to other clades.

Carambola isolates Cm1 and Cm3 resolved as sister to all other isolates of *C. gloeosporioides*, although this placement was not always statistically supported. It is possible that isolates of *C. gloeosporioides* causing anthracnose on carambola form another host-

specialized lineage that has diverged from the host-general isolates of *C. gloeosporioides* sensu lato. Further characterization of isolates from carambola is necessary to investigate this question.

Pathogenicity experiments supported the separation of the mango-specific from the host-general clades. Isolates from blossom blight and leaf anthracnose samples all fell in Clade 1, whereas isolates from all three clades were associated with fruit anthracnose. Since isolates in Clade 1 also caused severe symptoms on inflorescences in pathogenicity studies, this host-specific group appears to play a role in blossom blight development in South Florida. This contradicts previous reports from South Africa that attributed blossom blight of mango to *Neofusicoccum* spp., and indicated that *C. gloeosporioides* caused only small lesions on mango panicles (Darvas 1993, Lonsdale and Kotzé 1993).

Since the role of *Neofusicoccum* spp. in blossom blight development in South Florida has not been investigated, these fungi may also be involved with this disease here. However, based on the slight symptoms that *C. gloeosporioides* caused on mango panicles in South Africa, it is possible that host-general isolates outside Clade 1 were used in that work. Although additional work would be needed in South Africa to confirm this hypothesis, results from the present study illustrate the importance of cryptic lineages in the mango anthracnose and blossom blight pathosystems: erroneous conclusions about the etiology and epidemiology of these diseases are clearly possible when pathogens and nonpathogens are obscured and experimental work is conducted with generalist rather than host-specific isolates.

Arauz (2001) indicated that anthracnose on mango fruit is a monocyclic disease that begins with latent infection of immature fruit, similar to that described for avocado fruit (Prusky and Lichter 2007, 2008). On avocado, symptoms develop after fruit maturation in response to several pathogen and host factors, including the production of the enzymes laccase and pectate

lyase (Yakoby et al.2001b, Guetsky et al.2005, Kramer-Haimovich et al. 2006), and the decrease of antifungal compounds in the fruit pericarp (Guetsky et al. 2005).

In mango, Droby et al.(1986, 1987) described preformed antifungal resorcinols in the mango fruit pericarp that were associated with latent infections of *Alternaria alternata*, cause of black spot. Fungitoxic levels of two compounds, 5-12-cis-heptadecenyl resorcinol and 5-pentadecenyl resorcinol, were present in the peel of 'Haden' prior to ripening, and as ripening commenced, resorcinol concentrations declined and postharvest decay began (Prusky and Keen, 1993). To date, 15 resorcinols have been identified in mango peels (Knödler et al. 2007). Recently, Hassan et al. (2007) correlated concentrations of these compounds and the levels of anthracnose that developed on fruit of several different mango cultivars.

Because isolates in Clade 1 were the only ones that infected panicles, leaves and immature fruit, it is possible that they have a higher tolerance to these antifungal compounds than other lineages. Although it appears only isolates in Clade 1 latently infected immature fruit, isolates from all clades were recovered from, and caused anthracnose on, ripening fruit. Thus, anthracnose that is caused by isolates in Clades 2 and 3 does not appear to originate from latent infections. Work to confirm this observation with a larger set of isolates is warranted as it contradicts a long held view that anthracnose on mango fruit always results from latent infections. The sensitivity of isolates in the different clades to resorcinols that are found in immature mango peels should also be determined, as it may significantly clarify the etiology of this important disease. Furthermore, resorcinol concentrations in other mango organs (leaves and inflorescences) should be assessed to investigate whether they play a role in infection by isolates in Clades 1, 2 and 3 and the disease that they cause or do not cause on different mango tissues. For example, are Clade 1 isolates capable of causing blossom blight and leaf anthracnose due to

their insensitivity to high resorcinol concentrations that occur in those tissues? A more thorough understanding of these relationships is needed.

In addition to assessing resorcinol sensitivity in isolates in the different clades, other host-pathogen interactions should be investigated. The production of pectate lyase, alkalization of host tissue in the area of infection, and sugar and ammonia production have been reported as important factors in the infection of, and disease development on, avocado fruit (Yakoby et al.2001b, Guetsky 2005, Kramer-Haimovich et al.2006); whether they also play a role in the mango pathosystems should be examined. Whether isolates in Clade 1 produce a phytotoxin, such as that reported for the *C. gloeosporioides*/yam pathosystem (Abang et al. 2009), should also be assessed, as it could explain the severe symptoms that these isolates produce on mango panicles.

By combining results from isolation, pathogenicity and phylogenetic studies, the etiology, ecology and epidemiology of the mango pathosystems were clarified in the present work, and areas of research that were needed were indentified. Pathogenic species of *Colletotrichum* are considered hemibiotrophs, with a short period of biotrophic colonization after infection (with a longer biotrophic period when fruit are latently infected), followed by a switch to necrotrophic growth that coincided with symptom development (Bailey et al. 1992). Additionally, pathogenic species of *Colletotrichum* are often associated with endophytic or saprotrophic host colonization of alternative hosts (Perez et al. 2005, Freeman et al. 2001, Photita et al. 2005), indicating that individuals in this genus can alternate among endophytic, saprotrophic and pathogenic lifestyles.

Direct evidence supporting this hypothesis has been presented by several studies. In a phylogenetic study of *Colletotrichum* species isolated from *Magnolia lilifera*, there was no resolution separating pathogenic, saproptrophic, and endophytic strains, indicating that isolates

from different clades could fill different ecological niches depending on environmental factors (Promphutta et al. 2007).

Direct evidence for such flexibility was presented by Promphutta et al. (2007). In a phylogenetic study of *Colletotrichum* spp. that were isolated from *Magnolia lilifera*, pathogenic, saproptrophic and endophytic strains were not resolved, indicating that isolates from different clades could fill different ecological niches depending on environmental factors. A strain of *C. graminicola* that was transformed with the GFP protein colonized corn roots and systemically colonize aerial portions of the plant (Sukno et al. 2008), suggesting that anthracnose can also result from systemic infections. The asymptomatic root infection by this strain exhibited many similarities to infection by recognized root pathogens, suggesting that many fungi, even those recognized as foliar pathogens, maintain the ability for root infection. Another hemibiotrophic foliar pathogen, *Magnaporthe grisea*, also infected roots and systemically colonized rice plants. Many of the rice genes that were upregulated during this process are similar to those that are upregulated during colonization by endophytic arbuscular mycorrhizal fungi (Sesma and Osborne 2004, Güimil et al. 2005). Freeman and Rodriguez (1993) produced a single-gene mutant of *Colletotrichum magna*, path-1, that was nonpathogenic but still able to colonize plants endophytically. Thus, the genetic differenes between endophytes and pathogens may be slight, and pathogenicity and host colonization are controlled by different genes.

Based on the above studies, it appears that the distinction between endophyte and pathogen depends more on symptom development than on differences in host colonization. Indeed, Schulz et al. (1999) suggested that both endophyte-plant and pathogen-plant interactions are characterized by mutual-antagonism, but in the case of pathogens, the interaction is imbalanced and results in disease development. They reported a greater defense response in endophyte-host

reactions, suggesting endophytes are only able to overcome defenses to the point of infection and colonization, but not to the extreme of symptom development. Pathogens have an increased ability to overcome plant defenses (as in laccase production by *C. gloeosporioides* in avocado to overcome antifungal dienes in peels for development of fruit anthracnose; Guetsky et al. 2005). Better understanding is needed of the attributes and circumstances that distinguish the endophytic and pathogenic behavior of fungi.

The present study supports the hypothesis that strains of *Colletotrichum* are ecologically flexible. The recovery of an isolate in Clade 1, En2, from an asymptomatic, developing mango leaf suggests that pathogenic individuals may colonize mango as endophytes. In addition, non-host-specific individuals colonized necrotic mango tissue as saprotrophs. Not only were non-pathogenic isolates in the generalist clade recovered from leaf lesions, but *Colletotrichum* also sporulated on necrotic leaf lesions that were caused by other pathogens (data not shown). The same pattern was reported on coffee, where avirulent *C. gloeosporioides* was recovered along with *C. kahawae* from lesions of coffee berry disease (Beynon et al. 1995, Derso et al. 2003). Derso et al. (2003) suggested that *C. kahawae* had limited saprotrophic capabilities, based on its specialized carbon metabolism (Waller et al. 1993), and that it was displaced in lesions it caused on coffee berries by saprophytic strains of *C. gloeosporioides*. A similar situation may occur with the mango-specific Clade 1, as isolates from other clades were often recovered from old leaf lesions or ripening fruit, but not from immature fruit or panicles. The extent to which generalist, saprotrophic strains of *Colletotrichum* are able to colonize diverse hosts and host tissues warrants further investigation as this information could dramatically increase our understanding of the ecology and epidemiology of pathogenic and non-pathogenic interactions in this genus.

By identifying host-specific and host-general populations of *C. gloeosporioides* and the tissues that they colonize, the present study also sheds light on the development and epidemiology of important anthracnose diseases on mango. Most studies on tropical fruits that have investigated host range indicated that isolates were often most aggressive on the original host, but that generalists caused lesions on a wide range of hosts (Alahakoon et al. 1994, Hayden et al. 1994, Sanders and Korsten 2003). Although host range per se was not assessed in the present study, the phylogentic data corroborate the existence of a general, non host-specific population of *C. gloeosporioides* that causes anthracnose symptoms on diverse fruit hosts. Findings in this study also have ramifications for resistance assessments and which isolates of the pathogen should be used in artificial inoculations. Although it appears that only host-specific populations of *C. gloeosporioides* cause blossom blight and infect fruit latently, diverse populations are capable of causing anthracnose on mature and ripening fruit. Work is needed to assess the reservoirs and levels of the different generalist and host-specific populations of *C. gloeosporioides* that occur on mango and the relative threats that they pose to disease development on different organs.

The three DNA regions used in this study represented ones that have traditionally exhibited the ability to resolve clades at different levels of classification, as well as a third novel region chosen for its high number of polymorphic nucleotide sites. The ITS1-5.8-ITS2 rDNA region is most commonly used in phylogenetic studies of fungi, and is generally able to resolve clades at the species level (Hillis and Dixon 1991, Nilsson et al. 2008). While species can generally be resolved using the ITS region, intraspecific variation is very low, which should make the region a good indicator of cryptic speciation. Several studies suggested that sequence identity of ≥97% indicates an intraspecific relationship (Iwen et al. 2002, Ciardo et al. 2006, Nilsson et al. 2008).

However, assigning a quantitative limit on species definitions for all fungi is an oversimplification, as the amount of intraspecific variation at a specific locus can vary among genera (Bridge 2002).

Within a genus, the level of sequence identity at which two taxonomic units are considered to be distinct species there should be consistent. In this study, there were only 11 PIC within *C. gloeosporioides* sensu lato in the ITS phylogeny. Isolates of *C. musae* differed from the generalists in Clades 2 and 3 by only three nucleotides, and the mango-specific isolates in Clade 1 differed by only one to three nucleotides. Similarly, isolates of *C. fragariae* from strawberry, cyclamen and date palm differed by three nucleotides (MacKenzie et al. 2008), and isolates of *C. kahawae* differed by two nucleotides (Sreenivasaprasad et al. 1996a). Thus, the lack of polymorphic sites in the ITS region limits its utility to resolve such closely related lineages.

In contrast, ITS differentiated *C. gloeosporioides* sensu lato from other species of *Colletotrichum* species that were recovered from fruits in south Florida. For example, isolates of *C. acutatum* that were recovered from mango leaves (see Chapter 4) differed from *C. gloeosporioides* by 48 nucleotide sites and 11 indel sites, isolates of *C. capsici* that were recovered from papaya differed by 32 nucleotides and four indel sites, and isolates of *C. boninense* from passionfruit and *Piper betle* differed by 26 nucleotide substitutions and four indel sites (see Appendix B). A graphical representation is shown in Figure 3-14 of the numbers of polymorphisms that are present in the ITS regions between *C. gloeosporioides* and these taxa.

Mating type sequences have been used in phylogenetic analysis of many fungal taxa, including *Fusarium*, (O'Donnell et al. 2004), *Colletotrichum* (Du et al. 2005), *Ascochyta* (Barve et al. 2003), *Cochliobolus* (Turgeon 1998), and *Neurospora* and *Sordaria* (Poggeler 1999). The former four studies have all used the HMG-box region of the MAT1-2 idiomorph, and indicated

that this region contained more phylogenetically informative characters among closely related taxa than the ITS region. They suggested that this locus could be used to build highly resolved phylogenies among closely related accessions. While between-species variation was relatively high, intraspecific variation was very low, enabling further resolving power among species.

The MAT loci are unique in that instead of alleles, alternate forms of the locus are designated idiomorphs because the genes inhabiting identical positions on the chromosome are not similar and in fact encode different proteins (Moore 1998). The locus does not recombine, and its inheritance has been compared to the human Y chromosome (Merino 1996). The pattern of high interspecific and low intraspecific variability in mating genes is common in many eukaryotes, and rapid evolution in mating genes is thought to accompany speciation events (Ferris et al. 1997). Variation within a biological species is limited by recombination and selection mechanisms. Once speciation occurs and two populations no longer recombine, the MAT locus starts to diverge. Therefore, this region could be used to marry the phylogenetic and biological species concepts in classification. However, a disadvantage of using mating type genes in heterothallic species is that only one of the idiomorphs would be present in an individual and that the locus that is utilized would not be amplified in all mating populations.

A previous study evaluated the use of the HMG box region for phylogenetic analysis among *Colletotrichum* species (Du et al. 2005). They found that the MAT phylogenies followed previously reported patterns (high interspecific and low intraspecific variation), had a stronger phylogenetic signal than ITS, and was better able to resolve relationships among closely related accessions.

Since resolution of closely related strains of *C. gloeosporioides* was desired in the present work, a highly polymorphic locus was needed. To that end, the CGTT5 locus was cloned and

amplified as a sequence characterized amplified region (SCAR) marker (McDermott et al. 2004). BLAST searches indicated that this region displays homology to sequences coding for hypothetical proteins in *Gibberella zeae*, *Magnaporthe grisea*, and *Neurospora crassa*. The CGTT5 data set had 61 PIC among *C. gloeosporioides* isolates, compared to 13 and 11, respectively, for the MAT and ITS data sets. Clades resolved for the CGTT5 region also showed higher bootstrap support. In the resulting phylogeny, all but three isolates resolved into well-supported clades, compared to the ITS and MAT regions where large numbers of isolates did not resolve further into clades within the ingroup. This suggests that this region does provide a stronger phylogenetic signal, and this approach to developing loci is well-suited for analyses involving closely related accessions.

Martinez-Culebras et al. (2002), Du et al. (2005), Bridge et al. (2008), and MacKenzie et al. (2008), and the present results indicated that populations of *C. gloeosporioides* sensu lato have diverged several times to specialize on specific hosts or tissues; for example, *C. musae* on banana, *C. fragariae* on strawberry, *C. kahawae* on coffee and Clade 1 on mango. However, these lineages diverged relatively recently from *C. gloeosporioides*, and it is not entirely clear how and at what taxonomic levels they should be distinguished from this species.

Carbone and Kohn (2001) discussed a population-species interface that distinguishes between recent divergence of populations and older speciation events. Identifying the point on the evolutionary continuum at which populations become species will always be artificial. A consensus has not been reached on where species boundaries exist in *Colletotrichum*.

Taylor et al. (2001) suggested that phylogenies could be supplemented with biological data (ultrastructural characteristics, mating compatibility, pathogenicity) to erect evolutionary species, defined by Wiley (1978) as “single lineage of ancestral descendant populations of organisms

which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate." Biological characters may or may not be of evolutionary significance. For example, host-specificity could clearly influence the ecology of phytopathogens, and probably focused the evolution of the existence of the mango-specific taxon that was identified in the present study. And although conidial dimensions were not distinctive in the present work, hyphopodial morphology tended to distinguish isolates, in that those for isolates in Clade 1 were usually smooth and clavate-shaped vs the lobed appearance of those for isolates in Clades 2 and 3.

This study used a multifaceted approach combining phylogenetic, pathogenicity and morphology data to characterize several lineages that were associated with diseases of mango that are caused by *C. gloeosporioides* sensu lato in south Florida. The results provide new insight into, and suggest several new avenues for, research of the etiology and epidemiology of these diseases.

Table 3-1. Number of trees, samples and single-spore isolates collected in the hierachical sampling scheme from various mango organs and tropical fruit hosts.

	Inflorescences	Leaves	Immature fruit	Mature fruit	Peduncles	Avocado	Banana	Carambola	Guava	Papaya
# trees sampled	5	47	16	36	27	4	6	2	2	7
# organs sampled	11	104	26	60	52	9	6	2	3	7
# isolates stored	25	204	33	101	59	26	18	4	8	30

Table 3-2. Accessions included in study.

Accession	Tissue origin[a]	Recovery location	Clade[b]	Genbank accession number		
				ITS	MAT	CGTT5
En2	Leaf endophyte	TREC, 'Keitt'	1	GQ373201	GQ925061	GQ924960
Cg23	Leaf	TREC, mixed	---	GQ373208	---	---
Cg52	Leaf	TREC, mixed	1	GQ373210	GQ925017	GQ924968
Cg60	Leaf	TREC, 'Keitt'	---	---	GQ925019	---
Cg101	Leaf	TREC, 'Keitt'	3	GQ373215	GQ925023	GQ924974
Cg129	Leaf	TREC, 'Keitt'	2	GQ373218	GQ925026	GQ924977
Cg136	Leaf	TREC, 'Keitt'	1	GQ373225	GQ925032	GQ924982
Cg141	Leaf	USDA	1	GQ373228	---	GQ924985
Cg142	Leaf	USDA	1	GQ373229	GQ925035	GQ924986
Cg145	Leaf	TREC, 'Keitt'	1	GQ373230	GQ925036	GQ924987
Cg151	Leaf	TREC, mixed	3	GQ373234	GQ925042	GQ924991
Cg160	Leaf	TREC, mixed	3	GQ373243	GQ925049	GQ925000
Cg161	Leaf	TREC, 'mixed'	3	GQ373244	GQ925050	GQ925001
Cg162	Leaf	Commercial grove	---	GQ373245	GQ925051	---
Cg163	Leaf	USDA	1	GQ373246	GQ925052	GQ925002
Cg165	Leaf	Commercial grove	1	GQ373248	GQ925054	GQ924004
Cg166	Leaf	Commercial grove	---	GQ373249	---	---
Cg167	Leaf	Commercial grove	3	GQ373250	GQ925055	GQ925005
Cg170	Leaf	Commercial grove	1	GQ373252	GQ925057	GQ925007
Cg171	Leaf	Commercial grove	---	GQ373253	---	---
Cg137	Inflorescence	TREC, 'Keitt'	1	GQ373226	GQ925033	GQ924983
Cg138	Inflorescence	TREC, 'Keitt'	1	GQ373227	GQ925034	GQ924984
Cg147	Inflorescence	TREC, 'Keitt'	1	GQ373231	GQ925037	GQ924988
Cg148	Inflorescence	TREC, 'Keitt'	1	GQ373232	GQ925038	GQ924989
Cg34	Immature fruit	TREC, mixed	1	GQ373206	GQ925014	GQ924965
Cg35	Immature fruit	TREC, mixed	1	GQ373209	GQ925015	GQ924966
Cg36	Mummified fruitlet	TREC, mixed	1	GQ373207	GQ925016	GQ924967
Cg66	Immature fruit	TREC, 'Keitt'	1	GQ373212	GQ925020	GQ924970

Table 3-2. Continued.

Accession	Tissue origin[a]	Recovery location	Clade	Genbank accession number		
				ITS	MAT	CGTT5
Cg75	Immature fruit	TREC, mixed	1	GQ373214	GQ925022	GQ924973
Cg135	Immature fruit	TREC, 'Keitt'	1	GQ373224	GQ925031	GQ924981
Cg158	Immature fruit	Commercial grove	1	GQ373241	GQ925041	GQ924998
Cg131	Mature fruit	TREC, 'Keitt'	1	GQ373223	GQ925029	GQ924979
Cg132	Mature fruit	TREC, 'Keitt'	---	GQ373219	---	---
Cg152	Mature fruit	TREC, 'Keitt'	1	GQ373235	GQ925040	GQ924992
Cg153	Mature fruit	TREC, 'Keitt'	2	GQ373236	GQ925043	GQ924993
Cg154	Mature fruit	USDA	2	GQ373237	GQ925044	GQ924994
Cg155	Mature fruit	USDA	3	GQ373238	GQ925045	GQ924996
Cg157	Mature fruit	USDA	2	GQ373240	GQ925047	GQ924997
Cg169	Mature fruit	TREC, mixed	1	GQ373251	GQ925056	GQ925006
Cg16	Peduncle	TREC, mixed	---	GQ373204	---	---
Cg20	Peduncle	TREC, 'Keitt'	2	GQ373205	GQ925013	GQ924964
Cg55	Peduncle	TREC, 'Keitt'	2	GQ373211	GQ925018	GQ924969
Cg72	Peduncle	TREC, mixed	1	---	---	GQ924971
Cg73	Peduncle	TREC, 'Keitt'	2	GQ373213	GQ925021	GQ924972
Cg127	Peduncle	TREC, 'Keitt'	3	GQ373216	GQ925025	GQ924975
Cg128	Healthy peduncle	TREC, 'Keitt'	3	GQ373217	GQ925027	GQ924976
Cg130	Peduncle	TREC, 'Keitt'	3	GQ373222	GQ925028	GQ924978
Cg133	Healthy peduncle	TREC, 'Keitt'	---	GQ373220	---	---
Cg134	Peduncle	TREC, 'Keitt'	2	GQ373221	GQ925030	GQ924980
Cg149	Peduncle	TREC, 'Keitt'	1	GQ373233	GQ925039	GQ924990
Cg156	Peduncle	Commercial grove	3	GQ373239	GQ925046	GQ924995
Avo1	Avocado	TREC	3	GQ373189	GQ925008	GQ924951
Avo2	Avocado	TREC	3	GQ373190	GQ925009	GQ924952
Avo3	Avocado	TREC	---	GQ373191	---	---
Ban1	Banana	TREC	*C. musae*	GQ373192	GQ925010	GQ924953
Ban2	Banana	TREC	*C. musae*	GQ373193	GQ925011	GQ924954
Ban3	Banana	TREC	*C. musae*	GQ373194	---	GQ924955

Table 3-2. Continued.

Accession	Tissue origin	Recovery location	Clade	Genbank accession number		
				ITS	MAT	CGTT5
Ban4	Banana	TREC	3	GQ373195	GQ925012	GQ924956
Cm1	Carambola	TREC	carambola	GQ373196	GQ925058	GQ924957
Cm2	Carambola	TREC	3	GQ373197	GQ925059	GQ924958
Cm3	Carambola	TREC	carambola	GQ373198	GQ925060	GQ924959
Gua1	Guava	TREC	3	GQ373198	GQ925062	GQ924961
Gua2	Guava	TREC	3	GQ373199	GQ925063	GQ924962
Gua3	Guava	TREC	1	GQ373200	GQ925064	GQ924963
Pap16	Papaya	TREC	---	---	GQ925065	---
Pap17	Papaya	TREC	---	---	GQ925066	---
Pas4	Passionfruit	Farm	---	GQ373202	---	---
Piper3	*Piper betle*	Farm	---	GQ373203	---	---
C. acutatum (outgroup)	---	---	---	DQ003120	DQ002843	---
C. magna (outgroup)	---	---	---	DQ003104	DQ002828	---

[a] Unless another host is specified, tissue origin indicates mango organs from which an isolate was recovered. [b] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny

Table 3-3. Details of phylogenetic analyses.

	Data Set					
	ITS	MAT	CGTT5	ITS+MAT	CGTT5+MAT	ITS+CGGT5
# accessions	65	58	58	---	54	---
# characters	557	216	535	---	751	---
# PIC[a]	27	39	61	---	84	---
# PIC in ingroup[b]	11	13	61	---	---	---
# trees	11	28	2	---	63	---
# steps	112	146	73	---	224	---
CI	0.938	0.966	0.959	---	0.951	---
RI	0.949	0.981	0.996	---	0.986	---
ILD *P*-value[c]	---	---	---	0.008	0.002	0.002
I_{MF}[d]	---	---	---	0.38	0.36	0.52
Clade	Bootstrap support[e]					
1	49/*	72/*	*/*	---	99/97	---
1a	91/61	*/*	97/85	---	87/85	---
2	50/*	*/*	96/*	---	76/74	---
3	*/*	*/*	86/87	---	69/68	---

[a] Parsimony informative characters [b] Number of parsimony informative characters among accessions of *C. gloeosporioides* sensu lato (excluding outgroup taxa) [c] Incongruence length difference (ILD) [d] Mickevich-Farris index (I_{MF}) [e] Bootstrap support values for given clades, MP analysis/ML analysis, *<40%

Table 3-4. Number of isolates by tissue type and phylogenetic clade.

ITS data set						
Tissue	Clade 1			Clade 2	CG	Total
	1	1a	Clade 1 total			
Leaf	8 (39)[a]	4 (20)	12 (59)	5 (26)	2 (11)	19
Inflorescence	4 (100)	0 (0)	4 (100)	0 (0)	0 (0)	4
Immature Fruit	4 (57)	3 (43)	7 (100)	0 (0)	0 (0)	7
Mature Fruit	3 (27)	0 (0)	3 (27)	6 (55)	2 (18)	11
Peduncle	1 (11)	0 (0)	1 (11)	7 (78)	1 (11)	9
Mature fruit (other hosts)	0 (0)	0 (0)	0 (0)	3 (30)	7 (70)	10
Total	19	7	26	21	12	59

MAT1-2 data set				
Tissue	Clade 1	CG	---	Total
Leaf	10 (63)	6 (37)	---	16
Inflorescence	4 (100)	0 (0)	---	4
Immature Fruit	7 (100)	0 (0)	---	7
Mature Fruit	3 (33)	6 (67)	---	9
Peduncle	0 (0)	8 (100)	---	8
Mature fruit (other hosts)	0 (0)	8 (100)	---	8
Total	24	28	---	52

CGTT5 data set						
Tissue	Clade1			Clade2	Clade 3	Total
	1	1a	Clade 1 total			
Leaf	4 (27)	5 (33)	9 (60)	1 (7)	5 (33)	15
Inflorescence	2 (50)	2 (50)	4 (100)	0 (0)	0 (0)	4
Immature Fruit	5 (71)	2 (29)	7 (100)	0 (0)	0 (0)	7
Mature Fruit	2 (20)	1 (10)	3 (30)	3 (30)	4 (40)	10
Peduncle	1 (14)	0 (0)	1 (14)	4 (57)	2 (29)	7
Mature fruit (other hosts)	0 (0)	0 (0)	0 (0)	0 (0)	6 (100)	6
Total	15	9	24	8	17	49

[a] Number of isolates followed by percentage of total isolates from that tissue type

Table 3-5. Topography-based congruence analyses

Data sets with constraints	Tree length	Gain	Loss	Net	$P=$[a]
1. ITS	112				
a. MAT strict consensus tree	124	12	0	12	0.0023
b. MAT 70% boostrap	124	12	0	12	0.0023
c. MAT Clade 1	115	4	1	3	0.2568
d. MAT Clade 1, C. musae*, and Gua3 monophyletic*	124	12	0	12	0.0023
e. CGTT5 strict consensus tree	123	11	0	11	0.0384
f. CGTT5 70% bootstrap	123	11	0	11	0.0384
g. CGTT5 Clade 1	115	4	1	3	0.2568
h. CGTT5 Clade 1a	115	4	1	3	0.2568
i. CGTT5 Clade 2	112	0	0	0	---[b]
j. CGTT5 Clade 3	115	4	1	3	0.2568
2. MAT1-2	146				
a. ITS strict consensus tree	149	4	0	4	0.0455
b. ITS 70% booststrap	149	4	0	4	0.0455
b. ITS Clade 1	145	0	0	0	---
c. ITS Clade 1a	145	0	0	0	---
d. ITS Clade 2	149	4	0	4	0.0455
e. CGTT5 strict consensus tree	151	6	0	6	0.0339
f. CGTT5 70% bootstrap	148	3	0	3	0.1797
g. CGTT5 Clade 1	145	0	0	0	---
h. CGTT5 Clade 1a	145	0	0	0	---
i. CGTT5 Clade 2	145	0	0	0	---
j. CGTT5 Clade 3	147	2	0	2	0.1573
3. CGTT5	73				
a. MAT strict consensus tree	74	1	0	1	0.3173
b. MAT 70% bootstrap	74	1	0	1	0.3173
c. MAT Clade 1	73	0	0	0	---
d. MAT Clade 1, C. musae*, and Gua3 monophyletic*	77	5	1	4	0.1025

Table 3-5. Continued.

Data sets with constraints	Tree length	Gain	Loss	Net	$P=$[a]
e. ITS strict consensus tree	89	18	2	16	0.0003
f. ITS 70% bootstrap	89	18	2	16	0.0003
e. ITS Clade 1	75	2	0	2	0.1573
f. ITS Clade 1a	75	2	0	2	0.1573
g. ITS Clade 2	87	16	2	14	0.0010

[a] Wilcoxon Rank Sum test performed on # steps gained/lost to determine instances of topological incongruence; $P<0.05$ is significant

[b] Wilcoxon Rank Sum test not performed when gain/loss is equal to 0

Table 3-6. Morphological description of conidia and hyphopodia of 13 isolates.

Isolate	Clade [a]	Conidia shape	Conidia dimensions	Hyphopodia shape	Hyphopodia dimensions	# clavate	# irregular
Cg131	1	Cylindrical, sometimes constricted toward base or constricted	11 (14) 20 x 4 (4) 5	Scarce; clavate, smooth to rarely irregular, lobed	6 (10) 16 x 6 (7) 9	19	6
Cg135	1	Cylindrical, sometimes constricted toward base or constricted	13 (15) 18 x 4 (4) 5	Clavate, smooth to rarely irregular, lobed	5 (10) 14 x 5 (7) 13	23	2
Cg136	1	Cylindrical, sometimes constricted toward base or constricted	10 (13) 18 x 4 (4) 4	Abundant; irregular, lobed to rarely clavate, smooth	6 (9) 13 x 5 (7) 11	1	24
Cg138	1	Cylindrical, sometimes constricted toward base or constricted	11 (14) 20 x 3 (4) 5	Scarce; clavate, smooth to sometimes irregular, lobed	6 (8) 11 x 5 (6) 8	15	10
Cg141	1	Cylindrical, sometimes constricted toward base or constricted	13 (14) 21 x 4 (4) 6	Scarce; clavate, smooth to rarely irregular, lobed	8 (10) 13 x 5 (6) 8	19	2
Cg164	1	Cylindrical, sometimes constricted toward base or constricted	11 (14) 18 x 4 (5) 5	Very rare; clavate, smooth to irregular, lobed	6 (9) 11 x 5 (6) 6	2	2
Cg129	2	Cylindrical, sometimes constricted toward base or constricted	13 (14) 16 x 4 (4) 5	Abundant; irregular, lobed to rarely clavate, smooth	6 (8) 11 x 5 (6) 10	5	20
Cg134	2	Cylindrical, sometimes constricted toward base or constricted	10 (14) 18 x 4 (4) 5	Abundant; irregular, lobed to rarely clavate, smooth	6 (9) 14 x 4 (6) 8	2	23
Cg157	2	Cylindrical, sometimes constricted toward base or constricted	10 (13) 16 x 3 (4) 5	Abundant; irregular, lobed to rarely clavate, smooth	6 (9) 14 x 5 (7) 9	3	22
Cg128	3	Cylindrical, sometimes constricted toward base or constricted	10 (14) 19 x 3 (4) 5	Abundant; irregular, lobed to sometimes clavate, smooth	6 (9) 16 x 6 (9) 16	10	15

Table 3-6. Continued.

Isolate	Clade	Conidia shape	Conidia dimensions	Hyphopodia shape	Hyphopodia dimensions	# clavate	# irregular
Avo1	3	Cylindrical, sometimes constricted toward base or constricted	11 (14) 16 x 4 (4) 5	Abundant; irregular, lobed to slightly irregular	6 (8) 13 x 5 (7) 10	0	25
Cg156	3	Cylindrical, sometimes constricted toward base or constricted	11 (13) 15 x 3 (4) 5	Scarce; irregular, lobed to sometimes clavate, smooth	6 (8) 13 x 4 (7) 10	3	12
Cg161	3	Cylindrical, sometimes constricted toward base or constricted	11 (14) 20 x 3 (4) 5	Abundant; irregular, lobed	8 (9) 14 x 5 (7) 11	1	24

[a] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny

Table 3-7. Statistical comparison of conidia and hyphopodia dimensions.

Isolate	Clade [a]	Conidia			Hyphopodia		
		length	width	dimensions	length	width	dimensions
Cg131	1	14.05 ± 0.23 bcd[b]	4.30 ± 0.09 b	3.32 ± 0.07 de	10.11 ± 0.40 ab	6.94 ± 0.26 bc	1.47 ± 0.05 abc
Cg135	1	15.05 ± 0.19 a	4.03 ± 0.06 cde	3.77± 0.06 ab	9.90 ± 0.48 abc	7.40 ± 0.39 b	1.40 ± 0.08 abc
Cg136	1	12.73 ± 0.16 g	3.86 ±0.03 ef	3.30 ± 0.05 de	9.40 ± 0.32 abcd	7.00 ± 0.32 bc	1.40 ± 0.07 abc
Cg138	1	14.05 ± 0.23 bcd	3.90 ± 0.06 ef	3.63 ± 0.07 bc	8.42 ± 0.59 de	7.08 ± 0.45 d	1.29 ± 0.16 abc
Cg141	1	14.20 ± 0.22 bc	4.22 ± 0.09 bc	3.41 ± 0.07 d	10.36 ± 0.32 a	6.67 ± 0.16 bcd	1.58 ± 0.07 a
Cg164	1	13.60 ± 0.18 def	4.56 ± 0.08 a	3.04 ± 0.08 f	9.06 ± 1.07 bcde	5.94 ± 0.31 d	1.55 ± 0.22 a
Cg129	2	13.98 ± 0.15 bcde	4.23 ± 0.08 bc	3.36 ± 0.07 de	8.10 ± 0.29 e	6.40 ± 0.25 cd	1.28 ± 0.04 bcd
Cg134	2	14.05 ± 0.17 bcd	4.15 ±0.07 bcd	3.43 ± 0.07 cd	9.00 ± 0.36 bcde	6.60 ± 0.17 bcd	1.42 ± 0.11 abc
Cg157	2	13.45 ± 0.24 ef	3.94 ± 0.09 def	3.49 ± 0.10 cd	8.70 ± 0.40 cde	6.55 ± 0.25 bcd	1.37 ± 0.09 abc
Cg128	3	13.73 ± 0.20 cde	4.00 ± 0.09 de	3.51 ± 0.09 cd	9.49 ± 0.43 abcd	8.87 ± 0.43 a	1.11 ± 0.06 d
Avo1	3	13.90 ± 0.15 bcde	4.25 ± 0.08 b	3.34 ± 0.08 de	8.40 ± 0.27 de	7.00 ± 0.28 bc	1.26 ± 0.08 cd
Cg156	3	13.08 ± 0.14 fg	4.24 ± 0.09 bc	3.16 ± 0.08 ef	8.42 ± 0.59 de	7.08 ± 0.45 bc	1.29 ± 0.16 bcd
Cg161	3	14.39 ± 0.23 b	3.74 ± 0.07 f	3.90 ±0.09 a	9.80 ± 0.37 abc	6.60 ± 0.24 bcd	1.52 ± 0.07 ab

[a] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny [b] Means and standard errors are reported; means were compared using Fisher's LSD test; values in columns with same letter are not significantly different, $P<0.05$

Table 3-8. Area under the disease progress curve (AUDPC) and y_{max} values for blossom blight experiments, 2009.

		Exp. 1		Exp. 2		Exp. 3	
Isolate	Clade[a]	AUDPC	y_{max}[b]	AUDPC	y_{max}	AUDPC	y_{max}
Cg131	1	8.89 ± 2.49 ab[c]	0.53 ± 0.17 a	12.62 ± 2.35 ab	0.67 ± 0.10 ab	13.71 ± 2.02 a	0.80 ± 0.10 a
Cg135	1	11.22 ± 1.91 a	0.64 ± 0.12 a	14.64 ± 2.57 a	0.82 ± 0.09 a	13.28 ± 3.75 a	0.85 ± 0.11 a
Cg136	1	6.37 ± 0.12 b	0.18 ± 0.07 b	9.55 ± 1.13 b	0.55 ± 0.09 b	9.04 ± 2.15 ab	0.91 ± 0.08 a
Cg138	1	8.29 ± 0.99 ab	0.45 ± 0.06 a	12.31 ± 2.68 ab	0.80 ± 0.20 a	11.50 ± 2.63 a	0.74 ± 0.08 a
Cg141	1	8.34 ± 0.74 ab	0.51 ± 0.07 a	10.98 ± 1.75 ab	0.73 ± 0.13 ab	9.50 ± 1.90 ab	0.69 ± 0.17 a
Cg164	1	10.39 ± 1.68 a	0.53 ± 0.09 a	10.23 ± 2.96 ab	0.48 ± 0.13 b	6.51 ± 1.23 b	0.75 ± 0.25 a
Avo1	3	2.57 ± 0.63 c	0.13 ± 0.04 b	0.87 ± 0.17 c	0.06 ± 0.01 c	0.85 ± 0.70 c	0.07 ± 0.06 b
Cg156	3	2.61 ± 2.02 c	0.12 ± 0.09 b	2.06 ± 0.85 c	0.14 ± 0.08 c	0.89 ± 0.47 c	0.08 ± 0.05 b
Cg161	3	1.09 ± 0.51 c	0.05 ± 0.03 b	1.00 ± 0.17 c	0.06 ± 0.01 c	1.30 ± 0.38 c	0.09 ± 0.03 b
Cg128	3	2.09 ± 0.79 c	0.13 ± 0.06 b	0.51 ± 0.14 c	0.02 ± 0.01 c	0.42 ± 0.16 c	0.02 ± 0.01 b
Cg129	2	1.55 ± 0.78 c	0.08 ± 0.04 b	1.04 ± 0.42 c	0.10 ± 0.05 c	0.12 ± 0.05 c	0.01 ± 0.01 b
Cg134	2	2.58 ± 1.41 c	0.20 ± 0.14 b	0.72 ± 0.20 c	0.02 ± 0.00 c	0.18 ± 0.07 c	0.01 ± 0.01 b
Cg157	2	1.28 ±1.08 c	0.11 ± 0.10 b	2.09 ± 0.14 c	0.10 ± 0.07 c	1.29 ± 1.04 c	0.11 ± 0.07 b
Control	---	1.00 ± 0.49 c	0.04 ± 0.02 b	0.36 ± 0.28 c	0.03 ± 0.02 c	0.31 ± 0.10 c	0.02 ± 0.01 b
	LSD	3.53	0.231	4.66	0.249	4.91	0.234
	P=	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny [b] y_{max} is the severity value for panicles 28 days after inoculation [c] Values in columns with same letter are not significantly different based on Fisher's LSD, *P*=0.05.

Table 3-9. Area under the disease progress curve (AUDPC) and y_{max} values for leaf anthracnose experiments, 2009.

		Exp 1		Exp 2	
Isolate	Clade [a]	AUDPC	y_{max} [b]	AUDPC	y_{max}
Cg131	1	2.18 ± 0.31 a[c]	0.31 ± 0.05 a	2.91 ± 0.22 a	0.33 ± 0.03 a
Cg135	1	1.46 ± 0.39 ab	0.21 ± 0.04 ab	1.97 ± 0.25 b	0.21 ± 0.03 b
Cg136	1	1.61 ± 0.22 b	0.23 ± 0.03 ab	-	-
Cg138	1	0.71 ± 0.16 c	0.08 ± 0.02 d	1.28 ± 0.27 c	0.16 ± 0.03 bc
Cg141	1	1.52 ± 0.17 a	0.20 ± 0.03 abc	2.39 ± 0.24 b	0.29 ± 0.03 a
Avo1	3	0.42 ± 0 .07 c	0.07 ± 0.01 d	0.21 ± 0.06 d	0.04 ± 0.01 d
Cg156	3	0.18 ± 0.03 c	0.03 ± 0.00 d	0.31 ± 0.06 d	0.04 ± 0.01 d
Cg161	3	0.35 ± 0.09 c	0.06 ± 0.02 d	-	-
Cg128	3	0.48 ± 0.14 c	0.06 ± 0.02 d	0.14 ± 0.03 d	0.02 ± 0.00 d
Cg129	2	0.27 ± 0.06 c	0.04 ± 0.01 d	1.10 ± 0.15 c	0.13 ± 0.02 c
Cg134	2	0.75 ± 0.21 bc	0.12 ± 0.04 bcd	0.97 ± 0.21 c	0.13 ± 0.03 c
Cg157	2	0.54 ± 0.11 c	0.08 ± 0.02 d	-	-
Control	---	0.57 ± 0.10 c	0.09 ± 0.02 cd	0.30 ± 0.05 d	0.03 ± 0.01 d
	LSD	0.73	0.11	0.46	0.06
	P=	<0.0001	<0.0001	<0.0001	<0.0001

[a] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny [b] y_{max} is the severity value for leaves 14 days after inoculation [c] Values in columns with same letter are not significantly different based on Fisher's LSD, *P*=0.05.

Table 3-10. Anthracnose area under the disease progress curve (AUDPC) and lesion diameters on detached 'Tommy Atkins' fruit

		Exp 1		Exp 2	
Isolate	Clade [a]	AUDPC	diameter (mm)[b]	AUDPC	diameter (mm)
Cg131	1	23.38 ± 4.25 b[c]	12.38 ± 2.16 ab	19.06 ± 8.16	10.75 ± 4.25 a
Cg135	1	11.63 ± 2.70 c	7.75 ± 1.45 bc	33.50 ± 9.82	14.88 ± 3.06 a
Cg141	1	35.63 ± 5.61 a	17.38 ± 2.33 a	31.81 ± 3.81	16.75 ± 1.65 a
Cg129	2	21.44 ± 1.99 bc	10.50 ± 1.04 bc	26.38 ± 3.32	12.13 ± 2.19 a
Cg157	2	20.00 ± 4.74 bc	10.13 ± 2.35 bc	33.50 ± 10.87	16.63 ± 4.52 a
Cg128	3	14.25 ± 3.70 bc	8.67 ± 3.09 bc	27.75 ± 10.81	15.13 ± 5.21 a
Cg161	3	10.81 ± 3.47 cd	5.75 ± 1.48 c	36.13 ± 11.09	18.38 ± 4.51 a
Control	---	0.00 ± 0.00 d	0.00 ± 0.00 d	1.50 ± 0.87	0.25 ± 0.25 b
	LSD	11.39	5.73	24.14	10.46
	P=	0.0002	0.0004	0.1178	0.0390

		Exp 3		Exp 4	
Isolate	Clade	AUDPC	Diameter (mm)	AUDPC	Diameter (mm)
Cg131	1	14.94 ± 5.60	9.00 ± 2.89 bc	22.13 ±5.18 abc	10.25 ± 2.37 ab
Cg135	1	16.25 ± 6.65	10.00 ± 3.28 ab	23.56 ± 6.04 ab	11.63 ± 2.92 ab
Cg141	1	19.88 ± 5.11	15.00 ± 2.02 a	28.94 ± 1.60 ab	15.75 ± 0.72 a
Cg129	2	16.94 ± 2.67	8.38 ± 1.11 bc	27.83 ± 5.65 ab	12.33 ± 2.68 ab
Cg157	2	8.69 ± 2.95	4.33 ± 1.09 cd	30.94 ± 3.93 a	13.25 ± 0.92 ab
Cg128	3	13.00 ± 1.40	7.13 ± 0.83 bc	29.44 ± 7.40 ab	13. 38 ± 3.79 ab
Cg161	3	13.81 ± 2.54	7.13 ± 0.72 bc	14.75 ± 6.61 bc	6.88 ± 3.27 bc
Control	---	3.38 ± 3.38	1.50 ± 1.50 d	0.50 ± 0.35 c	0.50 ± 0.29 c
	LSD	11.05	5.50	15.09	7.18
	P=	0.1942	0.0067	0.0049	0.0056

[a] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny [b] Diameter measurements from14 days after inoculation [c] Values in columns with same letter are not significantly different based on Fisher's LSD, *P*=0.05.

Table 3-11. Area under the disease progress curve (AUDPC) and y_{max} values for attached fruit anthracnose experiments, 2009.

		'Haden'		'Tommy Atkins'		'Van Dyke'	
Isolate	Clade	AUDPC	y_{max}[b]	AUDPC	y_{max}	AUDPC	y_{max}
Cg131	1	1.22 ± 0.29 bc[c]	0.38 ± 0.11 bcd	0.61 ± 0.28	0.19 ± 0.06	0.42 ± 0.25 b	0.14 ± 0.05
Cg135	1	1.99 ± 0.49 b	0.49 ± 0.11 bc	1.08 ± 0.23	0.26 ± 0.05	0.28 ± 0.18 b	0.08 ± 0.05
Cg141	1	4.18 ± 1.01 a	0.87 ± 0.02 a	1.16 ± 0.55	0.34 ± 0.13	1.06 ± 0.47 a	0.26 ± 0.13
Cg129	2	0.76 ± 0.20 bc	0.23 ± 0.05 cd	0.51 ±0.24	0.16 ± 0.06	0.24 ± 0.08 b	0.07 ± 0.02
Cg134	2	1.98 ± 0.47 b	0.59 ± 0.12 ab	0.67 ± 0.09	0.24 ± 0.04	---	---
Cg157	2	---	---	---	---	0.25 ± 0.15 b	0.10 ± 0.05
Cg128	3	1.03 ± 0.40 bc	0.43 ± 0.14 bc	0.48 ± 0.17	0.18 ± 0.06	0.05 ± 0.03 b	0.03 ± 0.01
Cg161	3	0.87 ± 0.11 bc	0.30 ± 0.05 bcd	0.30 ± 0.11	0.13 ± 0.04	0.18 ± 0.05 b	0.06 ± 0.02
Control	---	0.36 ± 0.29 c	0.10 ± 0.07 d	0.35 ± 0.16	0.10 ± 0.03	0.10 ± 0.04 b	0.05 ± 0.02
	LSD	1.42	0.29	0.67	0.16	0.46	0.14
	P=	0.0013	0.0018	0.0896	0.1118	0.0224	0.0983

[a] Clade designations follow the CGTT5+MAT1-2 maximum parsimony phylogeny [b] y_{max} is the severity value for fruit 14 days after harvest [c] Values in columns with same letter are not significantly different based on Fisher's LSD, *P*=0.05.

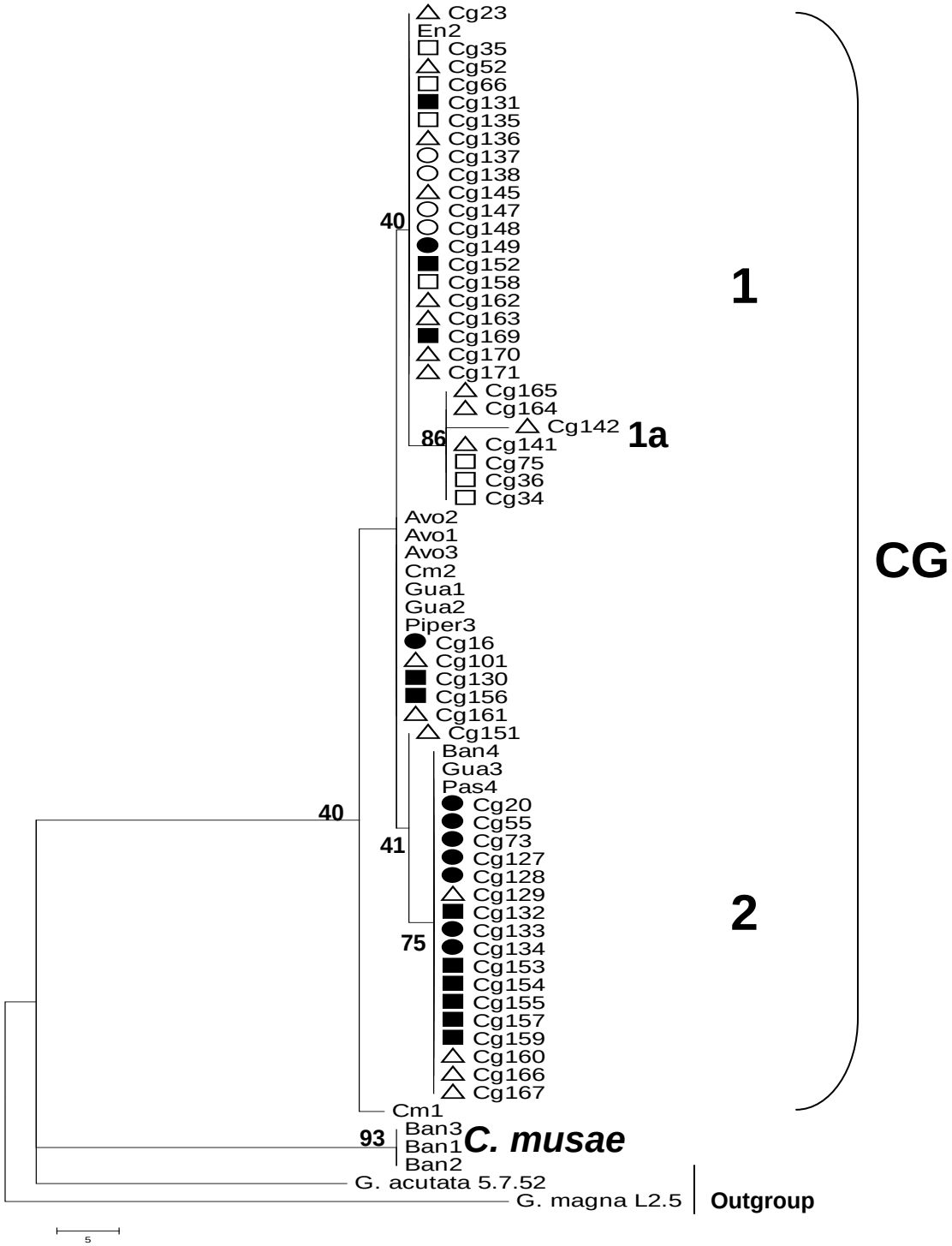

Figure 3-1. Phylogenetic relationships among 65 accessions of *Colletotrichum gloeosporioides* based on ITS sequence data. The phylogram represents one of 11 most parsimonious trees (112 steps, CI=0.938, RI=0.949). Support values are bootstrap values over 40%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

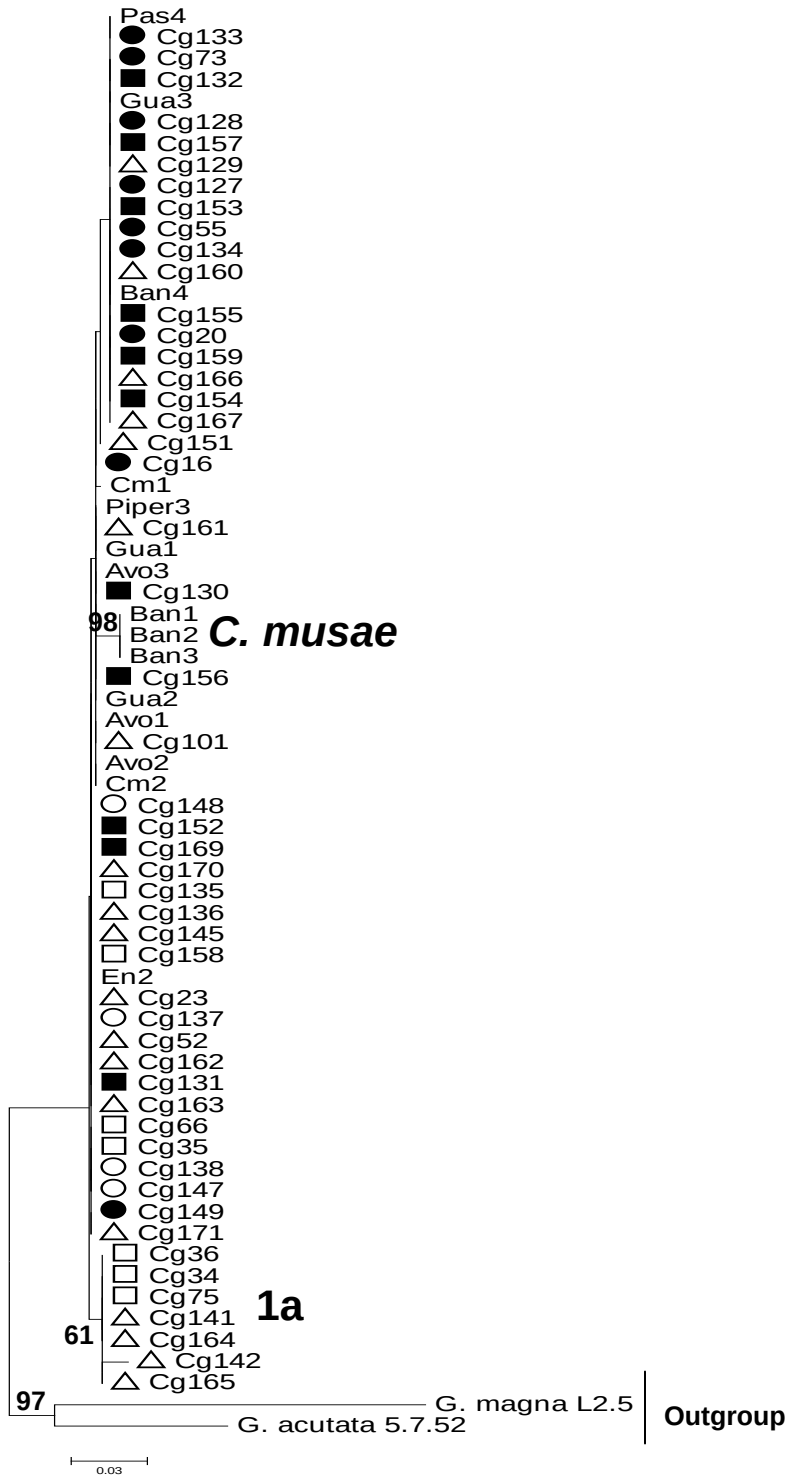

Figure 3-2. Phylogenetic relationships among accessions of *Colletotrichum gloeosporioides* using ML analysis of ITS sequence data, ln score = -1071.9. Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

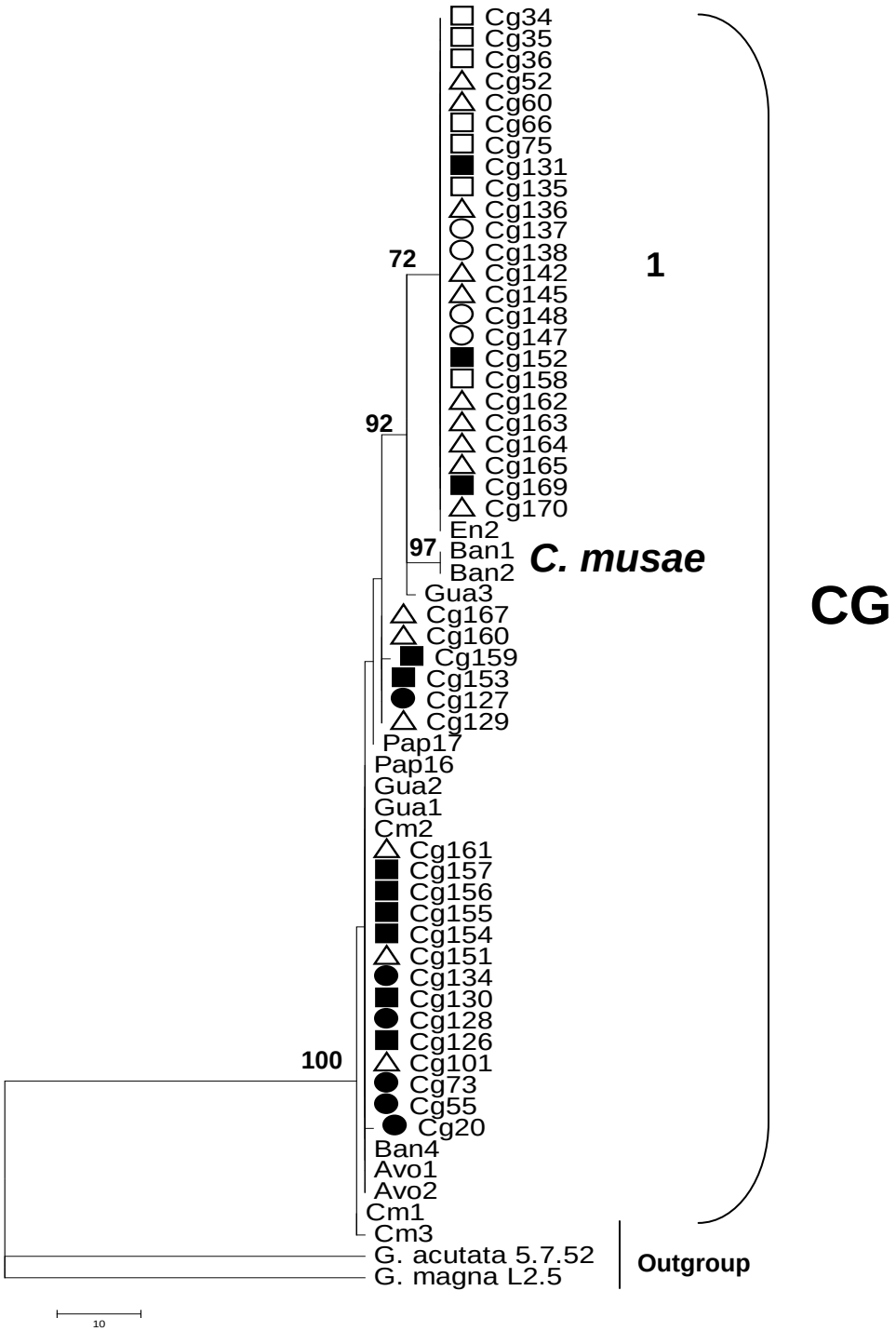

Figure 3-3. Phylogenetic relationships among 58 accessions of *Colletotrichum gloeosporioides* based on MAT1-2 sequence data. The phylogram represents one of 14 most parsimonious trees (216 steps, CI=0.966, RI=0.981). Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

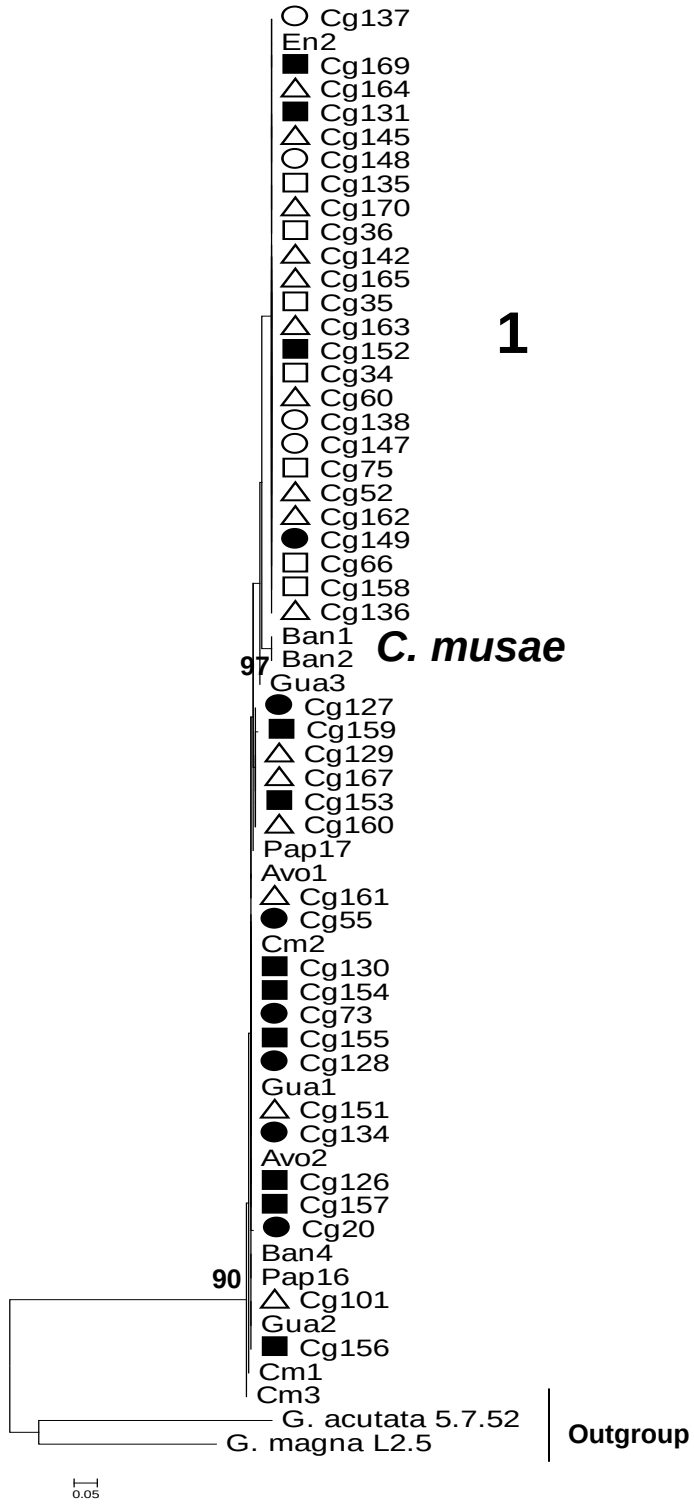

Figure 3-4. Phylogenetic relationships among accessions of *Colletotrichum gloeosporioides* using ML analysis of MAT1-2 sequence data, ln score = -821.25. Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

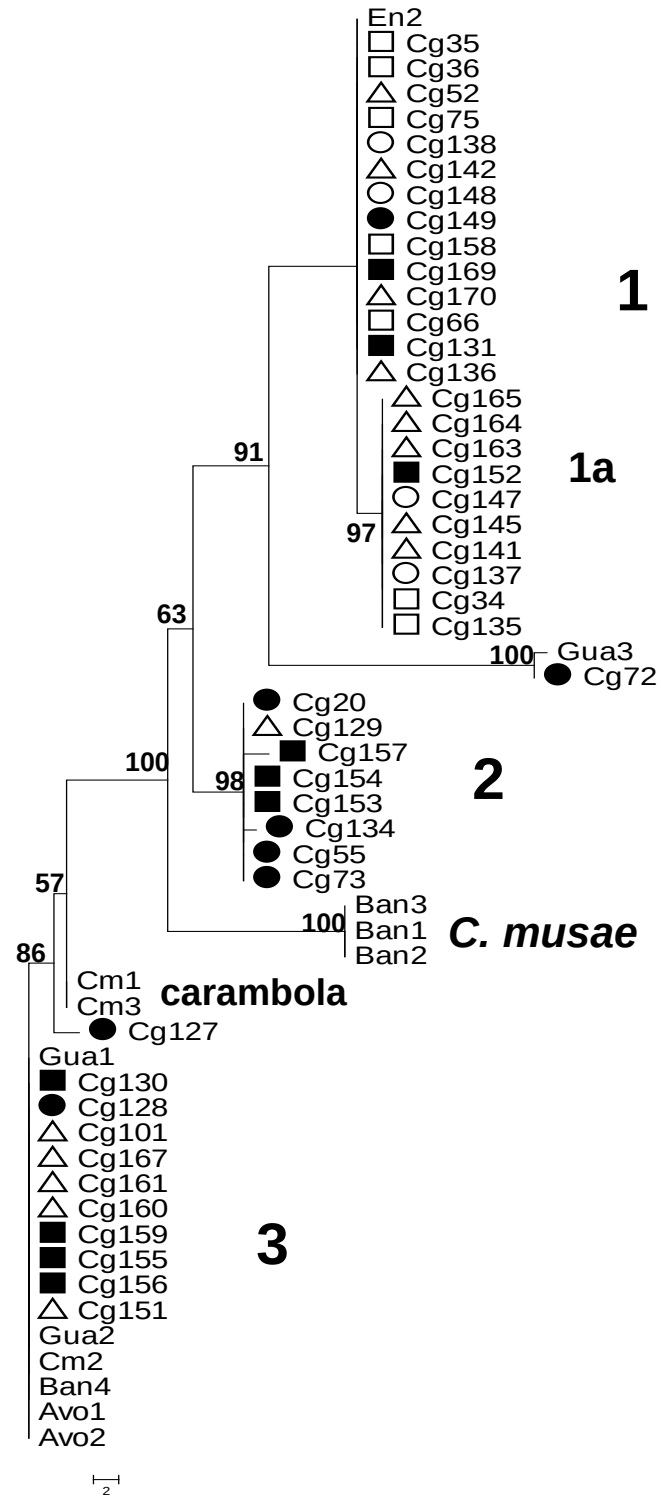

Figure 3-5. Phylogenetic relationships among 58 accessions of *Colletotrichum gloeosporioides* based on CGTT5 sequence data. The phylogram represents one of two most parsimonious trees (73 steps, CI=0.959, RI=0.995). Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

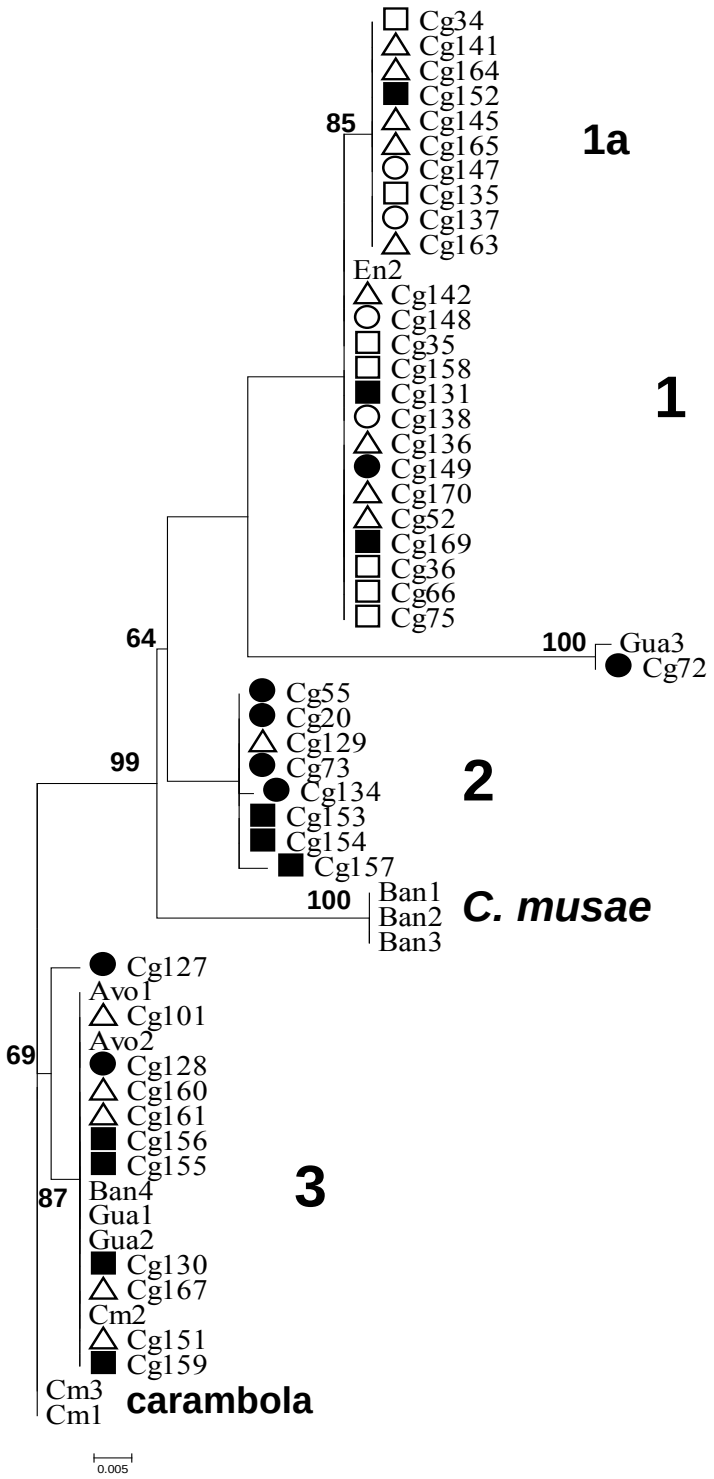

Figure 3-6. Phylogenetic relationships among accessions of *Colletotrichum gloeosporioides* using ML analysis of CGTT5 sequence data, ln score = -1166.49. Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

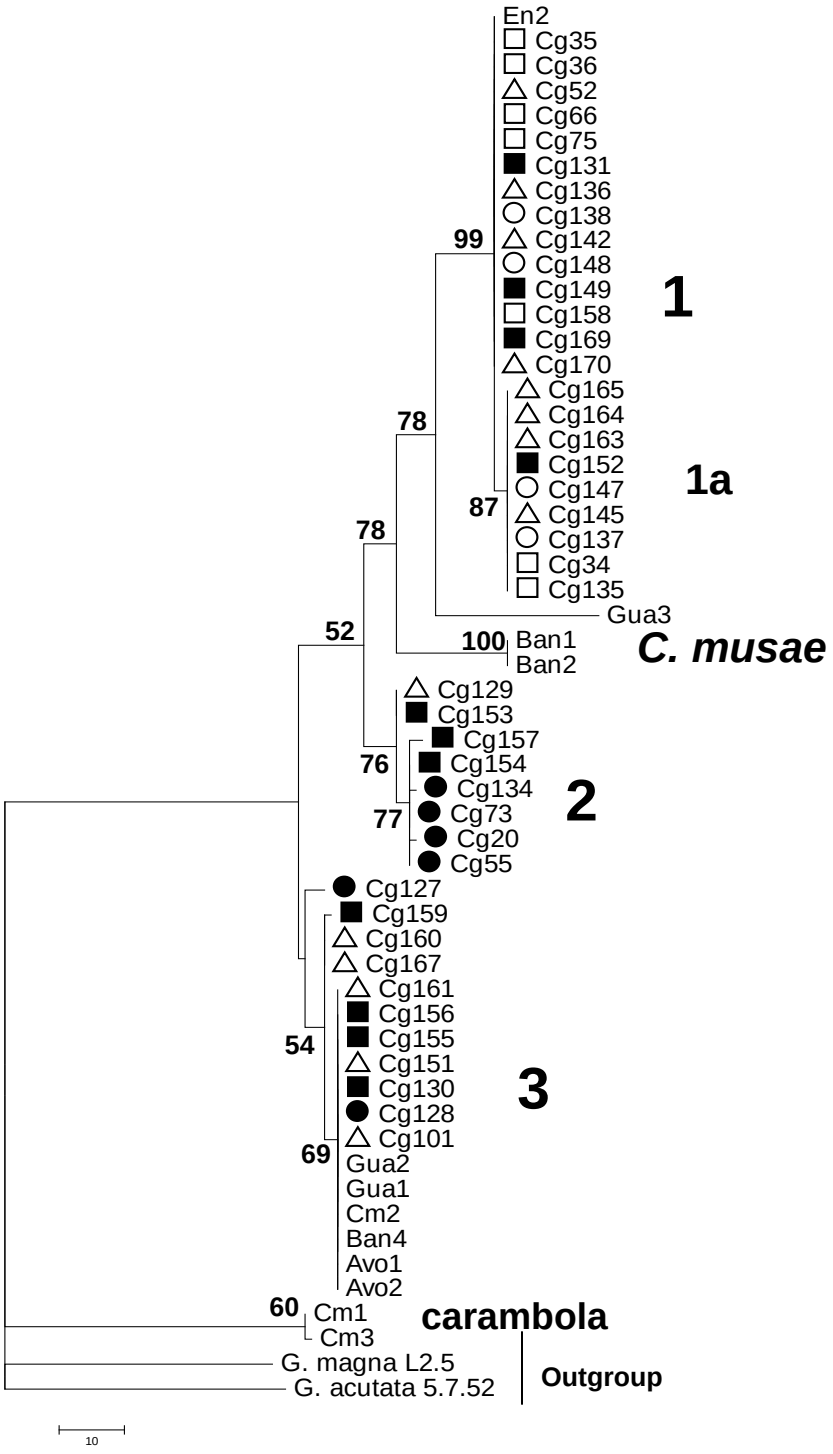

Figure 3-7. Phylogenetic relationships among 54 accessions of *Colletotrichum gloeosporioides* based on combined MAT1-2+CGTT5 sequence data set. The phylogram represents one of 63 most parsimonious trees (224 steps, CI=0.951, RI=0.986). Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

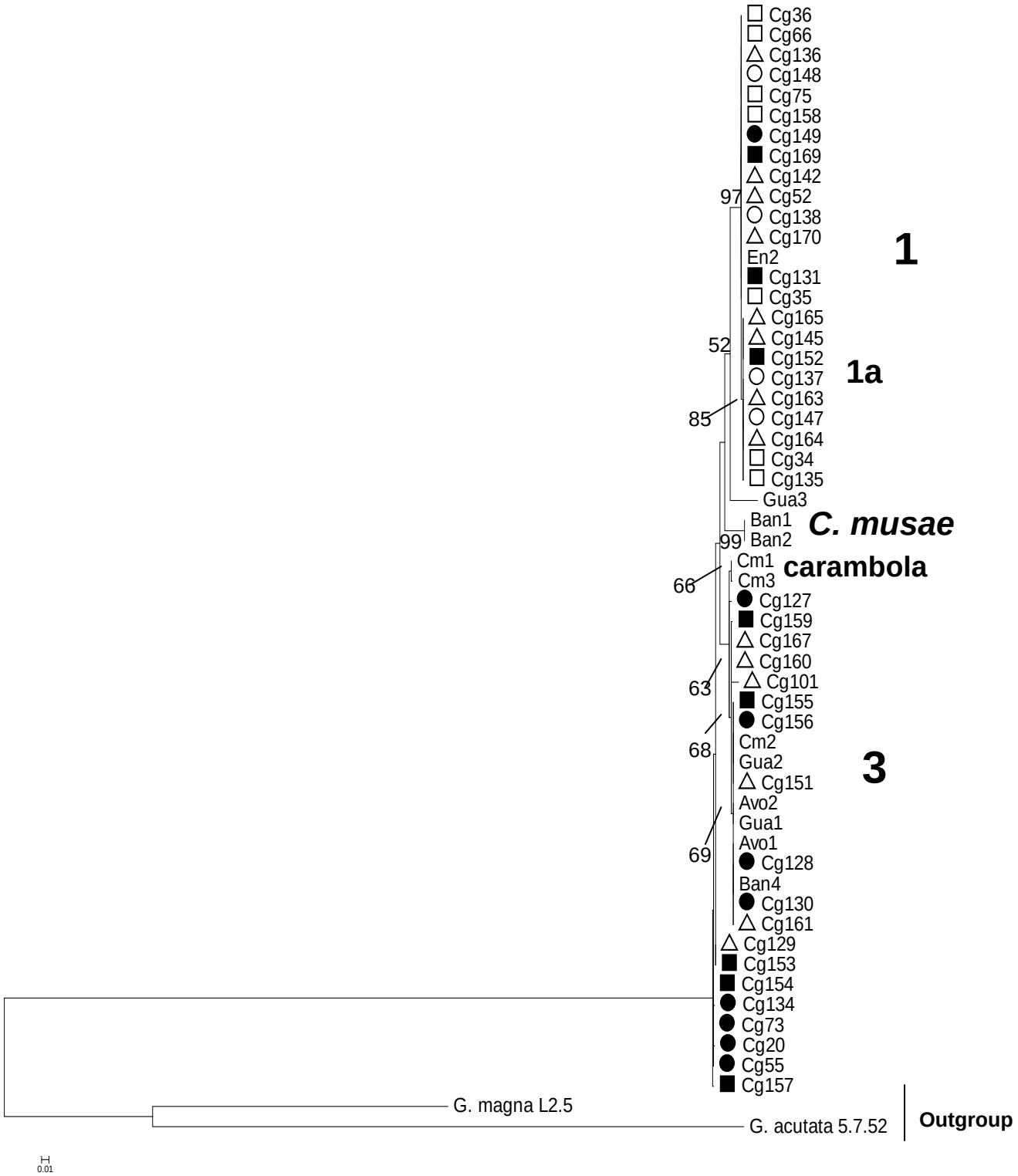

Figure 3-8. Phylogenetic relationships among accessions of *Colletotrichum gloeosporioides* using ML analysis of MAT1-2+CGTT5 data set, ln score = -2075.22. Support values are bootstrap values over 50%. Isolates are coded for host tissue as follows: open triangle=leaves, open circle=panicles, open square=immature fruit, closed circle=peduncles, closed square=mature fruit).

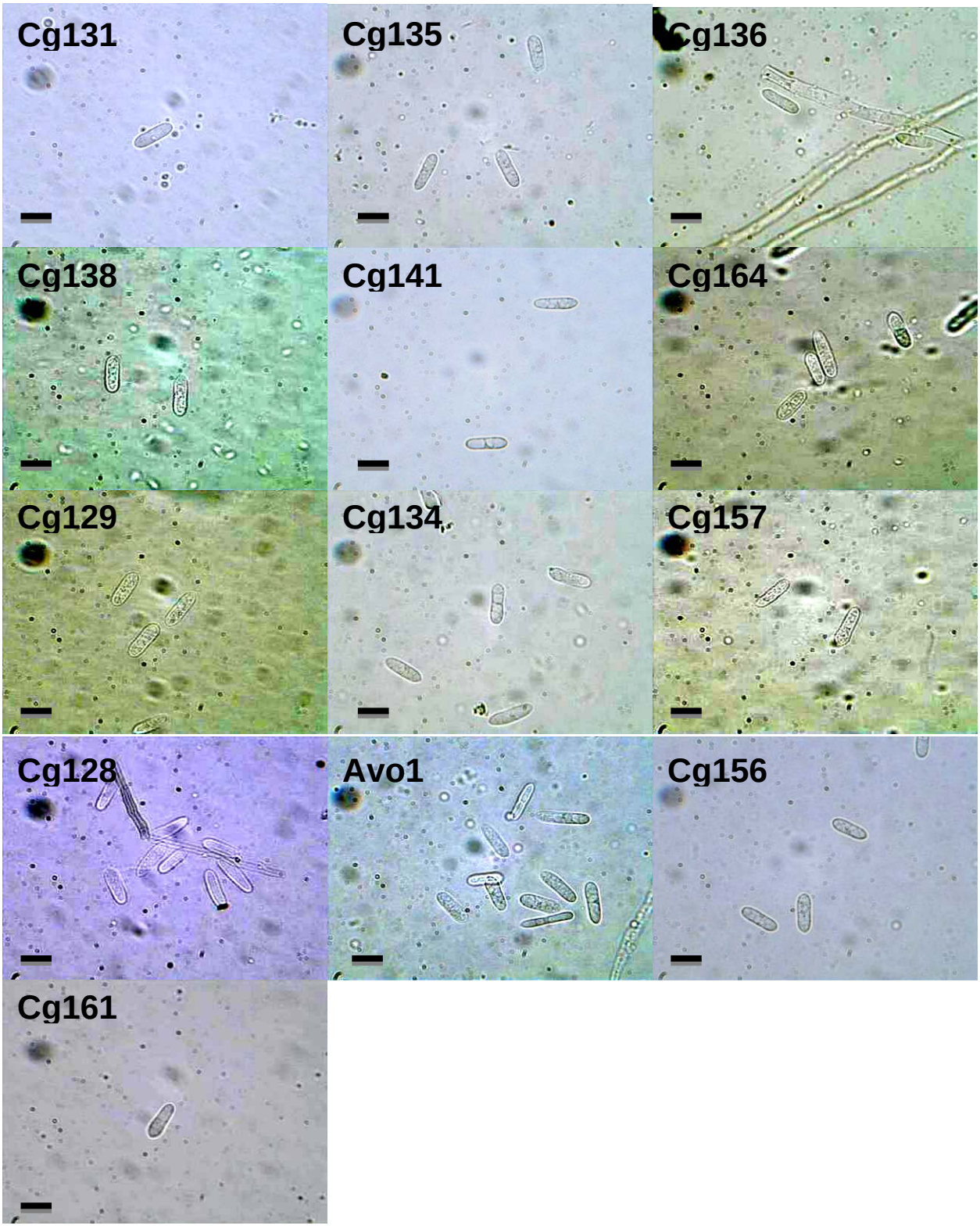

Figure 3-9. Micrographs of conidia of indicated isolates of *Colletotrichum gloeosporioides*. Bars=10 µm.

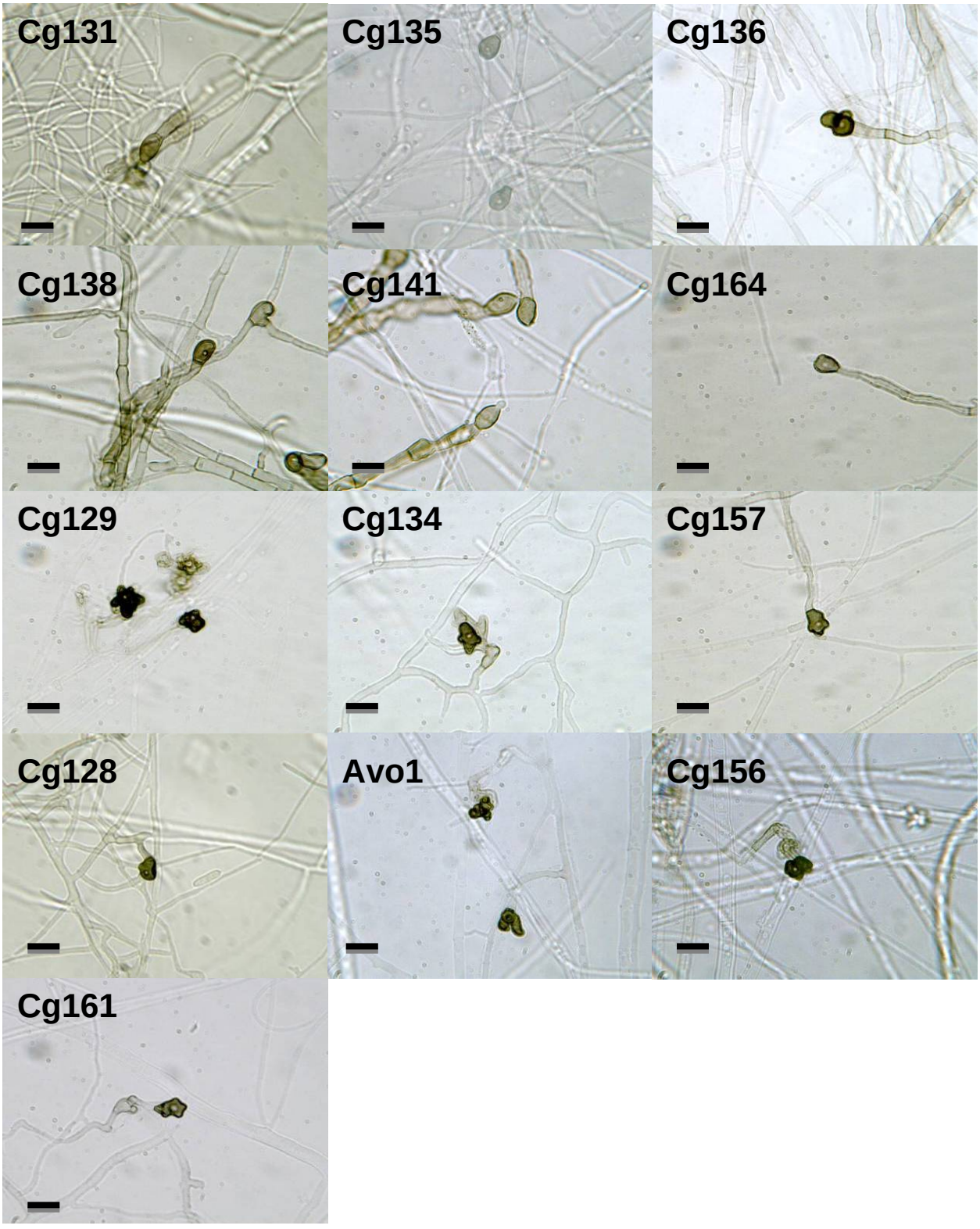

Figure 3-10. Micrographs of hyphopodia of indicated isolates of *Colletotrichum gloeosporioides*. Bars=10µm.

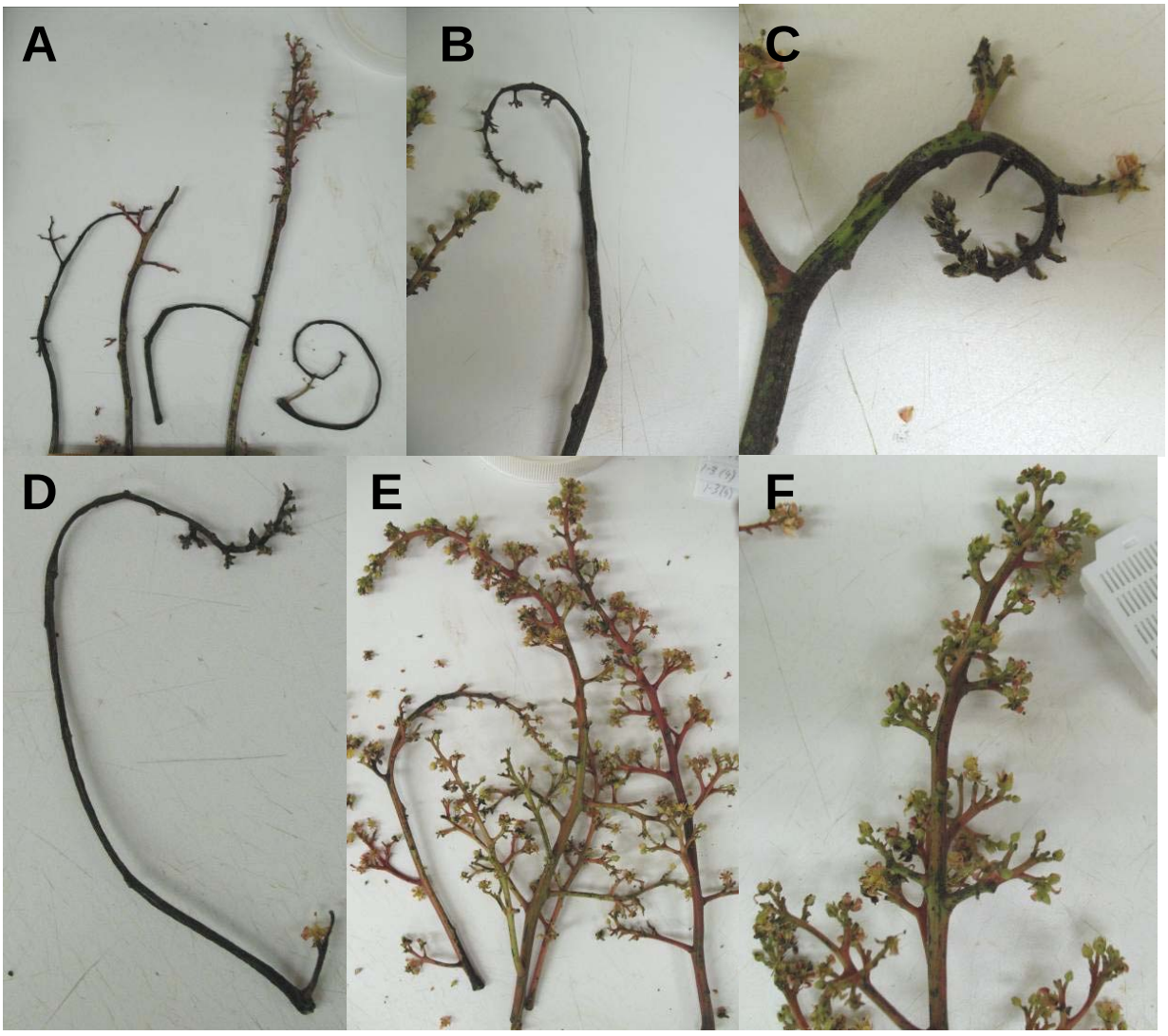

Figure 3-11. Blossom blight 28 days after inoculation. Severe symptoms were induced by isolates in Clade 1: A) Cg131, B) Cg164, C) Cg135 and D) Cg138. In contrast, minor or no symptoms were caused by isolates outside Clade 1: E) Cg129 and F) Cg161.

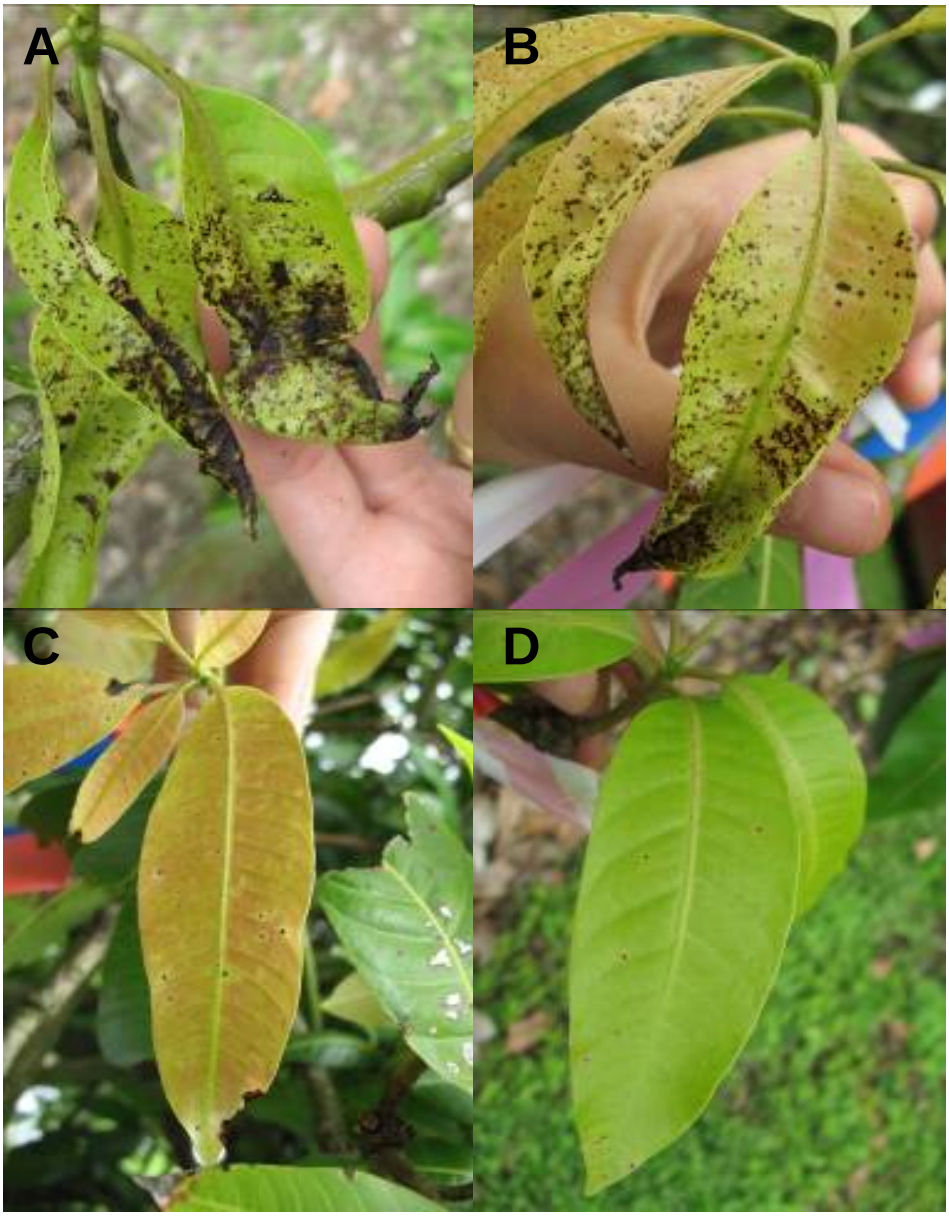

Figure 3-12. Leaf anthracnose 10 days after inoculation. Symptoms caused by isolates in Clade 1, A) Cg131 and B) Cg141, showing numerous lesions as well as large areas of necrosis and leaf distortion. Leaves inoculated with non-Clade 1 isolates, C) Cg128 and D) Avo1, showing few small lesions.

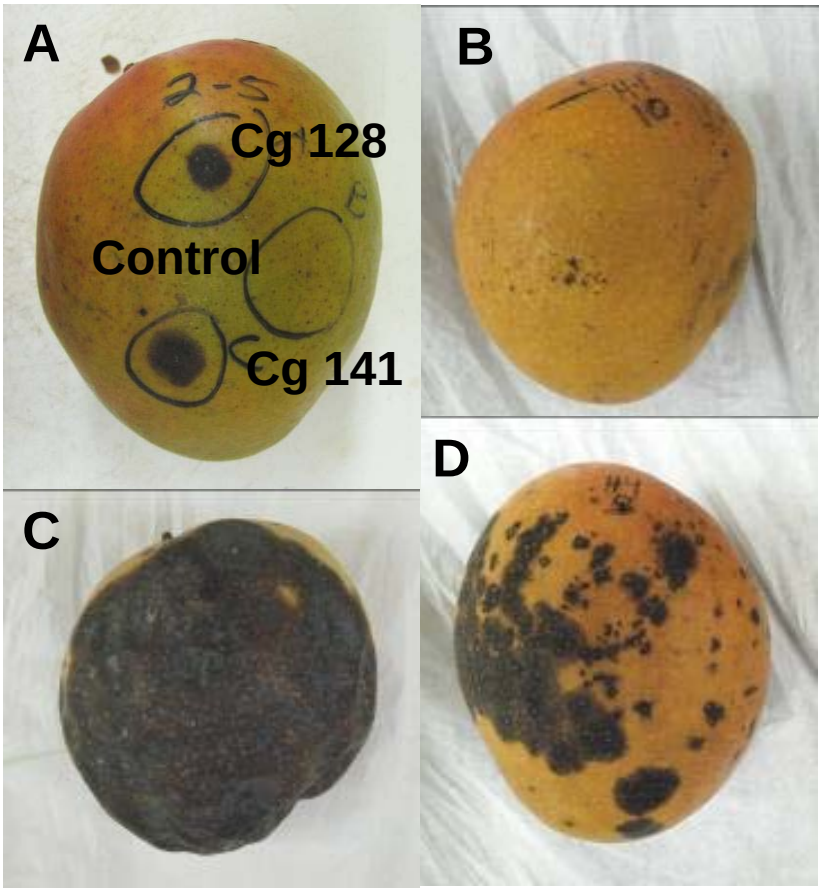

Figure 3-13. Lesion development on artificially inoculated fruit: A) 7 days after inoculation of a detached fruit, with isolates Cg128 and Cg141 compared to the water control; and attached fruit 14 days after harvest, B) water control, C) isolate Cg141 and D) isolate Cg161.

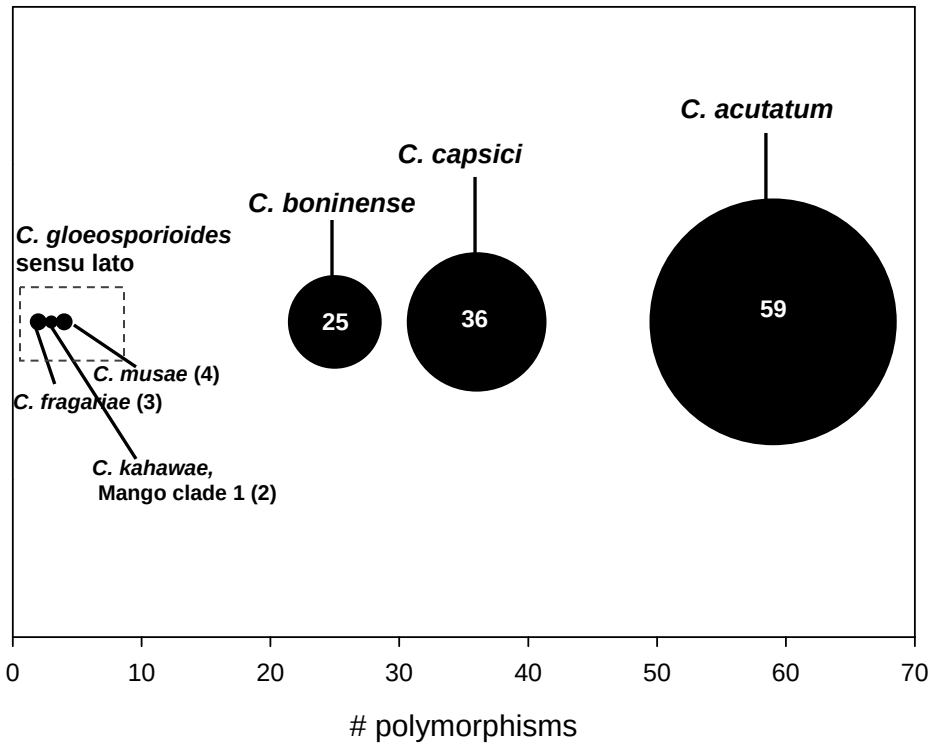

Figure 3-14. Graphical representation of the number of polymorphisms (including nucleotide and indel sites) among four taxa within *Colletotrichum gloeosporioides* sensu lato and three distantly related species, *C. boninense, C. capsici* and *C. acutatum*. Numbers are shown in parentheses or in white text within bubbles. Bubble size is proportional to the numbers of polymorphisms.

CHAPTER 4
INVESTIGATING THE ROLE PLAYED BY *COLLETOTRICHUM ACUTATUM* IN THE DEVELOPMENT OF BLOSSOM BLIGHT AND LEAF AND FRUIT ANTHRACNOSE OF MANGO IN SOUTHERN FLORIDA

Introduction

Anthracnose is the most impoortant disease of mango in wet climates. Three organs of the host are affected, resulting in blossom blight, leaf anthracnose, and fruit anthracnose. *Colletotrichum gloeosporioides*, *C. gloeosporioides* var. *minor* and *C. acutatum* have been associated with these diseases, but *C. gloeosporioides* is most prevalent. *Colletotrichum acutatum* has only been reported on mango in Australia (Fitzell 1979), Taiwan (Weng and Chuang 1995) and Homestead, Florida (Riveras-Varga et al. 2006). In south Florida, Riveras-Vargas et al. (2006) reported that *C. acutatum* comprised only 13% of the isolates that they recovered from mango, the rest of which were identified as *C. gloeosporioides*. Although they have been confused due to their similar morphologies, molecular markers are now available that identify *C. acutatum* sensu lato and *C. gloeosporioides* sensu lato (Mills et al. 1992, Sreenivasaprasad et al. 1996b).

Colletotrichum acutatum and *C. gloeosporioides* are distinct but diverse taxa that are now recognized as species complexes (Sutton 1980, Sreenivasaprasad and Talhinhas 2005). Sreenivasaprasad and Talhinhas (2005) reported eight distinct lineages in *C. acutatum* sensu lato based on phylogenetic analysis of the ITS1-5.8S-ITS2 region of 109 accessions. Several host specific lineages were identified, including A8 from *Cyphomandre* and A1 from *Lupinus* (*Colletotrichum lupini*). The type specimen from *Carica papaya* resolved in group A5. The species *C. lupini* and *C. phormii* resolve within *C. acutatum* sensu lato (Nirenberg et al. 2002, Farr et al. 2006).

In 2007, isolations were made from symptomatic leaves, blossoms and fruit from a cv.'Keitt' mango grove at the Tropical Research and Education Center (Homestead, FL). Although *C. gloeosporioides* was recovered from all blossom and fruit lesions, most of the leaf lesions yielded *C. acutatum* (Tarnowski and Ploetz 2008). Given its prevalence on 'Keitt' leaves and the infrequent reports of it as a mango pathogen, the present study sought to define and contrast the roles played by *C. acutatum* and *C. gloeosporioides* on mango in southern Florida. Although the work focused primarily on leaf anthracnose, blossoms and fruit were also studied as host organs.

Materials and Methods

Leaf Anthracnose Survey

Between July 2007 to May 2008, nine mango groves were surveyed, six of which were commercial plantings in the Redlands agricultural area, and three of which were experimental (University of Florida, Tropical Research and Education Center (TREC), Homestead, FL; USDA-ARS station, Miami, FL). Three of the commercial properties were exclusively of cv. 'Keitt', one was exclusively of cv. 'Tommy Atkins', and two contained blocks/rows of several cultivars, from which trees of 'Tommy Atkins' were sampled. Two plantings were studied at the TREC location, a solid block of cv. 'Keitt' and a mixed germplasm collection. The USDA location was a mixed germplasm collection. Lesions were sampled hierarchically and only from new, bright green and supple leaves. From past observation, *Colletotrichum* is most often recovered from new, actively growing lesions on young leaves, and becomes increasingly difficult to isolate in old lesions (data not shown). During the survey, the morphology of all lesions from which *Colletotrichum* was recovered was documented with digital photography. At each location, three to 17 trees were sampled depending on the abundance of leaf anthracnose symptoms. One to three symptomatic leaves were chosen from each tree, and one to three lesions

were chosen from each leaf. Lesions were cut from the leaf, surface disinfested (submersion for 60s in 10% bleach and rinsed with sterile water), and incubated in a moist chamber for 3 to 4 days. Conidia were then lifted from the acervuli that developed with a sterile needle and plated on PDA. The resulting colonies were identified as *C. gloeosporioides* or *C. acutatum* based on colony and conidium morphology (Figure 4-1). Colonies of *C. gloeosporioides* colonies were fast growing (6.72 ± 0.11 mm·day^{-1}), white to grey, fluffy with orange sporulation and produced straight and cylindrical conidia. Colonies of *C. acutatum* were slow growing (3.33 ± 0.31 mm·day^{-1}), grey to pink with pink reverse, and powdery, with bright salmon sporulation, and produced fusiform conidia. Isolates were single-spored and stored for future use in 10% glycerol at -80°C.

The above phenotypic identifications were corroborated with molecular diagnoses using *C. gloeosporioides*- and *C. acutatum*-specific primers. Isolates were grown for 3-5 days in PDB at room temperature (23-25ºC) on a shaker at ca. 80 rpm. Approximately 150 mg mycelia were harvested and triple rinsed with sterile deionized water and dried on sterile filter paper. DNA was extracted using a DNA genomic preparation protocol from the University of Wisconsin Biotechnology Center. Briefly, mycelia were ground in 500 µl Shorty DNA Extraction Buffer (0.2M Tris-HCl, pH 9.0, 0.4M LiCl, 25mM EDTA, 1% SDS) and incubated at 68°C for 10 minutes. Tissue was centrifuged at 14,000 rpm for 5 minutes, 400 µl of the supernatant was transferred to new tube and the DNA was precipitated with 400 µl 99% isopropanol. The tubes were centrifuged for 10 minutes at 14,000 rpm and the supernatant was decanted. The DNA pellets were air-dried for 5 minutes then resuspended in 400 µl TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) for 30 minutes at room temperature. 2µl DNA was used in PCR reactions.

The ITS1-5.8S-ITS2 ribosomal region was amplified via PCR in 50 µl reactions that contained 38.25 µl of sterile distilled, deionized water, 6.5 µl ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA), 1 µl 10 mM dNTP mix (New England Biolabs, Ipswich, MA), 0.25 µl Taq DNA polymerase (conc. 5,000 units/ml), 1 µl each of 15 µM primers and 2 µl DNA template using the following primers: CaInt (Sreenivasaprasad et al. 1996b) and CgInt (Mills et al. 1992) for *C. acutatum* and *C. gloeosporioides*, respectively, and ITS4 (White et al. 1990). Standard cycling parameters used a 55 °C annealing temperature.

The identity of five isolates of *C. acutatum* sensu lato from this study was confirmed by comparing their ITS sequences with those from previous studies. The entire ITS1-5.8S-ITS2 region of Ca3, Ca13, Ca26, Ca33, and Ca60 was amplified with primers ITS1 (Gardes and Bruns 1993) and ITS4, using a 55°C annealing temperature, and amplified products were sequenced by the Interdisciplinary Center for Biotechnology Research, Gainesville, FL. Sequences were compared with those of isolates of *C. acutatum*, *C. phormii* and *C. lupini* in Genbank from studies by Freeman et al. (2001), Nirenberg et al. (2002), Moriwaki et al. (2002), Saha et al. (2002), Vinnere et al. (2002), Afanador-Kafuri et al. (2003), Martinez-Culebras et al. (2003), Lubbe et al. (2004), Talhinhas et al. (2005), Than et al. (2006), Farr et al. (2006), and MacKenzie et al. (2009) (Table 4-1). The sequences represented different genetic groups in the *C. acutatum* species complex, as described in Sreenivasaprasad and Talhinas (2005) and including *C. phormii* and *C. lupini* (Farr et al. 2006), and different hosts of *C. acutatum* in Florida. Sequences were aligned with CLUSTALX (Thompson et al. 1994) and adjusted manually in Mega 4.0 using default parameters.

Maximum parsimony analysis was performed in Mega 4.0 using closest neighbor interchange (CNI) searches with 100 random taxon additions and gaps were treated as missing

data. Bootstrap analysis according to Felsenstein (1985) was performed using the above search criteria, with 500 repetitions. Maximum likelihood analysis was performed using GARLI v.0.946, with default parameters and a randomly generated starting topology. Analysis was stopped after $5x10^6$ generations or a 0.01 decrease in ML score. Three independent ML runs were performed and the resulting topologies were compared in TreeView v1.6.6. If similar topologies were generated, bootstrap analysis was performed using the same parameters with 500 repetitions. The *C. gloeosporioides* isolate Cg131 (*Mangifera indica*, Chapter 3), *C. magna* isolate L2.5, and *C. graminicola* M1.001 were used as outgroup taxa.

Pathogenicity Tests

Inoculum was prepared by growing isolates retrieved from -80°C storage on PDA for 7-10 days (Table 4-2). Conidia were harvested by flooding plates with sterile deionized water, scraping colonies with a sterile glass, and straining the suspension through a layer of sterile cheesecloth. Inoculum suspensions were adjusted to a concentration of 10^6 conidia·ml^{-1}.

Blossom blight tests

Attached panicles were inoculated in the field in January and February, 2008 and 2009. Seven treatments (three isolates of *C. acutatum*, three isolates of *C. gloeosporioides* and a water control) were applied to runoff with a handheld manual spray bottle to inflorescences at the mouse-ear to green-colored stage (florets extended but no pigment development, as described by Schoeman et al. 1995); conidium concentrations were 10^6 conidia·ml^{-1} sterile deionized water. Inflorescences were then covered for 48 hrs with a plastic bag inserted in a Kraft brown paper bag. A randomized complete block design (RCBD) was used, where experimental units were single inflorescences, treatments were replicated five times, and individual trees were blocks. The experiment was conducted three times in the same grove of cv. 'Keitt' at TREC.

Disease severity was rated six times for four weeks after inoculation, using a synoptic key (key 1.5; James, 1971). At the end of the experiment, lesion margins were excised from leaves, surface disinfested, and plated on PDA to confirm presence of the pathogen. Area under the disease progress curve (AUDPC) was calculated for each experimental unit, and y_{max} was disease severity at 28 days after inoculation. Analyses of variance of AUDPC and y_{max} were performed for each experiment, and mean separations were performed using least significant differences (PROC GLM, SAS).

Leaf pathogenicity

During the summers of 2008 and 2009, three attached leaf experiments were conducted in the same grove of cv. 'Keitt' that was used in the above blossom blight experiments. Seven treatments (three isolates of *C. acutatum*, three isolates of *C. gloeosporioides* and a water control) were applied to runoff with a handheld manual spray bottle to newly opened vegetative shoots. Conidium concentrations were 10^6 conidia·ml^{-1} sterile deionized water, and treated shoots were covered with Kraft paper bags for 48 hours. Treatments were replicated four times in a RCBD and trees were blocks. Experimental units were single shoots, and the five youngest leaves in a shoot were subsamples in disease analyses.

Disease severity was measured after 4, 7, 10, and 14 days using key 2.1.2 of James (1971). The experiments were stopped after 14 days due to severe defoliation on some shoots. Lesion margins were excised from leaves, surface disinfested, and plated on PDA to confirm presence of the pathogen. Analyses of variance of AUDPC and y_{max} were performed using PROC GLM in SAS. Mean separations were performed using least significant differences (PROC GLM, SAS).

Fruit pathogenicity

Mature fruit of cvs. 'Florigon', 'Haden', 'Saigon' and 'Turpentine' were harvested from a cultivar collection at TREC in July 2008. On each cv, five treatments (two isolates of *C.*

acutatum, two of *C. gloeosporioides* and a control) were imposed in a completely randomized design (CRD). Fifteen µl droplets of 0.3% water agar, without (control) or with 10^6 conidia·ml^{-1}, were applied to sites on the fruit surface that had been wounded with a sterile needle. Each fruit was treated in two or three locations, depending on fruit size, and treatments were replicated five times.

Fruit were incubated in plastic sweater boxes on wire mesh over moistened paper towels at 25°C in the dark, and disease was assessed after 4, 5 and 6 days. Lesion diameters were measured twice at right angles, and mean diameters were used in statistical analyses. After 6 days, lesion margins were excised from fruit, surface disinfested, and plated on PDA to confirm presence of the pathogen. The experiment was conducted three times. Analyses of variance of AUDPC and y_{max} were performed with PROC GLM in SAS. Mean separations were performed using least significant differences (PROC GLM, SAS).

Results

Survey Results

Colletotrichum spp. were not recovered from high proportions of lesions in the grove survey (4-22% recovery across locations), due to their age or other colonizing fungi; only lesions from which *Colletotrichum* was recovered are reported (Table 4-3). *Colletotrichum acutatum* was only recovered from leaves of 'Keitt' (three locations) and from an unknown cultivar in a mixed orchard; it was prevalent (>70% of all isolates of *Colletotrichum* spp. that were recovered) in only two locations. Fewer trees were sampled in 'Tommy Atkins' groves (3-9 trees) vs 'Keitt' groves (12-17 trees), due to lower incidences of leaf anthracnose, and only *C. gloeosporioides* was recovered from leaf lesions on 'Tommy Atkins'.

The leaf symptoms associated with and caused by *C. gloeosporioides* began as small pinpricks that often coalesced to cover large parts of the lamina (Figure 4-2A). In contrast, *C.*

acutatum caused fewer, angular lesions that grew to approximately 5-7 mm in diameter (Figure 4-2B and data not shown). Although they were larger than individual lesions caused by *C. gloeosporioides*, lesions caused by *C. acutatum* usually did not coalesce. Leaves affected by *C. acutatum* became puckered as healthy leaf tissue around lesions continued to grow. Necrotic areas in lesions eventually senesced and fell from the leaf surface resulting in a "shot-hole" appearance.

In phylogenetic analyses with isolates representing the known diversity of *C. acutatum*, as well as the closely related species *C. phormii* and *C. lupini*, mango isolates from south Florida fell in Sreenivasaprasad and Talhinhas's (2005) group A2 with 53% bootstrap support (MP analysis) (Figure 4-3). MP analysis of the 44 accessions resulted in 5,412 most parsimonious trees (length 65, CI=0.778, RI=0.966). ML analysis resulted in a tree (ln score=-1199.699) of similar topology to the MP majority consensus tree, except that some of the clades had lower bootstrap support (Figure 4-3). The same clades, *C. phormii* (A4, A6, A7), *C. lupini* (A1), A3, and A8, had bootstrap support >65% for both analyses.

Pathogenicity of *C. acutatum* and *C. gloeosporioides* on Mango Organs

Blossom blight

There was a significant isolate effect for AUDPC and y_{max} ($P<0.05$) for each of three experiments (Table 4-4); in each, all isolates of *C. gloeosporioides* caused significant blossom blight, compared with the water controls, whereas isolates of *C. acutatum* did so inconsistently. With few exceptions, isolates of *C. gloeosporioides* caused significantly higher AUDPC and y_{max} values than those of *C. acutatum*.

Leaf anthracnose

Although isolates of *C. acutatum* and *C. gloeosporioides* caused comparable levels of anthracnose on artificially inoculated leaves, disease development was inconsistent among

isolates and the three experiments (Table 4-5). Overall treatment effects were insignificant in experiment 1 (P=0.0640 and 0.0565 for AUDPC and y_{max}, respectively), and only one of the isolates, Ca2, caused AUDPC and y_{max} values significantly higher than the water control in all three experiments. Both species caused symptoms similar to those observed on naturally affected leaves in the field (Figure 4-2) and, in general, caused more severe disease than the water control.

Fruit anthracnose

All isolates produced significant lesions on wounded fruit of each of the four cvs that were tested (Table 4-6). On each cultivar, *C. acutatum* generally produced smaller lesions and lower AUDPC values than *C. gloeosporioides*, but these differences were often not significant.

Discussion

Fitzell (1979) first reported *C. acutatum* as a cause of mango anthracnose in New South Wales. He isolated *C. acutatum* from leaves and fruit, and reproduced anthracnose symptoms on leaves, panicles and fruit. *Colletotrichum acutatum* has also been reported on mango in Taiwan (Weng and Chang 1995) and Homestead, FL (Riveras-Vargas et al. 2006).

Riveras-Vargas et al. (2006) isolated *C. acutatum* only from mango flowers, peduncles and immature fruit. In contrast, in the present study *C. acutatum* was found most often on leaves, once on a panicle, and never on fruit (data not shown). Why such different results were obtained in generally the same production area is not clear. The previous study included a single location from Homestead, and the cultivars sampled were not reported. It is possible that the leaf isolates obtained from this location were not sampled from lesions caused by *C. acutatum.* Additionally, a population of *C. acutatum* other than group A2, as defined by Sreenivasaprasad and Talhinhas (2005), could have been involved in the previous study, different groups of cultivars may have been examined, or production practices or other conditions may have differed.

Regardless of the reason(s) for these differences, there was substantial agreement between results from the field survey and the artificial inoculations in the present study. Although complete data is available for only for 'Keitt', it appears that *C. acutatum* may be an important pathogen only on mango leaves, on which it causes symptoms that are distinct from those that are caused by *C. gloeosporiodes*. As reported in previous studies (Fitzell 1979, Freeman and Shabi 1996, Peres et al. 2002), *C. acutatum* also caused lesions on wounded fruit. However, during field surveys *C. acutatum* was recovered only once from naturally blighted inflorescences and it was never recovered from fruit lesions, even in locations where it was most prevalent on leaves. If *C. acutatum* plays a significant role in the development of blossom blight and fruit anthracnose on mango, it occurs on different cultivars or under different environmental conditions than were examined in the present study.

Colletotrichum acutatum causes blights and anthracnose diseases on leaves, fruit and inflorescences of other hosts. It is not a general necrotroph, and different host- and tissue-specific populations exist (Peres et al. 2005). For example, on citrus one lineage of *C. acutatum* causes key lime anthracnose and affects all young tissues of *Citrus aurantifolia*, and another causes post-bloom fruit drop on many citrus species and affects only young developing fruit (Agostini et al. 1992, Timmer et al. 1994, Peres et al. 2008). Therefore, it is not surprising that the mango population would show some tissue specificity.

Colletotrichum acutatum sensu lato is a species complex (Sreenivasaprasad and Talhinhas 2005). Although phylospecies within the taxon have not been thoroughly characterized, *C. lupini* and *C. phormii* have been described and resolved within *C. acutatum* sensu lato (Nirenberg et al. 2002, Farr et al. 2006). Mango isolates from the present study had identical ITS sequences and fell in Sreenivasaprasad and Talhinhas's (2005) A2 group. A2 is diverse and contains isolates

from *Capsicum*, *Coffea*, *Olea*, *Fragaria*, *Hevea* and *Citrus*. It is common in North American and Europe, and found in Florida on strawberry. Additional work is needed on the phylogeny and biology of the mango population of *C. acutatum* that was identified in this study and to determine its relationship to those in other locations.

Results from the leaf anthracnose survey suggest that the *C. acutatum* and *C. gloeosporiodes* are not uniformly distributed in south Florida. In each of the examined groves, one of the species prevailed (Table 4-3). *Colletotrichum acutatum* was recovered in only four four locations, and was prevalent in two. In addition, it was never recovered from 'Tommy Atkins' leaves.

Anthocyanins, the end-products of the flavonoid biosynthesis pathway, have been associated with disease resistance in other pathosystems (Hammerschmidt and Nicholson 1977, Kraft 1977, Wegulo et al. 1998). In mangos, anthocyanin content varies among different cultivars (based on color of emerging shoots) and as vegetative shoots develop, and increase as leaves emerge but decline as they expand (Ali et al. 1999). Whether the differential responses of 'Tommy Atkins' and 'Keitt' leaves to *C. acutatum* that were observed in this study are associated with anthocyanins should be investigated. If a relationship between anthocyanins and mango anthracnose exists, it would probably be complex. For example, the wide range in anthracnose responses among red fruited mango cultivars (e.g. fruit of 'Tommy Atkins' are tolerant but those of 'Sensation' are susceptible) suggests that different flavonoids would either be responsible for these interactions on fruit or that flavonoids are involved in anthracnose responses on some, but not all organs.

By comparing the geographic distribution of the two species reported in the USDA-ARS Fungal Database (http://nt.ars-grin.gov/fungaldatabases/index.cfm), it is apparent that *C.*

acutatum is most important in temperate regions and *C. gloeosporioides* is more common in the tropics. Thus, the subtropics may be a transition zone for the two species, and may be the only location where *C. acutatum* would be expected to be found on a tropical crop such as mango. To date, *C. acutatum* has only been found on mango in the subtropics (Fitzell 1979, Weng and Chang 1995, Riveras-Vargas et al. 2006).

Colletotrichum acutatum and *C. gloeosporioides* cause anthracnose on peach, apple, pecan, grape, almond, strawberry and other hosts, often in the same location (Kummuang et al. 1984, Smith and Black 1990, Bernstein et al. 1995, Shi et al. 1996, Freeman et al. 1998, Gonzalez and Sutton 2004, Hong et al. 2008). On some of these hosts, such as strawberry and apple (Smith and Black 1990, Ureña-Padilla et al. 2002, Gonzalez and Sutton 2004), the two species cause distinct symptoms similar to the situation on 'Keitt' leaves in the present study.

This study sheds new light on the role played by *C. acutatum* on mango. *Colletotrichum acutatum* affects newly emerged leaves of 'Keitt', but additional work is needed to fully understand its impact on other cultivars and organs of this important fruit crop.

Table 4-1. ITS sequences included in phylogenetic analysis

Accession	Species	Host	Origin	Genbank accession #	Group designation[a]	Published[b]
ATCC 56816[c]	*G. acutata*	*Carica papaya*	Australia	DQ286132	---	Farr et al. 2006
MEP1534	*G. acutata*	*Leucaena* sp.	Mexico	DQ286130	---	Farr et al. 2006
ATCC MYA-662	*G. acutata*	*Malus domestica*	USA	DQ286121	---	Farr et al. 2006
MEP1323	*G. acutata*	*Vaccinium*	New Zealand	DQ286124	---	Farr et al. 2006
AR2826	*C. lupini*	*Lupinus*	USA	DQ286119	---	Farr et al. 2006
AR2820	*C. lupini*	*Lupinus*	USA	DQ286117	---	Farr et al. 2006
AR3410	*C. phormii*	*Phormium cookianum*	South Africa	DQ286138	---	Farr et al. 2006
CBS198.35	*C. phormii*	*Phormium*	England	DQ286144	---	Farr et al. 2006
AR3787	*C. phormii*	*Phormium*	South Africa	DQ286146	---	Farr et al. 2006
CBS199.35	*C. phormii*	*Phormium*	England	DQ286142	---	Farr et al. 2006
MEP1334	*C. phormii*	*Phormium*	New Zealand	DQ286140	---	Farr et al. 2006
CBS199.35	*C. phormii*	*Phormium*	England	DQ286142	---	Farr et al. 2006
BBA 67435	*G. cingulata*[d]	*Sambucus nigrum*	Germany	AJ301931	A4	Nirenberg et al. 2002
BBA 62124	*C. acutatum*	*Coffea arabica*	---	AJ301924	A5	Nirenberg et al. 2002
BBA 71249	*C. lupini*	*Lupinus albus*	Canada	AJ301959	A1	Nirenberg et al. 2002
BBA 70358	*C. lupini*	*Lupinus albus*	Germany	AJ301933	A1	Nirenberg et al. 2002
BBA 70338	*C. acutatum*	*Tulipa*	---	AJ301910	A3	Nirenberg et al. 2002
STE-U 4459	*G. acutata*	*Protea*	South Africa	AY376507	A2	Lubbe et al. 2004
STE-U 164	*G. acutata*	*Pinus radiata*	South Africa	AY376498	A5	Lubbe et al. 2004
STE-U 5287	*G. acutata*	*Malus domestica*	USA	AY376509	A3	Lubbe et al. 2004
IMI 345026	*C. acutatum*	*Fragaria*	Spain	AJ536209	A4	Martinez-Culebras et al. 2003
IMI 345585	*C. acutatum*	*Fragaria*	New Zealand	AJ536213	A7	Martinez-Culebras et al. 2003
IMI 345027	*G. acutata*	*Fragaria*	France	AJ536199	A2	Martinez-Culebras et al. 2003
IMI 3600866	*C. acuatum*	*Fragaria*	Japan	AJ536205	A2	Martinez-Culebras et al. 2003
05-200	*G. acutata*	*Rumohra adiantiformis*	Florida	EU647311	---	MacKenzie et al. 2009
Ss	*G. acutata*	*Citrus aurantifolia*	Florida	EU647307	---	MacKenzie et al. 2009
STF-FTP-10	*G. acutata*	*Citrus sinensis*	Florida	EU647306	---	MacKenzie et al. 2009
OCO-ARC-4	*G. acutata*	*Citrus sinensis*	Florida	EU647305	---	MacKenzie et al. 2009
02-163	*G. acutata*	*Fragaria*	Florida	EU647302	---	MacKenzie et al. 2009
PD85-694	*G. acutata*	*Chrysanthemum*	Netherlands	AJ749675	A2	Talhinas et al. 2004, unpublished

Table 4-1. Continued.

Isolate name	Species	Host	Origin	Genbank accession #	Group designation	Published[a]
CBS193.32	*C. acutatum*	*Olea europaea*	Italy	AJ749688	A4	Talhinhas et al. 2005
PT250	*C. acutatum*	*Olea europaea*	Portugal	AJ749700	A6	Talhinhas et al. 2005
S8	*G. acutata*	*Rhododendron*	Sweden	AF411731	A4	Vinnere et al. 2002
IMI 117619[e]	*G. acutata*	*Carica papaya*	Australia	AF411701	A5	Vinnere et al. 2002
IMI 223120	*G. acutata*	*Anemone*	Australia	AF272783	A2	Freeman et al. 2001
PCN5	*G. acutata*	*Carya illionoinensis*	USA	AF272786	A3	Freeman et al. 2001
TOM-21	*G. acutata*	*Tamarillo*	Colombia	AF521196	A8	Afanador-Kafuri et al. 2003
TOM-9	*G. acutata*	*Tamarillo*	Colombia	AF521205	A8	Afanador-Kafuri et al. 2003
S2	*G. acutata*	*Fragaria*	Thailand	DQ454018	---	Than et al. 2006
IMI 383015	*G. acutata*	*Hevea*	India	AF488778	A2	Saha et al. 2002
Cooley2	*G. acutata*	*Fragaria*	---	AF489558	A3	Denoyes-Rothan et al. 2003
MAFF 306282	*G. acutata*	*Fragaria*	Japan	AB042300	A3	Moriwaki et al. 2002
Ca3	*C. acutatum*	*Mangifera indica*	Florida	GU045506	---	---
Ca13	*C. acutatum*	*Mangifera indica*	Florida	GU045507	---	---
Ca26	*C. acutatum*	*Mangifera indica*	Florida	GU045508	---	---
Ca33	*C. acutatum*	*Mangifera indica*	Florida	GU045509	---	---
Ca60	*C. acutatum*	*Mangifera indica*	Florida	GU045510	---	---
Outgroup						
Cg131[f]	*C. gloeosporioides*	*Mangifera indica*	Florida	GQ373223	---	---
L2.5	*C. magna*	*Citrullus lanatus*	USA	DQ003103	---	Du et al. 2005
M1.001	*C. graminicola*	*Zea mays*	Missouri	DQ003110	---	Du et al. 2005

[a] Group designation based on ITS analysis published in Sreenivasaprasad and Talhinhas 2005 [b] Publication in which ITS sequence was published. [c] Type speciment for *C. acutatum* [d] Species diagnosis in Genbank is questionable [e] Paratype specimen for *C. acutatum* [f] Outgroup taxon, recovered from *Mangifera indica* in Florida (see Chapter 3)

Table 4-2. Isolate, species and mango origin for isolates that were used in pathogenicity tests

Isolate	Species	Tissue origin	Organ on which isolate tested for pathogenicity
Ca1	*C. acutatum*	Leaf	Blossom, Leaf, Fruit
Ca2	*C. acutatum*	Leaf	Blossom, Leaf, Fruit
Ca3	*C. acutatum*	Leaf	Blossom, Leaf
Cg135	*C. gloeosporioides*	Immature fruit	Blossom, Leaf, Fruit
Cg136	*C. gloeosporioides*	Leaf	Blossom, Leaf
Cg131	*C. gloeosporioides*	Mature Fruit	Blossom, Leaf, Fruit

Table 4-3. Results for leaf anthracnose surveys

Location[a]	Cultivar(s)	# trees sampled	# lesions sampled[b]	*Colletotrichum gloeosporioides*[c]	*C. acutatum*
Grove A	Keitt	12	24	22 (92)	2 (8)
Grove B	Keitt	12	24	7 (29)	17 (70)
Grove C	Mixed	3	1	1 (100)	0 (0)
Grove D	Tommy Atkins	3	5	5 (100)	0 (0)
Grove E	Tommy Atkins	9	11	11 (100)	0 (0)
Grove F	Tommy Atkins	6	7	7 (100)	0 (0)
TREC A	Keitt	17	48	8 (17)	40 (83)
TREC B	Mixed	9	9	8 (89)	1 (11)
USDA	Mixed	17	18	18 (100)	0 (0)

[a] Groves A-F are commercial groves, TREC groves are located at the University Florida's Tropical Research and Education Center in Hometead, and the USDA grove is located at the USDA station in Miami. [b] The number of lesions from which *Colletotrichum* was recovered. [c] The numbers (percentages) of lesions that yielded a given species.

Table 4-4. Areas under the disease progress curve (AUDPC) and y_{max} values for blossom blight experiments, 2008-2009

	Exp1		Exp2		Exp3	
Isolate	AUDPC	y_{max}[a]	AUDPC	y_{max}	AUDPC	y_{max}
Ca1	6.92 ± 2.24 ab[b]	0.37 ± 0.09 ab	3.36 ± 0.86 b	0.18 ± 0.04 b	3.00 ± 0.84 bc	0.20 ± 0.07 cd
Ca2	3.62 ± 1.21 bc	0.20 ± 0.04 bc	3.69 ± 0.42 b	0.24 ± 0.04 b	3.14 ± 1.96 bc	0.21 ± 0.15 cd
Ca3	2.86 ± 1.11 c	0.20 ± 0.07 bc	2.53 ± 0.68 b	0.15 ± 0.03 b	5.76 ± 1.69 b	0.40 ± 0.10 bc
Cg131	---	---	---	---	11.38 ± 2.31 a	0.74 ± 0.11 a
Cg136	8.92 ± 1.89 a	0.56 ± 0.12 a	7.66 ± 1.72 a	0.49 ± 0.09 a	3.39 ± 0.29 bc	0.18 ± 0.03cd
Cg135	7.75 ± 2.22 a	0.51 ± 0.16a	8.43 ± 0.39 a	0.64 a	12.33 ± 1.53 a	0.69 ± 0.10a
Control	1.05 ± 0.28c	0.10 ± 0.02c	1.94 ± 0.52 b	0.15 ± 0.04 b	0.98 ± 0.27 c	0.06 ± 0.01 d
LSD	3.85	0.22	2.52	0.18	4.65	0.32
P-value	0.0001	<0.0001	0.0047	0.0032	<0.0001	0.0006

[a] y_{max} is the severity value for panicles 28 days after inoculation [b] Values in columns with same letter are not significantly different based on Fisher's LSD, *P*=0.05.

Table 4-5. Areas under the disease progress curves (AUDPC) and y_{max} values for leaf anthracnose experiments, 2008-2009

	Exp1		Exp2		Exp3	
Isolate	AUDPC	y_{max}[a]	AUDPC	y_{max}	AUDPC	y_{max}
Ca1	0.73 ± 0.20 ab[b]	0.10 ± 0.02 b	0.67 ± 0.17 a	0.11 ± 0.04 ab	0.87 ± 0.20 bc	0.13 ± 0.03 cd
Ca2	1.59 ± 0.67 a	0.34 ± 0.14 a	0.51 ± 0.16 abc	0.08 ± 0.03 abc	1.22 ± 0.18 b	0.21 ± 0.03 bc
Ca3	1.09 ± 0.41 a	0.15 ± 0.06 ab	0.59 ± 0.15 ab	0.12 ± 0.04 a	---	---
Cg131	0.92 ± 0.13 ab	0.13 ± 0.02 b	0.30 ± 0.10 bcd	0.04 ± 0.01 cd	2.10 ± 0.32 a	0.32 ± 0.05 a
Cg136	---	---	0.25 ± 0.04cd	0.04 ± 0.01 cd	1.48 ± 0.21 ab	0.23 ± 0.03 ab
Cg135	1.47 ± 0.45 a	0.19 ± 0.05 ab	0.37 ± 0.07 abc	0.07 ± 0.02 bcd	1.34 ± 0.24 b	0.20 ± 0.04 bc
Control	0.05 ± 0.02 b	0.01 ± 0.00 b	0.03 ± 0.02 d	0.01 ± 0.00 d	0.53 ± 0.09 c	0.10 ± 0.02 d
LSD	1.01	0.21	0.31	0.06	0.62	0.10
P-value	0.0640	0.0565	0.0050	0.0039	<0.0001	0.0002

[a] y_{max} is the severity value for panicles 28 days after inoculation [b] Values in columns with same letter are not significantly different based on Fisher's LSD, P=0.05.

Table 4-6. Areas under the disease progress curve (AUDPC) and y_{max} (lesion diameter) values for detached fruit experiments, 2008-2009

Isolate	'Florigon'		'Haden'	
	AUDPC	Lesion diameter (mm)[a]	AUDPC	Lesion diameter (mm)
Ca1	11.67 ± 1.48 ab[b]	9.67 ± 1.12 a	11.88 ± 4.72 ab	8.25 ± 2.56 a
Ca2	10.5 ± 1.88 b	8.20 ± 1.52 a	10.75 ± 3.38 ab	9.25 ± 2.82 a
Cg135	15.90 ± 1.97 a	12.80 ± 1.08 a	13.50 ± 1.77 a	10.00 ± 0.54 a
Cg131	15.00 ±2.77 ab	11.20 ± 2.20 a	19.69 ± 4.25 a	11.88 ± 2.37 a
Control	4.04 ± 0.04 c	2.08 ± 0.08 b	2.13 ± 0.13 b	1.00 ± 0.00 b
LSD	5.19	6.09	10.00	6.09
P-value	0.0006	<0.0001	0.0293	0.0167
	'Saigon'		'Turpentine'	
	AUDPC	Lesion diameter (mm)	AUDPC	Lesion diameter (mm)
Ca1	19.50 ± 2.01 ab	13.75 ± 0.83 ab	14.63 ± 3.61 b	12.00 ± 3.03 a
Ca2	15.19 ± 2.97 b	11.88 ± 1.23 b	14.19 ± 3.17 b	14.13 ± 2.22 a
Cg135	17.63 ± 1.13 b	13.75 ± 0.72 ab	25.63 ± 4.88 a	17.75 ± 3.25 a
Cg131	24.17 ± 1.74 a	16.33 ± 0.89 a	25.19 ± 2.25 a	17.63 ± 1.12 a
Control	2.75 ± 0.43 c	1.50 ± 0.29 c	2.00 ± 0.00 c	1.00 ± 0.00 b
LSD	3.75	3.75	3.33	3.33
P-value	<0.0001	<0.0001	0.0004	0.0004

[a] Lesion diameters 14 days after inoculation [b] Values in columns with same letter are not significantly different based on Fisher's LSD, P=0.05.

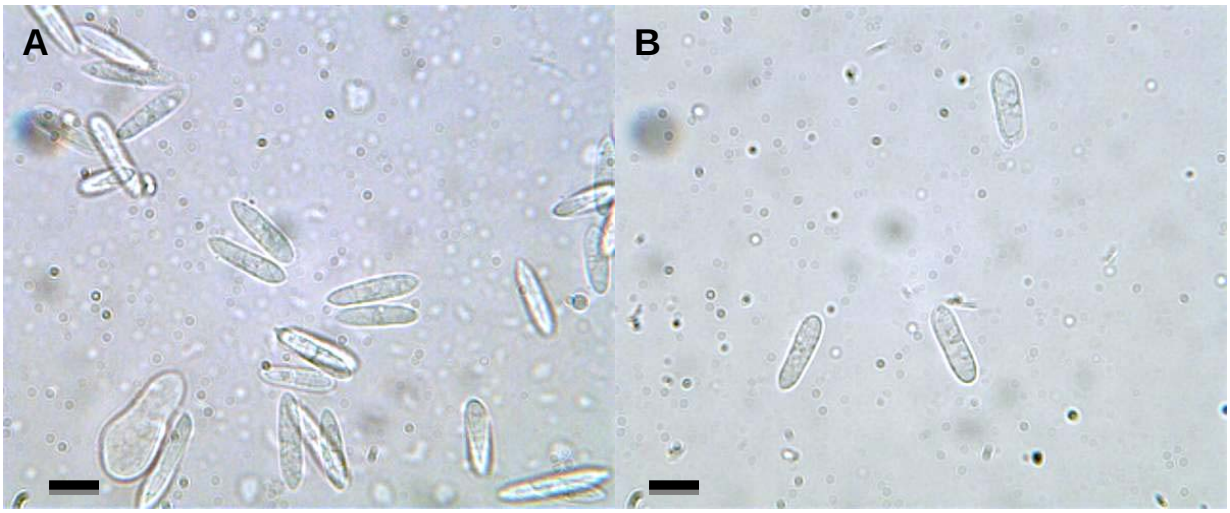

Figure 4-1. Conidia of A) *Colletotrichum acutatum* were slightly fusiform, whereas those of B) *C. gloeosporioides* had cylindrical tips. Bars=10µm

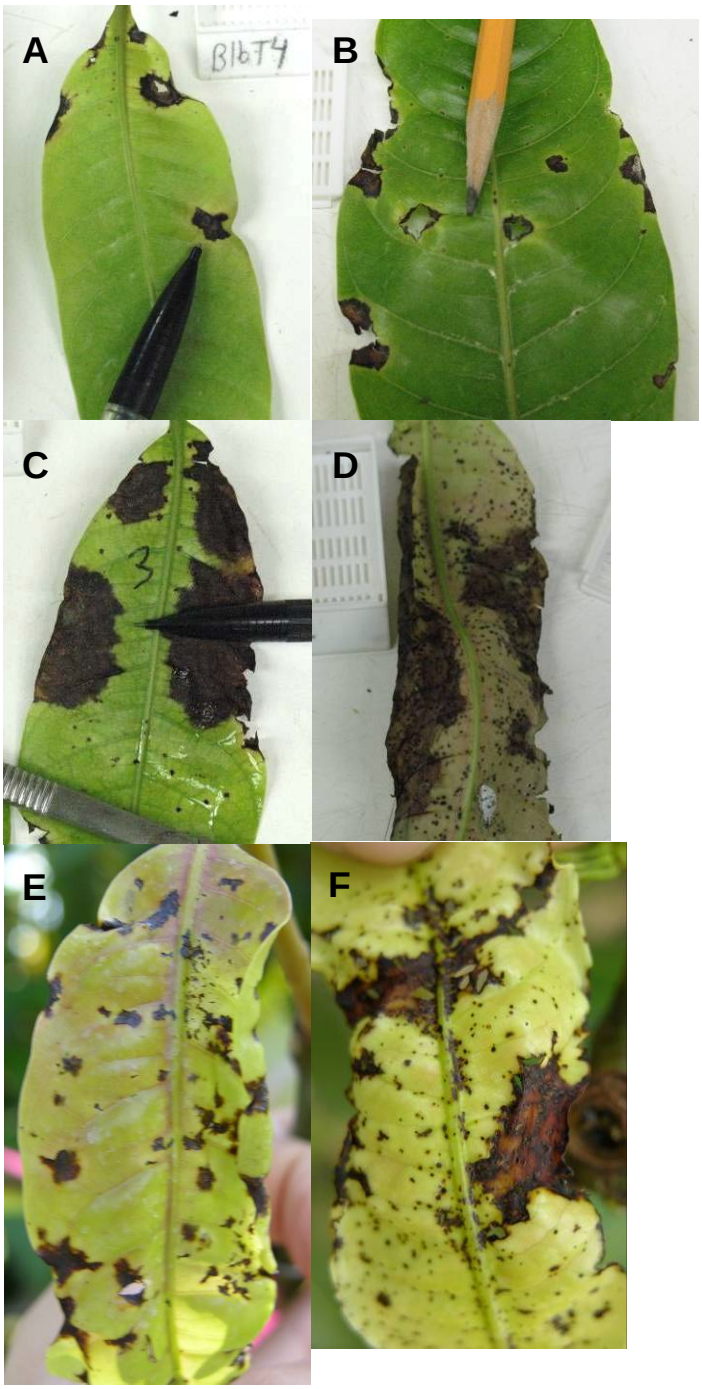

Figure 4-2. Anthracnose lesions collected during the grove survey (A-D) and lesions that were produced during pathogenicity tests (E-F). Lesions in A) and B) were associated with *Colletotrichum acutatum* and displayed an angular morphology and shot-holes. Lesions in C) and D) were associated with *C. gloeosporioides* and were initially small and numerous, but coalescenced to encompass large areas. After inoculation, with lesions were caused by E) *C. acutatum* and F) *C. gloeosporioides.* Note similar appearances of natural and artificially induced lesions caused by *C. acutatum* and *C. gloeosporioides.*

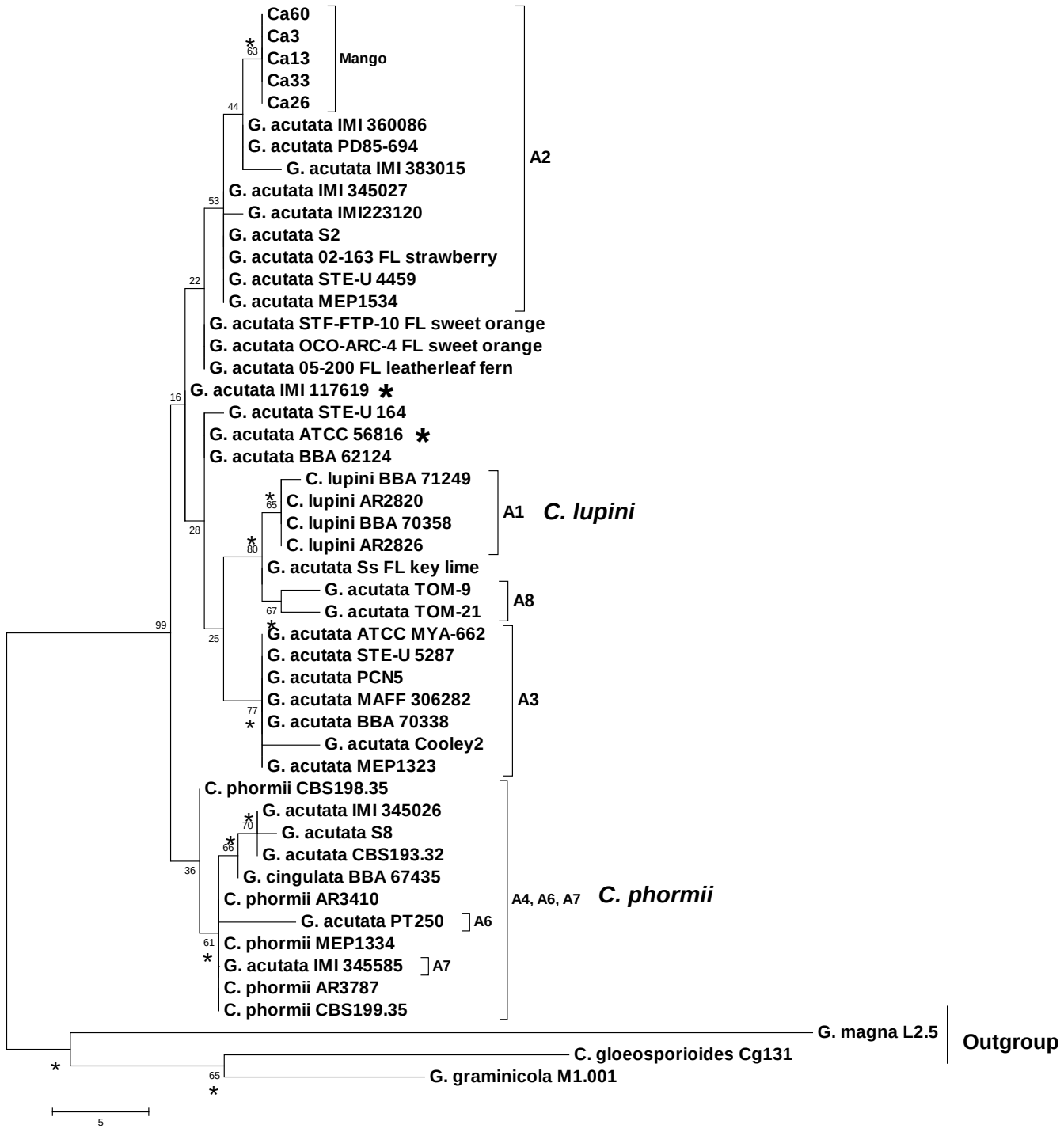

Figure 4-3. Phylogeny for 44 accessions of *Colletotrichum* based on ITS sequence data. The phylogram represents one of 5,412 MP trees (length 65, CI=0.778, RI=0.966). Bootstrap support values are shown at nodes. Nodes with asterisks were supported in maximum likelkihood analysis with >65% bootstrap support. Groupings defined by Sreenivasaprasad and Talhinhas (2005) are indicated with brackets. Type (ATCC 56816) and paratype (IMI 117619) strains of *C. acutatum* are marked with asterisks.

CHAPTER 5
CONCLUSIONS

This study attempted to answer hypotheses about host and tissue specificity of two tropical plant pathogens, *Moniliophthora perniciosa* and *Colletotrichum gloeosporioides*, using phylogenetic analysis of DNA sequence data. These questions were addressed with varying levels of success, and the importance of locus and taxon selection is illustrated in both phylogenetic studies.

In the first study, the utility of the host-based biotype classification used for *M. perniciosa* was evaluated. This biotype classification has traditionally been used to describe diversity within the species (de Arruda et al. 2005), but a complete phylogenetic analysis investigating relationships among biotypes has not been completed. Additionally, several new hosts have recently been identified for which no biotype has been designated (R. Barreto, personal communication). The hypothesis that previously described biotypes represent distinct evolutionary lineages that may comprise a species complex was investigated.

In *M. perniciosa*, phylogenies of the ITS, IGS, and RPB1 regions indicated a level of host-specificity, and the presence of several distinct clades. For this taxon, all regions provided sufficient phylogenetic signal to separate the isolates into several clades, although the RPB1 and IGS provided the strongest signal and the highest bootstrap support for the clades. The IGS was especially rich in indel sites that, when used as characters in the data set, increased support for for the major clades. Most published studies treat indels as missing data, potentially ignoring important data when constructing phylogenies.

The importance of taxon sampling is illustrated in this study, which would have benefited from the inclusion of more accessions of some biotypes. For example, many isolates from cacao and *Solanum* were included, but only one isolate from *Heteropterys acutifolia*, three from other

malpighiaceous hosts, and two from bignonaceous lianas were included. The limited numbers of these isolates hindered an examination of a distinct host-based lineage for this group. Future isolate collection in the pathogen's geographic range from different host species would enable a more robust analysis of the lineages that were identified in the present study.

The second study dealt with relationships among populations of *C. gloeosporioides* that are associated with mango. Previous studies indicated that a mango-specific population of *C. gloeosporioides* exists (Hodson et al.1993, Alakahoon et al.1994, Hayden et al.1994), as well as a level of tissue specificity on mango (Gantotti and Davis 1993, Davis 1999). These previous studies relied on a small number of isolates and outdated molecular data, and were not accompanied by robust statistical analyses. The present study re-investigated the lineages of this pathogen that are associated with anthracnose and blossom blight on mango with the specific hypotheses that: a) isolates from mango represent a distinct lineage within *C. gloeosporioides* sensu lato that differ from isolates that affect other tropical fruits; and b) these isolates display some level of tissue specificity.

In the *Colletotrichum* study, three regions were used to determine relationships among isolates. Three clades were resolved that supported the above hypotheses. Clade 1 contained only isolates from mango, and included isolates that caused blossom blight, leaf anthracnose, and fruit anthracnose. Clades 2 and 3 contained most fruit and peduncle isolates from mango and fruit isolates from other tropical hosts. They caused fruit anthracnose, but not leaf anthracnose and blossom blight, on mango. The three DNA regions provided varying levels of resolution. The ITS region was not very informative, with only 11 ingroup informative characters and low bootstrap support values for the clades. The MAT1-2 region, with 13 ingroup informative characters, resolved the mango clade (Clade 1), but not Clades 2 and 3. The CGTT5 region was

more informative, with 61 informative sites and resolution of the three clades. These clades were also resolved by combining the congruent MAT1-2 and CGTT5 data sets. The varying degree of resolution achieved by the different regions illustrated the importance of region selection during phylogenetic analyses.

Cryptic species that were found in the present studies are widespread and often associated with a specific host or ecological niche. As was demonstrated, molecular data can be especially useful for organisms with few morphological features. In *M. perniciosa* there was evidence for *Theobroma*-, *Solanum*-, and malpighiaceous-specific lineages, although these associations were not absolute. In *Colletotrichum*, a mango-associated lineage was revealed. Whereas Clade 1 contained only mango isolates, Clades 2 and 3 included generalists from many hosts. Tissue specificity was apparent in the latter study in that the mango clade contained most of the isolates from green portions of the tree (leaves, panicles, peduncles) and immature fruit, whereas isolates from mature fruit usually fell into other clades. Pathogenicity tests indicated that isolates in the mango clade cause anthracnose on all of the above organs, but that those from the general clades also cause anthracnose on ripe fruit. Investigations are warranted to determine why only isolates in Clade 1 are able to infect the above host tissues.

Tissue specificity is exhibited by other taxa and lineages of *Colletotrichum*. On strawberry, *C. acutatum* is the major cause of fruit anthracnose, whereas *C. gloeosporioides* and *C. fragariae* cause crown rot (Ureña-Padilla et al. 2002). And on citrus, two lineages of *C. acutatum* cause either key lime anthracnose, which affects all young tissues of key lime, or post-bloom fruit drop, which affects only young developing fruit of this and other *Citrus* spp. (Agostini et al. 1992, Timmer et al. 1994, Peres et al. 2008).

According to the phylogenetic species concept, phylogenetically distinct groups may be considered distinct species even if they do not possess defining morphological characters. As more molecular data becomes available and the power increases to resolve ever smaller groups, it will become increasingly important to establish the criteria for which species would be identified and what other characters might be considered when a new species is described. Adopting the evolutionary species concepts enable the incorporation of additional biological data to support the description of new species (Wiley 1978, Taylor et al. 2000). Agreeing upon and implementing such criteria would have tremendous academic and practical importance. There is a continous range of diversification between the individual and species. Carbone and Kohn (2001) discussed a "population-species" interface, wherein the threshold between populations and species can be somewhat arbitrary. In addition to monophyly, other criteria must be incorporated into a working species concept. For fungi, these include: morphological, ultrastuctural or biochemical characters; ecological niche; host range; substrate; mating population; virulence; and, as shown for the mango clade in Chapter 3, host and tissue specificity.

With the exception of the L-biotype reported from Ecuador (Griffith and Hedger 1994a,b), *M. perniciosa* utilizes a homothallic sexual reproductive strategy. In contrast, *C. gloeosporioides* appears to reproduce in the field only asexually. Whereas heterothallism enables adaptability via outcrossing and meitotic recombination, homothallism enables the fixation of genotypes that are well-suited to hosts and environments in clonal populations (Milgroom 1996). Entire genotypes rather than individual genes are propagated in clonal populations (Milgroom 1996), thus leading to the phylogenetic patterns observed in the present studies: closely related, host-associate lineages with low diversity within clades.

Homothallism confers the following advantages to a species. Because homothallic species are self-fertile, there is no need for different mating types and sexual reproduction can take place even in small populations. Homothallic species may become highly evolved in specific environments, and outbreeding may actually dilute these specific adaptations. In monocultures that prevail in agriculture, homothallism enables reproduction even when small numbers of individuals survive control measures; these populations can develop into highly adapted and stable genetic lines (Wheeler 1954, Anderson et al. 1992, Milgroom 1996).

Asexual pathogens, such as *C. gloeosporioides*, possess the same attributes. Given the prevalence of anamorphic plant-pathogenic genera (e.g. *Fusarium*, *Colletotrichum*, *Verticillium* and *Botrytis*), one might ask how asexual and homothallic sexual taxa differ and whether one reproductive strategy is superior to the other. Although both should result in identical offspring, there is some evidence that homothallic species can outcross occasionally, resulting in variation not seen in clonal species. Examples include many oomycetes (Cooke et al. 2005, Francis et al. 1994, Forster et al. 1994, Francis and St. Clair 1993), *Saccharomyces* (Johnson et al.2004), and, based on incongruence noted for isolates CPB7 and CPB9 in Chapter 2, possibly *M. perniciosa.* Retention of a sexual cycle has also been shown to reduce the accumulation of deleterious mutations in populations (Bruggeman et al. 2003). Asexual and sexual strains of *Aspergillus nidulans* were grown in culture for 38 generations and the growth rate of the 39th generation was used as a measurement of fitness. Although both types of strains lost fitness, the asexual strains were significantly less fit.

Phylogenomics will provide larger character sets for phylogenetic analyses, and enable genome evolution to be tracked between closely related species. For example, over 90% of the genomes of *Mycosphaerella graminicola* and a closely related pathogen of wild grass were

similar, but considerable rearrangement had occurred in their respective dispensable chromosomes (Stuckenbrock et al. 2009). A similar study has been undertaken comparing *M. perniciosa* and *M. roreri* (Meinhardt et al. 2008a). They report that 70% of the gene sequences in *M. perniciosa* and *M. rorei* had at least 80% sequence identity and that the species shared several pathogenicity factors. Genomic comparisons of biotypes and homothallic and heterothallic lineages of *M. perniciosa* would provide insight into the evolution of this pathogen, and could help define the role that host jumps, as opposed to coevolution, played in the development of different host associations and specificity in the species.

Additional work is needed to define *Moniliophthora* and the species it contains. The genus *Moniliophthora* now contains just six species (Aime and Phillips-Mora 2005, Kerekes and Desjardin 2009). *Crinipellis* species in section Iopodinae, such as *C. eggersii*, should be re-examined and included in molecular phylogenies to determine if they should be transferred to *Moniliophthora.* Additional collections in South America may identify new hosts of *M. perniciosa* and host relationships in the species and genus.

Three lineages of *C. gloeosporioides* sensu lato occur on mango in South Florida, one of which, Clade 1, could be considered a distinct species similar to *C. musae*, *C. kahawae,* and *C. fragariae*. Sequence data for the regions that were used in the present study should be obtained for the *C. gloeosporioides* type specimen, IMI 356878 (Cannon et al.2008), to determine if any of any these lineages are *C. gloeosporioides* sensu stricto. To fully understand variation in *C. gloeosporioides* sensu lato and the species that it contains, isolates from additional geographic regions and hosts should also be examined to determine their relationships to one another and to *C. gloeosporioides* sensu stricto. Currently, only the ITS sequence is available for the type. Although it was not included in the ITS analysis in the present study, when compared to

sequences for Clade1, 2, and 3 isolates, the type sequence differs by 5 bases (1.0%), 3 bases (0.6%), and 4 bases (0.8%), respectively (data not shown). Since these differences are comparable to those that separate *C. musae, C. kahawae,* and *C. fragariae,* the mango clade may represent a new species in the *C. gloeosporioides* sensu lato complex.

Studies like the one presented here are first steps in clarifying the complexity of *C. gloeosporioides* sensu lato. Once phylogenetic lineages are identified, additional host range, substrate, morphology, geographic and ecological characters are needed to understand the biological and evolutionary importance of these distinctions. One of the strengths of this study is that the identification of the mango and "general" clades is accompanied by pathogenicity and morphology data. Future investigations involving isolates belonging to these clades will add further understanding of the biology and ecology of evolutionary species of *C. gloeosporioides* sensu lato on mango

Work is needed on the infection strategies of the different clades on mango and on the physiology of the host-pathogen interaction. The nature of quiescent infections of fruit by *C. gloeosporioides* has been well characterized on avocado fruits, and include alkalization of host tissue, activation of pectate lyase, and production of sugars (Prusky and Lichter 2007, Kramer-Haimovich et al. 2006). Similar topics should be evaluated for the different clades that occur on mango. Microscopic study of the infection processes of isolates from different clades on leaves and fruits could clarify the physical nature of infection on different host tissues, and whether those from all lineages are able to infect and ultimately cause disease. The tolerance of isolates to antifungal resorcinols found in mango peels (Hassan et al. 2007, Knödler et al. 2007), as well as the levels of these compounds that occur in leaves and inflorescences should also be

characterized to determine the roles that resorcinols play in the development of disease on different organs.

These types of studies are a logical progression from phylogenetic studies that simply identify the existence of distinct clades. The next step is to understand the roles that the different lineages play in the cacao and mango agroecosystems, and to inform future ecological, physiological, pathological research.

APPENDIX A
IDENTIFICATION AND CHARACTERIZATION OF FIVE RANDOMLY AMPLIFIED DNA REGIONS FLANKING MICROSATELLITE REPEATS FOR USE IN PHYLOGENETIC ANALYSIS OF *COLLETOTRICHUM GLOEOSPORIOIDES*

Introduction

Colletotrichum gloeosporioides is a pathogen of hundreds of hosts worldwide. Tropical fruit, such as mango, avocado, papaya and banana, are among the most important hosts and can suffer immense losses to this pathogen. Information on the population structure and diversity in *C. gloeosporioides* would help understand the epidemiology of the important diseases it causes and could be used to improve management methods.

Various molecular markers have been used to study population structure and phylogenetics in *C. gloeosporioides.* Most common have been random amplified polymorphic DNAs (RAPDs) primed with commercially available RAPD primers. However, other markers have been obtained with arbitrarily primed-PCR (ap-PCR), random amplified microsatellite (RAMS), microsatellite-primed PCR (mp-PCR), and internal transcribed spacer (ITS) sequences (Freeman et al. 2000, Martinez-Culebras et al. 2002, Ureña-Padilla et al. 2002, Afanador-Kafuri et al. 2003, Mahuku and Riascos 2004, Xiao et al. 2004, Abang et al. 2005, Talhinas et al. 2005, Munaut et al. 2002, Denoyes-Rothan et al. 2003, Martinez-Culebras et al. 2003). While these studies have suggested high levels of diversity within the species, the development of better markers is needed to fully understand the extent and importance of variation in *C. gloeosporioides* sensu lato. For example, due to low numbers of informative sites, ITS sequence data do not give high resolution within *C. gloeosporioides* sensu lato, which includes *C. musae*, *C. fragariae* and *C. kahawae*. RAPD markers also have well-documented problems of repeatability and the equivocal identity of comigrating bands (Harris 1999).

More reliable and meaningful markers, such as simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) and polymorphic DNA sequences, have been applied to a wide range of biological questions (Agarwal et al. 2008). With regard to the DNA sequence data, polymorphic loci were identified and developed in the present study to construct phylogenies in *C. gloeosporioides* sensu lato. Bands that were generated with mp-PCR in isolates of *C. gloeosporioides* from mango were cloned and sequenced with the sequence characterized amplified region (SCAR) technique (McDermott et al.1994). Five new loci were developed, and the extent of polymorphism at each was determined in closely related strains.

Materials and Methods

RAPD analyses were conducted with 24 isolates of *C. gloeosporioides* from mango leaves and fruit. Isolates were grown for 3-5 days in potato dextrose broth (PDB) at room temperature on a shaker at approx. 80 rpm. Mycelia were harvested, triple rinsed with sterile deionized water, dried on sterile filter paper, and DNA was extracted using a DNA genomic preparation protocol from the University of Wisconsin Biotechnology Center. Briefly, mycelia were ground in 500 µl Shorty DNA Extraction Buffer (0.2M Tris-HCl, pH 9.0, 0.4M LiCl, 25mM EDTA, 1% SDS) and incubated at 68°C for 10 minutes. Tissue was centrifuged at 14,000 rpm for 5 minutes, 400 µl of the supernatant was transferred to new tube and the DNA was precipitated with 400 µl 99% isopropanol. The tubes were centrifuged for 10 minutes at 14,000 rpm and the supernatant was decanted. The DNA pellets were air-dried for 5 minutes then resuspended in 400 µl TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) for 30 minutes at room temperature. 2µl DNA was used in PCR reactions.

Three simple sequence repeat primers were used to produce RAPD profiles: $(TCC)_5$, $(GACA)_4$ and $(ACTG)_4$. PCRs were conducted in 50 µl reactions that contained: 39.25 µl of sterile distilled, deionized water; 6.5 µl of ThermoPol Reaction Buffer (New England Biolabs,

Ipswich, MA); 1 µl of 10 mM dNTP mix (New England Biolabs, Ipswich, MA); 0.25 µl of Taq DNA polymerase (conc. 5,000 units/ml); 1 µl of 15 µM primer; and 2 µl of DNA template. Standard cycling parameters with a 55°C annealing temperature were used. PCR products were separated on a 1.5% agarose gel at 60v for 60 min. RAPD band patterns for each primer x isolate combination were compared, and three bands that were present in all isolates were cloned and sequenced. Each of the bands from three different isolates (Cg43, Cg49, and Cg73) was cut from gels with a razor blade, and DNA was extracted with QIAQuick Gel Extraction Kit (Qiagen) following manufacturer instructions. Bands were cloned using pGEM®-T Easy Vector System (Promega). Plasmid DNA was extracted from the cloning vectors with QIAprep Spin Miniprep Kit (Qiagen), and DNA inserts for one or two isolates from each region were sequenced at the University of Florida ICBR. The homology of the resulting sequences was compared to others in GenBank with BLASTn.

SCAR primers were designed using Primer3 and tested on isolates of *C. musae*, *C. capsici*, *C. boninense*, *C. acutatum*, and of *C. gloeosporioides* from avocado, guava, carambola and mango. In addition, 11 mango isolates (Cg20, Cg66, Cg101, Cg127, Cg128, Cg129, Cg130, Cg131, Cg134, Cg135, Cg136) and one guava isolate (Gua1) were chosen for sequencing of the CGTT-3 to CGTT-6 (CGTT=*C. gloeosporioides* cloned by T. Tarnowski) loci to determine how polymorphic these loci were in closely related isolates of *C. gloeosporioides*.

Results and Discussion

RAPD banding patterns were variable among isolates with all three primers. From the $(GACA)_4$ profile, 950bp and 1,200bp bands were cloned, and from the $(TCC)_5$ profile a 1,000bp band was cloned. Two to three cloned inserts were sequenced for each band from which five distinct regions were identified (Table A-1). Clearly, comigrating RAPD bands can represent different DNA sequences. With the exception of the CGTT-1 primers, which produced a faint

additional 1500 bp band for some isolates, each primer pair generated clean, single-band products (Fig. A-1).

The CGTT-3 to CGTT-6 primers were used to directly sequence 12 isolates. The size and BLAST homologies of amplified products, as well as the number of polymorphic sites at each locus are recorded in Table A-2. All loci were amplified for isolates of *C. gloeosporioides* from avocado, as well as *C. musae*. For other *Colletotrichum* species, only the CGTT-6 region was successfully amplified (Figure A-2). Thus, CGTT-6 appeared to be conserved in several species, whereas the other loci were only present in *C. gloeosporioides* sensu lato. Only the CGTT-5 locus was homologous to other sequences of hypothetical proteins in *Gibberella zeae*, *Magnaporthe grisea*, and *Neurospora crassa* in GenBank (Table A-2).

Based on the large number of polymorphisms that were present, CGTT-3 to CGTT-6 should be useful regions for population and phylogenetic studies of *C. gloeosporioides* sensu lato. The number of polymorphisms among 12 isolates in this study ranged from 11-38, whereas the ITS and MAT1-2 regions yielded, respectively, only 11 and 13 informative characters in over 50 accessions in Chapter 3. In the present study the CGTT-5 locus discriminated lineages in this species complex, and increased resolution and internal support when used with data from other DNA regions (Chapter 3). Because *C. gloeosporioides* is a species complex, these regions would be useful to sort out closely related natural lineages in the complex. These regions can also be used in conjunction with already established loci to increase the resolution and internal support of phylogenies. The CGTT regions described have advantages over the RAPD type markers most often used in *Colletotrichum* studies in that they are universal (they can be used by any lab and are reproducible).

Table A-1. Origin of, and statistics for, CGTT loci.

	RAPD bands cloned		
	$(GACA)_4$	$(GACA)_4$	$(TCC)_5$
Size	950bp	1,200bp	1,000bp
# clone inserts sequenced	3 (2 from Cg49; 1 from Cg43)	3 (2 from Cg73; 1 from Cg49)	2 (from Cg49)
# loci sequenced	2 CGTT3 (860bp) CGTT4 (852bp)	1 CGTT1 (873 bp); 3' end of region not successfully sequenced)	2 CGTT5 (895bp) CGTT6 (891bp)

Three comigrating RAPD bands were cloned, and two to three inserts from each were sequenced. Five distinct loci were identified.

Table A-2. PCR primers, product size, homologies, and the extent of polymorphism of CGTT regions

Locus	Primers	PCR product length (bp)	Homology[a]	# polymorphism (% polymorphic)[b]
CGTT-1	Forward: 5'-AGGTGAGCCAACCTGTCAGT-3' Reverse: 5'-AGCAGTCACAGGCACACATC-3'	662	none	n/a
CGTT-3	Forward: 5'-CGATGACGATGATAAGGTG-3' Reverse: 5'-AGCAGTCACAGGCACACATC-3'	669	none	38 (5.6%)
CGTT-4	Forward: 5'-AGACTGATGAAATGCGATGC-3' Reverse: 5'-CTTACATGCCCCTGTTCCAT-3'	813	none	38 (4.7%)
CGTT-5	Forward: 5'-CCCTCAGATTTCAGCCAAAG-3' Reverse: 5'-GCTCTCTCGCTGCTTCATCT-3'	564	*Giberella zeae, Magnaporthe grisea, Neurospora crassa*, hypothetical protein	38 (6.7%)
CGTT-6	Forward: 5'-CGGGGCACACTAACGTAAAA-3' Reverse: 5'-TCATGGGGCTTCCTATTCAG-3'	560	none	11 (2.0%)

[a] Homology with sequences in Genbank using BLASTn algorithm. [b] Extent of polymorphism in a given locus for 12 isolates of *C. gloeosporioides* from mango and guava, followed by percent of polymorphic sites in parantheses

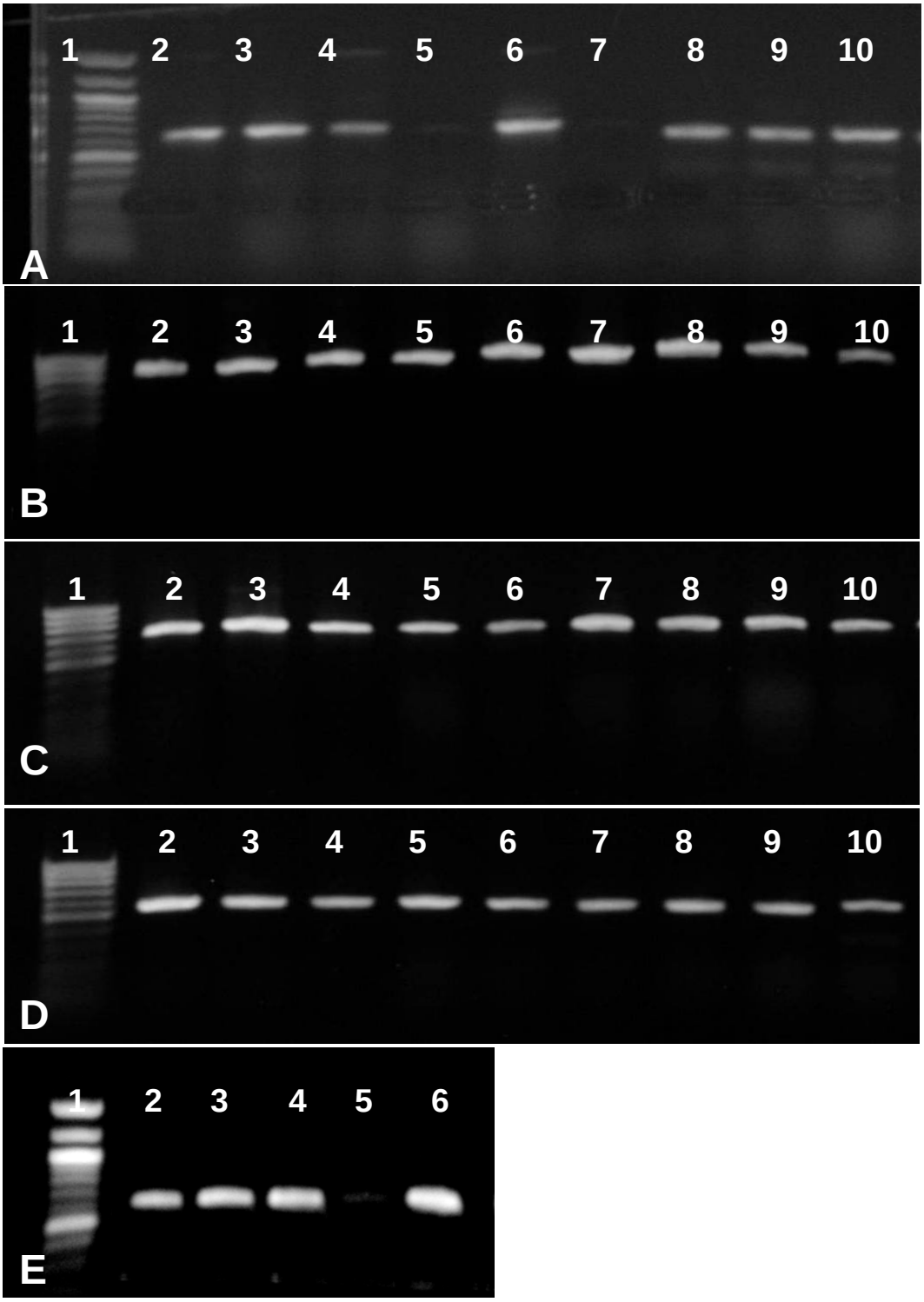

Figure A-1. Amplification of A) CGTT-1, B) CGTT-3, C) CGTT-4, D) CGTT-5, and E) CGTT-6 loci with primers described in Table 1. Lanes 1-10: 1=DNA ladder, 2=Cg20, 3=Cg66, 4=Cg101, 5=Cg127, 6=Cg127, 7=Cg128, 8=Cg129, 9=Cg131, 10=Cg134.

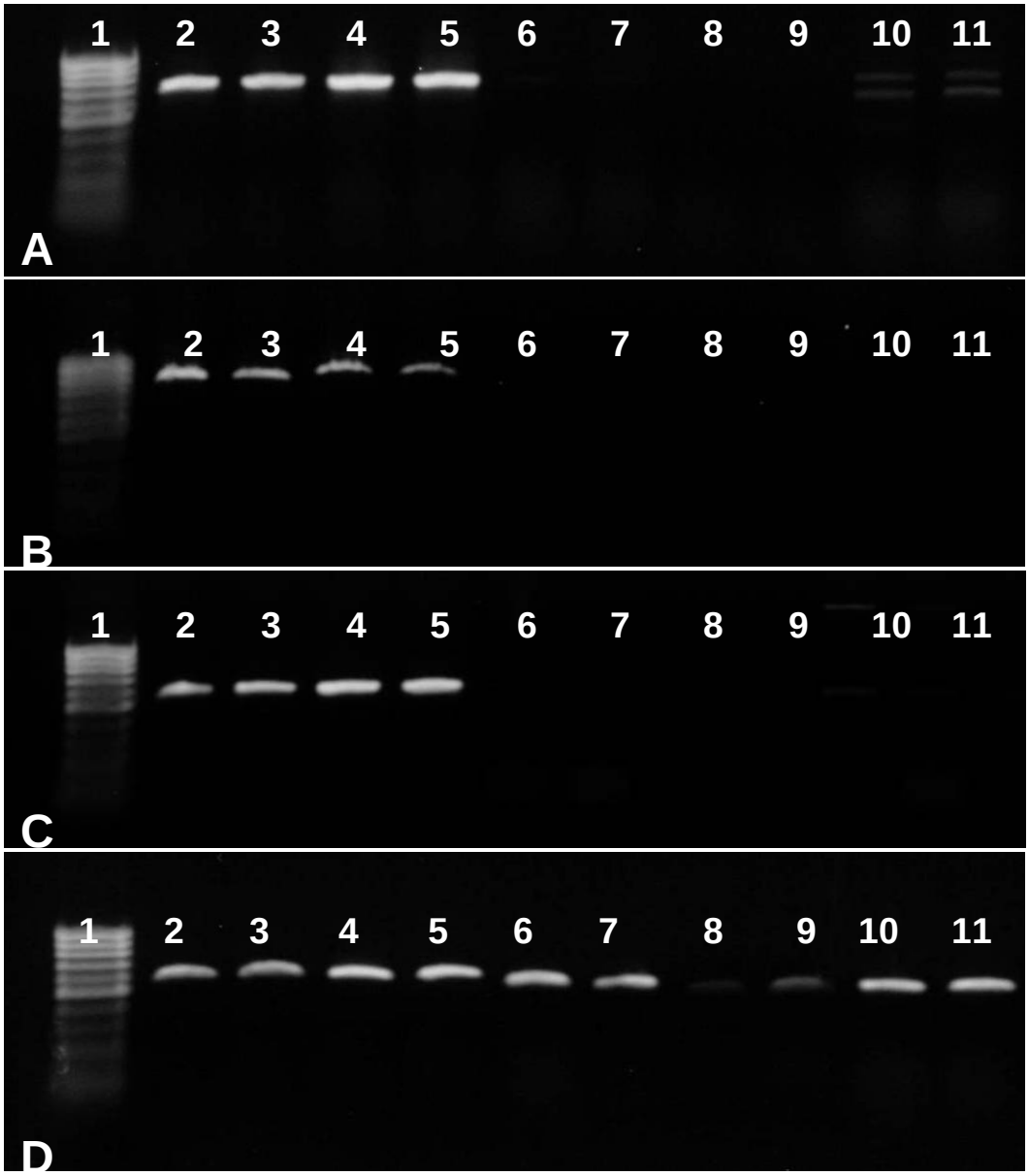

Figure A-2. Amplification of A) CGTT-3, B) CGTT-4, C) CGTT-5, and D) CGTT-6 loci in *C. gloeosporioides* isolates Avo1 and Avo2, lanes 2 and 3; *C. musae* isolates Ban1 and Ban2, lanes 4 and 5; *C. acutatum* isolates Ca3 and Ca13, lanes 6 and 7; *C. capsici* isolates Pap14, Pas5, lanes 8 and 9; *C. boninense* isolates Pas1, Piper, lanes 10 and 11. Lane 1 =DNA ladder.

APPENDIX B
FIRST REPORT OF *COLLETOTRICHUM CAPSICI* CAUSING POSTHARVEST ANTHRACNOSE ON PAPAYA *(CARICA PAPAYA)* IN SOUTH FLORIDA

Postharvest anthracnose of papaya is an important disease in most production areas worldwide (Persley and Ploetz 2003). Two types of symptoms have been associated with *Colletotrichum gloeosporioides*: 1) circular, sunken lesions with pink sporulation; and 2) sharply defined, reddish brown and sunken lesions, described as 'chocolate spot' (Dickman 1994, Persley and Ploetz 2003). Several *Colletotrichum*/*Glomerella* species were recently recovered from anthracnose lesions on papaya fruit from the University of Florida Tropical Research and Education Center (TREC), Homestead, FL in December of 2007, and from fruit imported to a local packinghouse from Belize in March of 2008. Eight lesions on four fruit from TREC and 19 lesions on eight fruit from Belize were examined and the incidence of *C. gloeosporioides*, *C. capsici*, and a *Glomerella* sp. were calculated (Table B-1). Lesions were variable and either: 1) sunken with darkly pigmented centers and masses of pink sporulation; or 2) initially brown and circular, becoming sunken with copious dark sporulation (Fig. B-1A-B).

Fungi were isolated from lesions by streaking sporulating acervuli on PDA and plating individual germinating spores. Species were identified using the morphology of colonies on PDA, conidium shape, whether acervuli were setose or glabrous, and whether a teleomorph formed in culture. Molecular identities were also obtained for representatives of the different morphological groups with ITS sequences that were aligned with sequences of several other *Colletotrichum* species in Genbank (Table B-2). Maximum parsimony analysis was performed in Mega 4.0 (Tamura et al. 2007) using closest neighbor interchange (CNI) searches with 100 random taxon additions and gaps were treated as missing data. Bootstrap analysis according to Felsenstein (1985) was performed using the above search criteria, with 500 repetitions.

Three taxa were identified: *C. gloeosporioides*, *C. capsici* and *Glomerella* sp. (Table B-1). Colonies of *C. gloeosporioides* were white to grey, fluffy with orange sporulation and straight and cylindrical conidia, and those of *C. capsici* had sparse, fluffy, white mycelia with setose acervuli and falcate conidia (Fig. B-2A-B). Colonies of *Glomerella* sp. were darkly pigmented and produced fertile perithecia after 7-10 days. The ITS-based phylogeny (Fig. B-3) identified setose, falcate-spored isolates from papaya as *C. capsici* (Pap14, Pap18), and isolates that formed a teleomorph as a nondescribed *Glomerella* species (Pap3-6, 8). *Colletotrichum capsici* was most often associated with lesion type 3.

Mature fruit (cv. 'Caribbean Red') obtained from Brooks Tropicals Inc. (Homestead, FL) were wounded with a sterile needle and inoculated with a 15µl drop of 0.3% water agar that contained 10^5 conidia ml^{-1} of a given isolate (Table B-3). Inoculated fruit were incubated in moist chambers at 25°C in the dark in a randomized complete block experiment, wherein four (exp1), five (exp2), or seven treatments (exp3) (two isolates of each taxon recovered, and a mock-inoculated control) were replicated six times on single fruit experimental units (=blocks). Experiments compared the pathogenicity of: 1) isolates of *C. gloeosporioides* to *Glomerella* sp.; 2) isolates of *C. gloeosporioides* to *C. capsici*; and 3) all three taxa. The diameters of developing lesions were measured after 7 days. Lesion margins were excised from fruit, surface disinfested and plated on PDA to confirm the presence of the inoculated isolate. Analysis of variance was performed on lesion diameter values using PROC GLM in SAS v9.1.3 (SAS Institute Inc., Cary, NC) and means were separated with Fisher's LSD.

In all experiments, *C. capsici* and *C. gloeosporioides* both produced lesions significantly larger than the water control and *Glomerella* sp. ($P<0.05$; Table B-3). *Glomerella* sp. does not appear to cause anthracnose on papaya, and it is not clear what role, if any, it played in the

development of the lesions from which it was recovered. The taxon has been reported most often as an endophyte of several hosts, including *Theobroma, Dendrobium*, and *Cattleya* (Farr et al. 2006), and causes anthracnose lesions on passionfruit (Appendix C). It is possible that the isolates of *Glomerella* sp. that were recovered from papaya lesions had colonized lesions that were caused by another pathogen. *Colletotrichum gloeosporioides* produced sunken lesions with dark grey centers and pink/grey sporulation, which are typical of and match previous descriptions for anthracnose on papaya (Fig. B-3A; Dickman 1994, Persley and Ploetz 2003). In contrast, *C. capsici* produced lesions with dark sporulation, due to copious setae in acervuli (Fig. B-3B). A similar type of lesion on papaya, caused by *C. capsici*, has been reported from the Yucatan Peninsula (Tapia-Tussell et al. 2008).

This is the first report of *C. capsici* as a cause of anthracnose on papaya in Florida, and perhaps Belize (fruit from which this pathogen was recovered may have been latently infected prior to importation). The only other known report of a falcate-spored *Colletotrichum* species on papaya in the United States is of *C. falcatum*, which caused a fruit rot in Texas (Anonymous 1960). *Colletotrichum capsici* has been reported as a causal agent of papaya anthracnose in Japan (Yagushi et al. 1998), Malaysia (Rahman et al. 2008), and in the Yucatan Peninsula, Mexico (Tapia-Tussell et al. 2008).

Table B-1. Number of lesions from which species were recovered from fruit from two locations.

	# lesions[a]	
Species	TREC	Imported
C. gloeosporioides	0	4 (21.1)
C. capsici	2 (25)	7 (36.8)
Glomerella sp.	2 (25)	0

[a] Eight lesions were sampled from from fruit fromTREC, and 19 were from imported fruit from Belize. Total numbers of lesions are followed parenthetically by percentages of lesions from which a species was recovered.

Table B-2. ITS sequences included in phylogenetic analysis.

Accession	Species	Host	Origin	Genbank accession #	Published[a]
Ban1	*C. musae*	*Musa*	Florida	GQ373192	Chapter 3
Ban2	*C. musae*	*Musa*	Florida	GQ373193	Chapter 3
Ban3	*C. musae*	*Musa*	Florida	GQ373194	Chapter 3
Cg101	*C. gloeosporioides*	*Mangifera indica*	Florida	GQ373215	Chapter 3
Cg129	*C. gloeosporioides*	*Mangifera indica*	Florida	GQ373218	Chapter 3
Cg131	*C. gloeosporioides*	*Mangifera indica*	Florida	GQ373223	Chapter 3
IMI 356878[b]	*C. gloeosporioides*	*Citrus sinensis*	Italy	EU371022	Cannon et al. 2009
Pap19	*C. capsici*	*Carica papaya*	Florida	GU045514	---
Papleaf	*C. capsici*	*Carica papaya*	Florida	GU045511	---
Pap14	*C. capsici*	*Carica papaya*	Florida	GU045512	---
Pap18	*C. capsici*	*Carica papaya*	Florida	GU045513	---
Pas5	*C. capsici*	*Passiflora*	Florida	GU045515	---
STE-U 5304	*C. capsici*	*Arachis hypogaea*	Tanzania	AY376526	Lubbe et al. 2005
AR4028	*C. capsici*	*Phaseolus lunatus*	USA	DQ286156	Farr et al. 2006
CBS120709[c]	*C. capsici*	*Capsicum frutescens*	India	EF683602	Shenoy et al. 2007, unpublished
MAFF 238714	*C. dematium*	*Raphanus sativus*	Japan	AB196301	Moriwaki et al. 2002
AR3563	*C. dematium*	*Lirope muscarii*	Mexico	DQ286154	Farr et al. 2006
AR2930	*C. agave*	*Agave*	Mexico	DQ286221	Farr et al. 2006
CBS318.79	*C. agave*	*Agave*	Netherlands	DQ286219	Farr et al. 2006
Pas1	*C. boninense*	*Passiflora*	Florida	GU045516	
MAFF 305972[d]	*C. boninense*	*Crinum asiaticum*	Japan	AB051400	Moriwaki et al. 2003
CB0616	*C. boninense*	*Glycine max*	Taiwan	EF503672	Chen et al. 2007, unpublished
CBS102667	*C. boninense*	*Passiflora*	New Zealand	DQ286172	Farr et al. 2006
AR3751	*C. boninense*	*Dracaena*	China	DQ286170	Farr et al. 2006
Pas3	*Glomeralla* sp.	*Passiflora*	Florida	GU045517	---
Pap6	*Glomeralla* sp.	*Carica papaya*	Florida	GU045522	---
Pap4	*Glomeralla* sp.	*Carica papaya*	Florida	GU045520	---
Pap5	*Glomeralla* sp.	*Carica papaya*	Florida	GU045521	---
Pap8	*Glomeralla* sp.	*Carica papaya*	Florida	GU045518	---
Piper5	*Glomeralla* sp.	*Piper betle*	Florida	GU045521	---

Table B-2. Continued.

Accession	Species	Host	Origin	Genbank accession #	Published
AC4-M-Mexico	*Glomerella* sp.	*Musa*	Mexico	AY566308	Espinoza-Ortega et al. 2004, unpublished
P060	*Glomerella* sp.	*Faramea occidentalis*	Panama	EF423535	Gilbert et al. 2007
MICH-3-345	*Glomeralla* sp.	*Annona cherimola*	Mexico	AY841136	Villanueva-Arce et al. 2004, unpublished
MCA2773	*Glomeralla* sp.	*Theobroma*	Costa Rica	DQ286217	Farr et al. 2006
AR3750	*Glomerella* sp.	*Dendrobium*	Thailand	DQ286215	Farr et al. 2006
AR3749	*Glomerella* sp.	*Cattleya*	Thailand	DQ286213	Farr et al. 2006
MAFF 305974	*Glomerella* sp.	*Passiflora*	Japan	AB042319	Moriwaki et al. 2002
Ca3	*C. acutatum*	*Mangifera indica*	Florida	GU045506	Chapter 4
Ca13	*C. acutatum*	*Mangifera indica*	Florida	GU045507	Chapter 4
Ca26	*C. acutatum*	*Mangifera indica*	Florida	GU045508	Chapter 4
ATCC 56816[e]	*G. acutata*	*Carica papaya*	Australia	DQ286132	Farr et al. 2006
CBS198.35	*C. phormii*	*Phormium*	England	DQ286144	Farr et al. 2006
Outgroup					
TomIGZ	*Verticillium dahliae*	---	---	GU060637	Bollig et al. 2009, unpublished
CBS 130.51	*V. albo-atrum*	---	---	DQ825977	Zare et al. 2007
Vt536	*V. tricorpus*	---	---	GQ258661	Carlucci et al. 2009

[a] Publication in which ITS sequence was published. [b] Type speciment for *C. gloeosporioides* [c]Type speciment for *C. capsicsi* [d] Type specimen for *C. boninense* [e] Type specimen for *C. acutatum*

Table B-3. Lesion diameters from pathogenicity experiments.

		Exp1	Exp2	Exp3
Isolate	Species	Lesion diameter (mm)[a]	Lesion diameter (mm)	Lesion diameter (mm)
Pap8	*Glomerella* sp.	0.63 ± 0.38 b	---	0.60 ± 0.60 c
Pap9	*Glomerella* sp.	0.25 ± 0.25 b	---	0.33 ± 0.33 c
Pap10	*C. capsici*	---	20.75 ± 0.75 bc	12.92 ± 1.80 b
Pap14	*C. capsici*	---	18.00 ± 3.66 c	10.00 ± 3.58 b
Pap11	*C. gloeosporioides*	18.25 ± 5.02 a	---	---
Pap16	*C. gloeosporioides*	---	25.67 ± 3.72 ab	22.42 ± 3.77 a
Pap17	*C. gloeosporioides*	---	29.25 ± 3.67 a	12.4 ± 6.00 b
Control	---	0.00 ± 0.00 b	0.00 ± 0.00 d	0.00 ± 0.00 c
	LSD	2.262	8.12	6.79
	P-value	0.0017	<0.0001	<0.0001

[a] Lesion diameters 7 days after inoculation [b] Values in columns with same letter are not significantly different based on Fisher's LSD, P=0.05.

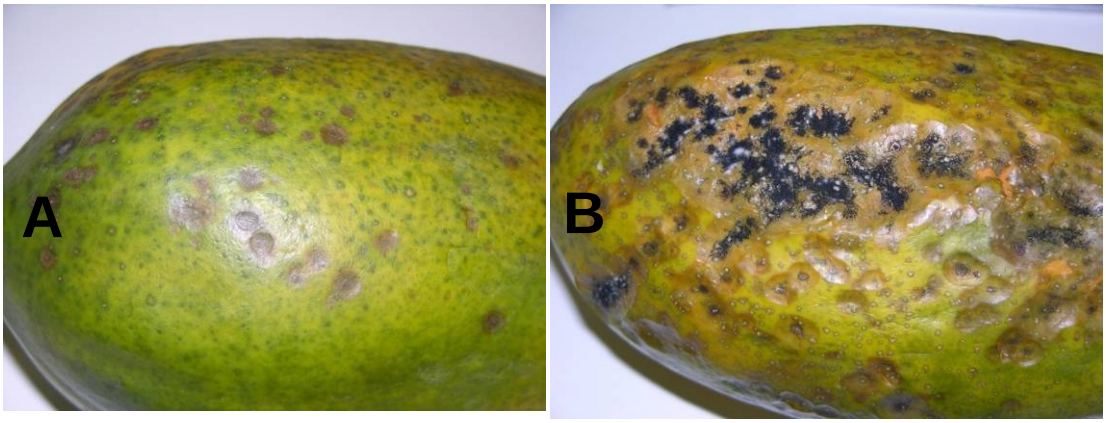

Figure B-1. Lesions associated with *Colletotrichum capsici* began as A) small brown and slightly sunken areas that became B) increasingly sunken and eventually covered with dark sporulation.

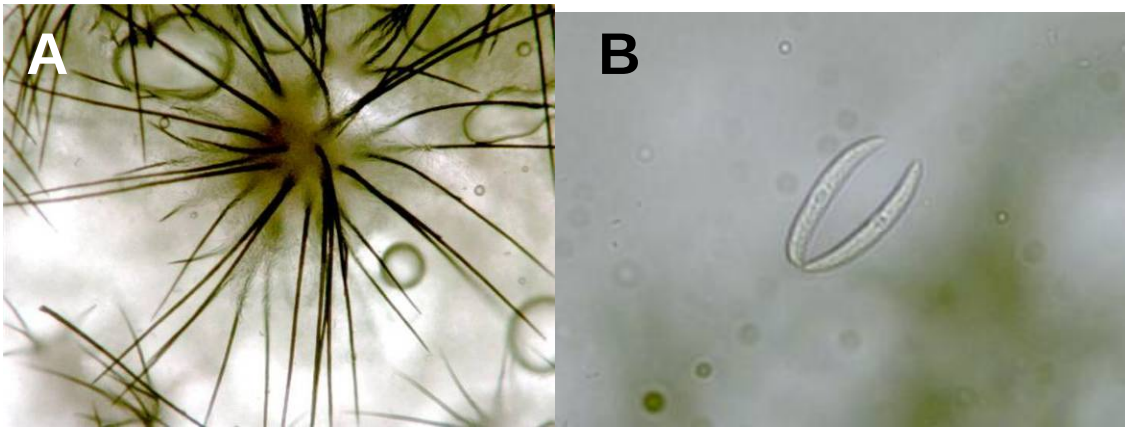

Figure B-2. Morphological characteristics of *Colletotrichum capsici* recovered from papaya: A) acervulus with setae, and B) falcate conidia.

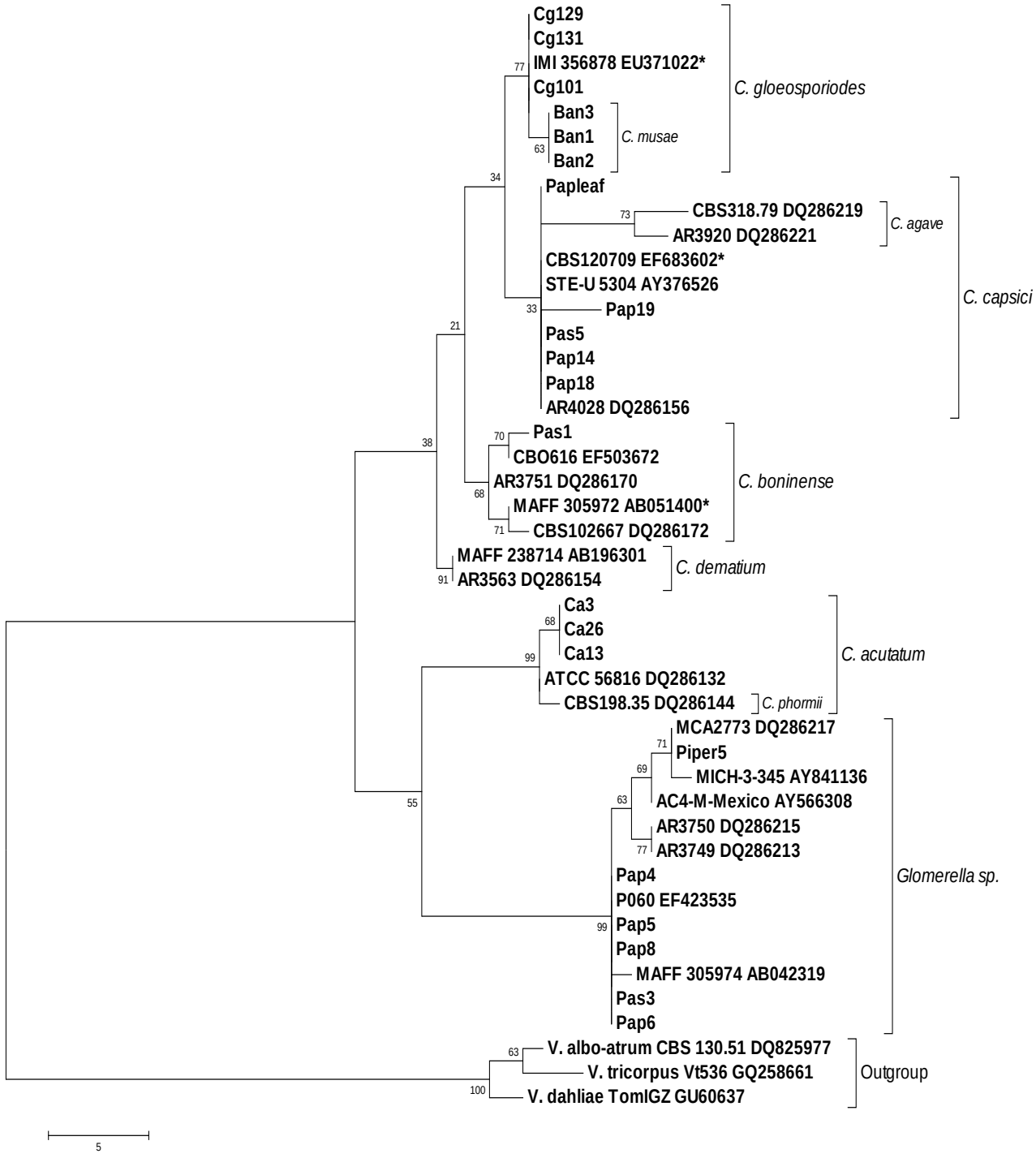

Figure B-3. One of 2,255 most parsimonious ITS-based trees (101 steps, CI=0.796, RI=0.967) for taxa recovered from papaya and other hosts. The data set contained 38 accessions with 322 characters, 53 of which were parsimony informative. Bootstrap values over 40% are listed at nodes. Isolates with an asterisk are types for a given species, and papaya isolates are designated with the prefix Pap, passionfruit with prefix Pas.

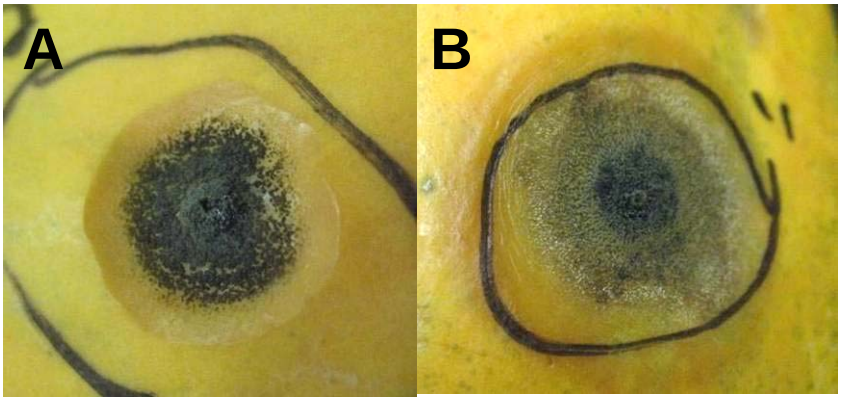

Figure B-4. Lesion produced after artificial inoculation with A) *Colletotrichum capsici* and B) *C. gloeosporioides*.

APPENDIX C
FIRST REPORT OF *COLLETOTRICHUM CAPSICI, COLLETOTRICHUM BONINENSE,* AND A *GLOMERELLA* SP. CAUSING POSTHARVEST ANTHRACNOSE ON PASSIONFRUIT (*PASSIFLORA* SPP.) IN FLORIDA.

Anthracnose, caused by several *Colletotrichum* species (Alfieri et al. 1994, Kobayashi and Okamoto 2003, Manicom et al. 2003, Moriwaki et al. 2003), is one of the most important diseases on leaves, flowers and fruit of passionfruit (Manicom et al. 2003). In October 2008, yellow and purple passion fruit (*Passiflora edulis* f. *flavicarpa* and *P. edulis*, respectively) with postharvest anthracnose were examined in Miami-Dade County, FL. Lesions began as light brown areas that expanded to cover half of the fruit surface in 3-4 days. The skin became papery, and some lesions developed a dark brown pigment. Pink to dark sporulation developed first in lesion centers, and then spread toward the margins.

Fungi were isolated from lesions by streaking sporulating acervuli on PDA and recovering individual germinating spores. Isolates were identified morphologically, based on colony appearance on PDA, conidium and ascospore shape, whether acervuli were setose or glabrous, and whether a teleomorph was formed in culture. Molecular identities were also obtained for single representatives of each of three morphological groups with ITS sequences. Sequences were aligned with those for several other *Colletotrichum* species in Genbank, and included the type strains of *C. acutatum* (ATCC 56816), *C. gloeosporioides* (IMI 356878), *C. boninense* (MAFF 305972) and *C. capsici* (CBS 120709). Maximum parsimony analysis was performed in Mega 4.0 (Tamura et al. 2007) using closest neighbor interchange (CNI) searches with 100 random taxon additions and gaps were treated as missing data. Bootstrap analysis according to Felsenstein (1985) was performed using the above search criteria, with 500 repetitions.

Four taxa were identified: *C. gloeosporioides*, *C. boninense*, *C. capsici* and *Glomerella* sp. Colonies of *C. gloeosporioides* were white to grey, fluffy with orange sporulation and straight and cylindrical conidia; those of *C. boninense* colonies were cream to orange and felted, with cream-colored sporulation and cylindrical conidia; those of *C. capsici* had sparse, fluffy, white mycelia with setose acervuli and falcate conidia; and those of *Glomerella* sp. were darkly pigmented and produced fertile perithecia after 7-10 days (Fig. C-2A-D). In the ITS-based phylogeny, a setose falcate-spored isolate, Pas5, was identified as *C. capsici*, a straight-spored isolate with cream-colored colonies was *C. boninense* Pas1, and an isolate that formed a teleomorph in culture was an undescribed *Glomerella* species (Pas3) (Appendix B, Figure B-3).

Inoculation studies were conducted with several isolates from each taxon, including several isolates of *Glomerella* sp. that were recovered from other hosts (Table C-1). Mature yellow passionfruit were wounded with a sterile needle and inoculated with a 15µl drop of 0.3% water agar that contained 10^5 conidia ml^{-1} of a given isolate. Inoculated fruit were incubated in moist chambers at 25°C in the dark. A randomized completely block design (RCBD) was used, where experimental units were single fruits inoculated with one of seven (Exp. 1) or 11 (Exp. 2) isolate treatments (Table C-1). Each isolate x treatment combination was replicated four times, and the two repetitions of the experiment were treated as blocks. Incidences of lesion development, which coincided with fruit softening, were recorded for 21 days after inoculation, where no lesion development was scored '0' and lesion development was scored '1'. Lesion diameters were not recorded because lesions developed at different times throughout the experiment, depending on maturity of individual fruit. Lesion margins were excised from fruit, surface disinfested and plated on PDA to confirm presence of the inoculated isolate. Analysis of

variance was performed for incidence values using PROC GLM in SAS v9.1.3 (SAS Institute Inc., Cary, NC) and means were separated with Fisher's LSD.

In both experiments, all accessions except those of *C. gloeosporioides* and Pas2 of *C. boninense* in Exp. 2 caused at least a 50% lesion incidence (Table C-1). The inoculated species were successfully recovered from lesions. Lesions that developed from inoculations with *Glomerella* sp. produced dark brown pigment in the center of the lesion, whereas lesions caused by other isolates were darkened due to extensive sporulation (Fig C-1A-C).

In the present study, three new species were identified as causes of fruit anthracnose of passionfruit in Florida. *Colletotrichum capsici* has been associated with leaf anthracnose of passionfruit in Florida and Japan (Alfieri et al. 1994, Kobayashi and Okamoto 2003), but has apparently not been associated previously with fruit anthracnose. *Colletotrichum boninense* is a relatively recently erected species (Moriwaki et al. 2003), and has been associated with passionfruit anthracnose in Japan (Moriwaki et al. 2003) and Colombia (Freeman, personal communication).

The undescribed *Glomerella* sp. caused anthracnose on passionfruit, but in other work caused no disease on papaya (Appendix B) and eugenia (Ploetz et al.2009), two other hosts in South Florida from which it has been recovered. This fungus produces the teleomorph readily in culture, as well as directly on sporulating lesions on passionfruit (Figs. C-2A-D). Almeida and Coêlho (2007) characterized a *Glomerella* sp. that caused anthracnose on passionfruit in Brazil, but DNA sequences of these isolates are not available. Additional work to characterize this apparently new species is underway.

Table C-1. Incidence of symptoms from two inoculation studies

			Incidence[a]
Isolate	Species	Host	Exp 1
Pas4	*C. gloeosporioides*	Passionfruit, *Passiflora edulis f. flavicarpa*	0.13 ± 0.13 bc[b]
Pap11	*C. gloeosporioides*	Papaya, cv. 'Caribbean Red'	0.00 ± 0.00 c
Pap8	*Glomerella* sp.	Papaya, cv. 'Caribbean Red'	0.75 ± 0.25 a
Pap9	*Glomerella* sp.	Papaya, cv. 'Caribbean Red'	0.63 ± 0.18 ab
Pas3	*Glomerella* sp.	Passionfruit, *P. edulis f. flavicarpa*	0.75 ± 0.16 a
07-598	*Glomerella* sp.	*Eugenia* endophyte	0.50 ± 0.29 abc
pGlom	*Glomerella* sp.	Passionfruit, *P. edulis f. flavicarpa*	0.50 ± 0.29 abc
Pas2	*C. boninense*	Passionfruit, *P. edulis f. flavicarpa*	0.75 ± 0.16 a
Pas1	*C. boninense*	Passionfruit, *P. edulis f. flavicarpa*	0.75 ± 0.25 a
Pas7	*C. boninense*	Passionfruit, *P. edulis f. flavicarpa*	0.75 ± 0.25 a
Pas5	*C. capsici*	Passionfruit, *P. edulis f. flavicarpa*	0.75 ± 0.25 a
Pas6	*C. capsici*	Passionfruit, *P. edulis f. flavicarpa*	0.50 ± 0.29 abc
Control	---	---	0.00 ± 0.00 c
		LSD	0.5664
		P=	0.0065

[a]Incidence was rated for each experimental unit as '0' for no lesion development, and '1' if a lesion developed for [b] Values in columns are means from two experiments that, when followed by the same letter, are not significantly different based on Fisher's LSD, P=0.05..

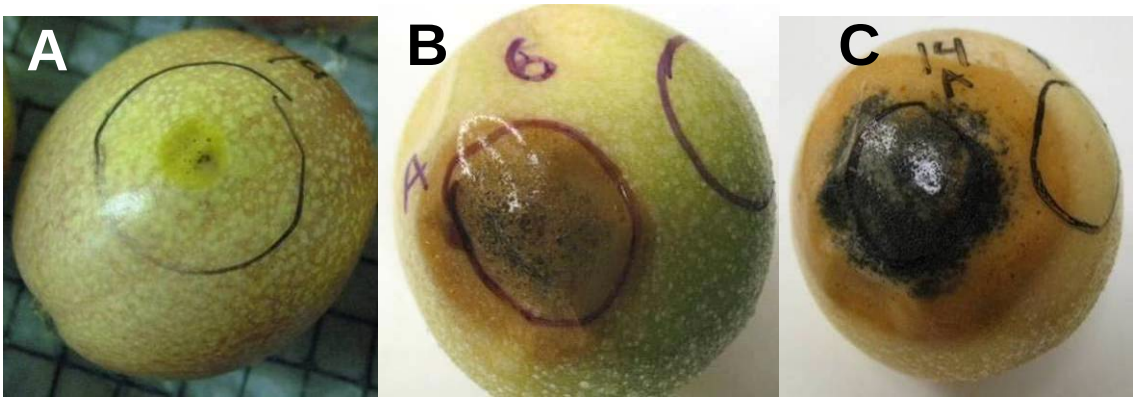

Figure C-1. Anthracnose lesion development on inoculated yellow passionfruit. A) young lesions start as circular light brown areas and quickly enlarge to B) large sporulating lesions developed on fruit inoculated with *Colletotrichum capsici*, and C) large sporulating lesions with dark brown pigment developed on fruit inoculated with *Glomerella* sp.

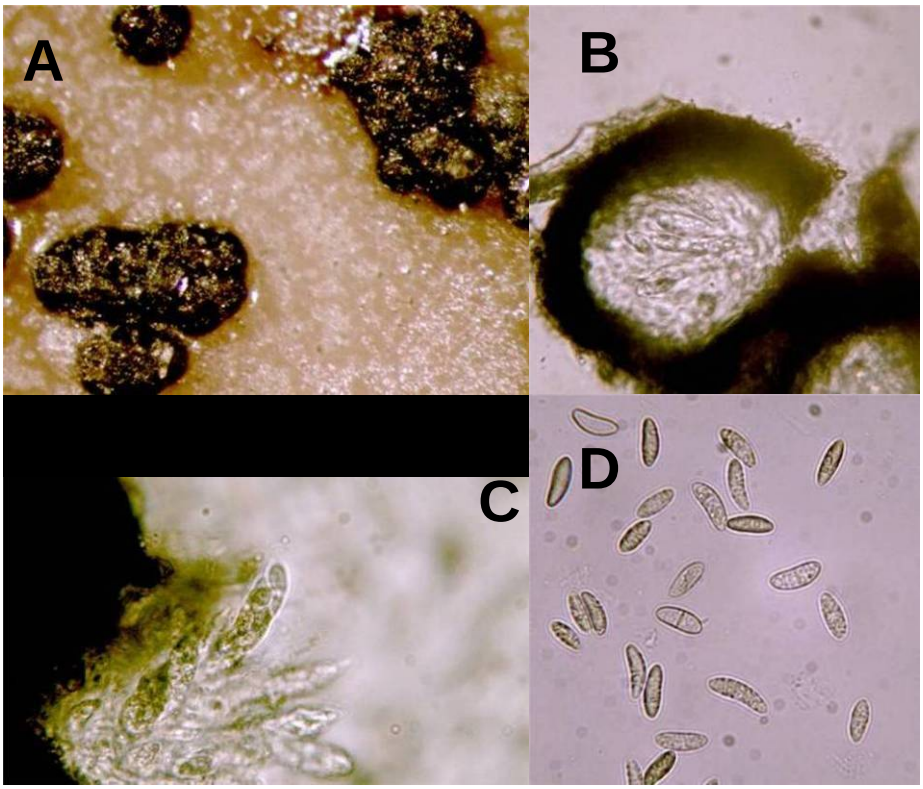

Figure C-2. Morphology of *Glomerella* sp. A) perithecia on fruit surface, B) cross-section of perithecium, C) asci erupting from perithecia, and D) ascospores

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BIOGRAPHICAL SKETCH

Tara Tarnowski is originally from Des Moines, IA. Although she is from an agricultural state, she grew up in the city. As a kid, she helped her mother in their large vegetable garden every summer, and it is here that her interest in plants and agriculture began. In high school, she had the opportunity to spend two summers in Costa Rica, and these trips sparked her interest in how people lived in different places in the world. She attended Iowa State University for her Bachelor of Science degree, where she majored in Plant Health & Protection and International Agriculture, and minored in Spanish. At Iowa State she had the opportunity to work with Dr. Mark Gleason on a range of fruit crop disease management research, and completed two independent research projects on sooty blotch and flyspeck on apple.

In 2003 she moved to Athens, GA to begin an MS in Plant Pathology. She studied with Dr. Harald Scherm, characterizing fungicide efficacy in blueberry flowers against *Monilinia vaccinii-corymbosi*, which causes mummy berry of blueberry. After completing her degree she moved south again to Gainesville to begin her PhD, and later to the Tropical Research and Education Center in Homestead, FL, to complete her dissertation research.