12-13-2008

Determination And Compatibility Of Putatively Hypovirulent And Virulent Isolates Of Cryphonectria Parasitica Collected From The Great Smoky Mountains National Park

David Franklin McNeill

DETERMINATION AND COMPATIBILITY OF PUTATIVELY HYPOVIRULENT AND VIRULENT ISOLATES OF *CRYPHONECTRIA PARASITICA* COLLECTED FROM THE GREAT SMOKY MOUNTAINS NATIONAL PARK

By

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A Thesis
Submitted to the Faculty of Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Plant Pathology
in the Department of Entomology and Plant Pathology

Mississippi State, Mississippi

December 2008

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Pages in Study: 101

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A two-year study was conducted to characterize isolates of the chestnut blight fungus (*Cryphonectria parasitica*) from the Great Smoky Mountains National Park (GRSM). Of 339 isolates, 54 had abnormal cultural morphologies and 3 contained dsRNA. Analysis of vegetative compatibility (VC) divided all isolates into 34 groups, 16 of which only contained one isolate. A total of 19 isolates and 3 controls were inoculated onto healthy American chestnut trees in the Nantahala National Forest, North Carolina, and data on canker growth and stromata production were obtained over six months. Results from the field trial indicated that five isolates were potentially hypovirulent. Based on those data, isolate, 236-1C, has the greatest potential for use as a biological control agent for the pathogen in the GRSM, but compatibility is limited to select VC groups. Additional hypovirulent isolates representative of the other VC groups must be identified before large scale biocontrol can succeed.

DEDICATION

Like all things in my life, this research is dedicated first to my Lord and Savior, to whom I am thankful for all of the many blessings in life. Second, to my parents Dave and Elizabeth McNeill for their unending support, without which this project would not have been possible.

ACKNOWLEDGEMENTS

I wish to extend sincere thanks to my major professor, Dr. Richard Baird, for guiding and supporting me through this project and for helping me prepare this document. I also wish to thank my committee members Clarissa Balbalian, Dr. Clarence Collison, Dr. Evan Nebeker, and Dr. Sead Sabanadzovic, and to extend special thanks to Dr. Sead Sabanadzovic for the many hours of assistance with dsRNA extraction and laboratory work.

Mark Alexnder, William Starrett, and Paul Scott deserve special recognition for providing encouragement and assistance in facing the rigors of graduate school. Special recognition is also due to Sergio Pichardo for help proofreading, editing, and formatting the document. Thanks to Isaac Jaben, Sarah McManus, and Katie Warner for providing assistance with fieldwork and for good company over many miles of trails. Additional thanks to Dr. Dennis Rowe, Dr. Thomas G. Matney, and Dr. John C. Schneider (Mississippi State University); Sandra Anagnostakis (Connecticut Experimental Station); Dr. Mark Double and Dr. William MacDonald (West Virginia University); Dr. Hill Craddock (University of Tennessee, Chattanooga).

This project would not have been possible without the assistance and support of Great Smoky Mountains National Park personnel including Kristine Johnson, Glenn

Taylor, Paul Super, and David Clark as well as Ted Oprean at the Pisgah National Forest and the staff of the Wayah District of the Nantahala National Forest.

In addition, multiple institutions and organizations assisted in making this study a success including the Mississippi State University Department of Entomology and Plant Pathology, the Great Smoky Mountains National Park, The Great Smoky Mountains Conservation Association, and the Highlands Biological Station. Additionally, thanks to the staff of the Cooper Creek Trout Farm and Ober Gatlinburg for logistical support.

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INTRODUCTION

The Great Smoky Mountains National Park (GRSM), which borders Western North Carolina and East Tennessee, contains approximately 2,200 km^2 of a very diverse assemblage of plant life including over 130 tree and 1,570 vascular plant species (Sharkey, 2001). Elevations within this unique geographical area range from 265 to 2,024 meters, and the park contains five major forest types ranging from low elevation cove hardwood and hemlock forests to high elevation spruce-fir forests (Great Smoky Mountains Institute at Tremont, unpub. data). Prior to 1920, the American chestnut (*Castanaea dentata* (Marsh.) Borkh.) composed approximately 65% of the overstory, or 25% to 40% of tree basal area, of what is now the GRSM (Ashe, 1911; Vandermast and Van Lear, 2002). Presently, however, this species has been reduced to an understory species that does not contribute to overstory composition.

***Cryphonectria parasitica* (Murr.) Barr.**

The chestnut blight fungus, belonging to the phylum Ascomycota and Order Diaporthales, is also parasitic on different species of chestnut trees, including European

chestnut (*Castanea sativa* Mill.), and Chinese chestnut (*Castanaea mollissima* Bl.). *Cryphonectria parasitica* has also been shown to be saprophytic on other hardwood species such as red oak (*Quercus rubra* L.) (Carey, 1985; Baird, 1991). Originally described as *Diaporthe parasitica* Murr., the fungus was soon renamed *Endothia parasitica* ((Murr.) P.J. & H.W. And.) (Kuhlman, 1978), then later *C. parasitica* in 1978 (Barr, 1978).

Ascospores of the fungus are most frequently wind disseminated, while asexual conidia are often spread by rain splash (Liebhold et al., 1995). The fungus enters healthy bark tissue through wounds or direct penetration, after which it spreads into inner bark tissues by formation of mycelial fans (Hebard et al., 1984). Infection leads to canker formation which is often accompanied by a darkening of external bark tissue that eventually becomes slightly raised and violet to red in color. The center of a canker often develops distinct cracking or splitting, and a number of orange stromata extrude from the bark surface at the outer margins of the canker (Turchetti, 1978; Elliston, 1982). Often, epicormic shoots form proximally to the canker in response to tissue death at the canker site (Turchetti, 1978; Jaynes and Elliston, 1982; Heiniger and Rigling, 1994).

The fungus does not infect the roots of chestnut trees, and will not survive in soil (Weidlich, 1978). The inability of the pathogen to persist in soil is believed to be due to the inhibitory effect of competition with other organisms, such as *Trichoderma* spp. (Weidlich, 1978). As a result of this antagonism, the roots of chestnut trees persist

after the pathogen has killed the entire aboveground portions of the host. No large, surviving American chestnut trees found to date have exhibited more than a low level of resistance to *C. parasitica* (Griffin et al., 1983; Kubisiak et al., 1997). Many surviving American chestnuts found in the forests of the eastern United States are located on desirable sites free from competition with other trees (Whittaker, 1956; Griffin, 1992).

Chinese chestnut (*Castanaea mollissima* Blume) generally shows greater resistance to *C. parasitica* than American chestnut, although it has been reported that Chinese chestnut exhibits a wide variation in resistance to the pathogen (Huang et al., 1996). Numerous programs have sought to breed American chestnut trees for resistance to *C. parasitica* through selective breeding and backcrossing with resistant species such as the Chinese chestnut (Given and Haynes, 1978; Anagnostakis, 1987; Anagnostakis, 1992; Kubisiak et al., 1997). However, since Chinese chestnut does not develop a straight, tall bole, it does not have the potential to become a dominant overstory species as the American chestnut. Therefore, breeding efforts have focused on selecting for as many characteristics of the American chestnut as possible in hybrid trees while maintaining resistance to the fungus (Jaynes, 1978). However, because the policies of the National Park Service (NPS) prohibit the introduction of material not endemic to the park, introduction of crosses into the GRSM would not be allowed.

Discovery of *Cryphonectria parasitica* in North America

Although the chestnut blight fungus is believed to have been introduced into eastern North America as early as 1890, the pathogen was originally reported in the United States at the New York Zoological Gardens in 1904 (Merkel, 1905; Elliston, 1982). During that year, cankers were observed on American chestnut trees in a few scattered locations throughout the park. Within a year, over 98% of the chestnut trees in the park showed symptoms of *C. parasitica* infection (Merkel, 1905). It was quickly recognized that the pathogen had the potential to eliminate the entire population of the American chestnut within the region, and that any healthy trees in blighted stands were not disease-resistant, but simply had not yet become infected (Merkel, 1905; Gravatt, 1926). By 1909, a majority of the American chestnut trees within 48 kilometers of New York City were infected, with the main disease center moving outward at approximately 16 to 38 kilometers per year and numerous spot infections developing up to 190 kilometers from the infection center (Kuhlman, 1978; Anagnostakis, 1987).

It was observed that 10 cm diameter stems could be killed within 21 days of the initial development of symptoms, which often included the formation of necrotic cankers on bark tissue (Merkel, 1905; Elliston, 1982). The pathogen is hard to detect in a stand where less than 1% of the trees are infected with the pathogen, but once established, the fungus spreads rapidly to other portions of infected trees and to the surrounding population (Gravatt, 1926).

Soon after discovery of *C. parasitica* in the United States, state and local authorities attempted to control the spread of the fungus through various means. Methods of control including cutting and burning of infected material in an attempt to reduce the concentration of inoculum in an infected stand, and spraying of live trees with fungicides such as a solution of copper sulfate were attempted as a measure to lessen the severity of the fungus on individual trees, though spraying proved to be difficult and expensive (Merkel, 1905). In 1911, the state of Pennsylvania allotted $275,000 to the Chestnut Blight Commission, and authorized the commission to cooperate with landowners in the removal of infected material. However, these efforts did little to either stop or noticeably impede the spread of the pathogen (Hepting, 1974).

Spread of Cryphonectria parasitica into Appalachia. The natural range of the American chestnut once extended from southwestern Maine to northern Georgia and Alabama following the Appalachian Mountains, where it composed up to 25% or more of standing timber in some areas (Elliston, 1982; Anagnostakis, 1987). Throughout the Appalachian region, the American chestnut was ecologically and culturally important. Many buildings, fences, and utility poles were constructed from chestnut logs, and the mast produced by mature trees was used to feed not only domestic animals such as hogs, but also wildlife such as squirrels and turkeys (Ashe, 1911; Hepting, 1974; Anagnostakis, 1982).

The pathogen spread rapidly to the southwest along the Appalachian and Allegheny Mountains (Gravatt, 1926). Within 20 years of it's initial discovery in the United States, *C. parasitica* could be found infecting 80 to 100% of American chestnut trees in counties as far south as western North Carolina (Gravatt, 1926). By 1938, approximately 12 years after initial discovery of the fungus in the GRSM, 85% of the American chestnut trees in the park had been destroyed by the pathogen (MacKenzie and White, 1998). Today, the American chestnut generally persists in the environment as sprout coppice that originates from roots of trees of which the above ground portion has been destroyed, with a small number of trees larger than 20 cm diameter breast height (dbh) persisting in scattered locations throughout the southern Appalachians (Jaynes and Elliston, 1982; Griffin et al., 1983; Carey, 1985; Griffin, 1992).

Following the loss of American chestnut in the park, succession in old-growth riparian areas led to a forest dominated by many species of oaks (*Quercus* spp.), while disturbed and logged sites are typically dominated by cove mesophytic species such as eastern hemlock (*Tsuga canadensis* L.) (Griffin, 1992; Vandermast and Van Lear, 2002). However, several hundred American chestnut trees still persist in the understory throughout the GRSM (Wood, 2003). These trees are a valuable reservoir of genetic material in the survival of the American chestnut.

Chestnut Blight in Europe

In 1938, *C. parasitica* was discovered near Genoa, Italy, and soon spread rapidly throughout Europe, threatening the European chestnut (*Castanaea sativa* Mill.) (Woodruff, 1946; Pavari, 1949; Anagnostakis, 1987). This tree species occurs in the Piedmont zone in northern Italy and throughout the middle mountain zone of the Apennine Mountains in Central and Southern Italy. In this region, European chestnut was an economically important species often grown in coppice stands with a rotation period of 15 to 30 years for use in the production of timber, tannin, and nuts (Mittempergher 1978; Heiniger and Rigling, 1994). By 1948, between 5% and 100% of chestnut trees in stands throughout northwest Italy showed symptoms of infection (Heiniger and Rigling, 1994). However, the fungus generally spread less rapidly throughout Europe than it had in the United States, and was found to be less virulent on European chestnut trees (Mittempergher, 1978; Heiniger and Rigling, 1994).

Discovery of Hypovirulence (hv)

In 1950, several abnormal cankers were noted on sprouts in the region around Genoa, Italy (Mittempergher, 1978; Elliston, 1982). These abnormal, superficial cankers did not completely girdle stems, and infection did not result in death of host tissue distal to cankers. A superficial canker is defined as having mycelial fans which do not penetrate the vascular cambium (Hebard et al., 1984), and is evidenced by the swelling of live bark tissue around the circumference of the canker and by the sparseness or

absence of stromata (Griffin et al., 1983; Carey, 1985; Griffin et al., 1993). This phenomenon was initially attributed to host resistance (Elliston, 1982), but the widespread, sudden appearance of superficial cankers on European chestnut trees throughout Italy and France ruled out this in favor of the hypothesis that superficiality of cankers resulted from a reduction in virulence of the pathogen (Turchetti, 1978). In 1965, *C. parasitica* isolated from these unusual cankers was described as lacking orange pigmentation (Grente and Berthelay-Sauret, 1978a). It was later reported that the proportion of white isolates recovered was proportional to the superficiality of a canker and that white isolates from these cankers were termed "hypovirulent" (Grente and Berthelay-Sauret, 1978a). These hypovirulent isolates, if inoculated onto an actively growing canker, often cause a reduction in normal colonization rate and can eventually lead to formation of callus tissue (Elliston, 1978; Grente and Berthelay-Sauret, 1978b; Heiniger and Rigling, 1994). Hypovirulent isolates vary in their degree of virulence, although many have been described as completely nonpathogenic (Day, 1978; Willey, 1980). Highly virulent isolates are able to penetrate the phellodermal generative layer of plant tissue that forms in response to infection, while hypovirulent isolates do not grow into this layer as quickly (Grente and Berthelay-Sauret, 1978b).

Hypovirulent forms of *C. parasitica* were found to rapidly spread throughout Europe. Within 15 years of the initial discovery of superficial cankers, hypovirulent forms of *C. parasitica* had become widespread throughout the region (Mittempergher, 1978). In Europe, the high incidence of hypovirulent forms of the pathogen did not decrease the

incidence of chestnut blight, but drastically reduced its severity. Although *C. parasitica* is still widespread on chestnut trees throughout Europe, the frequency of active and superficial or sealed cankers in a region can vary widely, with one study reporting that 5% to 72% of cankers in a given stand were superficial (Heiniger and Rigling, 1994).

*Hypovirulence Associated dsRNA viruses***.** In 1969, it was concluded that cytoplasmic agents, which could be transmitted through anastomosis, lowered the virulence and altered the morphology of isolates of the chestnut blight fungus *in vitro* (Grente and Berthelay-Sauret, 1978a; Elliston, 1982). Analysis of the cytoplasmic contents of hypovirulent isolates of *C. parasitica* revealed that, for all hypovirulent isolates examined, a double stranded ribonucleic acid (dsRNA) later found to represent a genome of *Cryphonectria hypovirus 1* (CHV-1; Shapira et al., 1991) was present in fungal cytoplasm. This finding led researchers to conclude that the determining factor for hypovirulence was the presence of this dsRNA virus (Anagnostakis, 1978; Dodds, 1978; Jaynes and Elliston, 1980; Elliston 1982). Through transformation experiments involving a vector containing the first open reading frame from a dsRNA known to induce hypovirulence, it was demonstrated that the phenotypic expression of hypovirulence is due to specific viral coding domains contained on this hypovirus rather than a general reaction of the fungus to the presence of replicating dsRNA strands (Choi and Nuss, 1992a). As noted by Elliston (1984), virulent isolates do not display wide morphological variation *in vitro*, while hypovirulent isolates exhibit a wide variety of

abnormal characteristics. In addition to a reduction in pathogenicity, the presence of dsRNA can result in an alteration of phenotypic characteristics such as reduced orange pigmentation, reduced sporulation, reduced growth rate (Elliston, 1982), and reduced laccase production (Choi and Nuss, 1992b). Hypovirulent isolates often show a lobate growth pattern on potato dextrose agar (PDA) (Enebak et al., 1994). Pigmentation in hypovirulent isolates varies, with some isolates remaining white and others developing cream, yellow, or light orange pigmentation, often in concentric rings, within three to five days following transfer (Elliston, 1982). Although the presence of dsRNA can lead to a reduction in virulence, some dsRNAs have been demonstrated to have little to no impact on pathogenicity *in vivo* or cultural morphology *in vitro* (Willey, 1980; Enebak et al., 1994; Smart et al., 1999).

Various dsRNAs have since been described from *Cryphonectria parasitica*. Many of these dsRNAs constitute the genomes of viruses belonging to the genus *Hypovirus* in the family *Hypoviridae* (Nuss et al., 2005). There are currently four different viral species described from *C. parasitica* belonging to this taxon and designated *Cryphonectria hypovirus 1* through *4* (CHV-1, CHV-2, CHV-3, CHV-4). Unlike a majority of viruses, no true virions are found associated with these viruses Instead, polymorphic vesicles containing dsRNA are present in fungal cytoplasm (Nuss et al., 2005). These dsRNAs may vary in size from approximately 9 kilobase pairs (kbp) (CHV-3 and CHV-4) to 13 kbp (CHV-1 and CHV-2) depending on the virus species. The effects of these viruses on *C. parasitica* vary by virus species. CHV-1, CHV-2, and

CHV-3 dramatically reduce fungal virulence, while most strains of CHV-4 do not debilitate the host isolates. The most studied member of this family is *Cryphonectria hypovirus 1* isolate EP713 (CHV-1/EP713), a European hypovirus that has become well established in fungal populations throughout Italy and France but has not become established in North America despite widespread release as biological control (Peever et al., 1997). Incidence of hypoviruses in *C. parasitica* population is higher in North America than in Asia (Peever et al., 1997; Peever et al., 1998). One study reported finding three hybridization groups throughout North America composed of different strains of hypoviruses that vary in their ability to induce hypovirulence in *C. parasitica,* including hypovirus CHV3-GH2 from Michigan and Ontario, (Hillman et al., 2000; Nuss et al., 2005), an SR2-type distributed throughout eastern North America, and a third hypovirus, CHV2, that was collected only in New Jersey with dsRNA incidence ranging from 6% to 100% in populations studied (Hillman et al., 1990; Peever et al., 1997; Linder-Basso et al., 2005). *Cryphonectria hypovirus 4* (CHV4) has been reported widely in the environment in West Virginia in both treated and untreated plots, although CHV4 is smaller in size and does not induce hypovirulence in *C. parasitica* (Liu et al., 2002). Hypovirus CHV-4 is reported to be widespread throughout the entire eastern United States, but CHV-1, despite having been deliberately released as a biological control of the chestnut blight fungus, is largely absent in North America while being prevalent in Europe (Linder-Basso et al,. 2005).

Horizontal transmission of this dsRNA has only been demonstrated to occur through the exchange of cytoplasmic material of its fungal host with another isolate through anastomosis, but it has not been shown to infect other fungi through extracellular means (Nuss et al., 2002). Cyclohexamide added to PDA media has been demonstrated to destroy dsRNA from hypovirulent isolates and resulted in the isolation of virulent dsRNA-free types from originally hypovirulent types. These results provided evidence as to the causal role of dsRNA in hypovirulence (Fulbright, 1984).

To date, *Cryphonectria* hypoviruses have never been observed to be disseminated through ascospores in nature, and can only effectively spread through conidia (Nuss et al., 2002). However, 10% to 90% of conidia produced by hypovirulent isolates have been found to be free of dsRNA (Nuss et al., 2002). It has been noted that, although sporulation is reduced in hypovirulent isolates of *C. parasitica,* dsRNAs have minimal impact on the viability of conidia (Peever et al., 2000). Although this dsRNA hypovirus does not contain a protein sheath and does not naturally occur outside of fungal cytoplasm, researchers have demonstrated techniques to isolate and identify dsRNA. Hillman et al. (1990) outlined a procedure for extraction and isolation of dsRNA from fungal tissue. An older procedure for isolation of dsRNA through column chromatography has been reported, but this method does not allow for accurate quantification of dsRNA present in a sample (Hillman et al., 1990). Following extraction, isolates of dsRNA have been examined using Restriction Fragment Length Polymorphism (RFLP) analysis and sequencing following Polymerase Chain Reaction

(PCR) amplification (Shapira et al., 1991; Alleman et al., 1999; Smart et al., 1999; Suzuki et al., 1999), and it has been reported that the observation of a band around 12.7 kb on agarose gel can serve as a means for verifying the presence of dsRNA (Shapira et al., 1991; Smart et al., 1999).

Vegetative Compatibility. Vegetative compatibility (VC) is believed to be the most significant factor impacting the conversion capacity of virulent isolates to hypovirulent forms. Two fungal isolates that are not vegetatively compatible will not undergo hyphal anastomosis, and instead a ridge of pycnidia often forms along the barrage between both isolates *in vitro* (Anagnostakis, 1978; Grente and Berthelay-Sauret, 1978b). As many as 95% of paired isolates that undergo anastomosis successfully transfer dsRNA to adjacent, uninfected isolates (Kuhlman et al., 1984). Vegetative compatibility has been shown to not simply be related to the overall genetic similarity between two isolates, but rather to the number of differences in *vic* alleles between two isolates (Liu and Milgroom, 1996; Nuss et al., 2002). As the number of identical alleles at these genetic loci decreases, the ability of two isolates to fuse and undergo anastomosis decreases (Nuss et al., 2002). Thus, VC types, or groups of isolates that will undergo anastomosis with one another, are determined by alleles at *vic* loci (Milgroom and Cortesi, 1999). Since horizontal transmission of *Cryphonectria* hypoviruses requires anastomosis, vegetative compatibility has the capacity to severely limit the spread of hypovirulent forms of the pathogen in the environment, and significantly reduce the ability of a

hypovirulent isolate to effectively convert a virulent form of *C. parasitica* present in an active canker (Anagnostakis, 1978).

Hypovirulence as a Biological Control Agent. In Europe, hypovirulent isolates have been applied extensively in orchards since 1974 as a biological control (Heiniger and Rigling, 1994; Bissegger et al., 1997; Robin et al., 2000). In addition, hypovirulent forms of *C. parasitica* containing dsRNA became well established in coppice forests throughout Europe, limiting the severity of the blight throughout the region (Robin et al., 2000). In the United States, although it has been demonstrated that treatment of active cankers caused by *C. parasitica* with hypovirulent isolates can be used effectively to treat individual American chestnut sprouts, hypovirulence has not been found to persist as well in the environment as it has in Europe (Jaynes and Elliston, 1982; Anagnostakis, 1987; Liu et al., 2002). Speculation as to the reasons for the greater severity of the blight in the United States in contrast to Europe include the greater abundance of virulent inoculum, the higher susceptibility of American chestnut trees to *C. parasitica* and the greater diversity of vegetative compatibility groups among *C. parasitica* in the United States (Bissegger et al., 1997; Robbins and Griffin, 1999).

The lower diversity and number of VC types in Europe compared to the United States is believed to be due to the founder effect, which is the reduction of genetic diversity of *vic* alleles in the European population of *C. parasitica* as a result of this population having been initially colonized by a relatively small number of individuals

(Milgroom and Cortesi, 1999). In addition, it has been determined that little gene flow occurs between subpopulations in Southern and Northern Italy, further limiting their diversity (Milgroom and Cortesi, 1999). Furthermore, as *C. parasitica* becomes established in a stand, active cankers are more prevalent, but if dsRNA are present, the proportion of active to superficial cankers decreases as hypovirulent forms of the fungus become established in the fungal population (Heiniger and Rigling, 1994).

A pathogen can benefit from the presence of hypovirulent forms of *C. parasitica* under certain conditions by not quickly killing the host (Taylor et al., 1998). Pathogens that do not quickly kill their hosts typically have higher reproductive success than highly virulent forms (Taylor et al., 1998). However, this phenomenon has not been shown to have occurred in the United States (Anagnostakis, 1981; Bissegger et al., 1997; Robbins and Griffin, 1999).

As stated previously, researchers have attempted to control the severity of *C. parasitica* infections by inoculation of active cankers with hypovirulent isolates (Jaynes and Elliston, 1978; Kuhlman et al., 1984; Robbins and Griffin, 1999). Unfortunately, in North America, vegetative compatibility limits the effectiveness of some hypovirulent isolates to transfer dsRNA into the virulent *C. parasitica* hyphae present on an active canker. To overcome this compatibility problem, most cankers are inoculated with a mixture containing hypovirulent isolates from several VC groups (Anagnostakis, 1978; Day, 1978; Jaynes and Elliston, 1982; Kuhlman et al., 1984). One common inoculation method involves creating a wound in canker tissue using a cork borer, then inserting a

plug of PDA agar containing fungal hyphae into this hole (Willey, 1980; Huang et al., 1996). Jaynes and Elliston (1978) have suggested that spraying an aqueous suspension of hypovirulent conidia onto the external surface of a canker may also be effective, but noted that this form of treatment is more effective if trees are wounded prior to inoculation. Field applications of hypovirulent isolates should be an effective means of increasing the dissemination of hypovirulent forms of the pathogen in the environment, having the potential to lessen blight severity (Kuhlman et al., 1984; Heiniger and Rigling, 1994).

Research in the Southeastern United States

Since many sprouts of the American chestnut are killed before they reach maturity, the relatively low number of trees of seed-bearing age now found in forests in the United States limits pollen sources, which minimizes sexual reproduction (Phares, 1978; Anagnostakis, 2001). This has generally limited the ability of the American chestnut to develop resistance to *C. parasitica*. With very little hope that natural resistance will enable reestablishment of American chestnut trees into areas such as GRSM, control methods must be attempted to enable populations of the tree to persist for future generations.

Survival of introduced hypovirulent isolates in the environment has been examined in the United States (MacDonald and Double, 1978; Carey, 1985; Peever et al., 1997). Griffin et al. (1978) reported that most isolates sampled from trees in West

Virginia and Virginia were highly or moderately pathogenic, with only one hypovirulent isolate identified. In a survey involving plots in two national forests in West Virginia, 179 of 202 isolates collected belonged to fourteen VC types, and plots containing a higher diversity of VC types also had higher incidence of infection (MacDonald and Double, 1978).

In a survey of large chestnut trees throughout western North Carolina, abnormal isolates were cultured from only two of 39 infested sprouts within 30 m of American chestnut trees larger than 20 cm dbh, while 10 of 18 chestnut trees larger than 20 cm dbh contained abnormal isolates (Carey, 1985). This led Carey to conclude that the presence of abnormal, possibly hypovirulent isolates on these larger trees was likely responsible for their survival. In another study, it was observed that 12 years after initial inoculation of hypovirulent isolates containing CHV-1 in forests throughout West Virginia, no CHV-1 was recovered from areas in which it had previously been released (Liu et al., 2002). The low incidence and poor persistence of hypovirulence was speculated to be due to the lack of competition between the American chestnut trees in this study and other associated hardwoods (Liu et al., 2002).

The GRSM, on the border of Tennessee and North Carolina and near the southern extent of the historic range of the American chestnut, is known to contain a remnant population of American chestnut. As previously mentioned, Wood (2003) located 288 surviving trees greater than 10 cm dbh throughout the park, but no studies have examined the conditions of these trees or the virulence of fungal isolates associated with

them. National Park Service management policy does not allow for the introduction of material not endemic within the boundaries of the national park and does not allow for breeding programs involving material not endemic to the park (National Park Service, Department of the Interior, 2004). Only fungal isolates collected within the park can be inoculated onto chestnut trees in the GRSM. Naturally occurring hypovirulent isolates present on one of the trees recorded in the previous study (Wood 2003) could potentially be used to control the severity of the disease on select large, nut-producing chestnut trees within the park. This would enable park personnel to maintain a population of larger trees for future generations to observe and provide seed for further studies. Therefore, the objectives of this study were to:

1. Culture hypovirulent and virulent isolates of *C. parasitica* from 10 cm dbh or larger American chestnut trees in the GRSM.
2. Evaluate collected isolates *in vitro* for the presence of dsRNA and rate for hypovirulent potential based on morphological characteristics and hypovirus presence.
3. Test isolates collected in objective 1 for VC types *in vitro* in order to determine the relative number and diversity of groups present in the GRSM.
4. Perform in-field inoculation screening of select isolates to determine their virulent or hypovirulent potential based on visual signs and symptoms.

CHAPTER 2

MATERIALS AND METHODS

Field Sampling in the GRSM

Surveying American chestnut trees. Bark samples from 81 of the 288 American chestnut trees documented during a survey in the GRSM (Wood, 2003) were collected from May 2006 through September 2007. Bark samples were removed from bark cankers formed by *Cryphonectria parasitica* on these trees. Universal Transverse Mercator (UTM) coordinates were obtained for these 288 trees (Wood, 2003) and a Garmin GPSMAP® 60CS Global Positioning System (GPS) (Garmin International Inc., Olathe, KS) was used to locate individual trees.

Table 1 lists all sampling dates in 2006 and 2007. In the course of this study, 201 of the 288 trees (Wood, 2003) were visited and 123 new trees were recorded in the GRSM, Nantahala National Forest, and Pisgah National Forest. A map of all trees visited in this study is shown in Figure 1.

Table 1. American chestnut bark sampling dates and localities in 2006 and 2007.

Sampling Date	Location	Number of trees
4/14/2006	Sugarland Mountain	4
5/12/2006	Cades Cove	6
5/13/2006	Cove Mountain	28
5/14/2006	Albert Mountain	5
6/17/2006	Andrew's Bald	2
6/19/2006	Smokemont	4
6/20/2006	Albert Mountain[a]	2
6/21/2006	Thomas Divide	4
6/22/2006	Sunkota Ridge	3
6/24/2006	Foothills Parkway	4
6/27/2006	Thomas Divide	39
6/28/2006	Whiteside Mountain[a]	6
7/17/2006	Cove Mountain	6
7/18/2006	Sugarland Mountain	11
7/19/2006	Newfound Gap Road	2
7/20/2006	Cataloochee Divide	40
8/7/2006	Noland Divide	17
8/8/2006	Balsam Mountain/ Beech Gap	4
8/12/2006	Curry Mountain	6
8/13/2006	High Rocks/ Welch Ridge	9
9/1/2006	Gregorys Bald	15
9/3/2006	Cataloochee Divide	1
4/20/2007	Lanier[a]	1
5/24/2007	Greenbrier Pinnacle	9
6/11/2007	Hyatt Ridge Trail	7
6/14/2007	Hemphill Bald Trail	3
6/15/2007	Thomas Divide	9
6/19/2007	Albert Mountain[a]	5
6/20/2007	Pisgah[a]	5
6/25/2007	Whiteside Mountain[a]	6
6/26/2007	Bull Head Trail	6
6/27/2007	Bote Mountain Trail	3
7/23/2007	Chestnut Top Trail/ Roundtop Trail	3
7/25/2007	Rich Mountain Loop	13
7/26/2007	Scaly Mountain[a]	5
8/14/2007	Albert Mountain[a]	14

[a] Sampling locations outside of the Great Smoky Mountains National Park from which isolates were obtained. These isolates were not used in subsequent studies.

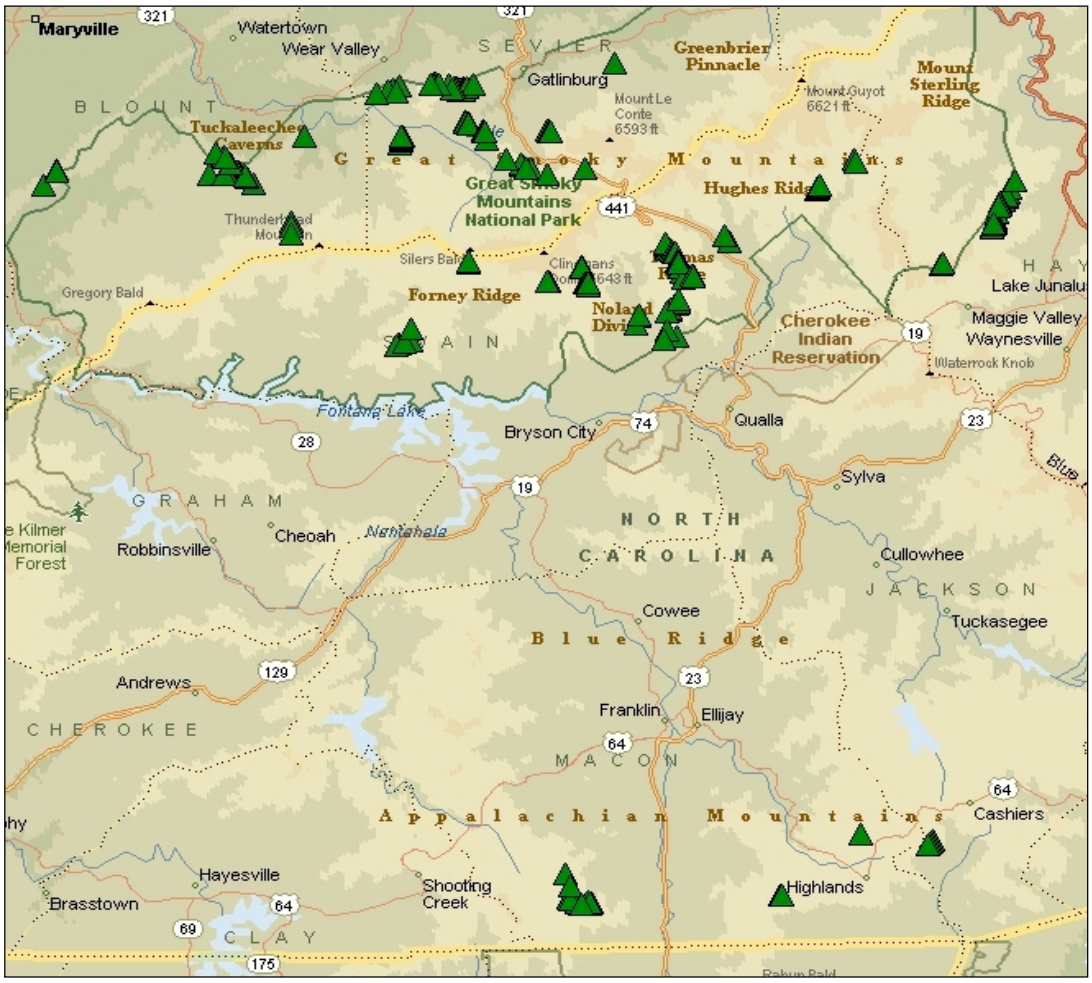

Figure 1. Locations of all American chestnut trees visited during the course of this study from May 2006 through September 2007.

For a given tree, sampling focused on all cankers between 0.5 and 2.5 meters from the ground. The condition of each canker sampled was noted based on guidelines outlined by Carey (1985). In his study, a canker is described as "diffuse" if necrotic tissue merges into surrounding bark with no visual evidence of callus formation, and rated as "swollen-diffuse" if the center portion is raised slightly in relation to surrounding healthy bark tissue while margins of the canker blend into surrounding bark as in a diffuse canker. The term "superficial" was used if the margin of cankered tissue appeared raised and swollen (Carey, 1985). Each sampled canker was numbered based on the tree it occurred on for future reference and location. Isolates obtained were assigned a number based on the specific tree and canker from which they were collected.

Sampling cankers. When sampling individual cankers, a 10 mm diameter cork borer was used to remove bark pieces. Samples were taken at five points within a canker (Figure 2). One bark plug was removed from the approximate center of the canker, and two each along the horizontal and vertical axes of the canker. These outermost samples were removed from within 1 cm of the edge of a canker. Samples were removed to the edge of underlying uninfected cortex tissue or up to 2 cm deep, whichever was shallower. A number of cankers were diffuse, having no clear boundary between healthy and infected tissue. In this case, the edge of stromata formation was considered the outermost limit of the canker. The cork borer was sterilized with 70% ethanol and flamed between each use.

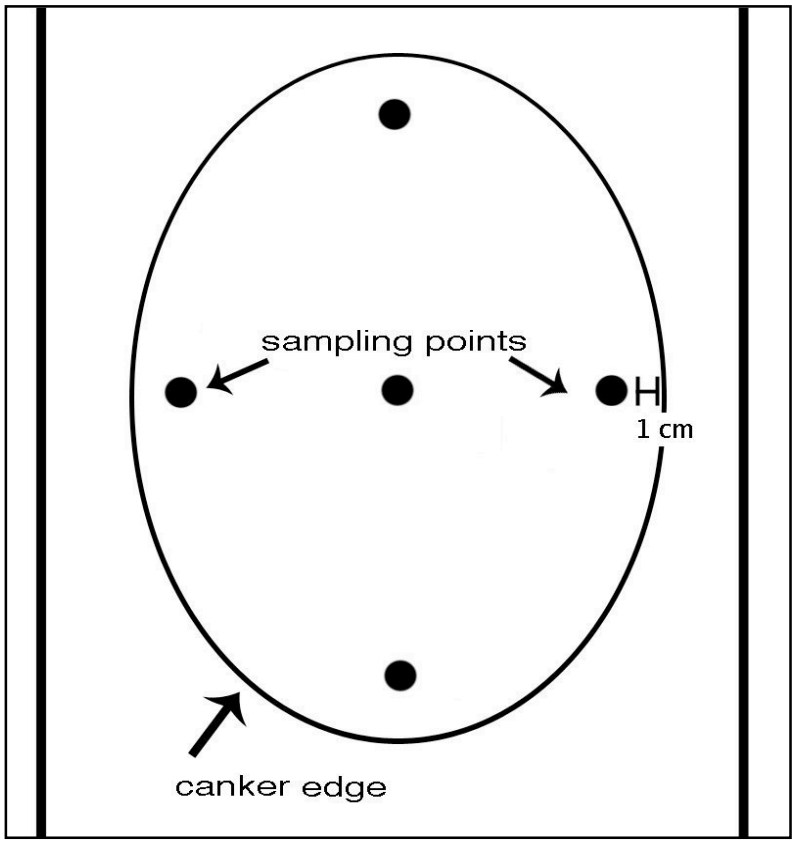

Figure 2. Locations of 10 mm sampling points on *C. parasitica* cankers of American chestnut trees.

Transport and short-term storage. The 10 mm bark samples were stored and transported in individual sterilized 2 ml cryogenic vials which were kept at ambient temperature for transport to a trailhead. The samples were then transferred to a cooler maintained between 0 and 10º C for transport to the laboratory at Mississippi State University, Mississippi State, MS. All samples were kept below 10º C until processed within 5 days as described below.

Determination of Virulence

*Culturing and subculturing of collected isolates***.** Bark samples were surface sterilized in a 10% sodium hypochlorite (w/v 0.534) solution for two minutes. Samples were then placed onto a Petri plate (10 x 100 mm) containing 16 ml of potato dextrose agar (PDA, Difco™, Detroit, MI) consisting of 39 g PDA in 1 l distilled water. Media was amended with chlorotetracycline (Sigma, St. Louis, MO; 50 mg/l) and streptomycin sulfate (Sigma, St. Louis, MO, 8 mg/l) to hinder bacterial growth. All cultural work conducted during this investigation employed PDA with antibiotics and all cultures were incubated at room temperature with alternating light-dark periods of 12 hours. After 7 to 14 days, any isolates identified as *C. parasitica* through observation of morphological characteristics were subcultured from the advancing edge of the colony to ensure that pure cultures were obtained. These isolates were placed onto a fresh Petri plate containing PDA as described above, and kept under the same environmental conditions.

Determination of cultural morphology. Visual analysis of cultural characteristics of all *C. parasitica* isolates obtained during this study was undertaken in order to determine the potential for hypovirus presence. All isolates obtained from the GRSM were cultured onto PDA as previously described with two replications prepared per isolate. After seven days, resulting colonies were subcultured on PDA by removing a 3 mm^3 plug of mycelia from their actively growing margins. Cultural morphological characteristics of all isolates were compared to a positive, hypovirulent control known to contain dsRNA, and a negative, or virulent control isolate that is dsRNA-free. Three control isolates were obtained from Dr. William MacDonald of the Department of Plant and Soil Science at West Virginia University and subcultured from vials stored at -80 º C at the Mississippi State University Department of Plant Pathology, Mississippi State, MS. Two of these isolates, designated CHV-1/Euro 7 (referred to as E96) and Hypovirulent #98 (referred to as 98+), contained the CHV-1 dsRNA. Isolate Virulent #97 (referred to as 97-) contained no dsRNA. A fourth dsRNA-free control isolate, EP-155, was obtained as an actively growing culture on PDA from Sandra Anagnostakis of the Connecticut Agricultural Experiment Station, New Haven, CT.

Examination of morphological characteristics. After 14 days growth, morphological characteristics such as colony size, pigmentation, and number of stromata of resulting colonies were analyzed and recorded (Turchetti, 1978; Willey, 1980). After 14 days growth, subcultured isolates were prepared for storage at -80º C by removing

ten 3 mm^3 plugs of mycelia from the actively advancing edge of each colony grown on PDA. The mycelial plugs were placed into a sterile 1.2 ml cryogenic vial (Corning, Acton, MA), and 800 µl of a glycerol solution (10% glycerol + 90% sterile distilled water) was added to completely cover the sample. Vials containing these isolates were stored at -80º C for use later in this study. Additional backup isolates were prepared in 15 ml screw top vials containing 5 ml of PDA. These cultures were grown for 72 hours at room temperature before being stored at 4 º C.

Extraction of dsRNA

In preparation for extraction of dsRNA, all isolates stored in vials were removed from -80º C and active cultures transferred to PDA using the methods described above for morphological comparison. An "EP complete" (*Cryphonectria parasitica*) liquid media described by Puhalla and Anagnostakis (1971) was prepared (Table 2), then divided into 150 ml aliquots, placed into 250 ml flasks, and autoclaved (Puhalla and Anagnostakis, 1971; Suzuki et al., 1999). Four 3 mm^3 plugs of mycelia from subcultures were extracted from the advancing edge of the colony and placed into the liquid media. Two replicates were prepared per isolate for dsRNA extraction, and combined into a single sample after incubation. The flasks were placed on a laboratory shaker (New Brunswick C-24 Environmental Incubator Shaker, Edison, NJ) and incubated with agitation for 14 days at room temperature with 12 hour alternating light and dark periods. After 14 days, the contents of the flask were poured into a Buchner

Table 2. Components of liquid media for culturing isolates of *C. parasitica* in preparation for dsRNA extraction (Puhalla and Anagnostakis, 1971).

Solution	**Component**	**Amount**
Base media	Glucose	10 mg
	Difco yeast extract	2.5 g
	Difco malt extract	7.5 g
	Thiamine	2 mg
	Salt solution (below)	62.5 ml
	Distilled water	937.5 ml
Salt solution	NH_4NO_3	2.5 g
	KH_2PO_4	1.6 g
	Na_2SO_4	4.0 g
	KCl	8.0 g
	$MgSO_4 \cdot 7H_2O$	2.0 g
	$CaCl_2$	1.0 g
	Trace element solution (below)	8 ml
	Distilled water (volume to)	1.0 l
Trace element solution	H_3BO_3	30 mg
	$MnCl_2 \cdot 4H_2O$	70 mg
	$ZnCl_2$	200 mg
	$Na_2MoO_4 \cdot 2H_2O$	20 mg
	$FeCl_3 \cdot 6H_2O$	50 mg
	$CuSO_4 \cdot 5H_2O$	200 mg
	Distilled water (volume to)	500 ml

funnel apparatus, and mycelia separated from liquid using 5.5 cm filter paper (Whatman #54), then finely ground in liquid nitrogen with a mortar and pestle. Ground mycelia were stored at -80º C until processed.

DsRNA extraction encompassed a multistep process beginning with a 4-step extraction process (mixing, cleaning, washing, elution) which was used to separate all nucleic acids from other cellular components. These steps were followed by a concentration step which reduced the volume of each sample, and an enzymatic digestion step which digested all DNA and single stranded RNA in a sample, leaving only dsRNA intact.

Mixing. Each ground sample was placed into a 250 ml beaker containing 15 ml of a 10% SDS (sodium dodecyl sulfate) solution, 1.0 ml of a 45 mg/ml bentonite suspension, and 1.0 ml of 2-mercaptoethanol in 45 ml of a 2x STE buffer. The 10X STE base solution was prepared from 58.44 g NaCl, 60.57 g Tris, and 3.72 g EDTA in H_2O to 1.0 l and brought to a pH of 7.5 with concentrated hydrochloric acid. The resulting mixture was stirred vigorously for 10 minutes until all mycelia had thawed, and then a mixture of 30 ml chloroform and 30 ml phenol was added. Each sample was then stirred vigorously for 45 minutes at room temperature using a magnetic stir bar.

Cleaning. After mixing, each sample was centrifuged in a Beckman J2-26 centrifuge for 10 minutes at 10,000 revolutions per minute (rpm). The aqueous phase

containing nucleic acids was recovered in a 100 ml graduated cylinder. The volume of each sample was brought to 84 ml by addition of a 1x STE solution, and then 16 ml of absolute ethanol was added to each sample to bring the volume to 100 ml. To each sample, 1.0 g of CF-11 cellulose powder (Whatman) was added, and samples were stirred for 120 minutes at room temperature to allow nucleic acids to bind to the cellulose.

Washing. Prior to the washing step, several liters of washing buffer were prepared from 100 ml of 10x STE, 165 ml ethanol, and water to 1 liter. Each sample was washed by centrifugation at 10,000 rpm for 7 minutes and the aqueous phase discarded, leaving a solid pellet of cellulose to which nucleic acids had bound in the presence of ethanol. Washings were repeated twice. After the final centrifugation, each sample pellet was then transferred to 50 ml of washing buffer and resuspended.

The resulting suspension was then poured into a 50 ml syringe, into which a small piece of Miracloth (Calbiochem, San Diego, CA) was inserted. The suspension was poured slowly to allow cellulose column formation, with excess liquid being filtered out the bottom by gravity. Several 30 ml aliquots of washing buffer were poured over the cellulose and allowed to completely drain in order to further wash the cellulose.

Elution. Following washing, any dsRNA bound to the cellulose was eluted with 30 ml of an STE 1x solution. The eluted portion was filtered using the apparatus

illustrated in Figure 3, and the eluted dsRNA portion was collected in a 125 ml flask containing 2.5 volumes of absolute ethanol and 0.1 volumes of a 3M sodium acetate solution at pH 5.5. Samples were stored for at least eight hours at -20º C to precipitate nucleic acids.

Concentration. At the end of the elution step, each sample volume was approximately 110 ml. This volume was reduced by centrifugation. Each sample was spun in a 50 ml centrifuge tube for 30 minutes at 10,000 rpm in order to precipitate all nucleic acids, as well as any remaining cellular debris. The aqueous phase was discarded, and each pellet was resuspended in 1.0 ml H_2O and subsequently divided into two aliquots of 500 µl each. To each aliquot, 1.0 ml ethanol and 40 µl of 3M sodium acetate were added to precipitate nucleic acids.

In order to remove any remaining cellular debris and cellulose residue, a mixture of equal volumes of a phenol and chloroform mixture was added. After two minutes of vigorous shaking, sample aliquots were centrifuged for three minutes at 13,000 rpm in order to separate the sample into an aqueous phase and a phenol phase. The aqueous phase was recovered, and dsRNA was precipitated in 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH 5.5) overnight.

Enzymatic digestion of nucleic acids. In order to separate any viral dsRNAs from other nucleic acids present in each sample, selective enzymatic digestion steps were

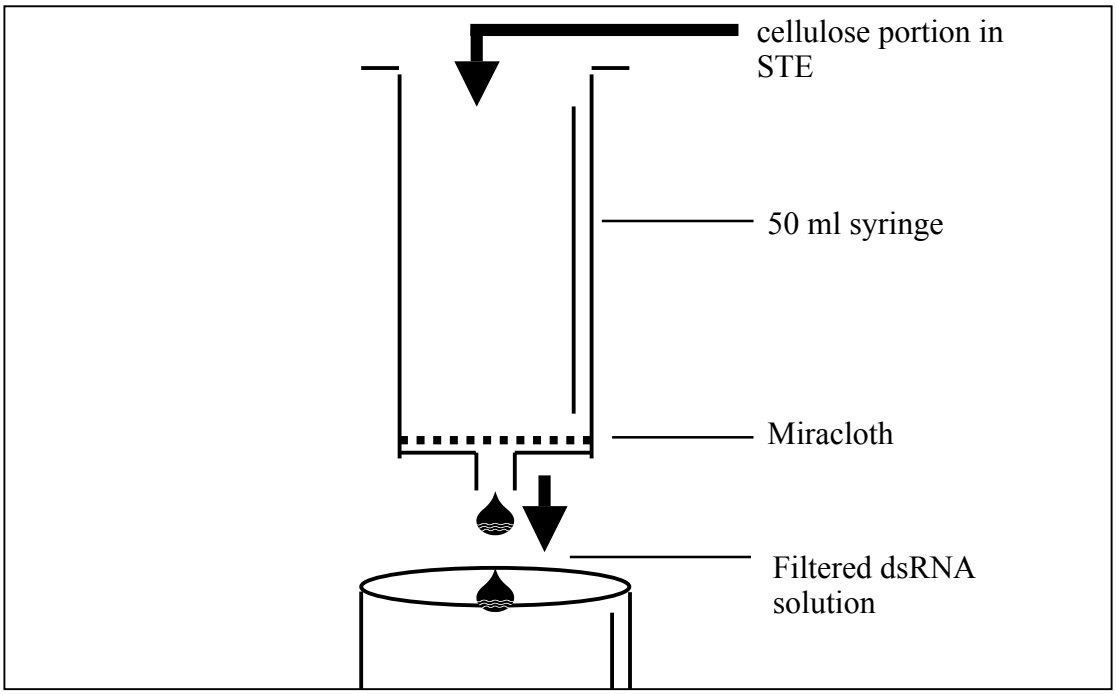

Figure 3. Elution of cellulose-bound dsRNA.

performed. First, each sample contained in 1.5 ml centrifuge tubes was centrifuged for 30 minutes at 13,000 rpm in order to precipitate all nucleic acids into a pellet. The aqueous phase was discarded, and each sample was allowed to air dry for 60 minutes. To each sample, the following was added: 99 µl H_2O, 1 µl $MgCl_2$ (1M), and a DNAse solution (2 units/µl). Each sample was incubated at 37 º C in order to digest all DNA in the sample. After 30 minutes, 3 µl diluted RNAse solution (25 ng/ml) was added under high salt conditions (SSC 2X). Each sample was again incubated for 30 minutes at 37 º C to digest remaining traces of single stranded RNA.

After 30 minutes, 3 µl of a proteinase K solution (3 µg/µl) was added to each sample in order to denature DNAse and RNAse and to halt further digestion of nucleic acids. Samples were incubated for an additional 30 minutes, after which time the following were added to each: 45 µl SDS 10%, 250 µl H_2O, 225 µl phenol, and 225 µl chloroform. Again, a mixture of 225 µl phenol and 225 µl chloroform was added to each sample aliquot. After two minutes of vigorous shaking, sample aliquots were centrifuged for three minutes at 13,000 rpm in order to separate the sample into an aqueous phase and a phenol phase. The lower phenol phase was discarded.

Visualization of extracted dsRNA. The product from the extraction procedure was separated by electrophoresis on a 1.0% agarose gel (1.0 g agarose (Difco™) in 100 ml Tris/Borate/EDTA (TBE) buffer) in order to determine the presence of any dsRNA in collected isolates. The TBE buffer (pH 8.3) was prepared from 60.55 g Tris base (0.5M),

25.68 g boric acid (0.4M), 1.86 g EDTA disodium salt (0.005M), and nanopure water to 1.0 l. Ethidium bromide was used to visualize bands.

Alternatively, dsRNAs were examined in 6% polyacrilamide gel electrophoresis (PAGE), and visualized using silver nitrate. Estimation of dsRNA size was made by comparison with high molecular weight markers (CHV-1 98+ isolate, *Peanut stunt virus* (PSV)). The data collected from the analysis of morphological characteristics and from the extractions of dsRNA was used to tentatively determine which isolates are considered to be virulent or hypovirulent.

Determination of VC groups

Isolates previously collected during the survey in the GRSM were tested against each other in order to determine the VC groups to which they belong (Appendix A1). A 3 mm^3 agar plug was removed from the actively growing colony edge of each subcultured isolate to be tested. Pairings of different isolates were placed approximately 5 mm apart onto Petri plates (10 x 100 mm) containing PDA prepared as previously described and amended with 50 mg/l bromocrestol green in order to more easily visualize weakly incompatible interactions (Powell, 1995) as well as 0.6 ml of Tween 20. Each pairing was replicated twice on individual plates (Liu and Milgroom, 1996).

After 7 days, anastomosis or barrage formation was noted. Paired isolates were observed daily for 14 days to determine any pycnidia formation along the barrage zone (Liu and Milgroom, 1996). Isolates which underwent anastomosis upon contact were

considered in the same VC group (Figure 4). Additionally, the control isolates obtained previously (WVU and CT) were paired with the ones collected from the GRSM to test for compatibility.

Verification of Hypovirulence Through Inoculation

In April 2007, a location in the Highlands Ranger District of the Nantahala National Forest (N35.08641, W83.22033) containing susceptible American chestnut trees between 5 and 10 cm in dbh was selected for *in situ* study. The site was a previously clearcut second growth mixed hardwood site approximately 1,300 meters in elevation. On this site, 54 healthy American chestnut trees greater than 4 cm dbh were located in an area approximately 4 hectares in size (Figure 5; Appendix A2).

On May 15, 2007, susceptible healthy American chestnut trees were inoculated using potentially virulent and hypovirulent isolates previously collected from the GRSM, as well as with virulent (dsRNA-free EP-155 and 97-) and hypovirulent (CHV-1-containing E96) control isolates. Four replicates of each isolate inoculated were transferred to PDA 14 days prior to inoculation. Selected trees were inoculated using a randomized design while ensuring that no replicate isolate was inoculated twice onto a single tree (Appendix A3). Stems greater than 5 cm dbh were inoculated twice, with one inoculation at 0.5 and another at 1.5 meters above the ground surface with each inoculation site aligned to one of four randomly assigned cardinal directions (north, east, south, or west).

Inoculations were performed with a 10 mm cork borer by punching a hole to the cambial layer of healthy bark tissue showing no external symptoms of *C. parasitica*

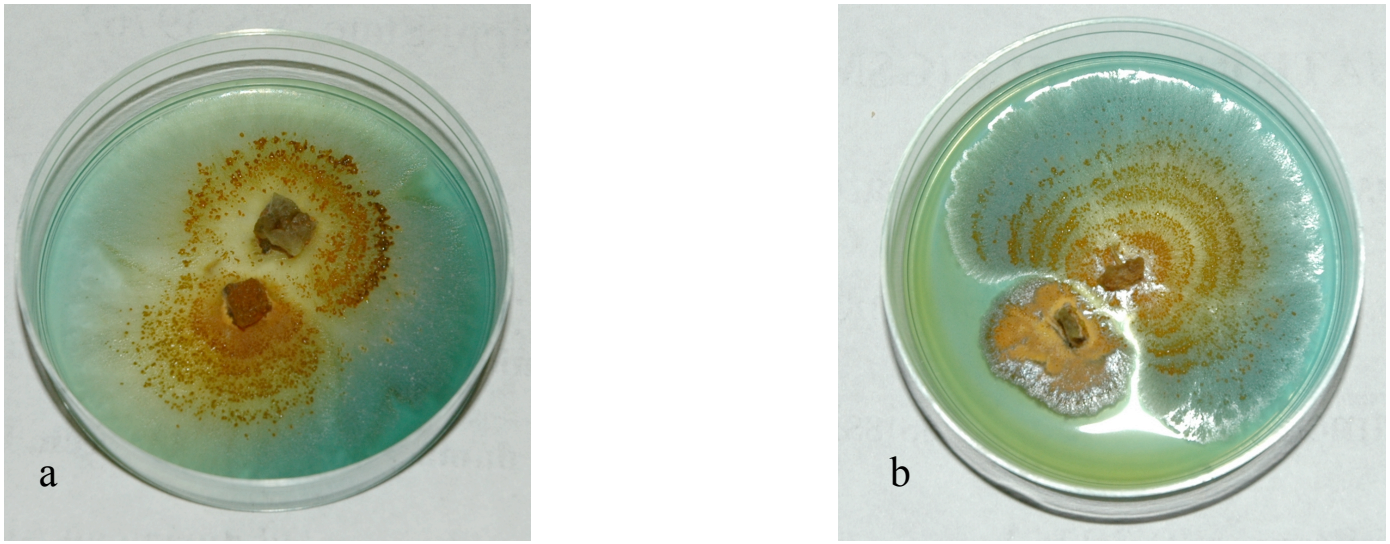

Figure 4. Anastomosis (a) and barrage formation (b) between paired isolates of *Cryphonectria parasitica* tested *in vitro*.[a]

[a] Anastomosis has occurred only in the two paired isolates in the left frame (a), indicating that these isolates are in the same VC group. Barrage formation and lack of interaction between other paired isolates (b) indicates that these isolates are in different VC groups.

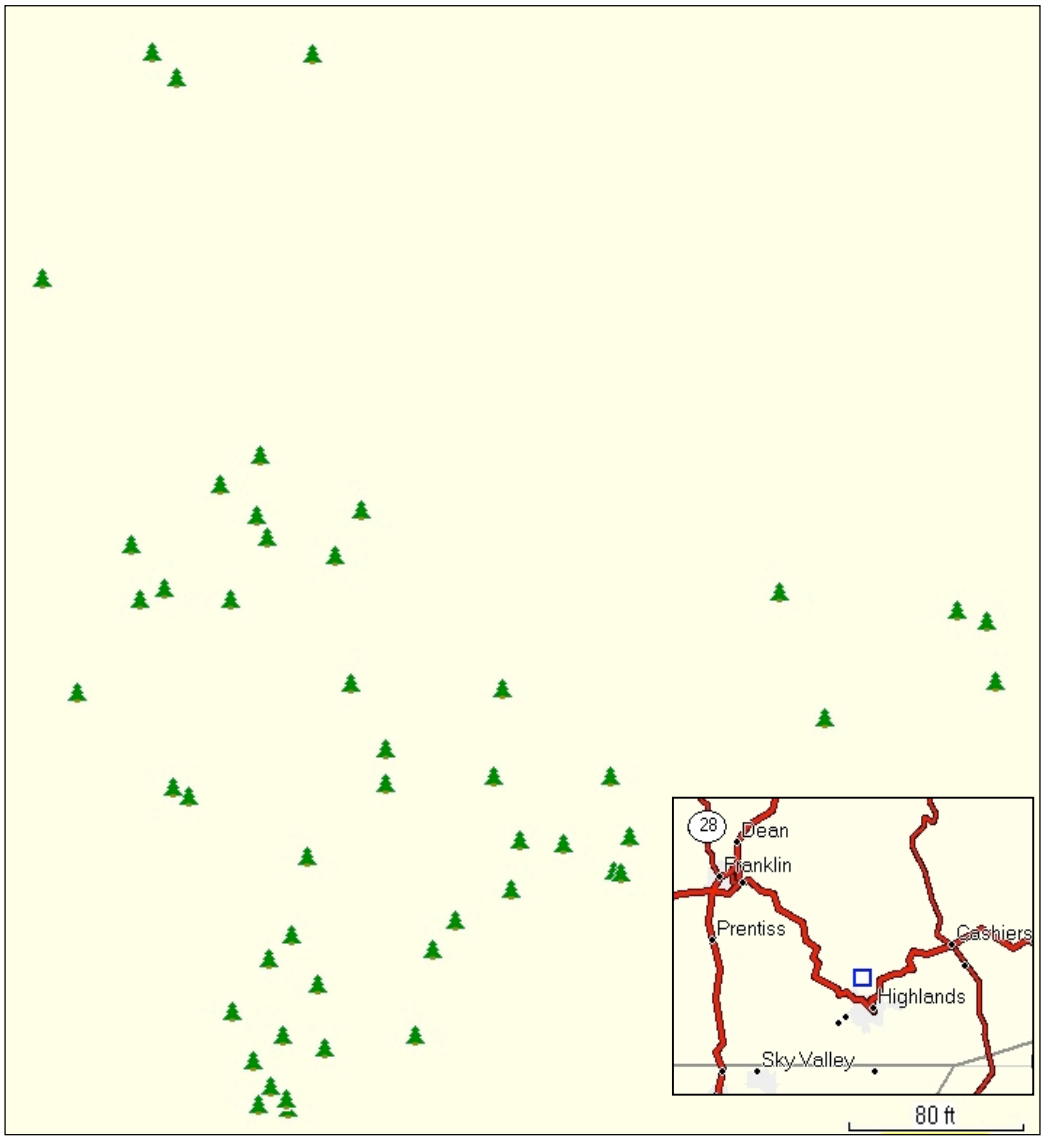

Figure 5. Plot of all healthy American chestnut trees used for field inoculations, Nantahala National Forest, Highlands, NC; 2007.

infection. A punch of equal diameter was also used to extract a plug of three day old fungal mycelium from the growing colony edge of the subcultured isolates on PDA, and inserted into the bark with the mycelial surface down (Willey, 1980), then covered with masking tape to prevent desiccation (Carey, 1985; Anagnostakis et al., 1998). In order to prevent cross contamination, the cork borer was surface sterilized using 70% ethanol and flamed prior to each inoculation.

Data Collection on inoculated American chestnut trees. Inoculation sites were checked monthly for canker growth. Observation dates in 2007 were as follows: June 28, July 24, August 15, September 30, and October 23. On each of these dates, pictures were also taken of each inoculation site. All masking tape was removed from inoculated trees during the first week of June. Stromata formation and canker width and height were recorded on each of these dates for any newly formed cankers at inoculation sites (Elliston, 1978; Griffin et al., 1978). Canker area (mm^2) was calculated by multiplying canker length by canker width. In cases where cankers completely encircled a stem, canker width was calculated as the circumference of the stem at approximately the vertical center of the canker. All stromata were counted in a 16 cm^2 area surrounding the inoculation site (Baird, 1980). In addition, qualitative analysis of canker superficiality was recorded using the same criteria for rating cankers previously employed in this study (Carey, 1985).

Re-isolation of C. parasitica. On October 23, all inoculation sites were sampled for *C. parasitica* using the methods employed previously in this study during tissue

collection from cankers on American chestnut trees. Following isolation of pure cultures on PDA, isolates were once again tested for the presence of dsRNA using the extraction method previously described. Any differences in dsRNA presence or cultural morphology were noted for an isolate after re-isolation from inoculated trees.

Statistical Analysis

Inoculation procedures were performed following a completely randomized design. Data on canker area and stromata production on cankers inoculated with hypovirulent isolates and dsRNA-free controls were analyzed using analysis of variance (ANOVA) and least squares means separation (LS Means). Pairwise comparisons of mean canker size were performed using Tukey's test. A method of population analysis, the Shannon Diversity Index (Robin et al., 2000):

$$H' = - \sum_{i=1}^{s} p_i \ln p_i \qquad (2\text{-}1)$$

was used to estimate the diversity of VC types present among the population sampled from the GRSM, where S is the total number of VC groups found in this study and p_i is the proportion of the total population composed of a given species. Statistical analyses were performed using the Statistical Analysis System (SAS) software package version 9.2 (SAS Institute, Cary, NC).

CHAPTER 3

RESULTS

Tree Locations and Sampling

A total of 67% of 288 known American chestnut trees greater than 10 cm dbh reported to occur in the GRSM (Wood, 2003) were located in 2006 and 2007. Additionally, 45 new trees were identified along trails while searching for documented trees. In total, 238 American chestnut trees were included in this study (Table A1), but only 81 trees contained one or more cankers. All cankers on the 81 infected trees were sampled in 2006 and 2007. An additional 48 trees were dead, and 20 trees from the Wood (2003) survey could not be located due to either tree mortality or incorrect coordinates. Coordinate data for all other trees were observed to be accurate to within approximately 30 m, with the majority of trees being found within 8 m of an indicated point.

Of all 238 chestnut trees confirmed in the GRSM, 195 were located in seven disjunct localities throughout the park, usually on high ridgetops separated by valleys including Gregory Bald, Rich Mountain, Cove Mountain, Sugarlands Mountain, Noland Divide, Thomas Divide, and Cataloochee Divide (Table 3, Figure 6). The remaining 43 trees were generally scattered in isolated spots throughout the park, with one to ten trees

occurring along a single trail or ridge. Trees ranged from 10 cm dbh to 26 cm dbh, with most being at the lower end of the range.

Gregory Bald. Trees at this site were sampled on September 1, 2006. The trees were located within a high elevation (1450-1500 m) hardwood forest along the edge of a grassy bald. This area was unique in that it contained 15 diseased trees, and none were dead (Table 3).

Rich Mountain. Trees at this site were sampled on May 12, 2006 and July 25, 2007 (Table A1). American chestnut trees occurred along the southern slopes of this area containing cove hardwood forests (850-1120 m). Rich Mountain contained a fairly even distribution of healthy, diseased, and dead trees, with six of 19 asymptomatic, four containing one or more cankers, and four dead. An additional five trees from the Wood (2003) survey could not be located (Table 3).

Cove Mountain. Trees at this location were sampled on May 13 and July 17, 2006. Chestnut trees were located on the Little Greenbrier Trail between Wear Cove Gap (560 m), the summit of Cove Mountain (1230 m), and on the Cove Mountain Trail along the GRSM boundary above Holy Butt (900 m). Habitat along these trails consisted of mixed pine-hardwood forest on south-facing slopes within the GRSM boundary. Bark samples were obtained from nine of 34 trees for isolation of *C. parasitica* (Table 3). An additional 11 trees were healthy, 10 were dead, and four could not be found. One healthy

American chestnut tree (#45) was the largest one encountered during this investigation at 26 cm dbh.

Sugarland Mountain. This site was sampled on April 14 and July 18, 2006. American chestnut were found throughout the lower half of this north-facing ridge (860-1370 m) among cove hardwood forests intermixed with eastern hemlock (*Tsuga canadensis* L.). A total of seven trees occurring above 950 m were healthy, while four diseased trees and one dead tree were found between 860 and 950 m.

Noland Divide. Trees at this site were sampled on August 7, 2006, and were located on a ridgetop containing 11 of 22 trees with no symptoms of *C. parasitica*. Along the lower elevations of this south-facing ridge, three dead trees were noted. Noland Divide was higher in elevation than the other six areas in the GRSM (1300-1830 m), with seven diseased trees occurring throughout this elevation range. Trees along upper portions of this ridge were found in northern hardwood stands bordering the lower elevations of red spruce (*Picea rubens* Sarg.) forest, while the lower elevations contained primarily American beech (*Fagus grandifolia* Ehrh.) and maple (*Acer* spp.).

Thomas Divide. This location (1250-1450 m) was sampled on June 21 and June 27, 2006, and on June 15, 2007. This site contained the greatest known concentration of trees in the park, with 50 trees visited along the Thomas Divide Trail between Newfound Gap Road and the Deeplow Gap Trail. Habitat along Thomas Divide consisted of primarily northern hardwood and cove hardwood forest along an exposed, south-facing

ridgeline. Thomas Divide also had the highest number of infected or dead trees of all seven locations, containing 14 diseased and 14 dead trees.

Cataloochee Divide. This geographical area (1250-1640 m) was sampled on July 20, 2006. This location had the highest proportion of healthy trees in the park, among an overstory of mixed hardwood and American beech. A total of 28 out of 40 trees were asymptomatic for *C. parasitica* (Table 3) while six trees contained one or more cankers. The remaining 6 trees were dead.

Isolation of *Cryphonectria parasitica*

Isolates identified as *C. parasitica* were obtained from 104 out of 121 cankers sampled from the GRSM. Of that total, isolates were obtained from 77 of the 88 cankers sampled from the seven locations in the GRSM (Table 3). From 11% of all cankers sampled, only saprophytic fungi or contaminants such as *Trichoderma* spp. or *Aspergillus* spp. were identified. From all cankers sampled in this study, 339 isolates of *C. parasitica* were obtained from the GRSM. Of this total, 231 isolates came from trees at the seven locations, while 108 were obtained elsewhere in the park (Table 3).

Morphological Characteristic Determination

All 339 isolates obtained from the GRSM were found to be one of three morphotypes as per cultural characteristics described by Anagnostakis (1988). A total of 278 cultures had a "normal" morphology and growth rate, in which fungi generally formed a radial growth pattern of white mycelia which completely colonized a 16 mm

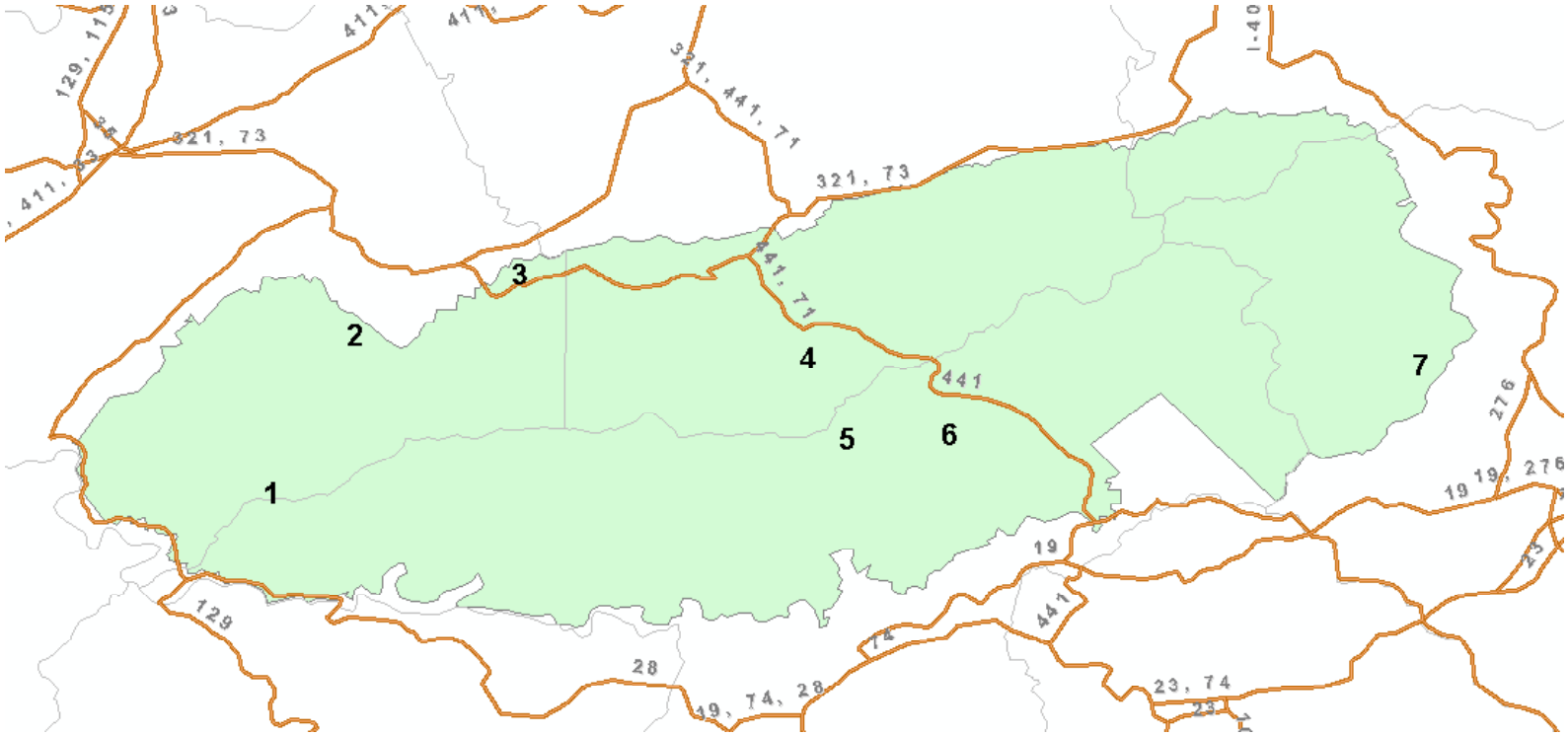

Figure 6. Locations in the Great Smoky Mountains National Park containing the greatest known concentrations of American chestnut trees, 2006-2007.[a]

[a] 1 = Gregory Bald; 2 = Rich Mountain; 3 = Cove Mountain; 4 = Sugarland Mountain; 5 = Noland Divide; 6 = Thomas Divide; 7 = Cataloochee Divide.

Table 3. Locations in the Great Smoky Mountains National Park (GRSM) containing the greatest concentrations of known American chestnut trees and their health status in 2006-2007.

Locality	Reference coordinates	Total trees	Healthy[a]	Diseased[b]	Dead[c]	Not found[d]	Cankers
Gregory Bald	N35.53030 W83.85628	**15**	0	15	0	0	22
Rich Mountain	N35.62727 W83.78499	**19**	6	4	4	5	4
Cove Mountain	N35.70109 W83.60471	**34**	11	9	10	4	17
Sugarlands	N35.64361 W83.54339	**15**	9	4	1	1	3
Noland Divide	N35.54874 W83.47392	**22**	11	7	3	2	8
Thomas Divide	N35.54270 W83.37456	**50**	19	14	14	3	17
Cataloochee	N35.59502 W83.07103	**40**	28	6	6	0	6
Total		**195**	84	59	38	14	77
Elsewhere in GRSM[e]		**63**	25	22	10	6	32
Total		**258**	109	81	48	20	104

[a] Trees were asymptomatic for *C. parasitica* infection.
[b] Trees containing one or more cankers caused by *C. parasitica* from which bark samples were obtained for fungal isolation.
[c] Trees originally reported in previous surveys which were no longer living.
[d] American chestnut trees which could not be located in the field using information and GPS coordinates from previous surveys within the park.
[e] See Table A1 for coordinates to other trees in the GRSM.

Petri plate within 14 days. Approximately seven to ten days after subculturing, numerous brightly colored orange pycnidia formed on the surface of these "normal" cultures, often in concentric rings (Figure 7). A total of 54 isolates (Table 5, Figure 8) were observed to have distinct cultural morphologies. These isolates exhibited a range of abnormal morphological characteristics including reduced orange pigmentation, and in some cases appeared light yellow or nearly white. Limited pycnidia production was also noted on these cultures, and these structures were more widely spaced than those which developed on normal isolates. However, this characteristic was not consistent among all abnormal isolates. Seven additional isolates had a slow growing orange morphology in which fungal tissue was densely packed, brightly colored, but only grew to approximately half the diameter of a 16 mm Petri plate after 14 days (Table 4, Figure 9). Stromata were absent from such isolates, which were similar in appearance to the *col1* or *flat* morphological type (Anagnostakis 1988). In no cases were cultures observed to have both a white color in conjunction with a slow growth rate.

Extraction and Analysis of dsRNA

Selection of isolates for dsRNA extraction was based on previous analysis of morphological characteristics (Tables 4, 5). Extraction of nucleic acids and enzymatic digestion was conducted on 20 of the 54 isolates which exhibited abnormal cultural characteristics as described previously, while another 34 could not be tested due to economic and time constraints. An additional 12 isolates with normal morphology were also chosen at random for comparison.

The presence of a high molecular weight dsRNA molecule resembling a hypovirus was observed in three of the tested isolates (236-1C, 324-1B, 325-1B). These isolates contained a major band which was calculated to be approximately 13,000 base pairs in size (Figure 10).

Extraction of dsRNA was performed on re-isolated cultures following the field trial in 2007. The presence of bands indicating a high molecular weight dsRNA molecule was observed in the three isolates following re-isolation from the field trial.

Vegetative Compatibility Group Determination

All 107 isolates paired with each other and the three controls were determined to belong to 34 distinct groups (Table 7). Among all isolates examined, the VC groups varied in size, with the smallest groups containing one isolate and the largest with 14 isolates. A total of 16 isolates did not undergo anastomosis with any other isolate. Such isolates were considered to belong to separate VC groups. On 27 trees which contained multiple cankers, isolates obtained from all cankers on a tree were vegetatively compatible with each other. For trees 175, 220, and 242, isolates from two different cankers on a single tree were not compatible. Among the seven different localities in the GRSM in which American chestnut trees were sampled, significant variation in the diversity of VC groups existed (Table 8).

Species diversity (Shannon Index) values ranged from a low value of 0.56 on Sugarlands Mountain, which had two VC groups occurring on three cankers, to a high

Table 4. Abnormal isolates of *C. parasitica* obtained from American chestnut bark samples collected from the Great Smoky Mountains National Park in 2006 and 2007.

Isolate numbers[a]	Morphology[b]	Location
20-1A, 1C	Abnormal	Cataloochee Divide
60-1B, 1C	Abnormal	Cove Mountain
60-2A, 2B, 2C, 2D	Abnormal	Cove Mountain
60-3A	Abnormal	Cove Mountain
62-1B, 1C	Abnormal	Cove Mountain
66-1A	Abnormal	Cove Mountain
73-1A, 1D	Abnormal	Cove Mountain
73-2B, 2C, 2D	Abnormal	Cove Mountain
75-1B	Abnormal	Curry Mountain
75-3B	Abnormal	Curry Mountain
83-1C	Abnormal	Gregorys Bald
88-1A, 1D	Abnormal	Gregorys Bald
88-2B, 2C, 2D	Abnormal	Gregorys Bald
90-2D	Abnormal	Gregorys Bald
93-1B, 1C	Abnormal	Gregorys Bald
98-2C	Abnormal	Gregorys Bald
101-1B	Abnormal	Gregorys Bald
101-3B, 3C	Abnormal	Gregorys Bald
183-1A	Abnormal	Noland Divide
214-2A, 2B, 2E	Abnormal	Smokemont
236-1A, 1B, 1C	Abnormal	Thomas Divide
242-2A, 2B	Abnormal	Thomas Divide
262-1B	Abnormal	Thomas Divide
291-1B	Abnormal	Cove Moutnain
291-2C	Abnormal	Cove Mountain
304-1B	Abnormal	Thomas Divide
308-1B, 1C	Abnormal	Thomas Divide
319-1A	Abnormal	Cataloochee Divide
329-1A, 1B	Abnormal	Gregorys Bald
16-1A, 1C	Slow orange	Cataloochee Divide
38-1B	Slow orange	Cataloochee Divide
49-1C	Slow orange	Cove Mountain
90-2C	Slow orange	Gregorys Bald
213-1A	Slow orange	Smokemont
291-2B	Slow orange	Cove Mountain
303-1A	Slow orange	Thomas Divide
320-1B	Slow orange	Noland Divide
325-1B	Slow orange	High Rocks

[a] Numbers ending in B and C indicate replicate isolates obtained from a single bark plug.

[b] Abnormal morphology similar to the phenotype observed in *C. parasitica in vitro* when associated with hypovirulence. Slow orange morphology indicates isolates exhibiting a phenotype similar in appearance to either the *col1* or *flat* morphological designation.

Table 5. Extraction of dsRNA associated with isolates of *C. parasitica* obtained from American chestnut trees in the Great Smoky Mountains National Park (GRSM) prior to and following a field trial in the Nantahala National Forest, Highlands, NC, 2007.

	GRSM isolations		**Field trial**[b]
Isolate	Cultural morphology	dsRNA[a]	Cultural morphology
20-1A	Abnormal	-	Abnormal
38-1B	Slow orange	-	Slow orange
45-1B	Orange	-	Orange
49-1C	Slow orange	-	Orange
60-2B	Abnormal	-	Abnormal
75-1B	Abnormal	-	Abnormal
88-1D	Abnormal	-	Abnormal
90-2C	Slow orange	-	Orange
101-3B	Abnormal	-	Abnormal
183-1A	Abnormal	-	Abnormal
192-1A	Orange	-	Abnormal
213-1A	Slow orange	-	Slow orange
236-1C	Abnormal	+	Abnormal
291-2B	Slow orange	-	Orange
303-1A	Slow orange	-	Abnormal
319-1A	Abnormal	-	Abnormal
320-1B	Slow orange	-	Orange
324-1B	Orange	+	Orange
325-1B	Slow orange	+	Abnormal

[a] (+) Detection of a nucleic acid band at approximately 13,000 kb following silver nitrate staining on polyacrylamide gel (SDS-PAGE); (-) no nucleic acid was detected in sample.
[b] Cultural morphology of a majority of replicate isolates after re-isolation following the field trial, 2007.

Figure 7. *Cryphonectria parasitica* collected from the Great Smoky Mountains National Park in 2006 (isolate 291-2C) exhibiting virulent cultural morphology after 14 days growth on potato dextrose agar.

Figure 8. *Cryphonectria parasitica* collected from the Great Smoky Mountains National Park in 2006 (isolate 304-1C) exhibiting abnormal cultural morphology after 14 days growth on potato dextrose agar.

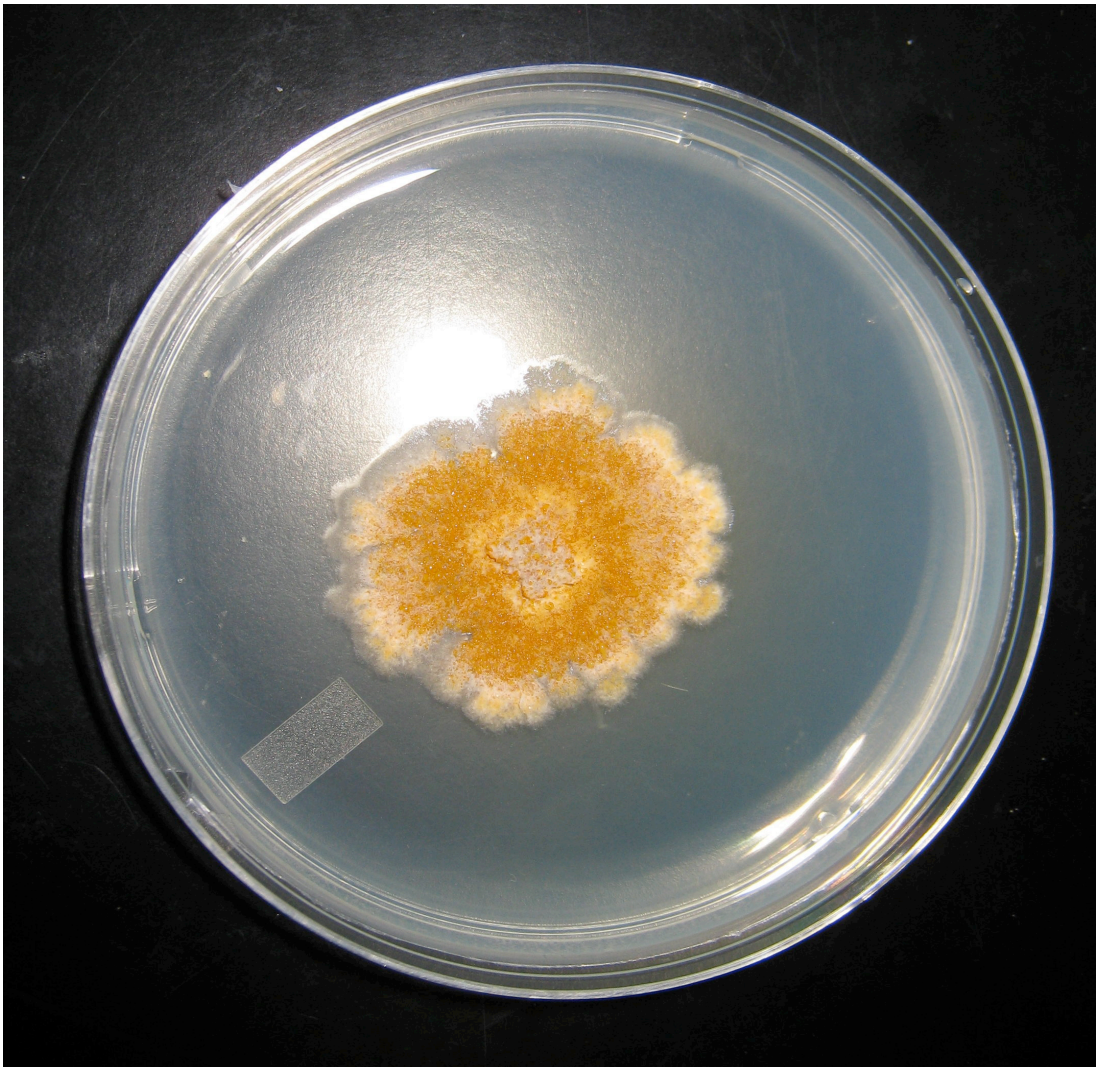

Figure 9. *Cryphonectria parasitica* collected from the Great Smoky Mountains National Park in 2006 (isolate 16-1A) exhibiting slow orange cultural morphology after 14 days growth on potato dextrose agar.

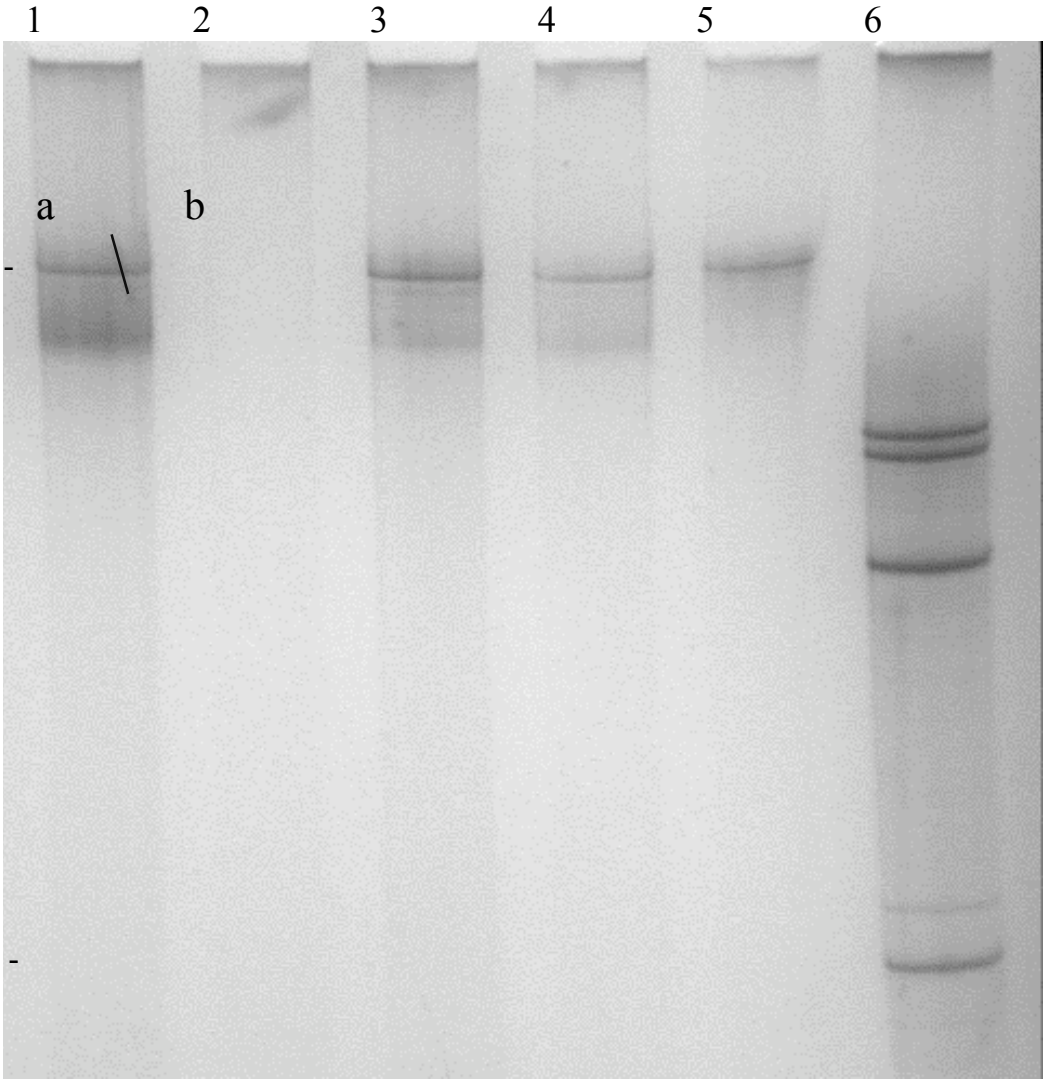

Figure 10. Electrophoretic profiles of dsRNA in PAGE showing both presence (a) and absence (b) of a dsRNA molecule approximately 13,000 base pairs in size.[a]

[a] Isolates of *C. parasitica* collected from the GRSM are in lanes 1-4: 236-1C (lane 1), 291-2B (lane 2), 324-1B (lane 3), 325-1B (lane 4). Isolate (E96) obtained from West Virginia University and containing CHV-1 dsRNA (lane 5) and replicative forms of *Peanut stunt virus* (lane 6) are used as reference markers.

diversity of 1.79 at Cataloochee Divide, having six separate VC groups on six individual cankers (Table 7). Only four VC groups were found among 22 cankers sampled on Gregory Bald, giving a diversity index value of 1.28. Unfortunately, comparisons of diversity between individual locations were not feasible because of wide differences in numbers of trees and sampled cankers (Thomas Matney, MSU, pers. comm.).

The largest two VC groups, 5 and 29, were each distributed over five of the areas in the GRSM (Table 7). The third largest group (8) occurred across three locations, whereas three other groups (2, 17, 25) were found at two sites each. The remaining 28 groups occurred only at a single location. Of these 28, five groups occurred on two separate cankers on a single tree (trees 73, 135, 177, 204, and 232).

Field Inoculation Trial

Two weeks after inoculation, all bark tissue was asymptomatic for the presence of *C. parasitica*. Although no data was collected on this date, the presence of fungal mycelia at the immediate inoculation sites provided external signs of active fungal growth. Four weeks after inoculation, 14% of inoculation sites had visible canker formation, 20% at eight weeks, and 90% at 20 weeks.

Canker formation. Presence of dsRNA in isolates had a significant correlation to canker size, with dsRNA-containing isolates being associated with smaller cankers (Tables 9, 10). Furthermore, canker sizes were similar when compared by isolate

morphology, which was not a significant indicator of hypovirulence. Analysis of variance of canker area data for all isolates, considering dsRNA presence, morphology, and time (Table 9) showed that time across sampling dates had a significant effect on canker area, with all cankers increasing in size per month.

When canker size was compared by isolate, means separation of canker size after three and five months indicated that four isolates (20-1A, 236-1C, 325-1B, E96), produced notably smaller cankers than the virulent controls (Table 10). Isolate 60-2B, a slow orange isolate without dsRNA, produced cankers which were significantly smaller at three months, but similar at 5. Cankers formed by isolate 325-1B, a slow orange dsRNA-containing isolate, were only significantly smaller than virulent controls after five months. Two isolates, 20-1A and 236-1C, formed cankers similar to the E96 hypovirulent control at both three and five months. Of these isolates, 20-1A and 325-1B had previously been found to be vegetatively incompatible with other isolates. However, isolate 236-1C was compatible with six other isolates in one group (Table 7), while 60-2B belonged to the largest VC group noted in this study and was compatible with 13 other isolates as well as the EP-155 virulent control (Table 7). These four isolates represent four of the 34 VC groups noted in this study.

Five months following inoculation, canker growth was not significantly different between the two virulent control isolates (EP-155 and 97-), while the hypovirulent control isolate E96 had significantly smaller cankers than the virulent controls (Table 11). In addition, five isolates (20-1A, 75-1B, 192-1A, 236-1C, 291-2B) caused

Table 6. Vegetative compatibility (VC) between isolates of *C. parasitica* collected from the Great Smoky Mountains National Park in 2006 and 2007, compared to three control isolates.

	Isolates		
Group	Isolate numbers	Isolates per group	Locations[a]
1	2-2	1	5
2	6-1, 39-1	2	7, 8
3	16-1	1	7
4	20-1	1	8
5	26-1, 60-1, 60-2, 60-3, 62-1, 66-1, 75-1, 78-1, 78-2, 119-1, 123-1, 214-1, 214-2, 270-1, **EP-155**[b]	15	2, 3, 6, 7, 8
6	38-1	1	7
7	45-1, 49-1	2	3
8	50-1, 50-2, 175-2, 192-1, 192-2, 192-3, **98+**	7	3, 6, 8
9	73-1, 73-2	2	3
10	79-1	1	8
11	82-1	1	8
12	83-1, 88-1, 88-2, 89-2, 89-3, 90-1, 90-2, 94,1, **E96**	9	1
13	93-1, 95-1, 95-2, 96-1, 98-1, 98-2, 99-1, 99-2	8	1
14	100-1, 101-1, 101-2, 101-3	4	1
15	130-1	1	2
16	135-1, 135-2	2	3
17	140-1, 210-1, 210-2, 213-1, 213-3	5	2, 8
18	175-1	1	6
19	177-1, 177-2	2	5
20	183-1	1	5
21	204-1, 204-2	2	2
22	220-1	1	4
23	220-2, 221-1, 221-2	3	4
24	232-1, 232-2	2	8
25	236-1, 277-1, 287-1, 291-1, 291-2, 292-2, 296-1	7	3, 8
26	242-1	1	6
27	242-2, 244-1, 244-2, 262-1, 262-2	5	6
28	277-2	1	8
29	304-1, 308-1, 319-1, 323-1, 323-2, 324-1, 326-1, 328-1, 329-1, 336-1	10	1, 5, 6, 7, 8
30	313-1	1	7
31	320-1	1	5
32	325-1	1	8
33	337-1, 337-2, 339-1, 340-1	4	6
34	341-1	1	8

[a] Locations from which representatives of VC groups were obtained; 1= Gregory Bald, 2= Rich Mountain, 3= Cove Mountain, 4= Sugarlands Mountain, 5= Noland Divide, 6= Thomas Divide, 7= Cataloochee Divide, and 8= elsewhere in GRSM.

[b] Control isolates are shown in bold print (hypovirulent E96 and 98+, virulent EP-155).

Table 7. Number of American chestnut trees and diversity of vegetative compatibility (VC) groups among *C. parasitica* isolates collected from seven geographically separated locations in the Great Smoky Mountains National Park in 2006 and 2007.

Locality	Total trees	Cankers sampled	VC groups (S)	Diversity (H')[a]
Gregory Bald	15	22	4	1.28
Rich Mountain	19	4	4	1.39
Cove Mountain	34	17	6	1.71
Sugarlands	15	3	2	0.56
Noland Divide	22	8	5	1.43
Thomas Divide	50	17	7	1.72
Cataloochee	40	6	6	1.79
Total[b]	195	77	34	

[a] Calculated using Shannon Diversity index (see equation 2-1) where S = number of VC groups per locality, ln = natural log, i = sum of all individuals belonging to each VC group, *n* = number of isolates in each VC group, *N* = total number of cankers sampled.

[b] Isolates collected elsewhere in GRSM were excluded from analysis.

significantly smaller cankers than the 97- virulent control. Of these isolates, two (75-1B, 192-1A) had normal morphology and no dsRNA. Isolate 291-1B formed a slow orange morphology. Only 236-1C, an abnormal isolate with dsRNA, had significantly lower canker size than the virulent controls but was not significantly smaller than the hypovirulent control (Table 11). Among seven isolates (20-1A, 88-1D, 90-2C, 101-3B, 303-1A, 320-1B, 324-1B) in which dsRNA had not been observed, only 20-1A formed cankers similar in size and morphology to the hypovirulent control. Canker growth rates for all other dsRNA-free isolates were significantly greater than the E96 hypovirulent control (Table 11), while being similar to both virulent control isolates (97-, EP-155). In total, eight isolates produced cankers statistically similar in size to the hypovirulent control, including 20-1A, 49-1C, 60-2B, 75-1B, 192-1A, 213-1A, 236-1C, and 291-2B. Three isolates (49-1C, 213-1A, 291-2B) had a slow orange morphology while another three (60-2B, 75-1B, 236-1C) had abnormal morphology. An additional isolate (192-1A) had a normal morphology.

The growth rates of cankers formed by two virulent control isolates (EP-155 and 97-) fit an increasing quadratic response (Table 12), as did cankers formed by 10 other isolates (183-1A, 213-1A, 319-1A, 320-1B, 324-1B, 325-1B, 38-1B, 49-1C, 75-1B, 88-1D). These 12 isolates led to the formation of cankers which expanded exponentially in area by month. Four of these isolates (183-1A, 319-1A, 88-1D, 97-) also showed an increasing linear trend in canker area, in which canker area did not expand as rapidly.

Surface stromata production. The number of stromata on each canker significantly increased between one and five months, but neither dsRNA presence nor cultural morphology had a significant effect on stromata production (Table 13). Because neither interaction was found to have a significant effect on overall stromata production, further analysis between individual isolates could not be conducted. However, three isolates (E96, 236-1C, 325-1B) consistently had numerically lower numbers of stromata than all other isolates (Table 13). Stromata were present at only 1% of inoculation sites in June, 8% in July, and 88% in September and October (Table 13). Only one isolate (97-) was found to fit an increasing quadratic response, with number of stromata increasing significantly between each month (Table 13).

Isolate morphology. Morphological characteristics were observed in isolates following isolation of the pathogen from newly formed cankers. Abnormal morphology was maintained in all eight isolates following reisolation. For three orange isolates, two had similar morphology (45-1B, 324-1B) while one isolate (192-1A) had an abnormal morphology in 50% of the replicates. Cultural morphology was least conserved among slow orange cultures, with six isolates out of eight (49-1C, 90-2C, 192-1A, 291-2B, 303-1A, 320-1B) either having different morphologies following inoculation or being the result of natural infection by *C. parasitica* from the environment. Of the six isolates, four had a normal orange morphology (49-1C, 90-2C, 291-2B, 320-1B), and two abnormal (303-1A, 325-1B) following reisolation.

Table 8. Analysis of variance of canker size between June and October 2007 following inoculation of *C. parasitica* onto American chestnut trees in the Nantahala National Forest, Highlands, NC, 2007.

Effect	df	F[a]	Pr > F
dsRNA[b]	**1**	**7.30**	**0.0073**
Morphology[c]	**1**	**0.70**	**0.4042**
dsRNA*Morphology	**1**	**0.80**	**0.3731**
Time[d]	**4**	**76.08**	**<0.0001**
dsRNA*Time	**4**	**1.90**	**0.1097**
Morphology*Time	**4**	**0.18**	**0.9477**
dsRNA*Morphology*Time	**4**	**0.87**	**0.4810**

[a] Numbers are significantly different at $P \geq 0.05$.
[b] Comparison between isolates with and without dsRNA as previously determined.
[c] Isolates exhibiting either normal (orange) or abnormal (white or slow orange) morphology.
[d] Monthly measurements taken over 5 months from June 2007 to October 2007.

Table 9. Evaluation of canker size after three and five months following inoculation of *C. parasitica* on healthy American chestnut stems at the Nantahala National Forest, Highlands, NC, 2007.

	Average canker area (mm^2)[b]		
Isolate[a]	August (3 months)	September (4 months)	October (5 months)
20-1A	1038 c-e	2925 e-g	9350 cd
88-1D	1325 a-d	8225 a-e	18075 ab
90-2C	2300 a	8900 a	10000 a-c
101-3B	1963 a-d	5850 a-g	10775 a-c
303-1A	3063 a-c	6250 a-f	12225 a-c
320-1B	1625 a-d	4525 a-g	15288 a-c
324-1B[c]	1956 a-c	5375 a-e	7575 a-c
38-1B	1588 a-d	4725 a-f	13975 a-c
45-1B	1688 a-d	2625 b-g	15050 a-c
49-1C	1650 a-d	2450 c-g	11100 a-c
60-2B	888 c-e	2425 d-g	12650 a-c
75-1B	1975 a-c	5450 a-e	9750 a-c
183-1A	1850 a-d	7175 a-d	14788 a-c
192-1A	1413 a-d	4975 a-g	10025 a-c
213-1A	1500 a-d	6250 a-e	16725 a-c
236-1C[c]	75 e	450 g	825 d
291-2B	1181 a-d	6275 a-e	9213 a-c
319-1A	1850 ab	7625 ab	15688 a
325-1B[c]	1100 a-d	4125 c-g	8825 b-d
EP-155	3050 a	8150 a-c	17875 a-c
E96[c]	475 de	550 fg	600 d
97-	1425 a-c	6250 a-e	17013 a

[a] Isolates obtained from American chestnut trees in the Great Smoky Mountains National Park, 2006-2007.

[b] Mean canker sizes followed by the same letter within a column are not significantly different ($P \geq 0.05$) following square root transformation of data and analysis using the Tukey test.

[c] Isolates in which dsRNA was detected.

Table 10. Comparisons of canker area with hypovirulent and virulent control isolates over five months following inoculation of *C. parasitica* on American chestnut stems in the Nantahala National Forest, Highlands, NC, 2007.

	Pr > |t|[a]		
	Virulent controls		Hypovirulent control
Isolate[b]	EP-155	97-	E96
20-1A	0.1660	**0.0348**	0.1249
88-1D	0.6117	0.2718	**0.0163**
90-2C	0.8815	0.7017	**0.0051**
101-3B	0.5123[b]	0.1864	**0.0215**
303-1A	0.6042	0.3171	**0.0179**
320-1B	0.5182	0.2410	**0.0221**
324-1B[d]	0.3392	0.1085	**0.0395**
38-1B	0.3507	0.1250	**0.0473**
45-1B	0.3660	0.1347	**0.0319**
49-1C	0.2295	0.0747	0.0924
60-2B	0.2330	0.0783	0.0880
75-1B	0.8200	**0.0128**	0.2456
183-1A	0.8927	0.4775	**0.0056**
192-1A	0.1191	**0.0301**	0.1730
213-1A	0.3002	0.1009	0.0591
236-1C[d]	**0.0028**	**0.0005**	0.8270
291-2B	0.0812	**0.0198**	0.2118
319-1A	0.9929	0.5957	**0.0065**
325-1B[d]	0.1689	0.0608	**0.0810**

[a] Least squares means analysis of canker size over five months at the $\alpha = 0.05$ level.
[b] Isolates obtained from American chestnut trees in the Great Smoky Mountains National Park, 2006 to 2007.
[c] Values in bold text indicate significant difference in canker area between individual isolate and control isolate.
[d] Isolates in which dsRNA was detected.

Table 11. Mean area of cankers associated with inoculation sites of *C. parasitica* on healthy American chestnut stems over five months following inoculation at Nantahala National Forest, Highlands, NC, 2007.

	Canker area (mm^2)[b]						
Isolate[a]	June	July	August	September	October	L[c]	Q[c]
20-1A	0	350	1038	2925	9350	NS	NS
88-1D	0	719	1325	8225	18075	**	**
90-2C	0	825	2300	8900	10000	NS	NS
101-3B	0	750	1963	5850	10775	NS	NS
303-1A	2	1575	3063	6250	12225	NS	NS
320-1B	0	913	1625	4525	15288	NS	**
324-1B[e]	0	950	1956	5375	7575	NS	**
38-1B	0	1188	1588	4725	13975	NS	**
45-1B	0	306	1688	2625	15050	NS	NS
49-1C	0	956	1650	2450	11100	NS	**
60-2B	0	463	888	2425	12650	NS	NS
75-1B	0	763	1975	5450	9750	NS	**
183-1A	0	563	1850	7175	14788	**	**
192-1A	0	475	1413	4975	10025	NS	NS
213-1A	0	400	1500	6250	16725	NS	**
236-1C[e]	0	150	75	450	825	NS	NS
291-2B	0	563	1181	6275	9213	NS	NS
319-1A	0	1206	1850	7625	15688	**	**
325-1B[e]	0	425	1100	4125	8825	NS	**
EP-155[d]	0	1250	3050	8150	17875	NS	**
E96[d]	0	225	475	550	600	NS	NS
97-[d]	2	413	1425	6250	17013	**	**

[a] Isolates obtained from American chestnut trees in the Great Smoky Mountains National Park, 2006-2007.

[b] Canker area is defined as width times height as measured through the center of each inoculation point.

[c] L refers to linear response, and Q to quadratic response of mean canker area among four replications; ** indicates a significant linear or quadratic response at $P \geq 0.05$, while NS indicates nonsignificance.

[d] Control isolates were EP-155 obtained from Connecticut Agricultural Experimental Station, New Haven, CT; E96 and 97- obtained from West Virginia University, Morgantown, WV.

[e] Isolates in which dsRNA was detected.

Table 12. Analysis of variance for area of number of stromata present within a 16 cm^2 area on cankers formed over five months following inoculation of *C. parasitica* onto American chestnut trees in the Nantahala National Forest, Highlands, NC, 2007.

source	df	F[a]	Pr > F
dsRNA[b]	**1**	**3.13**	**0.0777**
Morphology[c]	**1**	**0.27**	**0.6017**
dsRNA*Morphology	**1**	**0.46**	**0.4978**
Time[d]	**4**	**14.73**	**<0.0001**
dsRNA*Time	**4**	**0.40**	**0.8105**
Morphology*Time	**4**	**1.12**	**0.3451**
dsRNA*Morphology*Time	**4**	**0.74**	**0.5661**

[a] Numbers are significantly different at $P \geq 0.05$.
[b] Comparison between isolates with and without dsRNA as previously determined.
[c] Isolates exhibiting either normal (orange) or abnormal (white or slow orange) morphology.
[d] Monthly measurements taken over five months from June 2007 to October 2007.

Table 13. Mean number of stromata present within a 16 cm^2 area on cankers associated with inoculation sites on healthy American chestnut stems over five months in the Nantahala National Forest, Highlands, NC, 2007.

	Average number of stromata						
Isolate[a]	June	July	August	September	October	L[b]	Q[b]
20-1A	3.3	0	0	2.8	9.7	NS	NS
88-1D	0	0	1.3	5.8	11.0	NS	NS
90-2C	0	0	5.3	13.3	22.3	NS	NS
101-3B	0	0	4.3	7.3	13.0	NS	NS
303-1A	0	1.5	2.7	7.0	7.0	NS	NS
320-1B	0	0	6.0	14.3	20.3	NS	NS
324-1B[d]	0	0	3.0	7.0	16.0	NS	NS
38-1B	0	0.5	3.8	13.5	12.3	NS	NS
45-1B	0	0	3.5	3.8	11.0	NS	NS
49-1C	0	0	2.5	3.0	11.5	NS	NS
60-2B	0	0.8	3.5	3.3	13.3	NS	NS
75-1B	0	0	1.5	4.3	9.8	NS	NS
183-1A	0	0	0.8	8.0	17.5	NS	NS
192-1A	0	5.0	0	3.5	13.3	NS	NS
213-1A	0	0	4.8	4.0	9.3	NS	NS
236-1C[d]	0	0	0	0	0	NS	NS
291-2B	0	1.8	0	4.8	15.5	NS	NS
319-1A	0	3.3	2.0	14	21.0	NS	NS
325-1B[d]	0	0	0	2.0	5.3	NS	NS
EP-155[c]	0	1.8	5.5	6.5	17.8	NS	NS
E96[c]	0	0	0.8	2.3	2.8	NS	NS
97-[c]	0	0	1.0	2.0	14.7	NS	**

[a] Numbers refer to isolates obtained from American chestnut trees in the GRSM from 2006 to 2007.

[b] L refers to linear response, and Q to quadratic response of mean canker area among four replications; ** indicates a significant linear or quadratic response at $P \geq 0.05$, while NS indicates nonsignificance.

[c] Control isolates were EP-155 obtained from Connecticut Agricultural Experimental Station, New Haven, CT; E96 and 97- obtained from West Virginia University, Morgantown, WV.

[d] Isolates in which dsRNA was detected.

CHAPTER 4

DISCUSSION

This study sought to identify and characterize hypovirulent isolates in the GRSM, and to determine how these isolates may be used as a biological control of the chestnut blight pathogen. In working toward this goal, the first portion of this study sought to locate and sample as many American chestnut trees in the GRSM as was feasible. Although Wood (2003) reported the locations and reproductive potential of American chestnut in the GRSM, no data on disease incidence or impact was provided. Results of the tree survey and field study showed that incidence and severity of *C. parasitica* was very high in the GRSM. During the three years that elapsed between the two studies, 23% of 213 trees had been killed by the pathogen. An additional 29% contained one or more cankers, but were still living. This indicates that not only is disease incidence of *C. parasitica* in the GRSM high, but also that this pathogen has the potential to impact remaining trees within the park. Previous studies have also found that *C. parasitica* can persist as a saprophyte in an area even after numbers of American chestnut trees have been greatly reduced (Day, 1978; MacDonald and Double, 1978; Baird, 1991). Therefore, inoculum levels of this pathogen can potentially remain at high levels, quickly infecting new American chestnut sprouts (Merkel, 1905; Elliston, 1982).

Although incidence of *C. parasitica* was generally high throughout the study area, some variation existed among sites in the GRSM. Gregory Bald was unique in that every tree sampled showed signs of infection, but no trees were dead. However, a large number of dead trees were found at Rich Mountain, Cove Mountain, and Thomas Divide. Cove Mountain, the lowest elevation site, also had the highest disease incidence among all sites in the GRSM. In contrast, the higher elevation sites of Gregory Bald and Noland Divide had the fewest numbers of dead trees. This observation indicates a correlation between elevation and survival of American chestnut in the GRSM. It has been demonstrated that increased sunlight exposure leads to hotter, drier conditions which are generally not favorable to the growth of fungal pathogens, as is the case with the dogwood anthracnose pathogen (Redlin, 1991). The possibility exists that such a correlation is present between chestnut blight occurrence and light exposure, whereby the presence of associated overstory vegetation leads to decreased sunlight penetration. In general, trees at Gregory Bald were either located at the edges of a grassy bald, or were found at sites without overstory trees. Sugarland Mountain and Cataloochee Divide contained more exposed outcroppings and grassy areas in which chestnut composed the overstory. In contrast, Cove Mountain and Rich Mountain were dominated by a mixed hardwood and pine forest. This coincides with previous studies which have reported that American chestnut trees typically persist in areas such as along ridgetops and in canopy gaps in which this tree has a competitive advantage for sunlight (Woods and Shanks, 1959; Griffin, 1992).

All American chestnut trees included in this study were either found along the tops of ridges, or, in less than 10% of cases, along mountain slopes. No trees were found

along bottomland or riparian areas, with the exception of trees #191 and #192 which were located adjacent to a mown field along Newfound Gap Road. The American chestnut was once a dominant component of mountain slopes, but following the loss of mature trees due to *C. parasitica*, it was observed that other overstory species close canopy gaps more quickly at riparian sites and on slopes, while chestnut sprouts have been able to outcompete other species for sunlight on ridgetops (Whittaker, 1956; Vandermast and Van Lear, 2002). Survival of American chestnut in the GRSM will therefore be more likely in these ridgetop habitats. However, ridgelines throughout the park, while covered in trees, generally have a more open canopy than slopes or riparian areas (Whittaker, 1956). This may be why chestnut is found mostly along ridgetops, despite formerly also being found along slopes (Whittaker, 1956).

Although 14 of the 19 *C. parasitica* isolates tested from the GRSM were virulent, five isolates (20-1A, 236-1C, 291-2B, 324-1B, 325-1B) tested in the field trial were determined to exhibit hypovirulent characteristics with respect to canker growth. These five isolates were not obtained from a single location, but were scattered throughout the GRSM. Isolate 20-1A was recovered from Cataloochee Divide, while 291-2B was found on Cove Mountain, and isolate 236-1C was obtained from Thomas Divide. Isolate 324-1B was obtained from a tree on Noland Divide, and 325-1B along Welch Ridge north of High Rocks. With the exception of the tree on Cove Mountain, which was found in a low elevation (800 m) pine forest, each of these trees was located along an exposed ridgetop above 1500 m in elevation with little to no overstory vegetation greater than 15 m in height. Each of the trees from which these isolates were

obtained was located in an area which had one or more additional diseased chestnuts in proximity, although no hypoviruses were obtained from these adjacent trees.

Other researchers have reported hypovirulent isolates from elsewhere in the United States. A study in West Virginia reported that one out of 45 isolates obtained from a clearcut setting exhibited hypovirulent characteristics in a field trial (MacDonald and Double, 1978). Likewise, Carey (1985) identified 10 such isolates on large American chestnut trees in national forests of western North Carolina. In his study, large American chestnut trees yielded abnormal isolates more frequently than small ones, indicating that presence of these hypovirulent isolates may be correlated with tree survival. While these two studies concentrated on clearcut and open areas containing chestnut, such sites do not exist in the GRSM.

Vegetative Compatibility Group Determination

The number of vegetative compatibility groups among all 104 isolates tested in this study (34) correlates with other research. Typically, diversity of VC groups increases southward across the eastern United States. Speculation as to the reasons for this increase in diversity include factors such as secondary introductions of *C. parasitica* into the southern United States between the 1920's and 1950's on imported material from Asia (Sandra Anagnostakis, Connecticut Experimental Station, pers. comm.), although this has not been proven as a cause. In West Virginia, one study reported that 14 VC groups occurred within two clearcut plots 80 km apart (MacDonald and Double, 1978). In other studies throughout the United States, between 28 and 54 types have been identified during surveys (Anagnostakis 1977; Kuhlman et al., 1984; Anagnostakis,

1986; Robbins and Griffin, 1999). In contrast, only 13 groups are known to exist among populations of *C. parasitica* in Europe (Bissegger et al., 1997; Milgroom and Cortesi, 1999). The relatively greater number of VC groups observed in the southern Appalachian mountains compared to the northeastern United States presents a challenge in using dsRNA hypoviruses as a biological control, since it prevents hypovirus dissemination through anastomosis (Jaynes and Elliston, 1982; Anagnostakis, 1987; Robin et al., 2000; Liu et al., 2002).

A total of 16 isolates did not undergo anastomosis with any other isolate, and therefore were considered to be within their own VC group. This result has been observed in other similar studies, and may cause estimates of VC diversity to be high. Studies in the United States and in Europe have discovered isolates which never undergo anastomosis with other isolates (MacDonald and Double, 1978; Milgroom and Cortesi, 1999). However, it has been reported that differences in *vic* alleles do not always exist between incompatible isolates, suggesting that incompatibility is induced by additional factors and that the number of VC groups determined solely by VC groups is actually lower (Bissegger et al., 1997; Robin et al., 2000; Marra and Milgroom, 2001). This may be the case among the 16 isolates from this study, although this question lies outside the scope of this study.

Much greater variation in VC groups is observed when isolates are compared over a wide geographic area versus a single plot (MacDonald and Double, 1978; Robin et al., 2000). In general, a single locality in the GRSM contained multiple representatives of one VC group on different trees, while many groups were found to be present at only one geographic location. A total of 28 out of the 34 VC groups identified

in this study were each only found at one single location in the GRSM, and any isolates compatible with each of these 28 groups were not found across more than one geographic location. Of these 28 groups, 16 were single isolates and another 12 were groups containing more than one compatible isolate obtained from multiple trees at a single location. Cankers formed by the dissemination of conidia are compatible with one another indicating that multiple infections on adjacent trees can frequently originate from a single localized inoculum source (MacDonald and Double, 1978). Other research has indicated that the number of compatibility groups observed in surveys is greater when isolates are obtained from several geographic locations rather than within a single location, indicating that gene flow among populations of *C. parasitica* at a regional scale is somewhat limited (Milgroom and Cortesi, 1999; Robin et al., 2000, Marra and Milgroom, 2001).

Vegetative compatibility may limit the usefulness of the putatively hypovirulent isolates identified in this study. No hypoviruses were identified in isolates 20-1A or 291-2B, although they were present in the other three. Isolate 325-1A was not found to be compatible with any other isolate in this study. Isolate 324-1B was in the same VC group as isolates found on several trees at Thomas Divide and Gregory Bald, and one tree each at Cataloochee and Noland Divide. Isolates 236-1C and 291-2B had the most geographically widespread compatibility, being in the same VC group as isolates found at High Rocks, Thomas Divide, Cove Mountain, Smokemont, and the Foothills Parkway.

Extraction and Analysis of dsRNA

Presence of dsRNA can provide initial indication of potential hypovirulence in isolates. Extraction of dsRNA of the 19 isolates tested in the field trial indicated that three had evidence of dsRNA presence. However, previous studies have concluded that reliance on cultural morphology as an indicator of hypovirulence may cause some hypovirulent isolates to be overlooked (Peever et al., 1997). Hypovirulence in *C. parasitica* is most often associated with dsRNA hypoviruses in the family *Hypoviridae* (Enebak et al., 1994), but can also result from mutations in nuclear or mitochondrial DNA (Elliston, 1982). Also, since isolates with abnormal cultural morphology can still be virulent, morphological characteristics are not necessarily an indicator of hypovirulence. Therefore, further testing in the field is necessary to determine the hypovirulence of isolates with abnormal morphology or in which dsRNA presence has been confirmed (Choi and Nuss, 1992a; Robbins and Griffin, 1999; Nuss, 2002). Data collected from isolate morphological comparisons and field trial inoculations in this study confirm this lack of consistency between dsRNA presence, morphology, and hypovirulence. A majority of GRSM isolates exhibiting abnormal morphology (Table 5) were free of hypoviruses. In addition, many of these isolates were determined to be virulent in the field trial. In contrast, isolates 20-1A and 291-2B were determined to be hypovirulent, but lacked dsRNA. It is likely that other factors as discussed previously confer hypovirulent characteristics in these isolates.

A variety of morphological characteristics were observed in the three isolates containing dsRNA. 236-1C had a fast growing abnormal morphology which has previously been described in association with hypovirulent isolates (Elliston, 1982;

Anagnostakis, 1988). Isolate 324-1B had a normal, orange morphology, while 325-1B had orange pigmented, densely packed, slow growing mycelium.

Additional isolates collected in this study but not tested may also be hypovirulent. Ideally, in order to prevent such isolates from being overlooked, all 339 *C. parasitica* isolates need to be tested to determine dsRNA presence, including those with normal morphology. Because of economic constraints, not all isolates collected from the GRSM could be tested for dsRNA. Rather, isolates to be analyzed were chosen at random from those with abnormal morphological traits.

Several techniques have been used to verify dsRNA presence. The method used for this study, which involved a phenol-chloroform extraction, can provide diagnostic evidence of dsRNA presence but is not specific to viral species (Morris and Dodds, 1979). However, this method was chosen for its cost effectiveness as a straightforward method of determining dsRNA presence in a sample. The use of northern blot hybridization or reverse transcription polymerase chain reaction (RT-PCR), however, can be used to identify individual hypoviruses (Peever et al., 1997). These more specific techniques are useful to distinguish between dsRNAs associated with hypovirulence and other dsRNAs such as CHV-4, which is commonly found in *C. parasitica* throughout North America but does not confer hypovirulence (Enebak et al., 1994; Peever et al., 1997; Linder-Basso et al., 2005). However, these methods must take into account the large diversity that exists among dsRNAs associated with hypovirulence (Enebak et al., 1994; Peever et al., 1997). Other GRSM isolates not included in the dsRNA extraction portion of this study could be screened in the future using these diagnostic methods such as RT-PCR, applying specific primers for known C*ryphonectria* hypoviruses. Further

research of the three isolates in this study in order to determine the origin of associated dsRNA will be performed at a later date.

Field Inoculation Trial

All trees inoculated in this trial were four to 10 cm in diameter, and none of the trees died during the study period. Either natural or grafted trees about 10 cm in size have been shown to be ideal for studies spanning multiple years (Anagnostakis et al., 1998; Robbins and Griffin, 1999). However, stems smaller than 10 cm in diameter have been successfully employed in single season field trials (Jaynes and Elliston, 1978, Enebak et al., 1994; Robbins and Griffin, 1999).

Two factors, reduced canker size and reduced stromata production, have been used to establish hypovirulence in field trials (Griffin et al., 1983; Hebard et al., 1984; Carey, 1985; Elliston, 1984; Griffin et al., 1993). In this study, five out of 19 isolates had hypovirulent potential based on canker appearance, size, and stromata production. In general, canker morphology was found to be a good indicator of hypovirulence. This has also been demonstrated in previous research (Grente and Berthelay-Sauret, 1978b; Willey, 1980; Heiniger and Rigling, 1994). Either canker or callus tissue was observed at every inoculation point at six months after inoculation. While no inoculation sites had a callused morphology three months after inoculation, seven cankers had a callused appearance after five months. Six of these callused cankers had been inoculated with isolates which were putatively hypovirulent based on either cultural morphology or dsRNA presence. Cankers with this callused morphology have been associated with hypovirulence in previous research (Elliston, 1982, Kuhlman et al., 1984; Enebak et al.,

1994; Robin et al., 2000; Hogan and Griffin, 2002). This is consistent with prior studies, which reported that superficial callus tissue arises about five to six months after fungal growth reaches the cambial layer (Hebard et al., 1984; Griffin et al., 1993).

In this study, all isolates with a slow growing, densely packed orange morphology were determined to be virulent with the exception of 291-2B. Previous studies reported that slow orange cultural morphology may be the result of mitochondrial induced changes after repeated transfers (Anagnostakis, 1978) or may be indicative of hypovirulence. The shift of isolates to a normal growth pattern in culture after inoculation and re-isolation suggests either that this slow growing morphology may have been induced by cultural conditions (Anagnostakis, 1978), or that secondary infection by virulent *C. parasitica* present in the environment overtook inoculated isolates. In some cases, naturally occurring infections arose above inoculation sites during the field trial, although these cankers did not overrun inoculated cankers during the study period. Liebhold et al. (1995) reported that conidia can be washed down by rainwater and introduced into inoculation sites located below naturally occurring infection. Further testing of these isolates would be needed in order to verify that inoculated isolates were identical to those recovered following the field trial, rather than contaminates which entered the wound site following inoculation.

Results of this study suggest that five isolates (20-1A, 75-1B, 236-1C, 291-2B, 325-1B) have hypovirulent potential. Although isolate 20-1A and 75-1B had abnormal morphology in culture, these isolates have limited potential in future hypovirulence trials because they do not contain dsRNA. Only isolate 236-1C consistently exhibited hypovirulent characteristics in this study, forming distinct callused tissue and expanding

beyond the inoculation site in only one of four replications. In general, this isolate formed small, sunken cankers which developed a vertical split at five months after inoculation. This is consistent with the expected characteristics following inoculation of a hypovirulent isolate (Grente and Berthelay-Sauret, 1978a; Elliston, 1982; Hebard et al., 1984; Peever et al., 1997). Among all hypovirulent isolates confirmed in this study, this isolate has the greatest biological control potential, being vegetatively compatible with 16 other isolates present over a wide geographic range in the GRSM.

Although a limited number of potential hypovirulent isolates were identified in this study, further testing of other collected isolates must be done in order to confirm hypovirulent potential. In order for biological control efforts to be effective, isolates must be found which have potential to convert members of the most widely represented VC groups found within the GRSM. Although isolates such as 236-1C have potential for use as a biological control based on the number of vegetatively compatible isolates in the park, further testing to confirm this biological control potential is needed. However, because of vegetative compatibility, the isolates in this study would likely have selective usefulness to park personnel unless additional hypovirulent isolates representing more widespread VC groups are found.

CHAPTER 5

GENERAL CONCLUSIONS

Over the two year study, 58 unreported American chestnut trees were located in the GRSM, while 180 previously reported trees were visited. An additional 68 previously reported trees were either killed by the chestnut blight fungus or could not be located. Disease incidence of naturally occurring *C. parasitica* was high in the GRSM, with 104 out of 286 living trees containing one or more cankers caused by the pathogen. This high disease incidence indicates that the pathogen will continue to threaten remaining American chestnut trees in the southern Appalachians.

Isolates of *C. parasitica* were obtained in pure culture from 107 cankers on diseased trees. Of these isolates, 54 contained abnormal morphology, which is one visual indicator of potential hypovirulence. In 2007, 22 isolates (19 from the GRSM and three controls) were selected for further analysis in a field trial based on morphology and dsRNA results.

A higher number of VC groups have been observed in the southeastern United States compared to the northeastern portion of the country and Europe. All isolates from the GRSM were determined to belong to 34 distinct VC groups, with 16 groups only containing a single isolate and the largest two groups containing eight and 14 isolates.

Most of the VC groups in this study were not widely distributed, with 28 groups found at only one geographic location. However, biological control methods that rely on the natural dissemination of hypoviruses must focus on the more prevalent groups found in the GRSM.

Three factors were used to evaluate hypovirulence in the field trial including canker size, canker appearance, and stromata production. Canker area was found to be a significant indicator of potential hypovirulence, while stromata production on the surface of cankers was similar among all isolates tested. Five isolates were found to form cankers which were significantly smaller than virulent controls, indicating hypovirulent potential. One isolate, 236-1C, was identified to have the greatest hypovirulent potential based on analysis of canker area and morphology as well as dsRNA presence. This single isolate may have the greatest potential for use as a biological control agent in Great Smoky Mountains based on the laboratory and field results. Four additional isolates (20-1A, 75-1B, 291-2B, and 325-1B) also had hypovirulent characteristics in the field trial, although vegetative compatibility may limit the usefulness of these isolates. Overall, additional sampling must be done to increase the number of hypovirulent isolates that overlap the majority of VC groups present in the park. Obtaining a larger sample of hypovirulent isolates from the GRSM may identify additional VC affinities allowing for greater biological control potential of *C. parasitica* on American chestnut trees in the park.

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APPENDIX A.

LOCATIONS, SAMPLING DATES, TREE CONDITION, AND NUMBER OF *C. PARASITICA* CANKERS ON ALL AMERICAN CHESTNUT TREES VISITED DURING 2006 AND 2007 IN THE GRSM, NANTAHALA AND PISGAH NATIONAL FORESTS, TENNESSEE/NORTH CAROLINA.

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
1	35.53835	-83.49413	Noland Divide	6/17/2006	Not Found	0
2	35.53878	-83.49325	Noland Divide	6/17/2006	Sampled	2
4	35.63753	-83.20752	Beech Gap	8/8/2006	Not Found	0
5	35.63544	-83.20741	Beech Gap	8/8/2006	Healthy	0
6	35.66219	-83.49448	Bull Head Trail	6/26/2007	Sampled	1
7	35.66119	-83.49337	Bull Head Trail	6/26/2007	Healthy	0
8	35.66119	-83.49337	Bull Head Trail	6/26/2007	Healthy	0
9	35.66089	-83.49234	Bull Head Trail	6/26/2007	Healthy	0
10	35.66022	-83.4899	Bull Head Trail	6/26/2007	Not Found	0
11	35.57716	-83.73369	Bote Mountain Trail	6/27/2007	Healthy	0
12	35.58313	-83.73417	Bote Mountain Trail	6/27/2007	Healthy	0
13	35.58623	-83.73332	Bote Mountain Trail	6/27/2007	Healthy	0
16	35.58238	-83.07897	Cataloochee Divide	7/20/2006	Sampled	1
17	35.58222	-83.07909	Cataloochee Divide	7/20/2006	Healthy	0
18	35.58475	-83.07786	Cataloochee Divide	7/20/2006	Dead	0
19	35.58606	-83.07717	Cataloochee Divide	7/20/2006	Dead	0
20	35.63039	-83.04746	Cataloochee Divide	9/3/2006	Sampled	1
24	35.60729	-83.06562	Cataloochee Divide	7/20/2006	Healthy	0
25	35.60741	-83.06564	Cataloochee Divide	7/20/2006	Dead	0
26	35.60739	-83.06571	Cataloochee Divide	7/20/2006	Sampled	1
27	35.6071	-83.06568	Cataloochee Divide	7/20/2006	Healthy	0
28	35.60703	-83.06582	Cataloochee Divide	7/20/2006	Dead	0
29	35.60761	-83.06611	Cataloochee Divide	7/20/2006	Dead	0
30	35.60672	-83.06633	Cataloochee Divide	7/20/2006	Healthy	0
31	35.60447	-83.0688	Cataloochee Divide	7/20/2006	Healthy	0
32	35.60426	-83.06884	Cataloochee Divide	7/20/2006	Healthy	0
33	35.60428	-83.06881	Cataloochee Divide	7/20/2006	Healthy	0
34	35.60418	-83.06904	Cataloochee Divide	7/20/2006	Healthy	0
35	35.60424	-83.06896	Cataloochee Divide	7/20/2006	Healthy	0
36	35.58915	-83.07713	Cataloochee Divide	7/20/2006	Dead	0
37	35.58765	-83.07715	Cataloochee Divide	7/20/2006	Healthy	0
38	35.58814	-83.07718	Cataloochee Divide	7/20/2006	Sampled	1
39	35.58804	-83.07715	Cataloochee Divide	7/20/2006	Sampled	1
40	35.58797	-83.07717	Cataloochee Divide	7/20/2006	Healthy	0

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
41	35.58787	-83.07724	Cataloochee Divide	7/20/2006	Healthy	0
42	35.58771	-83.07716	Cataloochee Divide	7/20/2006	Healthy	0
43	35.58795	-83.07696	Cataloochee Divide	7/20/2006	Healthy	0
44	35.59476	-83.07546	Cataloochee Divide	7/20/2006	Healthy	0
45	35.697	-83.56998	Cove Mountain	5/13/2006	Sampled	1
46	35.69704	-83.57305	Cove Mountain	5/13/2006	Healthy	0
47	35.69497	-83.57277	Cove Mountain	5/13/2006	Healthy	0
48	35.69473	-83.57299	Cove Mountain	5/13/2006	Dead	0
49	35.69455	-83.57293	Cove Mountain	5/13/2006	Healthy	0
50	35.69468	-83.57297	Cove Mountain	5/13/2006	Sampled	2
51	35.69439	-83.57329	Cove Mountain	5/13/2006	Dead	0
52	35.69709	-83.56967	Cove Mountain	5/13/2006	Dead	0
54	35.69919	-83.56416	Cove Mountain	5/13/2006	Dead	0
55	35.69915	-83.60316	Cove Mountain	5/13/2006	Dead	0
56	35.70036	-83.60122	Cove Mountain	5/13/2006	Healthy	0
57	35.70049	-83.60117	Cove Mountain	5/13/2006	Healthy	0
58	35.69891	-83.59679	Cove Mountain	5/13/2006	Dead	0
59	35.69975	-83.59899	Cove Mountain	5/13/2006	Not Found	0
60	35.69879	-83.59682	Cove Mountain	5/13/2006	Sampled	3
61	35.69897	-83.59695	Cove Mountain	5/13/2006	Sampled	1
62	35.69905	-83.59653	Cove Mountain	5/13/2006	Sampled	1
64	35.69545	-83.58325	Cove Mountain	5/13/2006	Dead	0
65	35.69597	-83.58199	Cove Mountain	5/13/2006	Not Found	0
66	35.69607	-83.58146	Cove Mountain	5/13/2006	Healthy	0
67	35.69602	-83.58126	Cove Mountain	5/13/2006	Healthy	0
68	35.69625	-83.57883	Cove Mountain	5/13/2006	Dead	0
69	35.69599	-83.57867	Cove Mountain	5/13/2006	Dead	0
70	35.69541	-83.57858	Cove Mountain	5/13/2006	Not Found	0
71	35.6948	-83.57867	Cove Mountain	5/13/2006	Not Found	0
72	35.69465	-83.57809	Cove Mountain	5/13/2006	Healthy	0
73	35.69536	-83.57627	Cove Mountain	5/13/2006	Sampled	2
74	35.65628	-83.7214	Chestnut Top Trail	7/23/2007	Dead	0
75	35.6505	-83.63346	Curry Mountain	8/12/2006	Sampled	1
76	35.6532	-83.63197	Curry Mountain	8/12/2006	Healthy	0
77	35.65444	-83.63122	Curry Mountain	8/12/2006	Healthy	0
78	35.65557	-83.63084	Curry Mountain	8/12/2006	Sampled	2

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
79	35.65642	-83.62974	Curry Mountain	8/12/2006	Sampled	1
80	35.65603	-83.63062	Curry Mountain	8/12/2006	Not Found	0
81	35.61616	-83.96599	Foothills Parkway	6/24/2006	Dead	0
82	35.62766	-83.95341	Foothills Parkway	6/24/2006	Sampled	1
83	35.5194	-83.86761	Gregorys Bald	9/1/2006	Sampled	1
88	35.52129	-83.85787	Gregorys Bald	9/1/2006	Sampled	2
89	35.5213	-83.85797	Gregorys Bald	9/1/2006	Sampled	3
90	35.52138	-83.85779	Gregorys Bald	9/1/2006	Sampled	2
93	35.52127	-83.85782	Gregorys Bald	9/1/2006	Sampled	1
94	35.52136	-83.85777	Gregorys Bald	9/1/2006	Sampled	1
95	35.52157	-83.85766	Gregorys Bald	9/1/2006	Sampled	2
96	35.52136	-83.85779	Gregorys Bald	9/1/2006	Sampled	1
98	35.52167	-83.85752	Gregorys Bald	9/1/2006	Sampled	2
99	35.52158	-83.85757	Gregorys Bald	9/1/2006	Sampled	2
100	35.52162	-83.85752	Gregorys Bald	9/1/2006	Sampled	1
101	35.52135	-83.85754	Gregorys Bald	9/1/2006	Sampled	3
106	35.7187	-83.34576	Greenbrier Pinnacle	5/24/2007	Healthy	0
107	35.71864	-83.3456	Greenbrier Pinnacle	5/24/2007	Healthy	0
108	35.71769	-83.34699	Greenbrier Pinnacle	5/24/2007	Healthy	0
109	35.7175	-83.34759	Greenbrier Pinnacle	5/24/2007	Healthy	0
110	35.71823	-83.34851	Greenbrier Pinnacle	5/24/2007	Healthy	0
111	35.7182	-83.34845	Greenbrier Pinnacle	5/24/2007	Healthy	0
112	35.71989	-83.34963	Greenbrier Pinnacle	5/24/2007	Dead	0
113	35.71583	-83.34775	Greenbrier Pinnacle	5/24/2007	Dead	0
114	35.71543	-83.34603	Greenbrier Pinnacle	5/24/2007	Dead	0
115	35.5525	-83.12657	Hemphill Bald Trail	6/14/2007	Dead	0
116	35.55315	-83.1259	Hemphill Bald Trail	6/14/2007	Not Found	0
118	35.49989	-83.62112	High Rocks	8/13/2006	Dead	0
119	35.61522	-83.24205	Hyatt Ridge Trail	6/11/2007	Sampled	1
120	35.61656	-83.24116	Hyatt Ridge Trail	6/11/2007	Dead	0
121	35.62333	-83.77636	Rich Mountain Loop	7/25/2007	Dead	0
122	35.624	-83.77639	Rich Mountain Loop	7/25/2007	Not Found	0
123	35.62365	-83.77704	Rich Mountain Loop	7/25/2007	Sampled	1
124	35.62521	-83.77824	Rich Mountain Loop	7/25/2007	Dead	0
125	35.62733	-83.7834	Rich Mountain Loop	7/25/2007	Healthy	0
126	35.62802	-83.78466	Rich Mountain Loop	7/25/2007	Dead	0

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
127	35.62687	-83.79276	Rich Mountain Loop	7/25/2007	Not Found	0
128	35.62584	-83.7938	Rich Mountain Loop	7/25/2007	Healthy	0
129	35.62561	-83.79388	Rich Mountain Loop	7/25/2007	Healthy	0
130	35.625	-83.79598	Rich Mountain Loop	7/25/2007	Sampled	1
131	35.62448	-83.81015	Rich Mountain Loop	7/25/2007	Dead	0
135	35.69317	-83.63612	Cove Mountain	7/17/2006	Sampled	2
136	35.69344	-83.64386	Cove Mountain	7/17/2006	Healthy	0
137	35.69334	-83.64377	Cove Mountain	7/17/2006	Healthy	0
138	35.69515	-83.63878	Cove Mountain	7/17/2006	Healthy	0
139	35.69712	-83.63311	Cove Mountain	7/17/2006	Dead	0
140	35.69787	-83.63246	Cove Mountain	7/17/2006	Sampled	1
167	35.58462	-83.07788	Cataloochee Divide	7/20/2006	Healthy	0
168	35.58551	-83.07848	Cataloochee Divide	7/20/2006	Healthy	0
169	35.58544	-83.07842	Cataloochee Divide	7/20/2006	Healthy	0
170	35.58588	-83.07847	Cataloochee Divide	7/20/2006	Healthy	0
171	35.5433	-83.35843	Thomas Divide	6/27/2006	Dead	0
172	35.54394	-83.3607	Thomas Divide	6/27/2006	Dead	0
173	35.54373	-83.36059	Thomas Divide	6/27/2006	Dead	0
174	35.54266	-83.35857	Thomas Divide	6/27/2006	Healthy	0
175	35.54213	-83.35866	Thomas Divide	6/27/2006	Sampled	2
176	35.55082	-83.4631	Noland Divide	8/7/2006	Healthy	0
177	35.54977	-83.46183	Noland Divide	8/7/2006	Sampled	2
178	35.54962	-83.4622	Noland Divide	8/7/2006	Healthy	0
179	35.5494	-83.46191	Noland Divide	8/7/2006	Healthy	0
180	35.53781	-83.45906	Noland Divide	8/7/2006	Healthy	0
181	35.53749	-83.45842	Noland Divide	8/7/2006	Healthy	0
182	35.53772	-83.45838	Noland Divide	8/7/2006	Healthy	0
183	35.53652	-83.45663	Noland Divide	8/7/2006	Sampled	1
184	35.53655	-83.45641	Noland Divide	8/7/2006	Dead	0
185	35.53667	-83.45632	Noland Divide	8/7/2006	Healthy	0
186	35.53586	-83.45648	Noland Divide	8/7/2006	Healthy	0
187	35.53587	-83.4565	Noland Divide	8/7/2006	Healthy	0
188	35.53583	-83.45641	Noland Divide	8/7/2006	Dead	0
189	35.53575	-83.45618	Noland Divide	8/7/2006	Dead	0
190	35.5343	-83.45678	Noland Divide	8/7/2006	Healthy	0
191	35.63108	-83.45938	Newfound Gap Road	7/19/2006	Sampled	1

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
192	35.63126	-83.45916	Newfound Gap Road	7/19/2006	Sampled	3
204	35.63411	-83.79104	Cades Cove	5/12/2006	Sampled	2
205	35.63867	-83.79541	Cades Cove	5/12/2006	Not Found	0
206	35.63869	-83.79542	Cades Cove	5/12/2006	Not Found	0
207	35.6395	-83.79629	Cades Cove	5/12/2006	Healthy	0
208	35.63942	-83.79696	Cades Cove	5/12/2006	Not Found	0
209	35.64298	-83.80481	Cades Cove	5/12/2006	Sampled	1
210	35.6909	-83.65517	Roundtop Trail	7/23/2007	Healthy	0
211	35.68873	-83.6566	Roundtop Trail	7/23/2007	Healthy	0
212	35.57091	-83.3249	Smokemont	6/19/2006	Not Found	0
213	35.57577	-83.32946	Smokemont	6/19/2006	Sampled	3
214	35.57576	-83.32941	Smokemont	6/19/2006	Sampled	2
215	35.66876	-83.57135	Sugarland Mountain	4/14/2006	Dead	0
216	35.66846	-83.57083	Sugarland Mountain	4/14/2006	Sampled	1
217	35.66746	-83.56892	Sugarland Mountain	4/14/2006	Sampled	2
218	35.66701	-83.56818	Sugarland Mountain	4/14/2006	Not Found	0
219	35.66117	-83.55415	Sugarland Mountain	7/18/2006	Healthy	0
220	35.66072	-83.55392	Sugarland Mountain	7/18/2006	Sampled	2
221	35.66021	-83.5538	Sugarland Mountain	7/18/2006	Sampled	2
222	35.65424	-83.55087	Sugarland Mountain	7/18/2006	Healthy	0
223	35.63052	-83.51056	Sugarland Mountain	7/18/2006	Healthy	0
224	35.63064	-83.51399	Sugarland Mountain	7/18/2006	Healthy	0
225	35.63292	-83.5177	Sugarland Mountain	7/18/2006	Healthy	0
226	35.63297	-83.51834	Sugarland Mountain	7/18/2006	Healthy	0
227	35.63314	-83.51958	Sugarland Mountain	7/18/2006	Healthy	0
228	35.63398	-83.52001	Sugarland Mountain	7/18/2006	Healthy	0
230	35.63797	-83.53264	Sugarland Mountain	7/18/2006	Healthy	0
231	35.5038	-83.41159	Sunkota Ridge	6/22/2006	Dead	0
232	35.50995	-83.40942	Sunkota Ridge	6/22/2006	Sampled	2
233	35.51053	-83.40917	Sunkota Ridge	6/22/2006	Not Found	0
235	35.49888	-83.3829	Thomas Divide	6/21/2006	Healthy	0
236	35.49114	-83.38552	Thomas Divide	6/21/2006	Sampled	1
237	35.56914	-83.38504	Thomas Divide	6/27/2006	Healthy	0
238	35.56913	-83.38457	Thomas Divide	6/27/2006	Healthy	0
239	35.56883	-83.3848	Thomas Divide	6/27/2006	Healthy	0
240	35.56897	-83.38426	Thomas Divide	6/27/2006	Dead	0

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
241	35.56897	-83.38464	Thomas Divide	6/27/2006	Healthy	0
242	35.56487	-83.38322	Thomas Divide	6/27/2006	Healthy	0
243	35.56446	-83.3828	Thomas Divide	6/27/2006	Dead	0
244	35.56495	-83.37993	Thomas Divide	6/27/2006	Sampled	2
245	35.56428	-83.37934	Thomas Divide	6/27/2006	Not Found	0
246	35.51407	-83.38321	Thomas Divide	6/21/2006	Not Found	0
247	35.51631	-83.37943	Thomas Divide	6/15/2007	Sampled	1
248	35.51452	-83.37945	Thomas Divide	6/21/2006	Healthy	0
249	35.51977	-83.37404	Thomas Divide	6/15/2007	Dead	0
250	35.52166	-83.37177	Thomas Divide	6/15/2007	Healthy	0
251	35.56383	-83.37877	Thomas Divide	6/27/2006	Not Found	0
252	35.56366	-83.37848	Thomas Divide	6/27/2006	Healthy	0
253	35.56371	-83.37847	Thomas Divide	6/27/2006	Healthy	0
254	35.56372	-83.37786	Thomas Divide	6/27/2006	Healthy	0
255	35.56387	-83.37797	Thomas Divide	6/27/2006	Dead	0
256	35.5626	-83.37752	Thomas Divide	6/27/2006	Healthy	0
257	35.56256	-83.37744	Thomas Divide	6/27/2006	Healthy	0
258	35.56181	-83.37641	Thomas Divide	6/27/2006	Dead	0
259	35.56161	-83.37567	Thomas Divide	6/27/2006	Healthy	0
260	35.55692	-83.37228	Thomas Divide	6/27/2006	Sampled	1
261	35.55482	-83.37313	Thomas Divide	6/27/2006	Dead	0
262	35.55444	-83.3726	Thomas Divide	6/27/2006	Sampled	2
263	35.55394	-83.37272	Thomas Divide	6/27/2006	Healthy	0
264	35.55386	-83.37264	Thomas Divide	6/27/2006	Dead	0
265	35.55353	-83.3726	Thomas Divide	6/27/2006	Healthy	0
266	35.55108	-83.37141	Thomas Divide	6/27/2006	Dead	0
267	35.54974	-83.37067	Thomas Divide	6/27/2006	Dead	0
268	35.54976	-83.37062	Thomas Divide	6/27/2006	Dead	0
269	35.54149	-83.36705	Thomas Divide	6/27/2006	Dead	0
270	35.52398	-83.37192	Thomas Divide	6/15/2007	Sampled	1
277	35.49011	-83.62215	High Rocks	8/13/2006	Sampled	2
285	35.55466	-83.56781	Welch Ridge	8/13/2006	Healthy	0
286	35.55441	-83.56776	Welch Ridge	8/13/2006	Sampled	1
287	35.5545	-83.56772	Welch Ridge	8/13/2006	Sampled	1
288	35.55328	-83.56806	Welch Ridge	8/13/2006	Dead	0
291	35.69586	-83.57859	Cove Mountain	5/13/2006	Sampled	2

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
292	35.57105	-83.3233	Smokemont	6/19/2006	Sampled	2
293	35.05547	-83.47758	Albert Mountain	6/20/2006	Sampled	1
294	35.05539	-83.4775	Albert Mountain	6/20/2006	Healthy	0
295	35.63274	-83.94105	Foothills Parkway	6/24/2006	Healthy	0
296	35.6272	-83.95331	Foothills Parkway	6/24/2006	Sampled	1
297	35.08294	-83.13642	Whiteside Mountain	6/28/2006	Healthy	0
298	35.08121	-83.13827	Whiteside Mountain	6/28/2006	Healthy	0
299	35.0802	-83.13916	Whiteside Mountain	6/28/2006	Healthy	0
300	35.08017	-83.13934	Whiteside Mountain	6/28/2006	Healthy	0
301	35.07899	-83.14095	Whiteside Mountain	6/28/2006	Healthy	0
302	35.079	-83.14092	Whiteside Mountain	6/28/2006	Healthy	0
303	35.56768	-83.38235	Thomas Divide	6/27/2006	Sampled	1
304	35.56418	-83.37933	Thomas Divide	6/27/2006	Sampled	1
305	35.56371	-83.37858	Thomas Divide	6/27/2006	Sampled	1
306	35.54559	-83.36875	Thomas Divide	6/27/2006	Healthy	0
307	35.54437	-83.36451	Thomas Divide	6/27/2006	Healthy	0
308	35.54429	-83.36452	Thomas Divide	6/27/2006	Sampled	1
309	35.5986	-83.07366	Cataloochee Divide	7/20/2006	Healthy	0
310	35.59884	-83.07354	Cataloochee Divide	7/20/2006	Healthy	0
311	35.59943	-83.07303	Cataloochee Divide	7/20/2006	Healthy	0
312	35.59988	-83.07247	Cataloochee Divide	7/20/2006	Healthy	0
313	35.6002	-83.07217	Cataloochee Divide	7/20/2006	Sampled	1
314	35.60097	-83.07176	Cataloochee Divide	7/20/2006	Healthy	0
315	35.60166	-83.07196	Cataloochee Divide	7/20/2006	Healthy	0
316	35.60197	-83.07201	Cataloochee Divide	7/20/2006	Healthy	0
317	35.60222	-83.07191	Cataloochee Divide	7/20/2006	Healthy	0
318	35.60233	-83.07207	Cataloochee Divide	7/20/2006	Healthy	0
319	35.6023	-83.07191	Cataloochee Divide	7/20/2006	Sampled	1
320	35.54624	-83.46033	Noland Divide	8/7/2006	Sampled	1
321	35.54275	-83.4601	Noland Divide	8/7/2006	Healthy	0
322	35.54576	-83.46068	Noland Divide	8/8/2006	Sampled	1
323	35.56704	-83.48193	Noland Divide	8/8/2006	Sampled	2
324	35.56649	-83.47733	Noland Divide	8/13/2006	Sampled	1
325	35.63551	-83.20819	High Rocks	8/13/2006	Sampled	1
326	35.63526	-83.20792	High Rocks	8/13/2006	Sampled	1
327	35.54448	-83.84096	Gregorys Bald	9/1/2006	Sampled	1

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
328	35.52129	-83.85769	Gregorys Bald	9/1/2006	Sampled	1
329	35.52158	-83.85752	Gregorys Bald	9/1/2006	Sampled	1
331	35.61322	-83.24302	Hyatt Ridge Trail	6/11/2007	Sampled	1
332	35.61322	-83.24302	Hyatt Ridge Trail	6/11/2007	Healthy	0
333	35.61438	-83.24224	Hyatt Ridge Trail	6/11/2007	Healthy	0
334	35.61516	-83.24202	Hyatt Ridge Trail	6/11/2007	Healthy	0
335	35.61549	-83.24195	Hyatt Ridge Trail	6/11/2007	Healthy	0
336	35.51969	-83.37526	Thomas Divide	6/15/2007	Sampled	1
337	35.51959	-83.37376	Thomas Divide	6/15/2007	Sampled	2
338	35.52385	-83.37178	Thomas Divide	6/15/2007	Healthy	0
339	35.52392	-83.37188	Thomas Divide	6/15/2007	Sampled	1
340	35.52392	-83.37188	Thomas Divide	6/15/2007	Sampled	1
341	35.56016	-83.10793	Hemphill Bald Trail	6/14/2007	Healthy	0
344	35.66145	-83.49398	Bull Head Trail	6/26/2007	Sampled	1
345	35.61726	-83.76898	Rich Mountain Loop	7/25/2007	Healthy	0
346	35.62265	-83.77525	Rich Mountain Loop	7/25/2007	Healthy	0
ALB-01	35.05547	-83.47758	Albert Mountain	5/14/2006	Sampled	1
ALB-02	35.0554	-83.47755	Albert Mountain	5/14/2006	Sampled	1
ALB-03	35.04461	-83.47331	Albert Mountain	5/14/2006	Sampled	1
ALB-04	35.06641	-83.49554	Albert Mountain	5/14/2006	Sampled	1
ALB-05	35.06639	-83.49559	Albert Mountain	5/14/2006	Sampled	1
ALB-06	35.03069	-83.46198	Albert Mountain	6/19/2007	Sampled	2
ALB-07	35.03069	-83.46198	Albert Mountain	6/19/2007	Sampled	1
ALB-08	35.03376	-83.47226	Albert Mountain	6/19/2007	Healthy	0
ALB-09	35.04461	-83.47331	Albert Mountain	6/19/2007	Sampled	1
ALB-10	35.04458	-83.4733	Albert Mountain	6/19/2007	Sampled	1
ALB-11	35.03154	-83.45338	Albert Mountain	8/14/2007	Sampled	1
ALB-12	35.03061	-83.45516	Albert Mountain	8/14/2007	Sampled	1
ALB-13	35.0306	-83.45516	Albert Mountain	8/14/2007	Healthy	0
ALB-14	35.03096	-83.45504	Albert Mountain	8/14/2007	Sampled	1
ALB-15	35.03096	-83.45504	Albert Mountain	8/14/2007	Sampled	1
ALB-16	35.03098	-83.45508	Albert Mountain	8/14/2007	Healthy	0
ALB-17	35.03201	-83.45465	Albert Mountain	8/14/2007	Healthy	0
ALB-18	35.03201	-83.45465	Albert Mountain	8/14/2007	Healthy	0
ALB-19	35.03231	-83.45533	Albert Mountain	8/14/2007	Healthy	0
ALB-20	35.03165	-83.45784	Albert Mountain	8/14/2007	Healthy	0

Tree #[a]	Latitude	Longitude	Location	Date[b]	Condition[c]	Cankers[d]
ALB-21	35.03042	-83.46308	Albert Mountain	8/14/2007	Healthy	0
ALB-22	35.03036	-83.46437	Albert Mountain	8/14/2007	Healthy	0
ALB-23	35.03163	-83.46729	Albert Mountain	8/14/2007	Sampled	1
ALB-24	35.03502	-83.47415	Albert Mountain	8/14/2007	Sampled	1
BC01	35.62128	84.10672	Lanier	4/20/2007	Sampled	1
P01	35.25426	82.86064	Pisgah	6/20/2007	Healthy	0
P02	35.25426	82.86064	Pisgah	6/20/2007	Healthy	0
P03	35.25412	82.86077	Pisgah	6/20/2007	Sampled	1
P04	35.25413	82.86077	Pisgah	6/20/2007	Healthy	0
P05	35.25422	82.8607	Pisgah	6/20/2007	Sampled	2
WM01	35.08283	-83.13652	Whiteside Mountain	6/25/2007	Healthy	0
WM02	35.08292	-83.13655	Whiteside Mountain	6/25/2007	Healthy	0
WM03	35.08265	-83.1366	Whiteside Mountain	6/25/2007	Healthy	0
WM04	35.0806	-83.13896	Whiteside Mountain	6/25/2007	Healthy	0
WM05	35.08429	-83.13445	Whiteside Mountain	6/25/2007	Healthy	0
WM06	35.08305	-83.13622	Whiteside Mountain	6/25/2007	Healthy	0
ST01	35.0301	-83.28345	Scaly Mountain	7/26/2007	Sampled	2
ST02	35.02944	-83.2825	Scaly Mountain	7/26/2007	Sampled	1
ST03	35.04019	-83.27826	Scaly Mountain	7/26/2007	Sampled	1
ST04	35.0419	-83.27902	Scaly Mountain	7/26/2007	Sampled	1
ST05	35.04013	-83.28138	Scaly Mountain	7/26/2007	Healthy	0

[a] Tree numbers 1-288 were originally assigned by Wood (2003); all other designations were assigned to newly recorded trees, 2006 and 2007.
[b] Dates on which locations were visited.
[c] Healthy trees contained no outward signs of *C. parasitica* infection; sampled trees contained one or more cankers caused by *C. parasitica*; dead trees are those which had previously been reported but had subsequently been killed.
[d] Number of cankers caused by *C. parasitica*.

APPENDIX B.

LOCATIONS AND ELEVATION OF HEALTHY AMERICAN CHESTNUT TREES LOCATED AT HIGHLANDS PLOT, NANTAHALA NATIONAL FOREST, NORTH CAROLINA IN 2007.

Tree #	Latitude	Longitude	Altitude (m)
HL1	35.08719	-83.22019	1235
HL2	35.08718	-83.22014	1233
HL3	35.0871	-83.22013	1235
HL4	35.08721	-83.22046	1240
HL5	35.08706	-83.22039	1238
HL6	35.08699	-83.22072	1243
HL7	35.08688	-83.22072	1244
HL8	35.08692	-83.22069	1244
HL9	35.08691	-83.22079	1245
HL10	35.08691	-83.22086	1246
HL11	35.08685	-83.22087	1246
HL12	35.08698	-83.22107	1225
HL13	35.08702	-83.22107	1226
HL14	35.0868	-83.22121	1220
HL15	35.08674	-83.22117	1216
HL16	35.08678	-83.22099	1215
HL17	35.08659	-83.22122	1226
HL18	35.08666	-83.22116	1224
HL19	35.08668	-83.22102	1214
HL20	35.08682	-83.22096	1216
HL21	35.08699	-83.2209	1244
HL22	35.0871	-83.22112	1230
HL23	35.08725	-83.22114	1242
HL24	35.08728	-83.22125	1184
HL25	35.08731	-83.2211	1241
HL27	35.08734	-83.22132	1179
HL28	35.08722	-83.22141	1197
HL30	35.08727	-83.22146	1198
HL31	35.0872	-83.22144	1196
HL32	35.0872	-83.2213	1213
HL33	35.08709	-83.22154	1212
HL34	35.08698	-83.22139	1217
HL35	35.08697	-83.22137	1221
HL36	35.0873	-83.22127	1181

Tree #	Latitude	Longitude	Altitude (m)
HL37	35.08671	-83.2213	1220
HL38	35.08677	-83.22125	1220
HL40	35.08687	-83.22071	1243
HL41	35.08665	-83.22127	1221
HL42	35.0866	-83.22122	1221
HL43	35.08662	-83.22124	1221
HL44	35.08689	-83.22119	1226
HL45	35.08737	-83.22126	1179
HL46	35.08709	-83.22089	1241
HL48	35.08783	-83.22139	1221
HL49	35.08786	-83.22143	1221
HL50	35.08786	-83.22118	1221
HL51	35.08759	-83.22159	1223
HL53	35.08668	-83.22122	1220
HL54	35.08659	-83.22126	1222

APPENDIX C.

TREE ASSIGNMENTS AND INOCULATION LOCATION AND DIRECTION FOR ISOLATES OF C. PARASITICA INOCULATED ONTO HEALTHY AMERICAN CHESTNUT DURING A FIELD TRIAL, NANTAHALA NATIONAL FOREST, HIGHLANDS, NC; 2007.

	Upper[a]		Lower	
Tree	**Isolate**	**Direction[b]**	**Isolate**	**Direction**
HL1	101-3B	E	291-2B	N
HL2	45-1B	S	20-1A	N
HL3	303-1A	E	EP-155	W
HL4	88-1D	N	38-1D	W
HL5	E96	W	291-2B	S
HL6	60-2B	S	192-1A	E
HL7	325-1B	N	EP-155	S
HL8	319-1A	E	320-1B	W
HL9	90-2C	N	236-1C	W
HL10	E96	E	325-1B	N
HL11	20-1A	S	213-1A	S
HL12	303-1A	N	319-1A	W
HL13	88-1D	S	75-1B	N
HL14	192-1A	N	291-2B	S
HL15	183-1A	W	320-1B	N
HL16	49-1C	N	60-2B	W
HL17	90-2C	N	236-1C	S
HL18	45-1B	S	183-1A	E
HL19	97-	W	101-3B	S
HL20	324-1B	E	49-1C	N
HL21	324-1B	E	38-1B	W
HL22	75-1B	W	97-	N
HL23	75-1B	W	38-1B	S
HL24	325-1B	N	60-2B	S
HL25	97-	N	20-1A	S
HL26	236-1C	W	49-1C	S
HL27	320-1B	E	183-1A	W
HL28	101-3B	S	192-1A	E
HL29	EP-155	N	303-1A	W
HL30	45-1B	S	213-1A	W
HL31	88-1D	N	49-1C	E
HL32	319-1A	W	291-2B	E

Tree	Isolate	Direction	Isolate	Direction
HL33	324-1B	E	E96	N
HL34	38-1B	N	90-2C	S
HL35	213-1A	W	75-1B	E
HL36	97-	E	324-1B	N
HL37	236-1C	S	303-1A	W
HL38	101-3B	W	325-1B	N
HL39	319-1A	N	90-2C	E
HL40	320-1B	W	EP-115	S
HL41	88-1D	W	60-2B	N
HL42	20-1A	W	192-1A	E
HL43	183-1A	W	213-1A	N
HL44	E96	W	45-1B	E

[a] Upper isolates were inoculated at 1.5 m from the ground; lower isolates were inoculated at 0.5 m.
[b] Cardinal direction indicating side of tree on which inoculations were performed; N= north, E= east, S= south, W= west.