INFLUENCE OF MYCOVIRUSES ON THE POPULATION STRUCTURE OF THE CHESTNUT BLIGHT PATHOGEN, CRYPHONECTRIA PARASITICA AND RECOVERY OF AMERICAN CHESTNUT, CASTANEA DENTATA

By

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ABSTRACT

INFLUENCE OF MYCOVIRUSES ON THE POPULATION STRUCTURE OF THE CHESTNUT BLIGHT PATHOGEN, CRYPHONECTRIA PARASITICA AND RECOVERY OF AMERICAN CHESTNUT, CASTANEA DENTATA

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Double-stranded RNA (dsRNA) mycovirus hyperparasites can infect the chestnut blight pathogen, *Cryphonectria parasitica*, causing a reduction in virulence and inhibition of sexual reproduction in *C. parasitica*, which can lead to recovery of the blight pathogen's host, American chestnut (*Castanea dentata*). Studies to determine the level at which mycoviruses can influence population-level and genomic level diversity of *C. parasitica* have previously not been done. The main objectives of this dissertation are: 1) determine how mycovirus hyperparasites can alter vegetative compatibility group (VCG) diversity over long time periods, 2) evaluate the affect of mycoviruses on genome level diversity in *C. parasitica*, 3) test a hypothesis that mycovirus biological control agents would be most successful on American chestnut trees in the 1—10cm diameter at breast height (DBH) size class.

VCG studies suggest that the presence of mycoviruses influence *C. parasitica* population structure in Michigan. In *C. parasitica* populations where mycoviruses are present VCG diversity is low and VCGs generally unique to individual sites and where mycoviruses are absent VCG diversity is higher and VCGs are shared across these sexually reproducing populations.

Similarly, genome-wide diversity in *C. parasitica* also appears to be structured according to mycovirus presence and absence. Population genetic differentiation values (Φ PT) are higher for mycovirus-infected *C. parasitica* populations while lower values are found at mycovirus-free sites. The pattern of differentiation does not suggest any isolation by distance. The overall pattern suggests significant migration among epidemic populations of *C. parasitica* where mycoviruses are absent, while pathogen populations with mycoviruses are largely isolated. Thus, *C. parasitica* in Michigan is composed of a patchwork of sites whose dynamics appears to be governed by the presence/absence of mycoviruses.

Preliminary results of a long-term experiment using mycoviruses as biological control mechanism in *C. parasitica* is showing promising results. Trees in the 1—10cm DBH size class were tested for response to mycovirus infected strains of *C. parasitica*. Annual survivorship was 73.6% across three tree populations. Persistence of mycovirus in treated cankers however, was only around 50% from year to year suggesting that repeated introductions of mycoviruses as biological control agents may be necessary to achieve success. Further, local environmental conditions, tree genotype, and amount of competition from large overstory trees may play a role in American chestnut recovery.

This study investigates both phenotypic and genome diversity in *C. parasitica* relative to the presence and absence of naturally occurring mycovirus hyperparasites that are useful for biological control of the chestnut blight pathogen.

Dedicated to the memory of Bruce D. Parfitt (1952—2009)
My undergraduate mentor, friend, and fellow birder

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CHAPTER 1

INTRODUCTION

Plant pathogen interactions

Pathogens can play a major role in structuring plant populations on both a local and regional scale by reducing survivorship, growth and reproduction of infected hosts (Burdon 1987; Gilbert 2002). These interactions ultimately affect the evolutionary dynamics of plant populations (Price 1980; Burdon 1987; Gilbert 2002). Our understanding of community level effects is basic but growing (Dobson & Hudson 1986; Burdon et al. 2006). Weste (1980, 1981), McCormick & Platt (1980), and others have reported that introduced plant pathogens can alter the species interactions within a plant community. For example, *Phytophthora cinnamomi* Rands alters dominance relationships within Australian Eucalyptus forests resulting in a significant change in plant community structure (Weste 1980, 1981). Chestnut blight disease, caused by *Cryphonectria parasitica* (Murrill) Barr, eliminated the American chestnut (*Castanea dentata* (Marsh.) Borkh. from the overstory of eastern hardwood forests in North America, which allowed other species to increase in prevalence and dominance (McCormick & Platt 1980).

The fitness of plants after infection by a pathogen with high virulence is greatly reduced (Figure 1-1). However, Harper (1977) postulated that pathogens with such high virulence would quickly destroy their resource-base so that the evolution would favor reduced virulence. A reduction in pathogen virulence

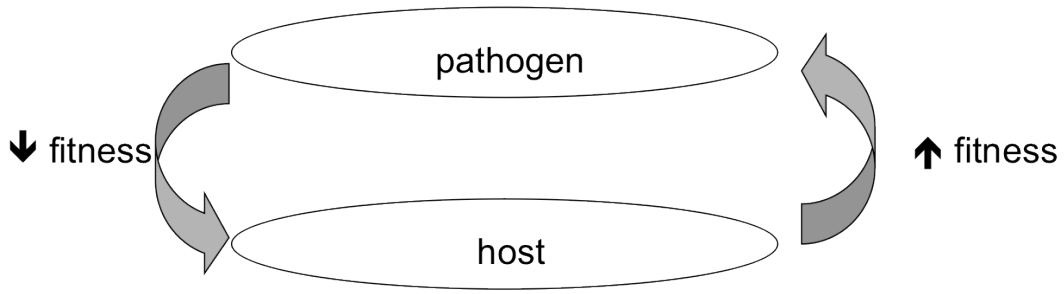

Figure 1.1. Generalized pathogen-host interaction. Pathogens generally reduce the fitness of their hosts and reduce biomass of the infected individual and population.

then, would lead to recovery of host populations so that the cycle of infection may continue. A well-known empirical example of the evolution of reduced virulence is found in the Myxoma virus-common rabbit (*Oryctolagus cuniculus* L.) system in Australia. Introduced for biological control of destructive rabbit populations, the virulent virus, causing myxomatosis, quickly reduced rabbit populations but also evolved reduced virulence while increased resistance and immunity to the virus evolved in rabbit populations. (Fenner & Ratcliffe 1965; Anderson & May 1982; Fenner 1983; May & Anderson 1983); the evolution of reduced virulence is not entirely common, however (Jarosz & Davelos 1995). A more reasonable model for the evolution of virulence was published by Lenski & May (1994), which predicted that intermediate levels of virulence should evolve over time due to ecologically important factors that affect density-dependent transmission of the pathogen. The Lenski and May model also focused attention on individual selection rather than the group selectionist argument hypothesized by Harper. The evolution of reduced virulence has been of some concern in situations of biological control since any loss of virulence in the biological control mechanism, can lead to a reduction in successful control of disease (Bryner & Rigling

2011, 2012). This is concerning since controlling invasive populations of plants with pathogens relies on the success of introducing biological control agents and realizing successful spread and persistence over long periods of time.

The fitness consequences of complex plant-pathogen interactions are often difficult to elucidate (Mordecai 2011) because reductions in survivorship and reproduction of infected plants is often conditioned by abiotic and biotic stresses that are being imposed on the plant (Jarosz & Davelos 1995; Gilbert 2002; Klironomos 2003). In the extreme, fungal infections of a plant can have negative fitness consequences under some conditions, but be mutualistic under others (Singh et al. 2011). For example, mycorrhizae may be mutualists in some conditions, but a change in the abiotic environment, such as temperature or amount of precipitation, may change the interaction to parasitism (Johnson et al 1997). Thus, host-pathogen interactions can have diverse consequences on both plant and pathogen populations but those interactions can help to maintain both species (Burdon 1987) and can be quite variable due to local conditions.

The influence of native pathogens on plant populations is often difficult to detect because the interaction between the plant and pathogen has been incorporated into the plant community for a considerable time period. In consequence, the pathogen's influence may be integrated into community and its influence is not obvious. However, the influence of some native pathogens has been studied and they have the potential to cause significant and wide-ranging effects on host populations (Burdon 1987). Native pathogens such as fusiform rust caused by the pathogen *Cronartium fusiforme* Hedg. & Hunt ex Cumm. in the southeastern United States on native pines increases in

prevalence when selective cutting of other tree species occurs Dinus (1974). *Uromyces ari-triphylli* (Schwein.) Seeler and can change the growth rates of Jack-in-the-pulpit (*Arisaema triphyllum* L.) populations (Baines et al. *in press*) and *Puccinia mariae-wilsonii* Clinton can cause widespread disease epidemics in *Claytonia* spp. while maintaining flower color variation in host plants over time (Frey 2004).

The influence of introduced pathogens is often easier to detect because their invasion into plant community can lead to a cascade of changes throughout the plant community (Vitousek at al. 1996, 1997; Lovett et al. 2006; Loo 2008). Fungal pathogens such as *Ophiostoma ulmi* (Dutch Elm Disease), *Phytophthora ramorum* (Sudden Oak death), *Nectria* spp. (Beech bark disease), and *Cryphonectria parasitica* (Chestnut blight), are all examples of introduced pathogens that have negatively affected their respective host species in ways that have changed the forest composition in areas where they have invaded (McCormick & Platt 1980; Swinton & Gilligan 1996; Paillet 2002; Griffin et al. 2003; Garnas et al. 2011; Cobb et al. 2012). In each of the four situations above, the introduced pathogens have greatly reduced host numbers, and caused disruptions of community composition. Additionally, pathogen effects can also cascade down to other members of the community such as birds and mammals by reducing food resource availability (*cf*. Monahan & Koenig 2006; Loo 2009).

Hyperparasites and Hyperparasitism

To make matters more complex, fungal pathogens can be infected with hyperparasites (i.e., parasites of pathogens or parasites and referred to as tri-trophic interactions), most frequently, viruses (Nuss & Koltin 1990). Hyperparasitism is not

confined to fungal pathogens of plants but is also well documented across taxa such as wasps and bacteria (Sullivan 1987; Tanaka et al 2007; Johnson et al 2010; Koskella et al 2011) and have far-ranging effects, many times acting as biological control agents. Viral hyperparasites of fungal pathogens (hereafter, mycoviruses) have been documented to decrease the virulence of fungal pathogens they infect, allowing plant hosts to grow at nearly a normal level (Liesebach & Zaspel 2004; Roossinck 2011). Mycoviruses can have other unexpected effects such as assisting fungal mutualists to confer thermal tolerance to plants, which allows the host plants to withstand higher temperatures near thermal pools in Yellowstone National Park (Redman et al. 2002).

Mycoviruses have the potential to act as top-down regulators in plant-pathogen systems (Taylor et al. 1998). The addition of mycoviruses into plant-pathogen interactions can quickly reduce pathogen virulence ecologically with their invasion; mimicking a slower evolutionary reduction in virulence. Mycovirus mediated reductions in virulence can often occur on a shorter time scale than expected with evolutionary change because mycoviruses can be transmitted both vertically (i.e., from parent to offspring) and horizontally (i.e., infectious spread to new individuals). Additionally, it appears that mycoviruses are common in nature, especially in fungi (Hollings 1982; Buck 1986; Roossinck 2011) and are often unencapsidated double-stranded RNA mycoviruses that can be found in Ascomycetous fungi (Oh & Hillman 1995), Oomycetes (Nuss & Koltin 1990), and Basidiomycetes (Nuss & Koltin 1990). However, it appears that mycovirus infection of a fungus often does not reduce virulence and are quite benign (Nuss 2005).

Ecological and evolutionary outcomes of plant-pathogen-mycovirus tri-trophic interactions may vary depending on location, environmental conditions within or among years, or the amount of genetic variation at all levels of the interaction. Thompson (1999) has termed this the geographic mosaic theory of coevolution (GMTC) where the interplay of organisms at differing hierarchical scales can result in vastly different outcomes at different temporal and geographic scales. For example, the moth *Greya politella* and its host plants *Lithophragma* spp. and *Heuchera* spp. have quite variable interactions, especially if competing pollinators of Greya moths, Bombyliid flies, are present in local populations. Bombyliid flies outweigh the commensalistic interaction that Greya moths can develop with the host plants, since the flies are effective pollinators that do not prey on developing seeds like Greya moths (Thompson & Pellmyr 1992; Thompson 1999). Benkman (1999) noted that red squirrels (*Tamiasciurus* spp.) changed the interaction between crossbills (*Loxia* spp.) and lodgepole pines (*Pinus contorta* Douglas) that led to development of different interactions across the geographic range of the species. When present, red squirrels preempt crossbills from feeding on cones of lodgepole pines and squirrel presence drives cone evolution. When red squirrels are absent from lodgepole pine forests, crossbills drive cone evolution and these particular coevolutionary hotspots can result in divergent selection, creating a variable selection mosaic across the landscape which, if strong enough can lead to speciation in both interacting members (Benkman 1999; Benkman et al. 2011). Further, another type of complex interaction involves a biological control mechanism of a pathogen: reductions in virulence caused by double-stranded RNA (dsRNA) mycovirus infection of *C. parasitica* are known to be

temperature sensitive (Bryner & Rigling 2012) and higher ambient temperatures could inhibit the ability of mycoviruses to promote recovery of host plant populations. Complex interactions like these can have far-reaching effects for biodiversity such as maintaining disease at low levels in plant populations and generating new species through tightly co-evolving species driven by strong local adaptation. Local, transient maladaptation may occur (Thompson 1999; Benkman 1999; Thompson & Pellmyr 1992; Thompson 2005) such as when the evolutionary forces genetic drift, recombination, and mutation (Hanski 1999; Thompson 1999 and references therein; Smith, Ericson, & Burdon 2003) can cause individuals' traits to be mixed within populations and when individuals subsequently migrate to nearby populations, thus creating a geographic mosaic of coevolution (Thompson 1999, Thompson 2005). Local populations of interacting species, as shown above, can behave very differently in isolated local populations. Invasion by additional species (such as dsRNA mycoviruses) may change the original interactions and may vary among sites within a metapopulation.

The question of scale and local interactions between species is becoming a major topic in ecology where localized populations of interacting species may, over short time periods develop very different dynamics that can change due to varying biotic and abiotic conditions and outcomes can be quite different even at small spatial scales (Hanski & Gilpin 1997; Hanski 1999; Thompson 1999; Thompson 2005).

The GMTC can be applied to the chestnut blight tri-trophic system discussed below. Gene flow, migration, recombination, genetic drift, and the local abiotic environment (and other unknown factors) may continuously rework local landscapes

within a metapopulation of the interacting species, continuously changing how species interact. Additionally, discussed in this dissertation is how the size (diameter) of individual hosts may influence the success of mycoviruses on the recovery of individual American chestnut trees (Chapter 4) and how diversity of the blight pathogen may affect spread of mycoviruses that lead to host tree recovery in local populations.

Chestnuts, Blight and Mycoviruses in Michigan

The chestnut, chestnut blight, and mycovirus interaction is a model tri-trophic system used for research in this dissertation. A generalized overview of the major, known players and their effects in this system is shown in Figure 1-2. A historically important forest species, *Castanea dentata*, the American chestnut was an important tree both to the natural ecosystem and to people who depended on the tree for its many uses. American chestnut was a tree that had more uses than its counterparts in the hardwood forests of the eastern United States (Griffin & Elkins 1986). Strong, rot-resistant lumber and edible nuts are two products that were important to not only rural families but throughout the country (Griffin & Elkins 1986). It appears that eastern Asia is the center of origin for the genus *Castanea* and subsequently spread to Europe and then North America (Lang et al 2006). American chestnuts, *C. dentata*, are highly susceptible to chestnut blight, while European chestnuts are somewhat less susceptible to *C. parasitica* than *C. dentata* (Graves 1950; Clapper 1952; Anagnostakis 1992). Asian chestnut species, such as *C. mollissima* and *C. davidii* have much higher resistance to blight than either American or European chestnuts. In fact, chestnut blight was not

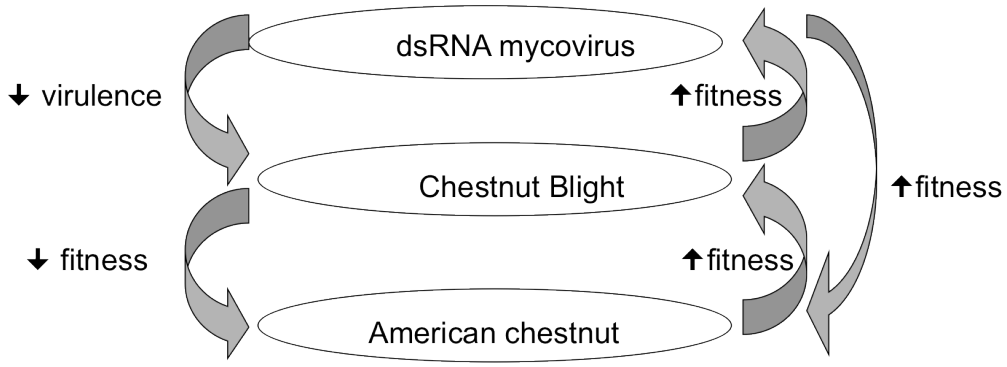

Figure 1-2. The three major players in the chestnut tri-trophic system. Arrow labels show general outcomes of interactions.

discovered in Asia until 1913, well after the American epidemic had begun (Fairchild 1913). Blight is present in both China and Japan but is rarely destructive (Fairchild 1913). Resistance in Asian chestnuts is probably due to its long coevolutionary history with blight (Anagnostakis 1992).

Cryphonectria parasitica (Murrill) Barr (previously *Endothia parasitica*) is a destructive non-native pathogen on chestnuts in North America and Europe. The blight pathogen decimated the once-common and beloved tree in the United States, *Castanea dentata* (Marsh.) Borkh., the American chestnut. A wound pathogen, *C. parasitica* quickly destroys vascular tissue of American chestnut, girdling branches and stems (Figure 1-3), killing all parts of the tree distal to the wound (Figure 1-4). The pathogen spread quickly throughout the range of American chestnut, just decades after first being introduced around New York City (Merkel 1905).

Contrary to popular belief, *C. parasitica* did not cause the extinction of the American chestnut but caused more than 3.5 billion main trunks to die back to ground level. American chestnuts once accounted for nearly 25% of all overstory trees in eastern hardwood forests, but blight has transformed these trees to understory

inhabitants. American chestnuts have persisted thanks to an interesting physiology; the blight pathogen does not infect the root tissues of the tree, allowing stump or root collar sprouts to grow for a period of time before becoming infected, thus creating a cyclical infection (Griffin et al. 1991). The only remaining disease-free American chestnuts occur outside of the native range (see Brewer 1995 for a list of populations in Michigan). These remaining populations are being colonized by blight as time passes. Indeed, the two disease-free sites we began monitoring in 1996 have become infected during the course of our work (Davelos and Jarosz 2004; see also Chapter 2).

Double-stranded (ds)RNA mycoviruses

Double-stranded RNA (dsRNA) mycoviruses (sometimes referred to as hypoviruses) have been documented to infect *C. parasitica* (Anagnostakis & Waggoner 1981; Fulbright et al 1988; Nuss 1990; MacDonald & Fulbright 1991; Nuss 1992; Milgroom & Cortesi 2004). Where mycoviruses have invaded the blight pathogen, the tree populations can exhibit significant recovery and regain its status as an overstory tree; mycovirus infection of *C. parasitica* allows trees to experience growth, survivorship, and reproduction that mimics uninfected trees (Davelos & Jarosz 2004). Mycovirus hyperparasite presence within the mycelia effects a noticeable change in phenotype of the fungal pathogen (Figure 1-5), which most importantly, results in a reduction in virulence. The decreased virulence of the fungal pathogen thus allows the tree host to respond more effectively to this now-debilitated pathogen and by extension the host tree can lay down callus tissue to effectively prevent a blight canker from girdling a

Figure 1-3. Typical result of *Cryphonectria parasitica* infection on American chestnut. Cankers normally expand concentrically from a wound site (branch point, left), collapsing vascular tissue as they expand (right). Orange pigmentation is evidence of disease. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

stem (Figure 1-6). Mycoviruses have no extracellular life-stage in which to spread of the infected pathogen (Milgroom & Cortesi 2004); they spread through vertical transmission by way of asexual conidia but not through sexually produced ascospores (Anagnostakis 1988). Another way mycoviruses spread, and important for their dissemination is through hyphal fusion (horizontal transmission through anastomosis of hyphae) when one fungal isolate infected with mycoviruses comes into contact with an uninfected strain and subsequently transmits mycoviruses through the cytoplasm

that stream between the newly connected hyphae. Huber (1996) described the influence of individual vegetative incompatibility (*vic*) loci in *C. parasitica* on the

Figure 1-4. Death of a distal end of an American chestnut branch from a girdling canker caused by *Cryphonectria parasitica*. Yellow, wilted leaves are evidence of disease.

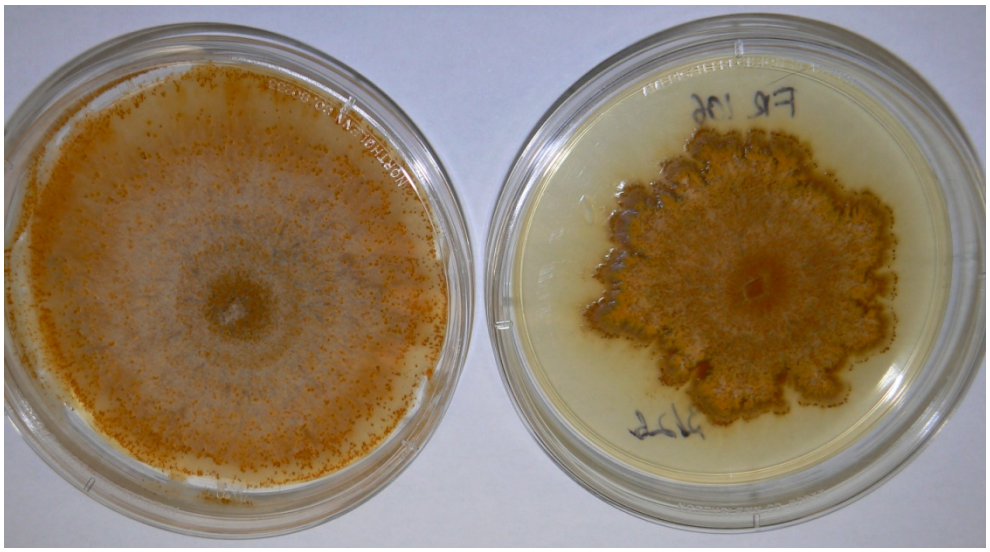

Figure 1-5. A mycovirus-free (left) and mycovirus infected (right) isolate of the chestnut blight pathogen *Cryphonectria parasitica*. Both fungal isolates are the same genotype and are both about one week old.

Figure 1-6. Examples of mycovirus infected *Cryphonectria parasitica* cankers on American chestnut trees. Note swollen nature of wounds. All parts distal to these cankers are alive.

transmission of mycoviruses between strains; mismatches at any of the seven known *vic* loci between two isolates of *C. parasitica* can significantly influence anastomosis (discussed later).

Mycoviruses are commonly found to be infecting *C. parasitica* in Europe but they have not spread to all regions of the continent. Chestnut blight is prevalent across the European continent but the condition of hypovirulence is variable. Detection of mycoviruses and healing cankers on chestnuts is normally found to occur around 10 years after the initial blight epidemic (Milgroom & Cortesi 2004); hypovirulence caused by mycoviruses is rare or absent in areas where *C. parasitica* has been recently spread such as northern Switzerland, northern France, Germany, and Turkey (Robin &

Heiniger 2001; Milgroom et al. 2004; Prospero & Rigling 2012). Italy, southern Switzerland, southern France, and Spain are areas where the CHV1 type of mycovirus is ubiquitous, where European chestnut trees are recovering and where chestnut blight is no longer a major concern (A. Vannini, pers. comm.). Five sub-types of CHV1 have been documented and in some areas, the subtype recovered after introduction of mycoviruses for biological control differs from the naturally occurring type, lending evidence of natural spread of hypovirulence in some forest (and orchard) situations. CHV1 has been found in Japan, Korea, and China but there is no known active research on biological control of *C. parasitica* in Asia since blight does not cause major problems except rarely in orchard situations where trees could be experiencing environmental stress (Jaynes 1975; Milgroom & Cortesi 2004).

Mycovirus induced hypovirulence in the United States is much less common. CHV1 has been introduced from Europe as a biological control mechanism but has failed to become established and spread beyond initial introductions (Peever et al. 1997). CHV1 causes female sterility in *C. parasitica*, severely debilitates the pathogen while reducing sporulation, pigmentation and virulence. These characteristics of CHV1 led to the thought that this would be a useful mycovirus for biological control on the North American populations of American chestnuts but was not successful. CHV2 has been found in New Jersey and is severely debilitating toward *C. parasitica*, reducing fungal development and fecundity (Hillman et al. 1992; 1994). Mycovirus GH2, a CHV3 type, is a naturally occurring mycovirus in Michigan, (discussed in Chapter 4). CHV3 type viruses have been found outside of Michigan in Kentucky and West Virginia but were most likely the result of their release for attempted biological control (Peever et al.

1997). Viruses similar to GH2 have been found across North America (Paul & Fulbright 1988; Peever et al. 1997) and in southern Ontario, Canada (Melzer & Boland 1999). Mycoviruses of the CHV3 type do not change morphology of *C. parasitica* but still reduces fungal virulence. SR2-type viruses, now included in the CHV4 type have been found in Maryland, Michigan (a second mycovirus type found at the Frankfort site), and throughout the Appalachian region but has little to no effect on virulence or morphology of *C. parasitica* (Enebak et al. 1994).

The unencapsidated dsRNA mycoviruses that infect *C. parasitica* are members of the monogeneric virus family Hypoviridae (Hillman et al. 1994, 1995). All CHV mycoviruses belong to the genus Hypovirus and are divided into species (and subtypes) depending on how each mycovirus genome is organized such as: the structure of its genetic sequence, and symptoms caused in infected isolates of *C. parasitica* (Gobbin et al. 2003). CHV1 contains a fragment 12.7kb long with two open reading frames with four subtypes that can vary in sequence divergence between 11 and 19%. CHV1 mycoviruses are similar to CHV2 viruses (Suzuki et al. 2003) with a total length of about 12.5 kb. CHV3 (specifically, GH2) contains three to four fragments, the largest at 9.8kb with others at 3.5kb and 1.0kb and a single large open reading frame; smaller ORFs are possible (Smart et al. 1999). CHV4/SR2 types, those least effective at decreasing virulence of *C. parasitica* also contains one ORF (Suzuki et al 2003) and a 9.1kb genome (Linder-Basso et al. 2005). Many regions of the genome of Cyphonectria hypoviruses have been tested for the specific actions they have on *C. parasitica* but it appears that regions known as p40 and p29 specifically affect blight virulence and transmission of mycoviruses into asexual conidia (Suzuki et al. 2003).

Vegetative incompatibility

Transmission of mycovirus hyperparasites from an infected fungal isolate to a mycovirus-free isolate can be limited through the formation of barrage zones when anastomosis of fungal hyphae fails (see Figure 2-3, Chapter 2). Failure of anastomosis occurs when two neighboring fungal colonies of the blight pathogen meet and subsequently die back due to programmed cell death or apoptosis. Mycovirus transfers (or conversions) occur when two fungal colonies meet and anastomose and subsequently transfer materials through cytoplasmic streaming. Liu and Milgroom (1996) have shown that the success of mycovirus transfer is negatively correlated with the number of vegetative incompatibility (*vic*) genes that differ between isolates that attempt to unite. Huber (1996) showed that individual alleles that control *vic* gene loci can have varying effects on mycovirus transmission; some *vic* loci are stronger at preventing mycovirus transmission than others. In this system there are between six and eight bi-allelic loci that control anastomosis. Loci are unequal in their effect, and multiple mismatches at weak loci may continue to allow mycovirus transmission while an allelic mismatch at one locus of strong effect can totally inhibit mycovirus transmission (Huber 1996).

Vegetative compatibility (VC) is a self/non-self recognition system in fungi and is thought to help maintain the integrity of individuals (Rayner 1991) and prevent the spread of alien nuclei and infectious elements such as mycoviruses. In *C. parasitica*, high vegetative compatibility group (VCG) diversity in has been linked to and blamed for the failure of mycoviruses to spread through *C. parasitica* populations in North America (Anagnostakis 1986; Anagnostakis & Kranz 1987). Vegetative compatibility groups

(VCGs) are relatively easy to determine in the laboratory (see Chapter 2) and have been used to assess diversity within fungal populations (Milgroom & Cortesi 1999). The use of vegetative compatibility (sometimes referred to as heterokaryon or somatic cell incompatibility) to determine population-level diversity is a common technique for fungi, especially filamentous Ascomycetes (Glass & Kuldau 1992; Leslie 1993), but other fungal taxa such as Basidiomycetes (Casselton & Economou 1985; Aimi et al. 2002), Zygomycetes (Griffin & Perrin 1960; Bayman & Bennett 1998), and Myxomycetes (Betterley & Collins 1984; Lane 1987; Clark & Haskins 2012) also exhibit vegetative incompatibility. Understanding fungal pathogen diversity within host populations is important to understanding temporal and spatial dynamics of the system and how the pathogen can affect host individuals and populations over time (Burdon 1987).

Mycoviruses as biological control agents

A common hypothesis for the successful invasion and spread of mycoviruses in populations of *C. parasitica* is that low diversity of vegetative compatibility groups of the blight pathogen allows easier spread of mycoviruses (Anagnostakis 1977; Anagnostakis et al. 1986; Milgroom et al. 1991; Milgroom and Cortesi 2004) since mismatches of alleles are less common. If mycoviruses can spread through *C. parasitica* populations, they can have significant effects on the fitness of the blight pathogen and the host tree, *Castanea dentata* (Griffin & Elkins 1986).

Mycoviruses have been championed as potential biological control agents of *C. parasitica*, but, their success in North America has been quite disappointing (Milgroom

& Cortesi 2004) while, in Europe, mycovirus hyperparasites are commonly found to be infecting *C. parasitica* on European chestnut host trees (Turchetti 1982; Heiniger & Rigling 1994; Robin & Heiniger 2001; Milgroom & Cortesi 2004). Mycovirus-mediated recovery of American chestnut populations has occurred naturally only at a number of sites in Michigan, (Fulbright et al. 1983;
MacDonald & Fulbright 1991; Brewer 1995; Milgroom & Cortesi 2004). At these sites, American chestnuts grow into the forest canopy, flower and reproduce at rates comparable to disease-free populations in Michigan (Davelos & Jarosz 2004).

The chestnut blight situation in Michigan

American chestnuts are native to the southeastern corner of Michigan's lower peninsula (Little 1971), but early settlers planted them widely across the lower peninsula of Michigan especially in areas where Lake Michigan modulates temperatures (Brewer 1995). In many areas, these planted trees have given rise to naturalized American chestnut populations once the original homesteads were abandoned.

Chestnut blight was discovered in Michigan around 1928 (Baxter and Strong 1931). As in the native range, many chestnuts in northwestern Michigan (see Chapter 2) have succumbed to blight. However, abnormal cankers were discovered to harbor mycoviruses around 1976 (Fulbright et al. 1983; Brewer 1995) and were naturally occurring since no mycoviruses were introduced to Michigan before their discovery (Fulbright et al. 1983). The state of Michigan contains the only known examples of successful natural invasion of mycoviruses in North America (Fulbright et al. 1983). Trees with cankers that contained mycovirus infected *C. parasitica* were discovered in

at least seven locations in Michigan. At least three mycovirus types exist in Michigan: Cryphonectria hypovirus 3 (CHV3), CHV4, and a unique type termed SR2 (Fulbright et al 1983; Fulbright et al. 1988; Paul and Fulbright 1988; Hillman et al. 1995; Peever et al. 1997, 1998) are all found in the state. CHV3 is the most common type that is associated with tree recovery, being found at Grand Haven (since removed for a housing development), County Line and Frankfort (Peever et al. 1997). Frankfort (discussed in Chapters 2 and 3 in this dissertation), is characterized by the presence of both CHV3 and SR2 (Peever et al. 1997). Thus, mycoviruses have naturally invaded in Michigan populations of *C. parasitica* and have allowed trees to survive there much longer than trees infected by only the blight pathogen (Fulbright et al. 1983; Davelos & Jarosz 2004). The extreme patchiness of recovery in Michigan hints at the possibility that genetic make-up of trees at these sites influences the eventual success or failure of mycoviruses as biological control agents (see Chapter 4).

Structure of this Dissertation

In the following pages of this dissertation, the impact of mycoviruses on populations of *C. parasitica* and recovery of *C. dentata* are explored. My work was designed to address the following questions and objectives:

A. How is population structure of the chestnut blight pathogen, *C. parasitica* influenced by the presence of mycoviruses with regard to vegetative compatibility group diversity of *C. parasitica*?

B. How does the presence of mycoviruses influence the population structure of the chestnut blight pathogen at the molecular level? Additionally, is the pattern of

molecular variability concordant with that observed for vegetative compatibility group diversity?

C. Implement a deployment strategy proposed by Davelos (1999) and Davelos & Jarosz (2004), which advocated that the best trees to treat with mycoviruses are sub-adults with 1—10cm DBH (diameter at breast height)

D. Are mycoviruses susceptible to degradation when stored as static cultures over long time periods?

My aim is to provide an understanding of the population structure of the chestnut blight pathogen in Michigan with the ultimate goal of further understanding the effect of mycovirus hyperparasites on fungal population structure. Additionally, this work aims to determine what strategies work best for deploying mycoviruses but these are long-term experiments that need long-term monitoring. This tri-trophic system can be used as a model system to understand of similar systems where techniques of biological control of pathogenic fungi can be applied.

In Chapter 2, results from a temporal comparison of vegetative compatibility group (VCG) diversity in the blight pathogen are presented. VCG data from 1996 (Davelos 1999) are compared with fungal isolates collected in 2009 to elucidate changes in population structure of *C. parasitica* in three Michigan population types: those infected for extended time periods with both *C. parasitica* and mycoviruses, those which only have had *C. parasitica* present for a long time period, and those populations of chestnut hosts recently infected by the blight pathogen. Data specific to the success of reviving stored cultures from 1996 collections are presented in Appendix A along with data that indicate the loss of mycoviruses during long term storage. In Chapter 3, I use

nine microsatellite loci to characterize molecular diversity within *C. parasitica* populations, and compare the pattern of diversity using microsatellites with VCG data from chapter 2.

Preliminary results of a long-term study of mycovirus introductions into three *C. parasitica* populations are presented in Chapter 4. Chapter 5 presents overall conclusions and discusses implications of the work for successfully managing or controlling the chestnut blight pathogen so that American chestnut trees may be restored to the eastern hardwood forests of the United States. Additionally, chapter 5 presents directions for future work on this important model tri-trophic system.

LITERATURE CITED

LITERATURE CITED

Aimi T, Yotsutani Y, and Morinaga T. 2002. Cytological Analysis of Anastomoses and Vegetative Incompatibility Reactions in *Helicobasidium monpa*. Current Microbiology. 44:148–152.

Anagnostakis SL. 1977. Vegetative Incompatibility in *Endothia parasitica*. Experimental Mycology. 1:306–316.

Anagnostakis SL. 1987. Chestnut Blight: The Classical Problem of an Introduced Pathogen. Mycologia. 79:3–37.

Anagnostakis SL. 1988: *Cryphonectria parasitica*: cause of chestnut blight. Advances in Plant Pathology. 6:123–136.

Anagnostakis SL. 1992. Chestnuts and the introduction of chestnut blight. Annual Report of the Northern Nut Growers Association. 83:39–42.

Anagnostakis SL, Hau B, and Kranz J. 1986. Diversity of Vegetative Compatibility Groups in *Cryphonectria parasitica* in Connecticut and Europe. Plant Disease. 70: 536–538.

Anagnostakis SL, Waggoner PE. 1981. Hypovirulence, Vegetative Incompatibility, and the Growth of Cankers of Chestnut Blight. Phytopathology. 71:1198–1202.

Anderson RM and May RM. 1982. Directly transmitted infectious diseases: control by vaccination. Science. 215:1053-1060.

Baxter DV and Strong FC. 1931. Chestnut blight in Michigan. Michigan Agricultural Experiment Station Circular Bulletin. 135:1-18.

Bayman P and Bennett JW. 1998. Nitrate-nonutilizing Mutants and Vegetative Self-incompatibility in *Cunninghamella Echinulata* var. *elegans*. Biotechnology Letters. 20:901–903.

Benkman CW. 1999. The Selection Mosaic and Diversifying Coevolution Between Crossbills and Lodgepole Pine. The American Naturalist. 153:S75—S91.

Benkman CW and Parchman TL. 2003. Reciprocal Selection Causes a Coevolutionary Arms Race Between Crossbills and Lodgepole Pine. The American Naturalist. 162:182–194.

Betterley DA and Collins OR. 1984. Vegetative incompatibility and myxomycete biology. Mycologia. 76:785–792.

Biraghi A. 1946. Il cancro del castagno causato da *Endothia parasitica*. Italia Agricola. 7:1–9.

Brewer LG. 1995. Ecology of Survival and Recovery from Blight in American Chestnut Trees (*Castanea dentata* (Marsh.) Borkh.) in Michigan. Bulletin of the Torrey Botanical Club. 122:40.

Bryner SF and Rigling D. 2011. Temperature-dependent Genotype-by-genotype Interaction Between a Pathogenic Fungus and Its Hyperparasitic Virus. The American Naturalist. 177:65–74.

Bryner SF and Rigling D. 2012. Virulence Not Only Costs but Also Benefits the Transmission of a Fungal Virus. *Evolution.* 66:2540–50.

Buck KW. 1986. Fungal Virology- an overview. Pages 1-84 in: Fungal Virology. K. W. Buck, ed. CRC Press, Boca Rotan, FL, U.S.A.

Burdon JJ. 1987. Diseases and plant population biology. Cambridge University Press, Cambridge.

Burdon JJ, Thrall PH, and Ericson L. 2006. The Current and Future Dynamics of Disease in Plant Communities. Annual Review of Phytopathology. 44:19–39.

Casselton LA and Economou A. 1985. Dikaryon formation. In Developmental biology of higher fungi (ed. D. Moore, L. A.Casselton, D. A. Wood & J. C. Frankland), pp. 213–229. Cambridge University Press.

Clapper RB. 1952. Relative blight resistance of some chestnut species and hybrids. Journal of Forestry. 50:453-455.

Clark J and Haskins EF. 2012. Plasmodial Incompatibility in the Myxomycetes: a Review. Mycosphere. 3:131–141.

Cobb RC, Filipe JN, Meentemeyer RK, Gilligan CA, and Rizzo DM. 2012. Ecosystem Transformation by Emerging Infectious Disease: Loss of Large Tanoak from California Forests. Journal of Ecology. 100:712–722.

Davelos AL and Jarosz AM. 2004. Demography of American chestnut populations: effects of a pathogen and a hyperparasite. Journal of Ecology 92:675-685.

Davelos AL. 1999. Double-stranded RNA mediated recovery of American chestnut populations: a demographic analysis. PhD thesis, Michigan State University, East Lansing, Michigan.

Dinus RJ. 1974. Knowledge about natural ecosystems as a guide to disease control in managed forests. Proceedings of the American Phytopathological Society. 1:184—190.

Dobson AP and Hudson PJ. 1986. Parasites, Disease and the Structure of Ecological Communities. Trends in Ecology & Evolution. 1:11–5.

Fairchild D. 1913. The discovery of the chestnut- bark disease in China. Science 38: 297-299.

Fenner F. 1983. Biological control as exemplified by small- pox eradication and myxomatosis. Proceedings of the Royal Society of London B 218:259-285

Fenner F and Radcliffe FN. 1965. 'Myxomatosis'. Cambridge University Press: Cambridge, UK.

Frey FM. 2004. Opposing Natural Selection from Herbivores and Pathogens May Maintain Floral-color Variation in *Claytonia virginica* (Portulacaceae). Evolution. 58:2426–37.

Fulbright DW, Weidlich WH, Haufler KZ, Thomas CS, and Paul CP. 1983. Chestnut Blight and Recovering American Chestnut Trees in Michigan. Canadian Journal of Botany. 61:3164–3171.

Fulbright DW and Paul CP, and Garrod SW. 1988. Hypovirulence: a Natural Control of Chestnut Blight *in* Biocontrol of Plant Diseases Volume II, Mukerji KG and Garg KL. Eds. CRC Press, Boca Raton, FL. pp.122–136.

Garnas J, Ayres M, Liebhold A, and Evans C. 2011. Subcontinental impacts of an invasive tree disease on forest structure & dynamics. Journal of Ecology. 99:532–541.

Gilbert GS. 2002. Evolutionary ecology of plant diseases in natural ecosystems. Annual Review of Phytopathology. 40: 13-43.

Glass NL and Kuldau GA. 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. Annual Review of Phytopathology. 30:201-224.

Gobbin D, Hoegger PJ, Heiniger U, and Rigling D. 2003. Sequence Variation and Evolution of Cryphonectria Hypovirus 1 (CHV-1) in Europe. Virus Research. 97:39–46.

Graves AH. 1950. Relative blight resistance in species and hybrids of Castanea. Phytopathology. 49:1125—1131.

Griffin DM and Perrin HN. 1960. Anastomosis in the Phycomycetes. Nature. 187:1039—1040.

Griffin GJ and Elkins JR. 1986. Chestnut blight. In: Chestnut Blight, Other Endothia Diseases, and the Genus Endothia. Ed. by Roane, MK, Griffin GJ, and Elkins JR. St Paul, MN: APS Press, pp. 1–26.

Griffin GJ, Smith HC, Dietz A, and Elkins JR. 1991. Importance of Hardwood Competition to American Chestnut Survival, Growth, and Blight Development in Forest Clearcuts. Canadian Journal of Botany. 69:1804–1809.

Griffin JM, Lovett GM, Arthur MA, and Weathers KC. 2003. The distribution and severity of beech bark disease in the Catskill Mountains, NY. Canadian Journal of Forest Research. 33: 1754–60.

Hanski I. 1999. Metapopulation ecology. Oxford: Oxford University Press.

Hanski I and Gilpin ME. (eds) 1997. Metapopulation Biology: Ecology, Genetics, and Evolution. Academic Press, San Diego, CA.

Harper JL. 1977. The Population Biology of Plants. London: Academic. 892 pp.

Heiniger U and Rigling D. 1994. Biological control of chestnut blight in Europe. Annual Review of Phytopathology. 32: 581.

Hillman BI, Tian Y, Bedker PJ, and Brown MP. 1992. A North American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA. Journal of General Virology. 73:681—686.

Hillman BI, Halpern BT, and Brown MP. 1994. A viral dsRNA element of the chestnut blight fungus with a distinct genetic organization. Virology. 201:241—250.

Hillman BI, Fulbright DW, Nuss DL, and Van Alfen NK. 1995. Hypoviridae, pp. 261–264. In Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MP, and Summers MD. (eds.), Sixth Report of the International Committee on the Taxonomy of Viruses. Springer-Verlag, New York, N.Y.

Hollings M. 1982. Mycoviruses and Plant Pathology. Plant Disease. 66:1106-1112.
Huber DH. 1996. Genetic analysis of vegetative incompatibility polymorphisms and horizontal transmission in the chestnut blight fungus *Cryphonectria parasitica*. PhD thesis. East Lansing, Michigan: Michigan State University.

Jarosz AM and Davelos AL. 1995. Effects of Disease in Wild Plant Populations and the Evolution of Pathogen Aggressiveness. New Phytologist. 129:371–387.

Jaynes R. 1975. Chestnut. In: Moore J (ed) Advances in fruit breeding. Purdue University Press, West Lafayette, Indiana. pp 490–503

Johnson NC, Graham JH, and Smith FA. 1997. Functioning of Mycorrhizal Associations Along the Mutualism-parasitism Continuum. New Phytologist. 135:575–585.

Johnson PTJ, Dobson A, Lafferty KD, Marcogliese DJ, Memmott J, Orlofske SA, Poulin R, and Thieltges DW. 2010. When Parasites Become Prey: Ecological and Epidemiological Significance of Eating Parasites. Trends in Ecology & Evolution 25:362–371.

Klironomos JN. 2003. Variation in Plant Response to Native and Exotic Arbuscular Mycorrhizal Fungi. Ecology. 84:2292–2301.

Koskella B, Thompson JN, Preston GM, and Buckling A. 2011. Local Biotic Environment Shapes the Spatial Scale of Bacteriophage Adaptation to Bacteria. The American Naturalist. 177:440–51.

Lang P, Dane F, and Kubisiak TL. 2006. Phylogeny of Castanea (Fagaceae) Based on Chloroplast trnT-L-F Sequence Data. Tree Genetics & Genomes. 2:132–139.

Lenski RE and May RM. 1994. The Evolution of Virulence in Parasites and Pathogens: Reconciliation Between Two Competing Hypotheses. Journal of Theoretical Biology. 169:253–265.

Leslie JF. 1993. Fungal vegetative compatibility. Annual Review of Phytopathology. 31:127–150.

Liesebach M and Zaspel I. 2004. Genetic Diversity of the Hyperparasite *Sphaerellopsis filum* on Melampsora Willow Rusts. Forest Pathology. 34:293–305.

Linder-Basso D, Dynek JN, and Hillman BI. 2005. Genome Analysis of Cryphonectria Hypovirus 4, the Most Common Hypovirus Species in North America. Virology. 337:192—203.

Little EL. 1971. Atlas of United States trees. Volume 1: Conifers and important hardwoods. USDA Miscellaneous Publication 1146, United States Department of Agriculture, Washington, D.C., USA. (Digital representation by U.S. Geological Survey. 1999.)

Liu YC and MG Milgroom. 1996. Correlation Between Hypovirus Transmission and the Number of Vegetative Incompatibility (vic) Genes Different Among Isolates from a Natural Population of *Cryphonectria parasitica*. Phytopathology. 86:79–86.

Loo J. 2009. Ecological Impacts of Non-indigenous Invasive Fungi as Forest Pathogens. Biological Invasions. 11:81–96.

Lovett GM, Canham CD, Arthur MA, Weathers KC, Fitzhugh RD. 2006. Forest ecosystem responses to exotic pests and pathogens in Eastern North America. Bioscience 56: 395–405.

MacDonald WL and DW Fulbright. 1991. Biological Control of Chestnut Blight: Use and Limitations of Transmissible Hypovirulence. Plant Disease. 75:656–661.

May R and Anderson RM. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. Proceedings of the Royal Society of Biology B. 219:281–313.

McCormick JF and Platt RB. 1980. Recovery of an Appalachian Forest Following the Chestnut Blight or Catherine Keever-You Were Right! American Midland Naturalist. 104:264–273.

Melzer MS and Boland GJ. 1999. CHV3-type dsRNAs and the GH2 Genotype in a Population of *Cryphonectria parasitica* in Ontario. Canadian Journal of Plant Pathology. 21:248—255.

Merkel HW. 1905. A deadly fungus on the American chestnut. Pages 97-103 in: Annual Report of the New York Zoological Society, 10th. New York Zoological Society, Bronx, NY.

Milgroom MG, MacDonald WL, and Double ML. 1991. Spatial Pattern Analysis of Vegetative Compatibility Groups in the Chestnut Blight Fungus, *Cryphonectria parasitica*. Canadian Journal of Botany. 69:1407–1413.

Milgroom MG and Cortesi P. 1999. Analysis of Population Structure of the Chestnut Blight Fungus Based on Vegetative Incompatibility Genotypes. *PNAS.* 96:10518–23.

Milgroom MG and Paolo Cortesi. 2004. Biological Control of Chestnut Blight with Hypovirulence: a Critical Analysis. Annual Review of Phytopathology. 42:311–38.

Monahan WB and Koenig WD. 2006. Estimating the Potential Effects of Sudden Oak Death on Oak-dependent Birds. Biological Conservation. 127:146–157.

Mordecai, E. 2011. Pathogen Impacts on Plant Communities: Unifying Theory, Concepts, and Empirical Work. Ecological Monographs. 81:429–441.

Nuss DL. 1992. Biological Control of Chestnut Blight: An Example of Virus-Mediated Attenuation of Fungal Pathogenesis. Biological Control. 56:561–576.

Nuss DL. 2005. Hypovirulence: mycoviruses at the fungal-plant interface. Nature Reviews in Microbiology. 3:632–642.

Nuss DL and Koltin Y. 1990. Significance of dsRNA Genetic Elements in Plant Pathogenic Fungi. Annual Review of Phytopathology. 28:37–58.

Oh CS and Hillman BI. 1995. Genome Organization of a Partitivirus from the Filamentous Ascomycete *Atkinsonella Hypoxylon*. Journal of General Virology. 76:1461–1470.

Paillet FL. 2002. Chestnut, history &ecology of a transformed species. Journal of Biogeography. 29:1517–1530.

Paul CP and DW Fulbright. 1988. Double-stranded RNA Molecules from Michigan Hypovirulent Isolates of *Endothia parasitica* Vary in Size and Sequence Homology. Phytopathology. 78:751–755.

Peever TL Liu YC, and Milgroom MG. 1997. Diversity of Hypoviruses and Other Double-Stranded RNAs in *Cryphonectria parasitica* in North America. Phytopathology. 87:1026-33.

Peever TL, Liu YC, Wang K, Hillman BI, Foglia R, and Milgroom MG. 1998. Incidence and Diversity of Double-Stranded RNAs Occurring in the Chestnut Blight Fungus, *Cryphonectria parasitica*, in China and Japan. Phytopathology 88:811-7.

Price PW. 1980. The evolutionary biology of parasites. Princeton University Press, Princeton, N.J.

Prospero S, and Rigling D. 2012. Invasion Genetics of the Chestnut Blight Fungus Cryphonectria Parasitica in Switzerland. Phytopathology. 102:73–82.

Rayner ADM. 1991. The Phytopathological Significance of Mycelial Individualism. Annual Review of Phytopathology. 29:305–323.

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, and Henson JM. 2002. Thermotolerance generated by plant/fungal symbiosis. Science. 298:1581.

Robin C, and Heiniger U. 2001. Chestnut Blight in Europe : Diversity of *Cryphonectria parasitica*, Hypovirulence and Biocontrol. Forest Snow and Landscape Research. 367: 361–367.

Roossinck MJ. 2011. The Good Viruses: Viral Mutualistic Symbioses. Nature Reviews. Microbiology. 9:99–108.

Singh LP, Gill SS, and Tuteja N. 2011. Unraveling the Role of Fungal Symbionts in Plant Abiotic Stress Tolerance. Plant Signaling & Behavior. 6:175–91.

Smart CD, Yuan W, Foglia R, Nuss DL, Fulbright DW, and Hillman BI. 1999. Cryphonectria Hypovirus 3, a Virus Species in the Family Hypoviridae with a Single Open Reading Frame. Virology. 265:66—73.

Smith DL, Ericson L, Burdon JJ. 2003. Epidemiological patterns at multiple spatial scales; an 11-year study of a *Triphragmium ulmariae—Filipendula ulmaria* metapopulation. Journal of Ecology. 91:890–903

Sullivan D. 1987. Insect Hyperparasitism. Annual Review of Entomology. 32:49–70.

Suzuki N, Maruyama K, Moriyama M, and Nuss DL. 2003. Hypovirus Papain-Like Protease P29 Functions in Trans To Enhance Viral Double-Stranded RNA Accumulation and Vertical Transmission. Journal of Virology. 77:11697–11707.

Swinton J and Gilligan C.A. 1996. Dutch elm disease and the future of the elm in the U.K., a quantitative analysis. Philosophical Transactions of the Royal Society London Series B. 351:605–615.

Tanaka S, Nishida T, and Ohsaki N. 2007. Sequential Rapid Adaptation of Indigenous Parasitoid Wasps to the Invasive Butterfly Pieris Brassicae. Evolution. 61: 1791–802.

Taylor, DR, Jarosz AM, Fulbright DW, and Lenski RE. 1998. The Acquisition of Hypovirulence in Host-pathogen Systems with Three Trophic Levels. The American Naturalist. 151: 343–355.

Thompson JN. 1999. Specific Hypotheses on the Geographic Mosaic of Coevolution. The American Naturalist. 153:S1–S14.

Thompson JN. 2005. The Geographic Mosaic of Coevolution. University of Chicago Press, Chicago, IL.

Thompson JN and Pellmyr O. 1992. Mutualism with pollinating seed parasites amid co-pollinators: constraints on specialization. Ecology. 73:1780–1791.

Turchetti T. 1978: Some observation on the ‘‘Hypovirulence’’ of chestnut blight in Italy. In: Proceedings of the American Chestnut Symposium. Ed. by MacDonald, W. L.; Cech, F. C.; Luchok, J.; Smith, C. Morgantown: WV University, pp. 92–94.

Vitousek PM, D’Antonio CM, Loope LL, Westbrooks R. 1996. Biological invasions as global environmental change. American Scientist. 84:468–478

Vitousek PM, D’Antonio CM, Loope LL, Rejmanek M, Westbrooks R (1997) Introduced species: a significant component of human caused global change. New Zealand Journal of Ecology. 21:1–16

Weste G. 1998. Vegetation changes as a result of invasion of forest on Krasnozem by *Phytophthora cinnamomi*. Australian Journal of Botany. 28:139—150.

Weste G. and Marks GC. 1987. The Biology of Phytophthora cinnamomi in Australian Forests. Annual Review of Phytopathology. 25:207—229.

CHAPTER 2

HYPERPARASITES INFLUENCE POPULATION STRUCTURE OF THE CHESTNUT BLIGHT PATHOGEN, CRYPHONECTRIA PARASITICA

ABSTRACT

Vegetative compatibility (VC) is commonly used to characterize structure and diversity in fungal populations. In the chestnut blight fungus, *Cryphonectria parasitica*, high VC diversity is hypothesized to be responsible for the failure of hyperparasitic mycoviruses to spread through pathogen populations in North America. To test this hypothesis, we assessed VC diversity at three recovering sites in Michigan where mycoviruses had invaded and compared them with four epidemic populations where mycoviruses were absent. VC diversity was assessed for samples collected in 1996 and 2009, which allowed us to determine how *C. parasitica* populations changed with time. Twelve VC types were found in 1996 while thirty were found in 2009; 75% of types overlapped between the sample dates. Sites where mycoviruses were present had unique VC structures with the exception of the recovering population site at County Line where the main VC group was also detected at two epidemic sites. *Cryphonectria parasitica* populations at epidemic sites usually had more VC groups and were more diverse. Mating type analyses of blight populations revealed that two of three recovering populations were significantly skewed for *MAT*2 suggesting asexual reproduction, while epidemic sites with a long history of blight infection had ratios near

50:50 suggesting sexual reproduction. We propose that selection in the largely asexual *C. parasitica* populations at two recovering sites favors the most-fit fungal genotype by mycovirus combination and results in reduced diversity relative to the sexually reproducing pathogen populations at epidemic sites.

INTRODUCTION

Introduced pathogens have a long history of causing extensive destruction to their plant hosts with trees being particularly vulnerable. For example, Dutch elm disease caused by *Ophiostoma ulmi* and *O. novo-ulmi* has changed the urban landscape in the United States and Europe by killing highly susceptible elms planted as shade trees (Brasier 1995, 2001; Potter et al. 2011; Santini et al. 2012). American chestnuts (*Castanea dentata* L.) have suffered a similar fate in North America. Prior to the 1900s, *C. dentata* composed nearly 25% of the canopy in eastern hardwood forests, but these natural populations were decimated when *Cryphonectria parasitica* Murrill (Barr.), the cause of chestnut blight, was accidentally introduced to the United States in the early twentieth century near New York City (Merkel 1906). *Cryphonectria parasitica* enters wounds on the trunk and branches of susceptible trees and causes a canker that expands in size until the branch or trunk is girdled, killing all plant material distal to the canker. Trees accumulate multiple infections during an epidemic and eventually all aboveground portions of the tree are killed. However, *C. parasitica* cannot enter the roots of infected trees and chestnuts can survive for considerable time as a live root system that periodically produces root collar sprouts. These sprouts grow for several seasons before being infected and killed by *C. parasitica*. Consequently, *C. dentata* is still

a common, persistent, understory tree in several areas (Anagnostakis 2001; Griffin & Elkins 1986; McCormick & Platt 1980).

Cryphonectria parasitica was also introduced in Europe in the 1930s and the initial stages of the European blight epidemic were similar to that in North America. However, European chestnuts (*Castanea sativa*) began recovering by the 1950s (Heiniger & Rigling 1994). Canker expansion on recovering trees was contained by wound callus tissue, which prevented girdling of the trunk and allowed infected trees to continue growing. Recovery was not due to resistance in European chestnuts (although their reduced susceptibility relative to American chestnuts may play a role), but was caused by reduced pathogen virulence induced by the presence of a double-stranded RNA mycovirus hyperparasite (Grente 1965; MacDonald & Fulbright 1991; Milgroom & Cortesi 2004). Mycoviruses reduce pathogen virulence by reducing fungal growth, decreasing conidia production, and inhibiting sexual reproduction (i.e., ascospore production) and therefore can act as a biological control mechanism against the blight pathogen (Anagnostakis 1987; Carbone et al. 2004; Elliston 1985; McCormick & Platt 1980). Over time, mycoviruses spread to many areas in Europe and are thought to be responsible for the recovery of *C. sativa*. With the exception of some isolated populations in Michigan, mycoviruses are relatively rare in North America and efforts to introduce them as biological control have been largely unsuccessful (Milgroom & Cortesi 2004).

Considerable speculation on why mycoviruses have not invaded North American populations of *C. parasitica* has centered on the higher diversity of vegetative compatibility (VC) groups in North America relative to Europe (Anagnostakis et al.

1986). VC groups are a consequence of a self/non-self recognition system found in many ascomycetes that are controlled by several unlinked vegetative incompatibility (*vic*) or heterokaryon incompatibility (*het*) loci (Glass et al. 2000). The incompatibility loci operate to detect non-self hyphae when the hyphal tips of adjacent colonies fuse (anastomosis). Mismatches at any incompatibility locus trigger apoptosis (programmed cell death) causing the connection between mismatched hyphae to die (for review, see Glass & Kaneko 2003). Apoptosis prevents alien nuclei from invading the fungal colony and reduces the transfer of cytoplasmic elements such as mycoviruses (Anagnostakis 2001; Bertrand 2000; Huber 1996; Liu & Milgroom 1996; MacDonald & Fulbright 1991; Milgroom & Cortesi 1999). Alleles at *vic* loci are thought to be under negative frequency dependent or balancing selection which maximizes the ability of individuals to detect non-self hyphae upon fusion (Glass & Kaneko 2003; Micali & Smith 2006; Mir-Rashed et al. 2000; Wu et al. 1998) and promotes high levels of diversity for VC groups within fungal populations (see Table 1 of Nauta & Hoekstra 1994). However, theoretical work by Taylor et al. (1998) and Morozov et al. (2007) suggest that the *vic* incompatibility system may not be a complete barrier to mycovirus invasion since mismatches at one or two *vic* loci reduces but does not entirely prevent transmission of mycoviruses between fungal colonies (Liu & Milgroom 1996).

Here we address a different aspect of the interaction between the chestnut blight pathogen and its mycoviruses by comparing seven *C. parasitica* populations in Michigan to determine how invasion by mycoviruses influences the VC structure of the pathogen. Mycoviruses have been present for an extended period of time (since at least the early 1980s) at three of our study sites: County Line, Frankfort and Roscommon (Brewer

1995; Davelos 1999; Fulbright et al. 1983), while they are largely absent at the other four sites (Davelos 1999). In 1996 when this study was initiated, greater than 90% of all *C. parasitica* samples from the three recovering sites contained mycovirus (Davelos 1999). Mycoviruses have a substantial positive influence on chestnut trees at the three sites by increasing tree survivorship, growth and reproduction resulting in a general "recovery" of these chestnut populations (hereafter recovering sites). Indeed, the population demographics at two of the recovering sites are similar to those of disease-free chestnut populations in Michigan (Davelos & Jarosz 2004). In contrast, trees at the four sites without mycoviruses have demographics similar to what is found in the main range of the American chestnut where all large trees are dying back as a result of the *C. parasitica* epidemics (hereafter epidemic sites).

What is not known is the extent to which invasions by mycoviruses influence the population structure of the *C. parasitica* pathogen. Mycoviruses would be expected to alter the population genetics of *C. parasitica* since they inhibit the pathogen's sexual cycle (Gobbin et al. 2003). The lack of a sexual cycle in mycovirus-infected individuals of *C. parasitica* would be expected to have multiple influences on pathogen population structure: 1. Diversity of VC groups should decrease because genetic recombination through sexual reproduction does not occur on a regular basis, 2. Selection will operate at the whole-genome level in asexual populations of the fungal pathogen because of the lack of recombination, 3. Dispersal of the fungal pathogen should be altered because the wind-dispersed sexual ascospores are not produced and the splash-dispersed asexual spores (*i.e.*, conidia) have a more limited dispersal distance.

Our objectives were to compare the VC structure of *C. parasitica* found at the three

recovering populations with that of the four epidemic populations to determine how mycoviruses influence VC structure over time. Additionally, we investigated the temporal pattern at five sites to determine if the pattern of variability differed between recovering and epidemic populations over the time period 1996 to 2009.

MATERIALS AND METHODS

Chestnut blight cankers were sampled from five blighted American chestnut populations in 1996 and seven in 2009. Thirty haphazard samples from each population were collected in 2009 while the number of samples used for comparison from 1996 was variable since we were unable to resurrect some samples from storage (see Chapter 5). Three of the blighted tree populations, County Line (CL), Roscommon (RC), and Frankfort (FR) have been invaded naturally by double-stranded RNA mycoviruses and the American chestnut trees at those sites are recovering from the blight epidemic (Davelos & Jarosz 2004; AM Jarosz and JC Springer, unpublished data). However, in recent years, many trees at the Frankfort site have died due to blight infections (AM Jarosz and JC Springer, unpublished data). The four remaining sites, Stivers (ST) and Missaukee Diseased (MD) Missaukee Healthy (MH) and Leelanau (LE), are experiencing blight epidemics that have severely debilitated the chestnut trees; mycoviruses have not been found at these sites at a level higher than 5% of cankers sampled (Davelos 1999; JC Springer, unpublished data). Population location coordinates can be found in Davelos and Jarosz (2004) for all sites except Roscommon. The Roscommon site is located at 44º 31' 28"N, -84º 30' 48"W (see Figs. 1 & 2 for general locations). Blight was first found at MH and LE in 1997; samples were obtained from

these sites only in 2009. Samples from 1996 were resurrected from sterile water storage vials inoculated with mycelial plugs; 200µl of water from each vial were plated onto potato dextrose agar (PDA) petri plates (Difco, Becton, Dickinson and Company; Sparks, MD). For 2009 samples, 2mm x 2mm subsamples of each bark collection were surface sterilized in 10% sodium hypochlorite solution for 20 seconds, rinsed in double distilled H_20 twice for 20 seconds; clean isolates were plated onto PDA.

Since the presence of mycoviruses can obscure VC testing, (DW Fulbright, pers. obs.) mycovirus-free isolates were obtained from all samples before testing for vegetative compatibility. Since, transmission of mycovirus to conidia is rarely 100% (Enebak et al. 1994; Wu et al. 1998) mycovirus-free isolates were obtained by plating serially diluted single conidia onto PDA plates and an isolate with a fast-growing virulent phenotype was chosen. Vegetative compatibility tests were performed on de-barked chestnut stem pieces embedded in PDA. Chestnut pieces were approximately one centimeter long, cut longitudinally and placed in standard 100 mm × 15 mm petri plates (Medegen; Gallaway, TN) according to Huber (1996). Isolates were allowed to grow across the chestnut stem pieces for approximately two weeks before compatibility reactions were scored as either compatible or incompatible using evidence of a solid mat of mycelia or barrage formation between isolates, respectively (Fig. 3). An internal control of two isolates from the same canker was included on each plate. Fresh isolates of similar age (usually less than one week old) were used for testing since they exhibited rapid, consistent growth on the petri plate. VC types were determined visually, since the formation of barrage lines is sufficient to determine that cultures differ for VC group.

For mating type analysis, isolates were grown on PDA plates overlaid with sterilized cellophane. After approximately two weeks, fungal mats were scraped off of the cellophane and dried at room temperature overnight. Isolates were ground to a fine powder in mortars with liquid nitrogen. DNA extractions were performed with 25—30mg of powdered fungus with a Qiagen plant mini kit (Qiagen corporation, Valencia, CA) according to manufacturer directions. Primer sequences for *MAT* 1 and *MAT* 2 and thermocycler protocol follow previous work of (Marra & Milgroom 2001). GoTaq Green master mix (Promega corporation, Madison, WI) was used according to manufacturer directions in place of separate PCR components. PCR products (5uL subsample) were run on a 1% agarose gel for 35 minutes at 90v and visualized under UV light and scored according to migration distance on the gel (Marra & Milgroom 2001). Chi-square tests

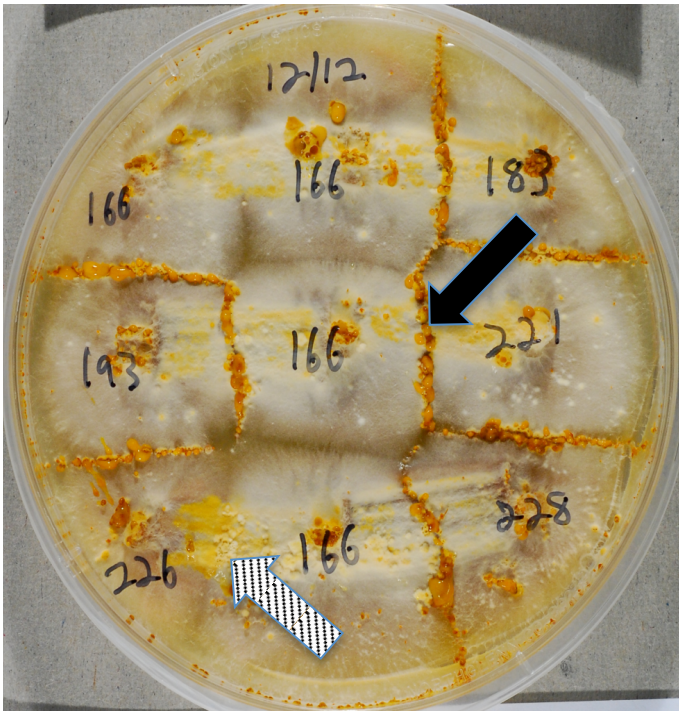

Figure 2-1. Vegetative compatibility testing on chestnut stem pieces. Compatible reaction (dashed arrow) between sample 226 and 166, incompatible reaction (solid arrow) between samples 166 and 221.

were performed on the frequencies of the two *MAT* alleles in each site to determine if populations diverged from the 50:50 ratio expected if the pathogen population was sexually reproducing.

DATA ANALYSIS

The lack of a sexual cycle in mycovirus-infected individuals of *C. parasitica* would be expected to have multiple influences on pathogen population structure: 1. Diversity of VC groups should decrease because genetic recombination does not occur on a regular basis, 2. Selection will operate at the whole-genome level in asexual populations of the fungal pathogen because of the lack of recombination, 3. Dispersal of the fungal pathogen should be altered because the wind-dispersed sexual ascospores are not produced and the splash-dispersed asexual spores (*i.e.*, conidia) have a more limited dispersal distance.

To specifically address these predictions, we asked the following questions: 1. Do populations with mycovirus have lower VC diversity than populations without mycovirus? 2. Does VC diversity decrease over time in populations with mycovirus? If yes, this result would be consistent with selection for a most fit fungus-mycovirus combination. 3. Are population structures similar in populations that are spatially close? If yes, this pattern would be consistent with limited dispersal of spores.

To address the first two questions, diversity measures were calculated. Alpha diversity, used here as population level VC richness, was calculated as the total number of VC groups within a population. Strong weighting to dominant members of the community

of interest is given by Simpson's index (Hill et al. 2003). Simpson's diversity index is based on the formula:

$D_S = 1-[\sum n_i(n_i-1)/N(N-1)]$,

where n_i is the number of isolates in a VC group and N is the total number of isolates. A moderate weighting to rare and intermediate species in comparison to dominant species is provided by the Shannon index. Thus, the Shannon index is more sensitive to changes in abundance of rare groups in comparison to other diversity indices (Hill et al. 2003). The Shannon diversity index is based on the formula:

$H = -\sum x_i/x_o)\ln(x_i/x_o)$,

where x_i is the number of isolates in a vc group and x_o is the total number of isolates. Diversity indices and standard deviations for epidemic and recovering populations were calculated using EstimateS (Colwell 2005).

Morisita's index of dispersion measures overlap of VC types among samples or populations (Magurran 1988) and can be thought of as a probability. Index values are generated using the formula:

$C_D = 2\sum^{S}{}_{i=1}\, x_i y_i/(D_x + D_y)\, XY)$

Where x_i represents how many instances species *i* is in the total X in one population, y_i how many instances VC group *i* is in the total Y from another population. *Dx* and *Dy* are replaced by Simpson's values according to population *x* and *y* and S is the number of species unique in a population. Morisita's index values range from 0 (no similarity between populations) to 1 (total similarity). The matrices of Morisita's index values and physical distances (in kilometers) for the seven populations were compared with

each other to determine if they were correlated using Mantel's test (Rohlf 1998). A positive correlation indicates that populations that are spatially close have similar VC population structures. The significance of the correlation was determined as described in Lapointe and Legendre (1992).

RESULTS

Vegetative compatibility group diversity

Twelve VC groups were detected among the five populations sampled in 1996 while 29 VC groups were found among seven populations in 2009. 75% (9 of 12) of the VC groups found in 1996 were detected again in 2009. The VC diversity and structure of *C. parasitica* populations was strongly influenced by the presence of mycovirus. Recovering sites, where mycoviruses are present, averaged 2.7 and 3.7 VC groups per site in 1996 and 2009, respectively. The increase in the number of VC groups at recovering sites in 2009 was due entirely to an increase in the number of VC groups at FR where the number of VC groups increased from four to eight, (Figs. 1 and 2). Further, the frequency of the 2 dominant VC groups at FR changed dramatically between the sampling dates; the most common VC group in 1996 was found at almost half the frequency in 2009 (80% versus 43%) and the second most common VC group in 1996 more than doubled in frequency in 2009 (13% versus 30%) (Figs. 1 and 2). CL and RC were both dominated by a single VC group in 1996, which remained dominant in 2009 (Figs. 1 and 2). However, the most striking feature of each recovering population was that all VC groups within a site were unique to that particular site (Figs. 1 and 2) aside from the main group at County Line (MI-5), which was also found at two of the epidemic

sites, MD and LE.

In contrast with the pattern in recovering populations, five out of the thirty different VC groups (17%) were found in multiple epidemic populations. Group MI-2, was detected at all four epidemic sites in 2009, while MI-1 and MI-5 were found at three sites and MI-3 and MI-4 were found at two sites (Figs. 1 and 2). Despite these shared VC groups, the overall pattern of VC diversity was not correlated with physical distance between populations ($r = 0.29$, $P < 0.25$). The lack of correlation is especially curious when considering two epidemic sites, MH and MD; the sites are separated by less than a kilometer but shared only a single VC group, MI-2 (Fig. 2). We have monitored the trees at MD and MH since 1996 (16, AM Jarosz and JC Springer, unpublished data) and the first infected tree at MH was detected in 1997. In the fifteen years since the first infection at MH VC diversity has increased to a level that is comparable with the MD site that has been infected since the late 1980s (DW Fulbright, pers. obs.). Infected trees were also noted in 1997 at LE and by 2009 the number of VC groups and overall VC diversity was similar to that found at MD and MH, sites that are in the main area of Michigan where chestnut blight is found. Generally, epidemic sites had more VC groups than recovering sites, 6.5 in 1996 and 6.75 in 2009 and, although not statistically significant, trends indicated higher overall diversity for VC groups in epidemic sites (Table 1). The number of VC groups increased from 9 to 11 at ST from 1996 to 2009, while the number of VC groups remained constant at MD.

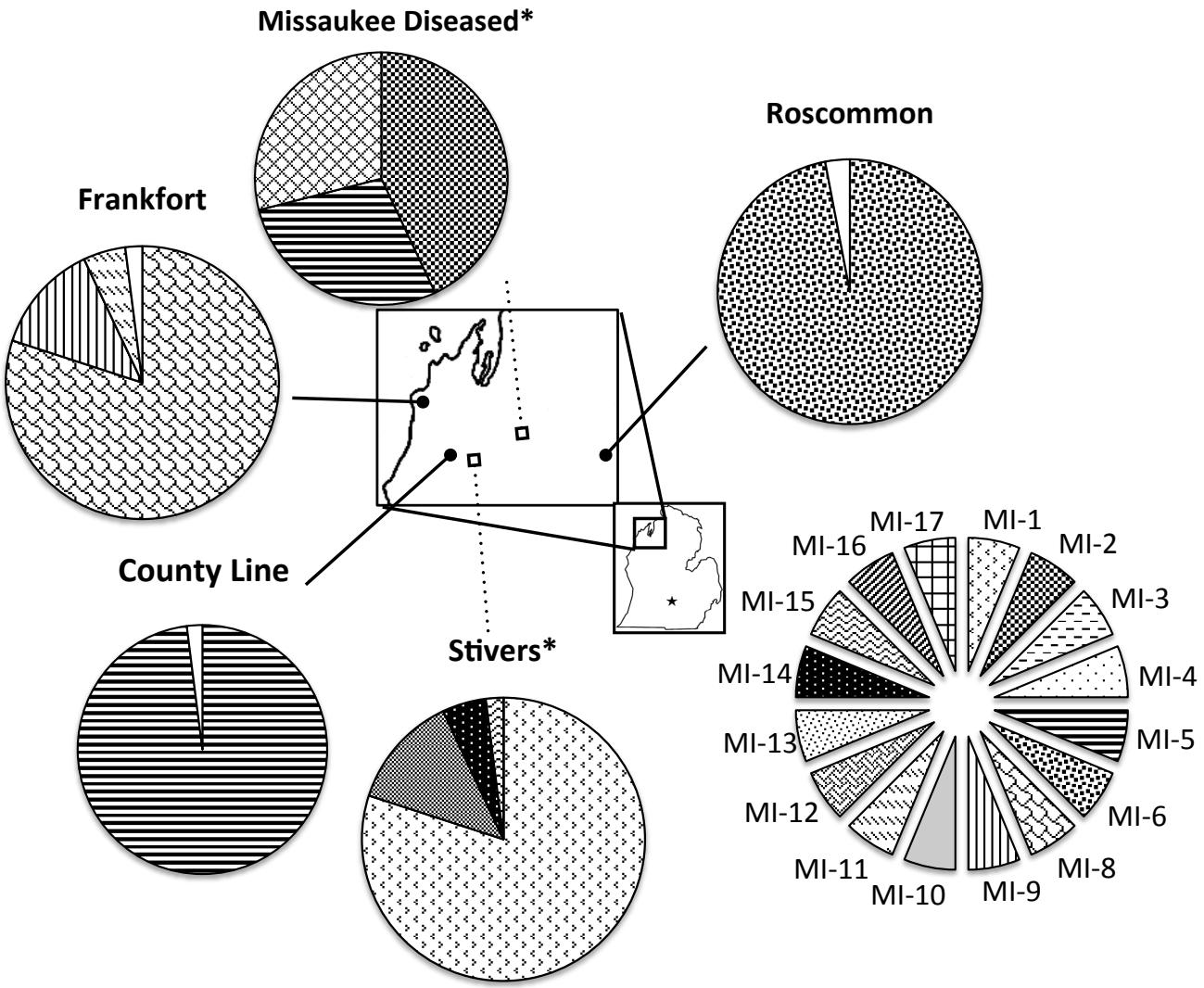

Figure 2-2. Vegetative Compatibility diversity for the year 1996 at five Michigan populations. Sizes of slices represent

Figure 2-2(cont'd). proportion of each VC type found at each site. A key to Michigan VC types is provided. Rare types at recovering and epidemic populations are represented by white and black slices, respectively, and in some cases more than one type is represented by the slice. *1996 Data for Stivers and Missaukee Diseased represent only those samples that could be resurrected from storage; see text for details.

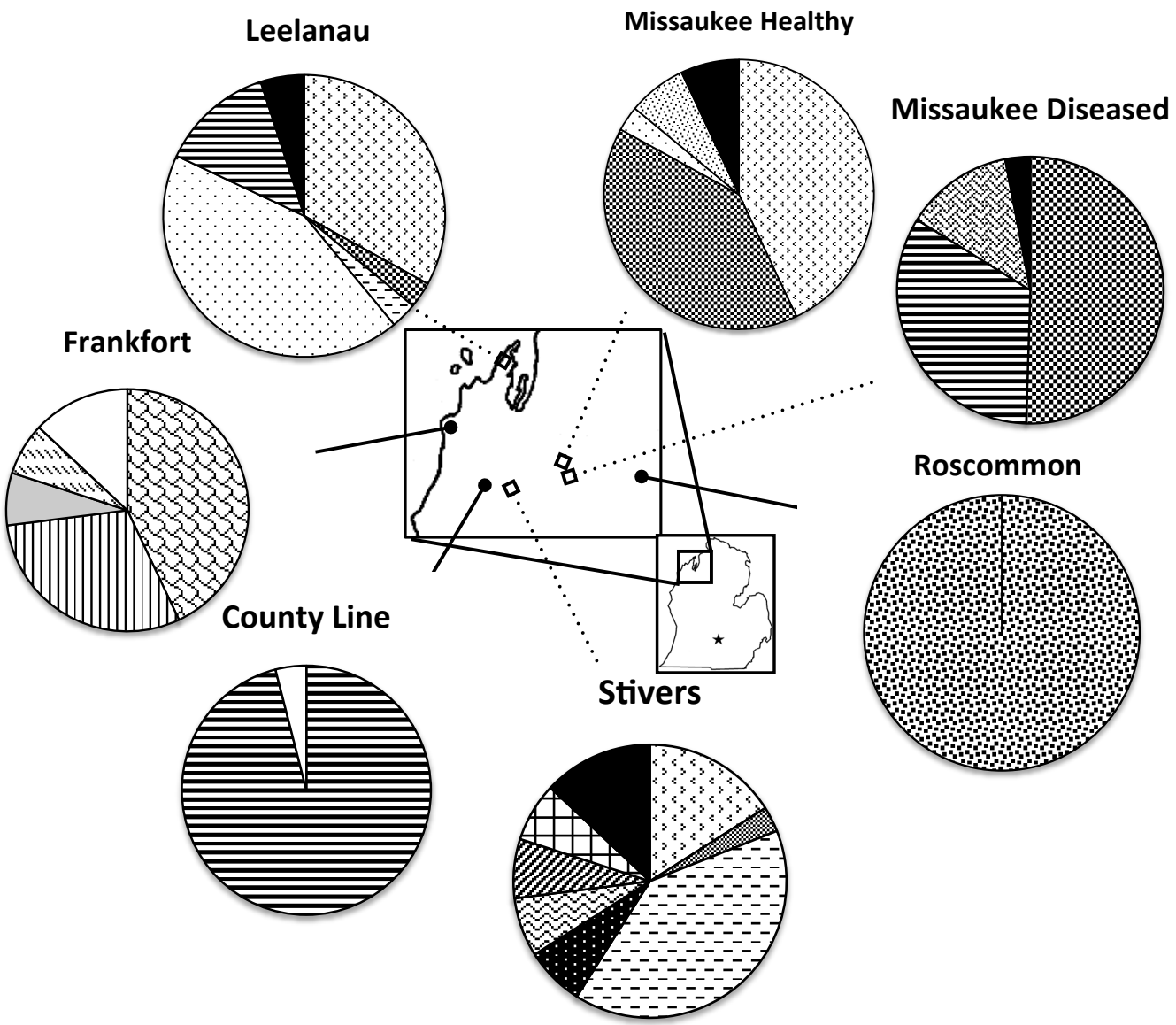

Figure 2-3. Vegetative Compatibility diversity data for the year 2009 at seven Michigan populations. Rare types at

Figure 2-3 (cont'd). recovering and epidemic populations are represented by white and black slices, respectively. Recovering sites are denoted with leader lines ending in solid circles, epidemic sites with solid squares, respectively.

Table 2-1. Summary diversity statistics for Epidemic and Recovering populations. VC richness represents the total time period 1996 to 2009.

Site Type	Total number of VC groups	Simpson± 2SE	Shannon ± 2SE	Maximum Shannon (% of max)
Epidemic (LE, MH, ST, MD)	19	5.67 ± 1.03	1.99 ± 0.36	2.94 (67.6)
Recovering (RC, CL, FR)	11	3.04 ± 0.65	1.25 ± 0.27	2.39 (52.1)

Mating type allele frequencies

Two recovering populations, CL and RC, had mating type allele frequencies that were highly skewed toward the *MAT*2 allele in both 1996 and 2009 (Table 2). These two populations deviated significantly from the expectation of a 50:50 mating type ratio that would be expected if these *C. parasitica* populations were regularly going through a sexual cycle. The third recovering population, FR, was skewed towards the *MAT*2 allele but frequencies were not as extreme as found at CL and RC, and allele frequencies did not deviate significantly from 50:50 in either 1996 or 2009. As explained below, we suspect that the *C. parasitica* population at FR is gradually escaping mycovirus infection. The mating type allele frequencies at the epidemic populations that were infected before 1980 (ST and MD) did not differ significantly from a 50:50 mating type ratio in either 1996 or 2009. Ratios were closer to 50:50 in 2009 at both sites. Contrary to recovering populations, ST and MD displayed a slight, but persistent skew towards *MAT*1 at both sampling periods. Mating type ratios deviated significantly from 50:50 at MH and LE in 2009. Trees at the MH and LE sites were disease-free in 1996 and *C. parasitica* was first noted at both sites in 1997. Despite the fact that both MH and LE

deviate from a 50:50 mating type ratio, MH was skewed toward *MAT*1, while the *C. parasitica* population at LE was skewed toward *MAT*2. The excess of *MAT*1 at MH could possibly be due to its juxtaposition to MD, which is only 300M away from MH and displayed a slight excess of *MAT*1 in both 1996 and 2009.

Table 2-2. Distribution of mating types within *Cryphonectria parasitica* populations in Michigan. Mixed indicates number of samples in which both mating types were present. The chi-square (X^2) value tests whether the population is at the 50:50 mating frequency expected if sexual recombination is occurring regularly.

	1996				2009			
Population	*MAT*1	*MAT*2	Mixed	X^2	*MAT*1	*MAT*2	Mixed	X^2
Recovering								
CL	2	12	1	7.14 *[a]	3	18	1	10.71*
RC	4	15	0	6.37 *	5	21	0	9.85 *
FR	9	15	3	1.50 n.s	12	17	3	0.86 n.s.
Epidemic								
ST	10	5	0	1.67n.s.	15	13	1	0.14 n.s.
MD	11	4	0	3.27 n.s.	15	11	7	0.62 n.s.
MH	---[b]	---	---	---	20	8	1	5.14 *
LE	---	---	---	---	9	20	2	4.17 *

a: Critical chi-square for p =0.05 is 3.84, with one degree of freedom=1.
b: MH and LE were blight-free in 1996

DISCUSSION

Vegetative incompatibility systems in fungi are thought to operate as self/non-self recognition systems that function to thwart the invasion of alien nuclei and mitochondria as well as parasitic elements (e.g., mycoviruses) that inhabit the cytoplasm (Anagnostakis 1977; Nauta & Hoekstra 1994; Lawrence et al. 1988; Rayner 1991; Bertrand 2000; Glass & Kaneko 2003; Glass & Dementhon 2006; Burdon & Thrall 2008; Roper et al. 2011). To minimize the probability of invasion by alien entities, negative frequency-dependent selection should operate to maximize variability at loci controlling self-incompatibility within fungal populations (Nauta & Hoekstra 1994; Milgroom & Cortesi 1999; Brusini et al. 2011). If this hypothesis is correct then we expect the number of VC groups within a population to increase over time and overall VC diversity should also increase. High VC diversity is expected to slow or even stop the spread of mycoviruses within a fungal population.

Patterns of VC diversity in *C. parasitica* have been used to explain the success or failure of mycovirus mediated recovery in European and American chestnut. Mycovirus invasion has been common in Europe where VC diversity is low (Milgroom & Cortesi 2004), perhaps due to low diversity among the founders of European populations. For example, VC type EU-1 is found at high frequency in *C. parasitica* populations on the Black Sea coast of Turkey (Akilli et al. 2009), while EU-2, and EU-5 are common in northern Italy, southern France, Switzerland, and eastern Spain (Robin & Heiniger 2001). Another VC type, EU-12, is dominant in Bulgaria, Sicily, Romania, southern Italy, and, Greece comprising 82% of isolates collected while VC type EU-1 is present at low frequency, around 1%. Similar situations can be found elsewhere in Europe where it

appears that a few founders at a site results in the proliferation of one to three groups (Sotirovski et al. 2006). In contrast to the successful spread of mycoviruses in Europe, the phenomenon is rare in North America; the only known natural invasions occurring in Michigan (Fulbright et al. 1983; Peever et al. 1997; Milgroom & Cortesi 2004;). Several authors have hypothesized that the inability of mycoviruses to gain a foothold in North American populations is due to the relatively high VC diversity (Anagnostakis 1986; Cortesi et al. 2001; Heiniger & Rigling 1994; Milgroom & Cortesi 2004; Nuss 1992; Robin et al. 2000). Our results are superficially consistent with this this hypothesis. There was a tendency for a greater number of VC groups and higher VC diversity within *C. parasitica* populations where mycoviruses were absent, although the values were not statistically significant (Table 1). In addition, mating type allele frequencies suggested that the pathogen populations at the two epidemic populations with a long history of infection (MD and ST) were sexually reproducing. However, if strong negative frequency dependent selection was operating, the number VC groups and evenness of their distribution should increase over time. This was not the case at the ST and MD epidemic sites where the number of VC groups increased slightly at ST and not at all at MD (Figs 1 and 2). Further, VC diversity actually declined at both ST and MD between 1996 and 2009. We suggest an alternate hypothesis in which mycovirus invasion actually leads to a reduction in *C. parasitica* VC diversity over time. In the absence of mycovirus, *C. parasitica* would be expected to regularly undergo sexual reproduction resulting in recombining of genes controlling vegetative compatibility. However, mycoviruses are known to inhibit sexual reproduction in *C. parasitica* (Anagnostakis 1987; Griffin & Elkins 1986; Milgroom & Cortesi 2004). Work

over the past 25 years with mycoviruses found at the CL site and another mycovirus found at Grand Haven, Michigan confirm that the CHV3 mycoviruses do reduce pathogen growth, conidia production, and largely inhibit sexual reproduction (D. Fulbright and ML Double; personal observation). Further, these CHV3 mycoviruses are transmitted by hyphal anastomosis in a manner similar to that of CHV1 mycoviruses from Europe (Huber 1996). The persistent and significant skewing of mating type allele frequencies at CL and RC in 1996 and 2009 also suggest that the *C. parasitica* populations at these two sites are not reproducing via sexual reproduction (Table 2). This trend is important because selection at these two recovering sites would then operating on asexual populations in which the most fit pathogen multilocus genotype by mycovirus combination would be favored. In consequence, the number of VC groups should decline over time due to the combined processes of selection and genetic drift. We also suggest that the process is reversible and mycoviruses can be lost from *C. parasitica* populations when conditions are not favorable for the maintenance and spread of mycoviruses. We suspect that *C. parasitica* at FR may be shedding its mycovirus since the percentage of isolates containing mycovirus declined from greater than 90% in 1996 (Davelos 1999) to 76% in 2009 (JC Springer, unpublished data). The decline in mycovirus frequency is paralleled by a doubling in the number of VC groups from four to eight between 1996 and 2009 and an increase in Shannon diversity (Table 1). Perhaps in consequence, trees at FR have begun to die back over the past four years, which contrasts to the gradual size increase occurring at CL and RC, the other two sites where mycoviruses occur at high frequency (JC Springer and AM Jarosz, unpublished data).

Results from this study suggest that mycoviruses have a significant influence on the VC structure of *C. parasitica* populations. When mycoviruses attain high frequency, the processes of sexual reproduction and selection in the *C. parasitica* pathogen population may be altered in a manner that leads to reduced VC diversity over time. Selection may act to favor a single most fit mycovirus x fungal genotype combination, while the inhibition of the sexual cycle reduces the formation of new VC groups. Thus, VC diversity within *C. parasitica* populations will be influenced by conditions that favor invasion of mycoviruses into pathogen populations and the concomitant changes in pathogen virulence. What is not known at present is how the underlying *vic* locus diversity is influenced by mycovirus invasion. We hypothesize that *vic* loci that have a strong influence on mycovirus transmission will be influenced more than *vic* loci that have little influence on mycovirus transmission (see Huber 1996). Based on this hypothesis, mycovirus invasion may influence genome-wide diversity within *C. parasitica* populations at recovering sites in Michigan.

LITERATURE CITED

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Akilli S, Katircioglu YZ, and Maden S. 2009. Vegetative Compatibility Types of *Cryphonectria parastica* Causal Agent of Chestnut Blight, in the Black Sea Region of Turkey. Forest Pathology. 39: 390–396.

Anagnostakis SL. 1977. Vegetative incompatibility in *Endothia parasitica*. Experimental Mycology. 1:306-316.

Anagnostakis SL. 1987. Chestnut blight: the classical problem of an introduced pathogen. Mycologia. 79:23-37.

Anagnostakis SL. 2001. American chestnut sprout survival with biological control of the chestnut-blight fungus population. Forest Ecology and Management. 152:225–233.

Anagnostakis SL, Hau B, and Kranz J. 1986. Diversity of vegetative compatibility groups in Cryphonectria parasitica in Connecticut and Europe. Plant Disease. 70:536-538.

Bertrand H. 2000. Role of Mitochondrial DNA in the senescence and hypovirulence of fungi and potential for plant disease control. Annual Review of Phytopathology. 38:397-422.

Brewer LG. 1995. Ecology of survival and recovery from blight in American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) in Michigan. Bulletin of the Torrey Botanical Club. 122:40–57.

Burdon JJ and Thrall PH. 2008. Pathogen evolution across the agro-ecological interface: implications for disease management. Evolutionary Applications. 1:57-65.

Brasier CM. 1995. Episodic selection as a force in fungal microevolution with special reference to clonal speciation and hybrid introgression. Canadian Journal of Botany. 73:S1213–S1221.

Brasier CM. 2001. Rapid evolution of introduced plant pathogens via interspecific hybridization. Bioscience. 51:123–133.

Brusini J, Robin C, and Franc A. 2011. Parasitism and maintenance of diversity in a fungal vegetative incompatibility system: the role of selection by deleterious cytoplasmic elements. Ecology Letters. 14:444-452.

Carbone I, Liu Y, Hillman BI, and Milgroom MG. 2004. Recombination and migration of Cryphonectria hypovirus 1 as inferred from gene genealogies and the coalescent. Genetics. 166:1611–1629.

Colwell RK. 2005. EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's Guide and Application published at: http://purl.oclc.org/estimates

Cortesi P, McCulloch CE, Song HY, Lin HQ, and Milgroom MG. 2001. Genetic control of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*. Genetics. 159:107–118

Davelos AL. 1999. Double-stranded RNA mediated recovery of American chestnut populations: a demographic analysis. PhD thesis, Michigan State University, East Lansing, Michigan.

Davelos AL, and Jarosz AM. 2004. Demography of American chestnut populations: effects of a pathogen and a hyperparasite. Journal of Ecology. 92:675-685.

Elliston JE. 1985. Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology. 75:151–158.

Enebak SA, MacDonald WL, Hillman BI. 1994. Effect of dsRNA associated with isolates of *Cryphonectria parasitica* from the central Appalachians and their relatedness to other dsRNAs from North America and Europe. Phytopathology. 84:528-534.

Fulbright DW, Weidlich WH, Haufler KZ, Thomas CS, Paul CP. 1983. Chestnut blight and recovering American chestnut trees in Michigan. Canadian Journal of Botany. 61:3164-3171.

Glass NL and Dementhon K. 2006. Non-self recognition and programmed cell death in filamentous fungi. Current Opinions in Microbiology. 9:553-558.

Glass NL, Jacobson DJ, Shiu KT. 2000. The genetics of hyphal fusion and vegetative compatibility in filamentous ascomycetes. Annuual Review of Genetics. 34:165-186.

Glass NL and Kaneko I. 2003. Fatal Attraction: Non-self recognition and heterokaryon incompatibility in filamentous fungi. Eukaryotic Cell. 2:1-8.

Gobbin D, Hoegger PJ, Heiniger U, Rigling D. 2003. Sequence Variation and Evolution of Cryphonectria Hypovirus 1 (CHV-1) in Europe. Virus Research. 97:39–46.

Grente J, 1965. Les formes hypovirulentes d'Endothia parasitica et les espoirs de lutte contre le chancre du châtagnier. Academie de l'agriculture de France. 1033–1037.

Griffin GJ, Elkins JR. 1986. Chestnut blight, other endothia diseases, and the genus Endothia. St. Paul, Minnesota. American Phytopathological Society Press. 53 pp.

Heiniger U and Rigling D. 1994. Biological control of chestnut blight in Europe. Annual Review of Phytopathology. 32:581–599.

Hill TCJ, Walsh KA, Harris JA, Moffett BF. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiology and Ecology. 43:1-11.

Huber DH. 1996. Genetic analysis of vegetative incompatibility polymorphisms and horizontal transmission in the chestnut blight fungus, *Cryphonectria parasitica*. PhD Dissertation. Michigan State University, East Lansing, Michigan.

Lapointe, F. J. and Legendre, P. 1992. Statistical significance of the matrix correlation coefficient for comparing independent phylogenetic trees Syst. Biol. 41:378–384.

Lawrence GJ, Boelen MG, and Pryor A. 1988. Transmission of double-stranded RNAs in flax rust, *Melampsora lini*. Canadian Journal of Botany. 66:61–66.

Liu YC, and Milgroom MG. 1996. Correlation between hypovirus transmission and the number of vegetative incompatibility (vic) genes different among isolates from a natural population of *Cryphonectria parasitica*. Phytopathology. 86:79–86.

MacDonald WL and Fulbright DW. 1991. Biological control of chestnut blight: use and limitations of transmissible hypovirulence. Plant Disease. 75:656–661.

Magurran A. 1988. Ecological diversity and its measurement. Princeton University Press, Princeton, NJ.

Marra RE and Milgroom MG. 2001. The mating system of the fungus *Cryphonectria parasitica*: selfing and self-incompatibility. Heredity. 86:134–143.

McCormick JF and Platt RB. 1980. Recovery of an Appalachian forest following the chestnut blight or Catherine Keever-you were right! American Midland Naturalist. 104:264–273.

Merkel HW. 1906. A deadly fungus on the American chestnut. 10th Annual Report of the New York Zoological Society. The Crow Press. New York, New York.

Micali CO and Smith ML. 2006. A nonself recognition gene complex in *Neurospora crassa*. Genetics. 173: 1991–2004.

Milgroom MG and Cortesi P. 1999. Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility genotypes. P. Natl. Acad. Sci. USA. 96:10518-10523.

Milgroom MG and Cortesi P. 2004. Biological control of chestnut blight with hypovirulence: a critical analysis. Annual Review of Phytopathology. 42:311-38.

Mir-Rashed N, Jacobson DJ, Dehghany MR, Micali OC, Smith ML. 2000. Molecular and functional analyses of incompatibility genes at het-6 in a population of *Neurospora crassa*. Fungal Genetics and Biology. 30:197-205.

Morozov AY, Robin C, Franc A. 2007. A simple model for the dynamics of a host-parasite-hyperparasite interaction. Journal of Theoretical Biology. 249:246-253.

Nauta MJ and Hoekstra RF. 1994. Evolution of vegetative incompatibility in filamentous ascomycetes. I. Deterministic models. Evolution. 48:979-995.

Nuss DL. 1992. Biological Control of Chestnut Blight: An Example of Virus-Mediated Attenuation of Fungal Pathogenesis. Biological Control. 56:561–576.

Peever TL, Liu YC, Milgroom MG. 1997. Diversity of hypoviruses and other double-stranded RNAs in *Cryphonectria parasitica* in North America. Phytopathology. 87:1026-1033

Potter C, Harwood T, Knight J, Tomlinson I. 2011. Learning from history, predicting the future: the UK Dutch elm disease outbreak in relation to contemporary tree disease threats. Philosophical Transactions of the Royal Society B. 366:1966-1974.

Rayner ADM. 1991. The phytopathological significance of mycelial individualism. Annual Rev. Phytopathology. 29: 305-323.

Robin C, Anziani C, and Cortesi P. 2000. Relationship between biological control, incidence of hypovirulence and diversity of vegetative compatibility types of *Cryphonectria parasitica* in France. Phytopathology. 90:730–737.

Robin C. and Heiniger U. 2001. Chestnut Blight in Europe: Diversity of *Cryphonectria parasitica*, Hypovirulence and Biocontrol. Forest Snow and Landscape Research. 367:361–367.

Rohlf FJ. 1998. NTSYSpc. Numerical taxonomy and multivariate analysis system. Version 2. User Guide. Applied Biostatistics, Inc., Setauket, NY.

Roper M, Ellison C, Taylor JW, and Glass NL. 2011. Nuclear and genome dynamics in multinucleate ascomycete fungi. Current Biology. 21:R786-R793.

Russin JS and Shain L. 1985. Disseminative fitness of *Endothia parasitica* containing different agents for cytoplasmic hypovirulence. Canadian Journal of Botany. 63:54-57.

Santini A, Pecori F, Pepori A, Brookes A. 2012. Morfeo Elm: a new variety resistant to Dutch elm disease. Forest Pathology. 42:171—176.

Sotirovski K, Milgroom MG, Rigling D, and Heiniger U. 2006. Occurrence of Cryphonectria Hypovirus 1 in the Chestnut Blight Fungus in Macedonia. Forest Pathology. 36:136–143.

Taylor DR, Jarosz AM, Lenski RE, Fulbright DW. 1998. The acquisition of hypovirulence in host-pathogen systems with three trophic levels. American Naturalist. 151:343-355.

Wu J, Saupe SJ, Glass NL. 1998. Evidence for balancing selection operating at the het-c heterokaryon incompatibility locus in a group of filamentous fungi. PNAS. 95:12398-12403.

CHAPTER 3

AN ANALYSIS OF MICROSATELLITE LOCI FROM POPULATIONS OF THE CHESTNUT BLIGHT PATHOGEN, *CRYPHONECTRIA PARASITICA*, IN MICHIGAN

ABSTRACT

Vegetative compatibility group diversity in populations of *Cryphonectria parasitica*, the fungus causing chestnut blight, varies according to mycovirus presence or absence in Michigan. In populations where mycoviruses are present, VCG diversity is generally low and, with one exception, each VCG is found at only a single site. In populations where mycoviruses are absent, VCG diversity is higher and several VCGs are shared among sites. Since mycovirus presence prevents sexual reproduction in *C. parasitica*, selection may act to sort through local combinations of mycovirus and blight pathogen to favor a single most-fit, locally adapted combination. Microsatellite markers developed for European populations of *C. parasitica* are used here to determine if genome-wide diversity is concordant with the pattern found for VCGs. Analysis of molecular variance (AMOVA) found significant differentiation among populations, with 15% of the total genetic variance being partitioned among the six *C. parasitica* Michigan population. Average ΦPT values for mycovirus-free sites was 0.079 while mycovirus-infected sites was 0.219. These values are consistent with the idea that gene flow is more limited among mycovirus-infected *C. parasitica* populations. In contrast, populations fell into two broad categories for within population genetic diversity that did not correspond with presence or absence of mycoviruses. The three high diversity populations had Shannon values of 0.37 or greater and a percentage of polymorphic loci of 63% or greater, while the three low diversity populations had Shannon values of 0.26

or lower and percentages of polymorphic loci of 40% or lower. The largest, mycovirus-free population at Stivers displayed low within population diversity, while Frankfort, a mycovirus infected populations displayed high within population diversity. Recent observations suggest that the mycoviruses at the *C. parasitica* population at Frankfort may be shedding its mycoviruses.

INTRODUCTION

In Chapter 2, I investigated diversity in Michigan populations of *C. parasitica* by identifying vegetative compatibility groups within and among seven populations. The presence of mycoviruses in some blight populations has seemingly influenced the number and diversity of vegetative compatibility groups (VCGs). VCG diversity is generally lower in *C. parasitica* populations where mycoviruses are present compared to populations where they are absent. Sexual reproduction is known to be inhibited in *C. parasitica* infected by mycoviruses (Bryner & Rigling 2012), which changes two important factors that would influence the genetic structure of *C. parasitica* populations. First, with no recombination in the pathogen, selection will tend to favor the most-fit pathogen genotype by mycovirus combination. Second, pathogen spread will be limited to asexual, splash-dispersed conidia. These two factors lead to the prediction that populations with mycoviruses should have a different structure relative to *C. parasitica* populations where mycoviruses are absent. In the presence of mycoviruses I expect lower overall genomic diversity and the possibly high levels of differentiation among mycovirus-infected populations because of asexually reproducing clonal populations. In contrast, I expect higher diversity within *C. parasitica* populations where

mycoviruses are absent since sexual reproduction is most likely occurring at those sites and lower differentiation among populations due to increased gene flow.

Although commonly used for determining diversity in populations of *C. parasitica* throughout its range in North America and Europe, VCG analyses have several drawbacks since the technique is time consuming (Breuillin et al. 2006) and it is difficult to score some pairings (see Chapter 2). Additionally, VCG are controlled by six to eight *vic* loci that are thought to be under negative frequency dependent selection (Huber 1996; Cortesi & Milgroom 1998). Further, determining the genetic basis of each VCG requires pairing to genetically characterized testers (Cortesi & Milgroom 1998). Additional problems occur because the six characterized *vic* loci do not encompass all of the *vic* loci controlling VCGs, and in consequence, many studies have reported isolates that do not pair with any of the characterized testers. (Milgroom & Cortesi 1999; Robin & Heiniger 2001; Braganca et al. 2007). The cumbersome method of pairing isolates together to determine VCG or genotypic information is useful but time-consuming and interactions are difficult to understand, thus my use of molecular markers to determine genomic diversity.

Molecular markers such as microsatellites can be used to efficiently characterize population genetic structure of various organisms (Selkoe & Toonen 2006). Understanding diversity at the genetic level allows ecologists to answer key questions about population structure of organisms across diverse taxa e.g., California Channel Island Foxes, (Goldstein et al. 1999); Cuban cattle, (Acosta et al. 2013); Lemba fruit, (Babaei et al. 2012); Baobabs, (Munthali et al. 2013); chickpea blight, (Peever et al. 2004); and the chestnut blight pathogen, (Breuillin et al. 2006). Microsatellite markers

are widely distributed throughout the genome of eukaryotes; they are co-dominant, and extremely variable which makes them very useful in genetics studies (Shikano et al. 2010). Microsatellites, a type of variable number tandem repeats, are highly variable short segments of DNA, (sometimes called simple sequence repeats [SSRs] or short tandem repeats [STRs]), usually 1—6 base pairs long, many times repeated up to 60 times (Goldstein & Pollock 1997). Use of these neutral markers can provide an opportunity to determine population genetic structure of organisms in regions of their genome that are independent of loci normally under selection.

The use of microsatellite markers has become ubiquitous as a tool for investigating the population structure of plant pathogenic fungi (Goldstein & Pollock 1997) and can help detect patterns of genetic variability within and among populations. Plant pathogens are great model systems to study how evolutionary forces affect population structure over short time periods and at various spatial scales (Holderegger & Wagner 2008; Linde 2010; Rieux et al. 2013). Since plant pathogens (and other organisms) are heavily influenced by evolutionary forces such as: selection, drift, migration, recombination, and mutation (McDermott & McDonald 1993), we can better understand how these forces have acted in the past (McDonald & Linde 2002) by obtaining information about their genetic structure. Additionally, spatially separated populations of plant pathogens can allow us to infer how geographic scale, local environmental conditions, biotic interactions, or in the case studied below: how the presence of double-stranded RNA (dsRNA) mycovirus hyperparasites can influence population structure of *Cryphonectria parasitica*.

This study is the first to investigate how mycoviruses influence the genetic structure in *C. parasitica* populations in Michigan. This chapter will: 1) investigate how mycoviruses alter the genetic variability of *C. parasitica*, 2) help us further understand the patterning of genetic differentiation among *C. parasitica*, populations, 43 investigate potential factors that influence the patterning of geographic variation (e.g., isolation by distance or the presence/absence of mycoviruses), and 4) provide a base-line population structure of *C. parasitica* that will allow tracking of genetic changes over time as three populations *C. parasitica* respond to the invasion of mycoviruses (see Chapter 4).

METHODS

Isolates collected in 2009 and used previously to characterize seven *C. parasitica* populations in Michigan for vegetative compatibility group diversity (Chapter 2) were grown in culture for microsatellite analysis. Thirty isolates from each of seven populations were grown on PDA petri plates overlaid with uncoated, sterilized cellophane. After approximately three weeks of growth, fungal mats were scraped off the cellophane, dried in mortars at 65^{o}C and ground to a fine powder with liquid nitrogen. Whole DNA extractions were performed with the Plant Mini Kit (Qiagen, Maryland, USA) with 25—30mg of dried tissue following manufacturers' directions. Extracted DNA was stored in microcentrifuge tubes at -20^{o}C until PCR could be performed.

Polymerase chain reactions were performed with GoTaq Green Master Mix (Promega Corporation, Madison, WI) in a total volume of 25uL as recommended by the manufacturer. The thirteen primers developed by Breuillin et al. (2006) were tested using DNA from ten random isolates to determine if the primers would amplify *C. parasitica* from Michigan. Ten of the primers were chosen for further analysis of the seven Michigan *C. parasitica* populations (Table 1). PCR was run according to the protocols of Breuillin et al. (2006); 9 cycles at 94^{o}C for 40s, annealing temperature decreasing from 69^{o}C to 65^{o}C (0.5^{o}C per subsequent cycle) for 30s, and 72^{o}C for 1min. PCR was then run for 30 cycles at 94^{o}C for 40s, 65^{o}C for 30s and 72^{o}C for 1min and, a final extension at 72^{o}C for 30 min then held at 10^{o}C until frozen. PCR products were stored in 96 well plates at -20^{o}C until fragment sizes could be analyzed on an Applied Biosystems ABI 3130 genetic analyzer. Fragment peak sizes were called manually using PeakScanner v1.0 (Applied BioSystems). Fragment sizes were rounded to whole numbers using power functions in the tandem software version 1.09 (Salzburger 2009) after being converted to the proper file format using CREATE (Coombs et al 2008).

Amplification was not consistent either within a locus across isolates or across loci for an individual *C. parasitica* isolate. Second and some third attempts to amplify these missing data all failed. In consequence, the final data set has a considerable number of missing data (approximately 40% of data are missing from 2,100 data points expected), which reduced the power of analysis considerably. Population MH had the lowest amplification success and was dropped from the final analyses. In addition, locus *CPG*5

amplified poorly across all populations, and it was also dropped from the final analyses. Finally, all loci that had sample sizes ≤5 for a population were removed from subsequent analyses since they provided little information to the dataset and hindered some population genetic tests in GenAlEx 6.5. Finally, poor amplification across loci for all samples precluded an analysis of multilocus genotypes and level of linkage disequilibrium in the six remaining populations.

Data were then analyzed with GenAlEx software version 6.5 (Peakall & Smouse 2006, 2012) where frequencies of alleles, private alleles, and pairwise population comparison tests across all alleles were performed. ΦPT, an analogue of the F_{ST} fixation index, was used to characterize the level of population divergence among the six *C. parasitica* populations. Pairwise genetic difference between individuals within populations was calculated in GenAlEX 6.5 by counting the number of genetic differences between all combinations of individuals across populations. Isolation by distance was tested by comparing the genetic distance matrix with the geographic distance matrix using a Mantel test. GenAlEX 6.5 was also used to calculate the average number of migrants per generation (N_m), number of alleles (N_a), and number of effective alleles in each population (N_e).

RESULTS

Ten of the thirteen microsatellite markers developed for European populations by Breuillen et al. (2006) successfully amplified in Michigan samples of *C. parasitica* (Table 3-1). The number alleles detected and size of individual alleles differed between

Europe and Michigan. One locus (CPG5) that appeared to be fixed for one allele of 220bp in Europe was much more variable in Michigan populations and had four alleles (Table 3-1). Five loci amplified with Michigan isolates matched the number of alleles found in European populations. Loci *CPE*1 and *CPE*8 had fewer alleles in Michigan isolates with three and two, respectively, compared to five each in European populations. Allele sizes were similar between Michigan and European populations and overlapped in number of base pairs except for locus *CPE*8 that was shifted to a smaller size in tested Michigan isolates (Table 3-1). Table 3-2 lists all alleles per locus for each of the seven *C. parasitica* populations in Michigan. As explained above, the MH population and CPG5 locus were dropped from population level analyses because of poor amplification and the resultant small sample size that precluded population level analyses.

A molecular analysis of variance (AMOVA) across the nine microsatellite loci for the six Michigan *C. parasitica* populations found that 15% of the genetic variation was partitioned among populations, while 85% was found within populations (Table 3-3). Populations fell into two categories with regard to within population variability. MD, LE and FR were relatively more variable than ST, CL and RC (Table 3-4). Between 63 and 70% of the loci were polymorphic in the three more variable populations, which also averaged between 1.88 and 2.00 alleles per locus and had Shannon diversity values between 0.37 and 0.45. In contrast, only 30 to 40% of the loci were polymorphic in the low variability group. The average number of loci at ST, CL and RC averaged between 1.18 and 1.35 alleles per locus and Shannon diversity were between 0.16 and 0.26.

Table 3-1. Range of allele sizes in base pairs and number of alleles amplified for thirteen loci of *C. parasitica* in Europe (Breuillin et al 2006) and ten in Michigan.

Locus	European Range in base pairs	European Alleles	Michigan Range in base pairs	Michigan Alleles
CPG1	226--228	2	225--229	3
CPG2	152--156	3	150--156	3
CPG3	188-262	8	--[a]	--
CPG4	188--208	4	183--203	5
CPG5	220	1	198--218	4
CPE1	131--149	5	124--140	3
CPE2	140--144	2	--	--
CPE3	176--194	4	182--194	4
CPE4	224--280	6	--	--
CPE5	256--265	3	248--258	3
CPE6	110--112	2	105--107	2
CPE7	176--191	3	182--186	3
CPE8	107--122	5	111--113	2

a: these primers were not amplified

Table 3-2 Summary table of population level diversity of alleles at each of ten loci. Data in gray boxes are shown for comparison and were not included in population level analyses due to poor amplification and resulting small sample size.

	CPG1	CPG2	CPG4	CPG5	CPE1	CPE3	CPE5	CPE6	CPE7	CPE8
MH	227, 229	152, 156	203	218	124	190	253	107	184	111
MD	225, 227	150, 152	199, 203	218	124	182, 186, 190	253	105, 107	184, 186	111, 113
ST	225, 227	150, 152	187, 203	218	138	190	258	107	186	111
LE	225, 227, 229	150, 152	187, 203	198, 218	124, 138	190	253, 258	107	184, 186	111
CL	227, 229	152	203	218	124	190	248, 253	107	182, 184	111
FR			183	206	124	190	253	107	184	111
			199	218	138		258		186	
			203		140					
RC	225, 227	152	187, 191	214, 218	138	194	248, 253	107	186	111
Total alleles	3	2	5	4	3	4	3	2	3	2

Within population variability was not associated with the presence or absence of mycovirus, since two of three epidemic populations, MD and LE (i.e., those with no mycovirus) were in the group with higher variability and the third population, ST, was in the low variability group. The opposite was true for the recovering population where mycoviruses were present. FR was in the more variable group, while CL and RC were in the low variability group. As discussed below, the *C. parasitica* population at FR appears to be shedding its mycoviruses, which may explain the higher variability at

Table 3-3. AMOVA statistics for tests of genetic differentiation within and among populations of *C. parasitica* in Michigan. Data were calculated without site MH, locus CPG5, and any sample where no loci amplified.

Source	df	SS	MS	Molecular Var.	ϕPT	Prob.
Among Pops.	5	62.31	12.46	15%	0.147	<0.001
Within Pops	174	351.83	2.02	85%		
Total	179	414.13		100%		

this site. Pair-wise comparisons between populations indicated considerable differentiation among populations. ϕPT values ranged over nearly an order of magnitude from 0.029 to 0.275 (Table 3-5). The only pair-wise comparison that was not significantly different from zero was between MD and LE (Table 3-5). These two epidemic site *C. parasitica* populations are separated by 43 kilometers. Pair-wise comparisons did follow a pattern with comparisons among *C. parasitica* populations from epidemic sites (Comparisons with the diagonal hatching in Table 3-5) having

Table 3-4. Average number of alleles per locus, effective number of alleles per locus, percentage of loci that were polymorphic and Shannon diversity for each population. Standard errors of the mean are in parentheses.

Population	Average number of alleles per locus	Average number of effective alleles per locus	% loci Polymorphic	Shannon Diversity
MD	2.00 (0.26)	1.38 (0.15)	70	0.37 (0.11)
ST	1.50 (0.22)	1.22 (0.11)	40	0.21 (0.09)
LE	1.90 (0.23)	1.54 (0.15)	70	0.45 (0.11)
CL	1.40 (0.22)	1.18 (0.12)	30	0.16 (0.09)
FR	1.88 (0.29)	1.49 (0.17)	63	0.41 (0.13)
RC	1.40(0.16)	1.35(0.15)	40	0.26 (0.11)

lower ϕPT than comparisons among *C. parasitica* populations from recovering sites (Comparisons with cross hatching in Table 3-5; see also Table 3-6). Indeed, the comparisons with the two highest ϕPT values involved comparisons between recovering sites (CL vs. RC and CL vs. FR). The level of differentiation among recovering site populations also tended to be higher than the comparisons between epidemic and recovering sites (Table 3-6). The average number of haploid migrants per generation (N_m) across all populations was 2.91, but the predicted number of migrants differed with population type. Epidemic populations had predicted migration rates ranging from 3.8 to 17 per generation with an average of 8.6 (Table 3-7). Migration was predicted to be much lower between recovering populations with a range from 1.3 to 2.4 and an average of1.9. All sites except LE most likely meet the assumption of large, stable population size, the LE site is experiencing annual expansion of disease on previously uninfected trees (AM Jarosz, ALD Baines, and JC Springer pers. obs.).

Table 3-5. Pairwise population differentiation values (ϕPT; below the main diagonal) based on nine loci across six *C. parasitica* populations; all probabilities of obtaining ΦPT values were significant at $p<0.05$ except LE x MD ($p=0.096$). Straight-line distances between populations, in km, are shown above the diagonal. Light shading indicates comparisons between populations without mycoviruses while darker shading indicates comparisons between populations with mycoviruses.

	MD	ST	LE	CL	FR	RC
MD	--	56.7	76.1	71.7	88.2	51.9
ST	0.117	--	67.2	15.2	34.1	107.7
LE	0.029 n.s.	0.092	--	69.8	67.6	103.4
CL	0.104	0.194	0.185	--	20.2	122.3
FR	0.107	0.155	0.050	0.214	--	136.9
RC	0.172	0.173	0.124	0.275	0.170	--

n.s. indicates the only pair-wise comparison that is not significantly different from 0.

Table 3-6. Average ϕPT and number of haploid migrants (N_m) based on population type, three epidemic sites and three recovering sites (described in Chapter 2). Standard errors are in parentheses.

	Average ϕPT	N_m
Epidemic	0.079 (0.026)	8.560 (4.216)
Recovering	0.219 (0.030)	1.860 (0.325)
Between pop. types	0.140 (0.017)	3.700 (0.778)

Table 3-7. Average number of migrants per generation of *C. parasitica* between six Michigan populations. Values are estimated based on haploid *C. parasitica* populations and on ϕPT values using GenAlEx 6.5. Shading is the same as in Table 3-5.

	MD	ST	LE	CL	FR	RC
ST	3.778	--				
LE	16.969	4.946	--			
CL	4.306	2.074	2.204	--		
FR	4.152	2.732	9.516	1.831	--	
RC	2.400	2.392	3.531	1.321	2.448	--

A test for isolation by distance between populations was carried out using a Mantel test to determine if geographic distance (in kilometers; Table 3-5) and Nei's (1987) genetic distance were correlated. The $R^2 = 0.0005$, P=0.09 indicated little, if any, geographic patterning of genetic variation.

Missing data precluded any multilocus genotype analyses within or among populations, nor could linkage disequilibrium values be calculated; key values important for understanding the level of asexual reproduction.

DISCUSSION

A previous study that investigated the pattern of vegetative compatibility group (VCG) diversity found that the presence of mycovirus affected the structure of *C. parasitica* populations (see chapter 2). Two populations with mycovirus, CL and RC, displayed low VCG diversity while populations where the mycovirus was absent (i.e., epidemic populations) generally had more VCGs, and also displayed higher diversity for VCGs. *Cryphonectria parasitica* populations also shared several VCGs which suggested

that *C. parasitica* from epidemic sites experienced more gene flow; although the CL recovering site shared a VCG with two epidemic sites. Although commonly used to detect patterns of *C. parasitica* diversity in Europe and North America (Anagnostakis et al. 1986; Bissegger et al. 1987), vegetative compatibility is cumbersome and time consuming. In this study, microsatellite markers were used to investigate whether the VCG pattern extended across the *C. parasitica* genome. The pattern of microsatellite variability supported some but not all of the VCG findings. Three epidemic populations, ST, LE and MD, were less differentiated than the three recovering sites. Indeed, genetic distance among the recovering sites indicated they are not closely related. It is also known that the mycoviruses at the three recovering sites differ significantly with CHV3 at CL, CHV3 and CHV4 at FR and SR2 at RC (see Chapter 1; Tartaglia et al. 1986; Paul & Fulbright 1988; Durbahn 1992; Smart et al. 1994). Taken together, this information suggests that both the mycovirus and *C. parasitica* colonized each recovering site independently and possibly has remained distinct from other *C. parasitica* populations in Michigan over the twenty plus years since mycoviruses invaded.

The contrasting pattern for epidemic populations of *C. parasitica* suggested more gene flow among these sites. This makes sense biologically, since frequencies for mating type alleles indicated that *C. parasitica* populations at two long-infected sites (ST and MD) were sexually reproducing, while *C. parasitica* at two recovering sites (CL and RC) displayed skewed allele ratios that indicated asexual reproduction (see Chapter 2). Mycoviruses are known to inhibit the sexual cycle in *C. parasitica* and, thus, reproduction occurs via asexually produced conidia (Zhang et al. 1998; Gobbin et al.

2003). Sexually produced ascospores are wind-dispersed and known to be more mobile than splash dispersed conidia (Heald et al. 1915).

Patterns of microsatellite variation only lent partial support to the hypothesis that epidemic populations are largely sexual and more variable genetically than the asexually reproducing *C. parasitica* populations at recovering sites. As expected, the CL and RC recovering sites had *C. parasitica* populations with low microsatellite diversity, but the FR recovering site displayed relatively high genetic diversity at microsatellite loci (Table 3-4). High diversity at FR might be due to the fact that the *C. parasitica* population appears to be in the process of escaping mycovirus infections, since >90% of isolates obtained from the site in 1996 harbored mycovirus (Davelos 1999) and only 76% of isolates collected in 2009 were infected with mycoviruses (JC Springer, unpublished data). Epidemic populations also provided only partial support for the hypothesis that they were sexually reproducing and expected to display more genetic variability. The MD and LE populations were both relatively diverse, which supported my hypothesis. However, the ST epidemic population had low diversity, despite the fact that *C. parasitica* has been resident at this site for over 20 years (DW Fulbright, pers. comm.), and with greater than 1100 trees, it has the largest American chestnut population of any we have monitored since 1996 (Davelos and Jarosz 2004).

These results suggest that *C. parasitica* across Michigan is organized into a patchwork of populations that differ in their level of sexual reproduction, level of genetic variability and level of migration among patches. Some of this patchwork can be explained by the presence of mycoviruses in some populations, but not others. However, we know little about environmental conditions that led to mycoviruses

invading in some populations and not others. It is tempting suggest that the presence of mycoviruses is simply the result of colonization of events at some locations and not others. However, attempts to introduce mycoviruses into *C. parasitica* populations across North America have generally met with failure, which suggests that other factors, such as abiotic conditions or tree genetics are also influencing mycovirus spread. These factors remain to be explored, but it is becoming increasingly evident that *C. parasitica* in Michigan, at least superficially, represents as geographic mosaic of coevolutionary interactions (see Thompson 1999). There are obvious hotspots where the mycoviruses are important and their presence has led to recovery of chestnut populations (Davelos & Jarosz 2004). But interspersed among these recovering sites are populations where *C. parasitica* continues to decimate the local *C. dentata* populations. Many questions remain to be answered before we can predict whether mycoviruses will ultimately spread more widely and cause a general recovery of American chestnut within Michigan.

Four populations studied in France to understand both VCG and microsatellite diversity, all infected with mycoviruses, were considered to be isolated by distance (Breuillin et al. 2006). Biologically this is a reasonable expectation given considerations of how mycoviruses affect sexual reproduction in *C. parasitica*. Michigan *C. parasitica* populations although not isolated by distance do however appear to be structured according to mycovirus presence as shown with VCG data in Chapter 2. Further, Breuillin et al. (2006) showed, based on measures of genetic diversity, that populations in France exhibited reduced gene flow between populations probably since chestnut forests there are fragmented across the landscape and are managed as orchards, similar

to the fragmented patchwork of chestnut populations in Michigan. VCG diversity in French populations was much higher (range 6—16 groups) compared to populations in Michigan that are also infected with mycoviruses (range 2—8 groups; see Chapter 2); it is unknown how long mycoviruses have been present in the four French populations. Shannon diversity across populations was much higher in France than in Michigan populations, average 2.32 and 0.31. Similarly, the average number of alleles per locus in French populations was higher than that in Michigan, average 2.10 and 1.68, respectively. In *C. parasitica*, VCG diversity (and by extension genomic diversity) may increase after colonization events by way of sexual reproduction (similar to diversification in many other plant pathogens when they are introduced). As mycoviruses invade, VCG and genomic diversity may decline through the effects of genetic drift and natural selection. However, a reduction in diversity may not be maintained given variable environmental conditions, biotic interactions, and human influence (e.g., accidental introduction of new VCG types or by changing of forest structure due to logging) thus creating boom and bust cycles of VCG and genomic diversification, mycovirus invasion, and eventual loss of mycoviruses caused by sexual reproduction in rare individual VCGs that escape mycovirus infection

As shown in Chapter 2 mycoviruses have the ability, to alter natural selection by favoring the most fit fungal genotype by mycovirus combinations,. Breuillin et al. (2006) suggested that further genetic analyses should be done throughout the geographic distribution of *C. parasitica*, especially where populations are under the influence of different mycovirus types which can provide informative results regarding the effects of mycovirus hyperparasites on the genetic structure of individual pathogen

populations. Although different mycoviruses exist at each of three Michigan sites, my work demonstrates that genomic diversity within mycovirus-infected populations is often low but these same populations are highly differentiated from one another.

Future work on microsatellite analyses in Michigan and throughout the range of *C. parasitica* in North America should first focus on optimizing PCR and amplification for current primers and loci along with developing markers unique to North American populations and second, further sampling determining structure of the pathogen population across the geographic range in North America. Phylogenetic studies as extensions of this work will allow an understanding of how *C. parasitica* spread and will help us understand how mycoviruses can influence blight pathogen population structure where they have been introduced for biological control of *C. parasitica* (e.g., West Salem, WI and the three populations discussed in Chapter 4). Since we have isolates of C. parasitica from populations where I have introduced dsRNA mycoviruses (MH, ST, and LE discussed in Chapter 4), studies of how genetic structure changes in response to mycovirus invasion might be possible in the future.

LITERATURE CITED

LITERATURE CITED

Acosta AC, Uffo O, Sanz A, Ronda R, Osta R, Rodellar C, Martin-Burriel I, and Zaragoza P. 2013. Genetic Diversity and Differentiation of Five Cuban Cattle Breeds Using 30 Microsatellite Loci. Journal of Animal Breeding and Genetics. 130:79–86.

Anagnostakis SL, Hau B, and Kranz J. 1986. Diversity of Vegetative Compatibility Groups in *Cryphonectria parasitica* in Connecticut and Europe. Plant Disease. 70:536–538.

Babaei N, Abdullah NAP, Saleh G, and Abdullah TL. 2012. Isolation and Characterization of Microsatellite Markers and Analysis of Genetic Variability in *Curculigo latifolia* Dryand. Molecular Biology Reports. 39:9869–9877.

Bissegger M, Rigling D, and Heiniger U. 1997. Population Structure and Disease Development of *Cryphonectria parasitica* in European Chestnut Forests in the Presence of Natural Hypovirulence. Phytopathology 87:50–59.

Breuillin F, Dutech C, and Robin C. 2006. Genetic Diversity of the Chestnut Blight Fungus *Cryphonectria parasitica* in Four French Populations Assessed by Microsatellite Markers. Mycological Research 110:288–96.

Bryner SF and Rigling D. 2012. Virulence Not Only Costs but Also Benefits the Transmission of a Fungal Virus. Evolution. 66:2540-2550.

Coombs JA, Letcher BH, and Nislow KH. 2008. CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. Molecular Ecology Resources. 8:578-580

Durbahn CM. 1992. Molecular characterization of dsRNA associated hypovirulence in Michigan isolates of *Cryphonectria parasitica*. Ph.D. dissertation. Michigan State University, East Lansing.

Goldstein DB and Pollock DD. 1997. Launching Microsatellites: A Review of Mutation Processes and Methods of Phylogenetic Inference. Journal of Heredity. 88:335–342.

Goldstein DB, Roemer GW, Smith DA, Reich DE, Bergman A, and Wayne RK. 1999. The Use of Microsatellite Variation to Infer Population Structure and Demographic History in a Natural Model System. Genetics. 151:797–801.

Heald FD, Gardner MW, and Studhalter RA. 1915. Air and wind dissemination of ascospores of the chestnut blight fungus. Journal of Agricultural Research. 3:493–526.

Holderegger R and Wagner HH. 2008. Landscape Genetics. BioScience 58:199.

Li YC, Korol AB, Fahima T, Nevo E, 2004. Microsatellites within genes: structure, function, and evolution. Molecular Biology and Evolution. 21:991–1007.

Linde CC. 2010. Population genetic analyses of plant pathogens: new challenges and opportunities. Australasian Plant Pathology. 39:23-28.

McDonald BA and Linde C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology. 40:349–379.

McDonald BA and McDermott JA. 1993. Population genetics of plant pathogenic fungi. BioScience. 43:311–319

Munthali CRY, Chirwa PW, Changadeya WJ, and Akinnifesi FK. 2012. Genetic Differentiation and Diversity of *Adansonia digitata* L. (baobab) in Malawi Using Microsatellite Markers. Agroforestry Systems. 87:117–130

Paul CP and Fulbright DW. 1988. Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology. 78:751-755.

Peakall R and Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes. 6:288-295.

Peakall R and Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics. 28:2537-2539.

Peever TL, Salimath SS, Su G, Kaiser WJ, and Muehlbauer FJ. 2004. Historical and Contemporary Multilocus Population Structure of *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) in the Pacific Northwest of the United States. Molecular Ecology. 13:291–309.

Rieux A, De Lapeyre De Bellaire L, Zapater M-F, Ravigne V, and Carlier J. 2013. Recent Range Expansion and Agricultural Landscape Heterogeneity Have Only Minimal Effect on the Spatial Genetic Structure of the Plant Pathogenic Fungus *Mycosphaerella fijiensis*. Heredity. 110:29–38.

Raymond M & Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity. 86:248-249

Rousset F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular and Ecological Resources 8:103-106.

Matschiner M and Salzburger W. 2009. TANDEM: integrating automated allele binning into genetics and genomics workflows. Bioinformatics. 25:1982-1983.

Selkoe KA and Toonen RJ. 2006. Microsatellites for Ecologists: a Practical Guide to Using and Evaluating Microsatellite Markers. Ecology Letters. 9:615–629.

Shikano T, Ramadevi J, Shimada Y, and Merilä J. 2010. Utility of Sequenced Genomes for Microsatellite Marker Development in Non-model Organisms: a Case Study of Functionally Important Genes in Nine-spined Sticklebacks (*Pungitius pungitius*). BMC Genomics. 11:334.

Smart CD, Nuss DL, and Fulbright DW. 1994. Partial sequence analysis of the dsRNA associated with hypovirulence in a Michigan strain of the chestnut blight fungus, *Cryphonectria parasitica*. Pages 8- 10 in: Proc. Intl. Chestnut Conf. M. L. Double and W. L. MacDonald, eds. West Virginia University Press, Morgantown.

Tartaglia J, Paul CP, Fulbright DW, and Nuss DL. 1986. Structural properties of double-stranded RNAs associated with biological control of chestnut blight fungus. PNAS. 83:9109-9113.

Zhang L, Baasiri RA, Van Alfen NK. 1998. Viral repression of fungal pheromone precursor gene expression. Molecular and Cellular Biology. 18:953–959.

CHAPTER 4

BIOLOGICALLY CONTROLLING CHESTNUT BLIGHT PATHOGEN POPULATIONS IN MICHIGAN USING A NATIVE MYCOVIRUS: INITIAL RESULTS OF A LONG-TERM EXPERIMENT

ABSTRACT

Mycovirus hyperparasites were introduced over a three-year period into three populations of American chestnuts (*Castanea dentata* (Marsh.) Borkh.) infected with chestnut blight caused by *Cryphonectria parasitica* (Murrill) Barr.). The GH2 mycovirus, which is native to Michigan, was used to determine whether American chestnuts in the size class 1—10cm diameter at breast height (DBH) would provide population level response to mycovirus treatment of blight cankers. Persistence of mycovirus after introduction was slightly over 50% for all three sites. Treated trees did not survive any better than untreated control trees (74% versus 73%, respectively, over the three year period). Survivorship did differ among sites with 87% of the treated trees at Stivers surviving, followed by Leelanau (72%), and Missaukee Healthy (65%). Survivorship of treated trees with a DBH less than 3cm DBH was very low (40%). Future work should consider only treating trees with DBHs above 3cm DBH. Growth of treated trees was low at Leelanau but treated trees grew better than untreated trees at both Stivers and Missaukee. Environmental conditions or tree genetics may play a role in the recovery of treated trees at each site.

INTRODUCTION

Biological control of non-native and invasive species has become an important management tool in ecological and agricultural systems (McEvoy & Coombs 1999 and references therein) especially since the use of biological control agents to control non-native species may be less harmful to the environment and human health than chemical control (Simberloff & Stiling 1996) and may be more stable evolutionarily than other methods of control since invasive species may not evolve resistance as quickly as with chemical means (Holt & Hochberg 1997). The management of non-native, invasive pests and pathogens with biological control organisms (sometimes in combination with other control techniques) has become a common method to preserve biodiversity (deBach 1974; Jezorek et al. 2012). Biological control organisms must meet rather specific needs because they must control the target organism while at the same time avoid being a pest in their own right. Examples of biological control schemes failing are common (e.g., parasitoid wasps failed to spread in Florida to control invasive bromeliad-eating weevils: Cooper et al. 2011; failure of pathogens to control termites: Chouvenc et al. 2011), and there are also examples where the biological control agent have become a dangerous pests (e.g., cane toads, *Bufo marinus* L. in Australia; Shanmuganathan et al. 2010). The spread of biological control organisms can also be detrimental when they begin to affect native species (a host shift), especially those considered federally threatened e.g., the seed-eating weevil *Larinus planus* Fabricius becoming a seed predator of Pitcher's Thistle, *Cirsium pitcheri* (Torr. ex Eat.) Torr. & A. Gray in Michigan and Tracy's thistle, *Cirsium undulatum* (Nutt.) Spreng. var. *tracyi* (Rydbg.) S.L. Welsh in Colorado after it was introduced to control invasive thistles elsewhere (Louda &

O'Brien 2002; Havens et al. 2012). Additionally, biological control organisms may compete with native species leading to changes in community structure (Simberloff & Stiling 1996). Simberloff and Stiling (1996) provide a detailed, useful review of the risks of biological control such as instances of predation, herbivory, and parasitism on non-target species across diverse taxa.

The success of biological control schemes is, however, sometimes difficult to ascertain even with repeated introductions of the control organism (e.g., Milgroom & Cortesi 2004). In at least one instance (*Chondrilla juncea* L., Skeletonweed in Australian agricultural fields), biological control was successful against one genotype of the invasive plant but has failed to control other genotypes of *C. juncea* that increased in frequency when the common form of the weed was controlled by the pathogen, *Puccinia chondrillina* Bubak & Syd. (Burdon et al. 1981). However, examples of successful biological control can also be found. Two popular examples of success are the control of lepidopterans affecting agricultural crops, using endoparasitoid stingless wasps, *Trichogramma* spp. (Smith 1996) and control of invasive Purple Loosestrife, *Lythrum salicaria* L. by using *Galerucella* spp. leaf beetles (Grevstad 2006 and references therein; Yeates et al. 2012). Mixed success of biological control are also quite common (*cf*. Murdoch et al. 1985).

Potential difficulties in using biological control include understanding which size of organisms to treat or protect to increase population size, timing of treatment or transmission of control mechanisms (Chua 1978; Reilly & Hajek 2012), and how often to treat (Crow 2005) which could all be contributing factors to the success or failure of biological control (Milgroom & Cortesi 2004). Additionally, long-term monitoring of

systems where biological control has been implemented is essential to determining long-term quantitative success but has rarely been done (McEvoy & Coombs 1999; Barton et al. 2007; Grevstad 2006). It is also difficult to evaluate the success of a biological control program because most studies do not incorporate comparison control sites where biological control agents are not released (Morin et al. 2009).

Biological control of chestnut blight in Europe has been successfully managed by naturally occurring and deliberately introduced mycoviruses that infect the mycelium of the blight fungus, *Cryphonectria parasitica* (Murrill) Barr. The success of mycoviruses in Europe has led to attempts to use mycoviruses to control blight on American chestnuts, *Castanea dentata* (Marsh.) Borkh., but these efforts have been largely unsuccessful. Why mycoviruses control blight in Europe but not North America remains an open question, but researchers have proposed several explanations including the complex vegetative compatibility structure of North America *C. parasitica* populations (Anagnostakis et al. 1986; Anagnostakis & Kranz 1987), differences in the susceptibility of chestnuts in Europe and North America (Griffin 1986), and treating trees inefficiently (Davelos and Jarosz 2004).

Blight cankers quickly girdle American chestnut trees infected with *C. parasitica*. Girdling results in the death of all tree material distal to the canker. Naturally occurring double-stranded (ds) RNA mycoviruses first discovered in blighted populations of European chestnuts with abnormal cankers (Biraghi 1953; Grente 1965) were later found to be naturally occurring in some Michigan populations of the chestnut blight pathogen (Elliston et al 1977; Fulbright et al. 1983). Mycoviruses alter the virulence of *C. parasitica* in such a way that can allow the host trees to tolerate infection by blight (see

Chapter 1). This condition, known as hypovirulence, is a textbook example for biological control and has been known in the chestnut-chestnut blight system for over 60 years (Milgroom & Cortesi 2004). The success of biological control in Europe has escalated interest in using mycoviruses to mange blight in North America (Van Alfen et al. 1975; Van Alfen 1982; Anagnostakis 1987; Fulbright & Paul 1988; MacDonald & Fulbright 1991). A goal in chestnut blight research is to successfully control or manage the disease of American chestnuts with mycoviruses (*cf*. MacDonald & Fulbright 1991) so they might again attain significant stature in the eastern hardwood forests of the United States and in areas where they and their hybrids have been introduced for nut crop production (DW Fulbright pers. comm.).

What trees to treat?

Demographic analyses have been used often to determine population viability and to identify critical life-history stages important for protecting at-risk populations of endangered or threatened organisms (Silvertown et al. 1993; Crowder et al. 1994, 1995; Kephart & Paladino 1997; Fujiwara & Caswell 2001; Andrieu et al. 2013). This information can be used when designing conservation schemes (Caswell 2010).

A demographic analysis of American chestnut populations in Michigan proposed that treating the largest blight-infected trees is not the most efficient way to achieve recovery of American chestnuts (Davelos & Jarosz 2004). Matrix projection models and sensitivity analyses proposed that treating sub-adult trees with a diameter at breast height (DBH) between 1 and 10cm would be the most efficient way of introducing

mycovirus since these trees contribute significantly to the overall chestnut population's growth, leading to faster recovery (Davelos 1999; Davelos & Jarosz 2004).

A Michigan mycovirus used for biological control

The naturally occurring Cryphonectria mycovirus, a CHV3 type (hereafter GH2, see Chapter 1) used in this study was discovered near Grand Haven, Michigan in the late 1970s (Elliston et al. 1977; Fulbright et al. 1983). Chestnut trees at the site were recovering from blight until the trees were removed for a housing development (DW Fulbright, pers. comm.). GH2 is of moderate virulence regarding its effect on the blight pathogen compared to similar mycoviruses found in Michigan and those in Europe (Fulbright et al. 1983; Enebak et al. 1994); the average size of cankers produced by GH2 infected *C. parasitica* are approximately one-third the size of cankers produced by *C. parasitica* that is not infected with GH2 (Fulbright et al. 1983). Other mycoviruses may debilitate the pathogen so much that asexual, conidia production nearly ceases while other mycoviruses have little affect on canker expansion (Fulbright et al. 1983; MacDonald & Fulbright 1991; Dawe & Nuss 2001; Milgroom & Cortesi 2004). It should also be noted that since mycoviruses inhibit sexual reproduction of *C. parasitica*, they only spread via asexual conidia that can cause new cankers (vertical transmission) or converting existing cankers via hyphal anastomosis (horizontal transmission) (Anagnostakis 1984; Fulbright & Paul 1988; Zhang et al. 1998; Chapters 1 & 2).

My reasons for using GH2 were twofold: 1) slow canker expansion so that cankers do not girdle stems or branches and b) allow the pathogen to grow and produce mycovirus containing asexual conidia, to promote mycovirus spread. The ideal

mycovirus, which effectively reduces symptoms of chestnut blight while still spreading efficiently through the blight population, might be considered a "Goldilocks mycovirus" (see Chapter 1 for a discussion of how different mycoviruses affect *C. parasitica*).

This study details results for the first three years of implementing Davelos and Jarosz's assertion that mycovirus introductions should concentrate on trees between 1 and 10cm DBH and investigates how trees in that size class have performed at three sites since mycovirus introduction. Success or failure of this biological control mechanism cannot be fully determined in three years; a long-term approach to recovery is imperative as is other biological control situations (Havens et al 2012).

METHODS

Three blight-infected populations of American chestnuts were used for mycovirus introductions. Site locations and descriptions of Missaukee Healthy, Stivers, and Leelanau (MH, ST, and LE, respectively) can be found in Davelos & Jarosz (2004). Two populations (MH and LE) became infected with blight in 1997 (Chapter 2; AL Davelos Baines and AM Jarosz, unpublished data) while the ST site has been infected by the blight pathogen since the 1980s (DW Fulbright pers. comm.). The presence of mycoviruses at these sites prior to my mycovirus introductions was less than 5% (Davelos 1999, and this study).

Bark samples from thirty cankers in each population were collected early in 2009 and the three most common vegetative compatibility groups (VCGs) were determined using the techniques of Huber (1996; and see Chapter 2). A representative

from each VCG at each site was paired with a GH2 infected *C. parasitica* isolate revived from filter paper long-term storage.

Mycovirus transfer to the uninfected isolate was evident by a change in phenotype (Figure 4-1) including slowed growth rate relative to uninfected isolate and scalloped margin of the newly infected isolate. For each VCG, a slurry of each mycovirus containing isolate was created using one liter of water agar, one liter sterile water, and 20—25 petri plates containing the GH2 infected isolate and blending it into a consistency similar to applesauce.

Trees in the 1—10cm DBH size class were chosen randomly from a pool of available trees in each population; comparative performance of trees above 10cm was not possible since there are few trees available at the three sites in that size class. Total numbers of trees and cankers treated varied across the three-year mycovirus deployment period. Both infected and disease-free tree were inoculated with mycovirus. A mixture of mycovirus infected isolates representing the three most VCGs (see Chapter 2) were used to inoculate trees. At the ST population, cankers were treated with the VC type matching the VCG, or with a mixture of VC types if canker VCG was unknown or if new cankers were created.

APPLICATION OF GH2

For the 2009 and some 2010 applications, a cordless drill with a 0.6 or 1 cm drill bit was used to create holes around the canker circumference in uninfected tissue (the smallest trees were injured with the smaller drill bit and *vice versa,* Figure 4-2a). Canker conversion and canker initiation rates were very low using this method so a scratching

method was used in subsequent applications. In May of 2010 and 2011 the margins of existing cankers were scratched with a small wooden paddle with lag screws attached at one end (Figure 4-2b). Threads of the lag screws allowed the bark to be wounded sufficiently to expose both the phloem and cambium leading to a larger amount of surface area available to infect than drill bits. A thick slurry of mycovirus infected mycelia 'applesauce' was applied with a laboratory squirt bottle and paintbrush. In 2009 the treated and created cankers were covered with laboratory bench paper for two weeks to maintain a humid environment but was not used in subsequent treatment years.

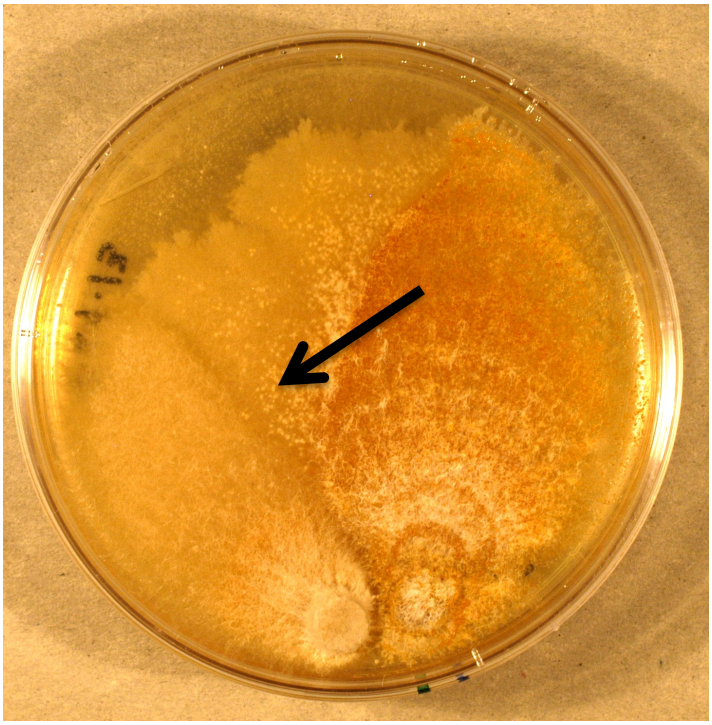

Figure 4-1. Representative mycovirus transfer plate. A GH2 infected donor strain, left; note flattened appearance of mycelia with a virulent recipient strain, right; note raised appearance. Successful conversion of virulent strain occurred near arrow. A change in phenotype similar to the left isolate shows successful transfer of GH2 from donor to recipient strain.

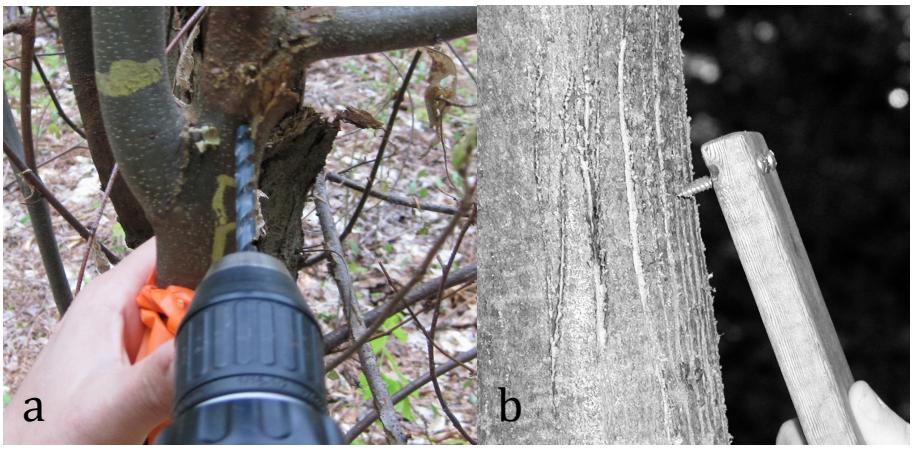

Figure 4-2. Two forms of treating cankers used in this study. a) drill bit created holes at canker margins where mycovirus inoculum could be introduced and b) "scratcher" with lag screws to wound canker and surrounding area; more surface area was able to be treated with this method. Figure 2b photo credit: AM Jarosz.

DETERMINING MYCOVIRUS PREVALENCE

Trees were visited during August of each year to determine the level of mycovirus transfer to existing cankers and creation of hypovirulent cankers on uninfected trees. Each treated tree was measured at DBH (at a height of approximately 1.3 meters). In 2011 before mycoviruses were applied and in June of 2012, previously treated cankers were sampled by removing bark tissue at canker margins.

Subsamples of 2x2mm were removed from the larger bark sample and surface sterilized in 10% sodium hypochlorite for 15s, repeated, then washed in ddH20 for 15s. Bark subsamples were placed onto petri plates with potato dextrose agar (PDA). When chestnut blight fungus began to grow, cultures were hyphal tipped and placed onto new PDA plates. After 2 weeks, isolates were scored for the presence of mycovirus based

culture morphology. A scalloped margin on the culture indicated that mycoviruses were present.

Average DBH for treated trees was calculated for surviving trees at each annual time step between 2009 and 2012 (Table 4-2). Diameter at breast height was used as a proxy for tree size; growth from year to year was calculated only for trees that remained alive during each time-step. The survivorship of main stems across the time period was also determined at both treated and control populations.

Two control populations, Missaukee Diseased (MD), a population 300 meters north of MH and Stivers North (SN) did not receive mycovirus treatments. Cankers were sampled in 2012 at these two control sites to determine the prevalence of naturally occurring mycovirus. Additionally, a cohort of 30 trees in the 1—10cm diameter at breast height (DBH) size class was tracked through the time period 2009—2012 for comparison with the mycovirus treated populations

These initial studies focus on survivorship and growth of the main stem, future studies will consider the fate of individual genets and whether the GH2 mycovirus has spread to other cankers on inoculated trees and to new trees within the populations.

RESULTS

Mycovirus prevalence after treatments

Greater than 50% of the mycovirus treated cankers had mycovirus in May 2011 and June 2012 (Table 4-1). Mycovirus presence increased slightly at LE from 2011 to 2012, which is curious given the performance of treated trees at that site, but decreased at the two remaining sites. Only one of 19 cankers contained mycovirus at the

Missaukee Diseased control site, is similar to the prevalence reported by Davelos (1999) in the 1990s.

Annual survivorship of all 1 to 10cm DBH trees monitored across all five sites was 74%. However, annual survivorship varied among treated sites with average survivorship of treated trees being highest at ST (87%), intermediate at LE (72%), and MH (65%) having the lowest. Control sites MD and SN had survivorship annual survivorship at 77% and 69%, respectively. Nearly half of the first cohort of 13 trees treated at LE in 2009 died by 2010 (Fig. 4-3). A second cohort of 44 trees was treated at LE in 2010 and monitored until 2012. Survivorship trends for both cohorts from 2010 to 2012 were roughly similar (Figure 4-3), suggesting that survivorship may vary from year to year due to environmental conditions—see discussion. Twenty-eight trees treated at MH beginning in 2009 were compared to a similar number of untreated trees of approximately the same size from MD site, which is only 300M to the northwest. Treated trees at MH displayed lower survivorship from 2009 to 2011 compared to MD, but survived much better from 2011 into 2012 (Fig. 4-4). The trend was more consistent at Stivers where the cohort of 87 treated trees survived better than untreated trees from 2009 to 2011. Unfortunately, I lost access to this site in 2012 and was unable to continue monitoring.

Davelos (1999) found that the success of mycovirus treatment was dependent on the size of the treated branch with small branches succumbing to disease at a much higher rate than larger branches. In consequence, I expected a pattern where larger trees would survive better after treatment than smaller trees. This pattern was observed only at Leelanau where treated trees that survived were larger, when

measured as DBH, than trees that died (Table 4-2). There was no pattern among the treated trees at MH with surviving trees having a larger DBH in 2009-10, smaller in 2010-11 and similar in 2011-12. Somewhat surprisingly, surviving treated trees at ST had smaller diameters than trees that died in both 2009-10 and 2010-11.

Trees at LE displayed little to no growth over the course of the experiment with the exception of trees in cohort 2 in from 2010 to 2011 (Figure 4-6) which is also evident in the average increase in diameter over all time periods and cohorts at that site. In contrast, trees at MH, MD and ST exhibited strong growth over the course of my monitoring and this represents a possible positive effect of mycovirus treatment (Figures 4-7and 4-8). Aside from trees at the LE site, which performed poorly throughout the course of the experiment (Figure 4-6), treated trees at MH and ST increased in diameter faster than trees at control sites except for the period 2011-2012 at the MD site. The second cohort at MH consisted of only five trees, four of which survived to 2012. There was also suggestion of a significant positive effect of mycovirus treatment on growth at ST, where treated trees in the southern portion of the population grew faster than untreated, control trees from the northern portion in both 2009-10 and 2010-11 (Figure 4-8).

Table 4-1. Percentage of treated cankers at three sites that contained mycovirus. Presence of mycovirus was determined based on culture morphology.

	N	Percentage with mycovirus
Missaukee Healthy		
2011	27	0.67
2012	38	0.55
Leelanau		
2011	73	0.75
2012	64	0.8
Stivers		
2011	42	0.57
2012	69	0.55
Missaukee Diseased		
2012	19[a]	0.05

a: Cankers were not treated with mycovirus at Missaukee Diseased. Thus, values represent natural levels of mycovirus prevalence at this site.

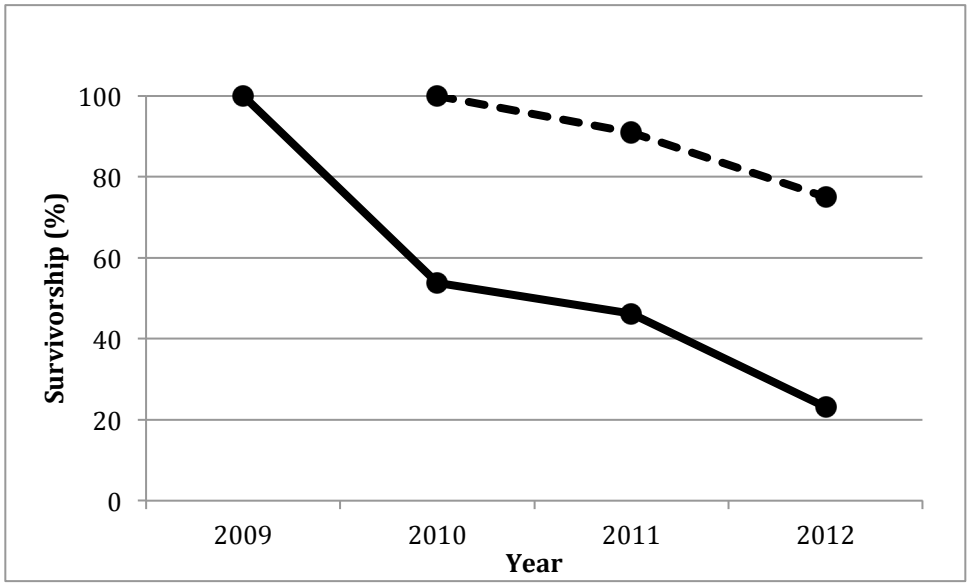

Figure 4-3. Survivorship of treated American chestnut trees at the Leelanau site. Cohort 1 (solid line) was composed of 13 trees that were treated initially in 2009. The second cohort (dashed line) consisted of 44 trees that were first treated in 2010.

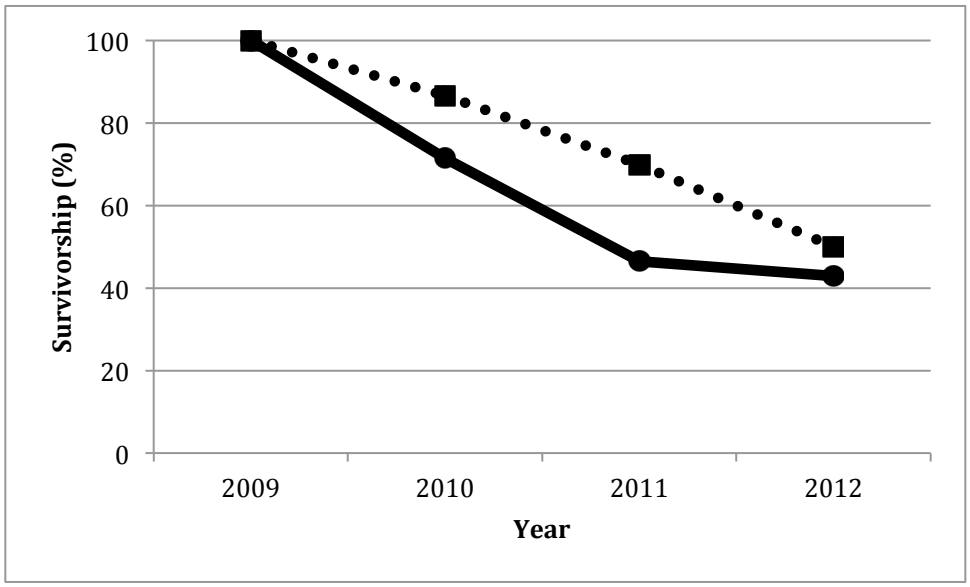

Figure 4-4 Survivorship of 28 American chestnut trees treated with mycovirus at Missaukee healthy (solid line) and compared to 30 untreated control trees at Missaukee Diseased (dotted line) over the time period 2009 to 2012. The MH and MD sites are only 300 meters apart. Cohort two of five trees at MH is not shown.

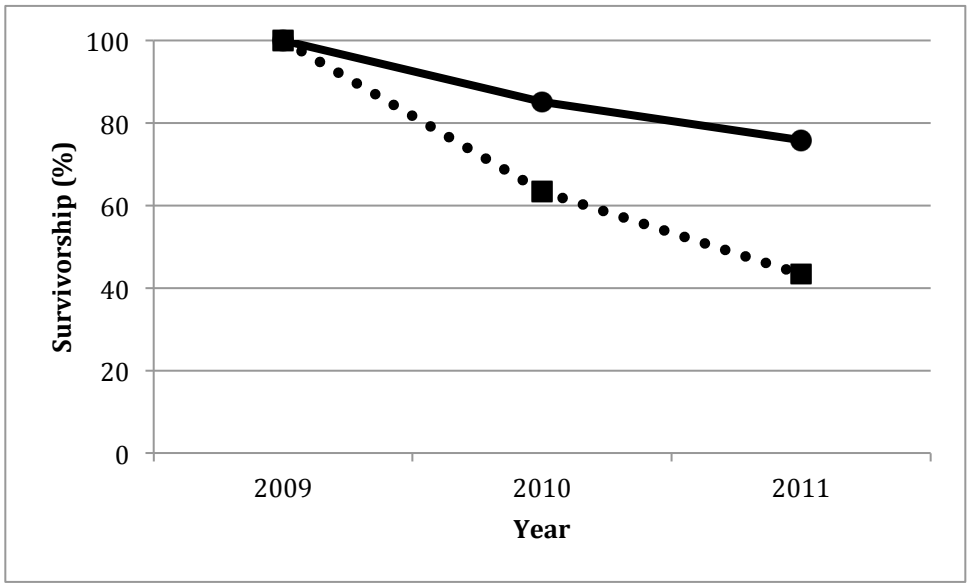

Figure 4-5. Survivorship of 87 American chestnut trees at the treated ST south trees (solid line) and 30 untreated control trees in the northern portion of the site (dotted line) from 2009 to 2011.

Table 4-2. Average size (DBH) of trees that survived and trees that died over each time step. Missaukee Diseased and Stivers North data are control populations. Standard errors are in parentheses.

	2009-10		2010-11		2011-12	
	Alive	Dead	Alive	Dead	Alive	Dead
Leelanau–LE (treated)	4.7 (1.7)	4.8 (1.9)	5.4 (0.8)	4.3 (1.9)	5.6 (0.8)	4.3 (1.1)
Missaukee Healthy–MH (treated)	3.3 (0.7)	2.6 (0.9)	3.9 (0.9)	4.2 (1.5)	3.9 (0.8)	3.9 (2.3)
Missaukee Diseased–MD (control)	2.5 (0.5)	2.4 (1.2)	2.9 (0.6)	2.6 (0.8)	3.1 (0.8)	3.2 (0.8)
Stivers–ST (treated)	2.3 (0.4)	4.4 (1.6)	3.9 (0.9)	4.2 (1.3)	na*	na
Stivers North-SN (control)	3.0 (0.7)	2.3 (0.7)	3.1 (0.9)	4.1 (1.0)	na	na

*Data for this time step are not available for these sites.

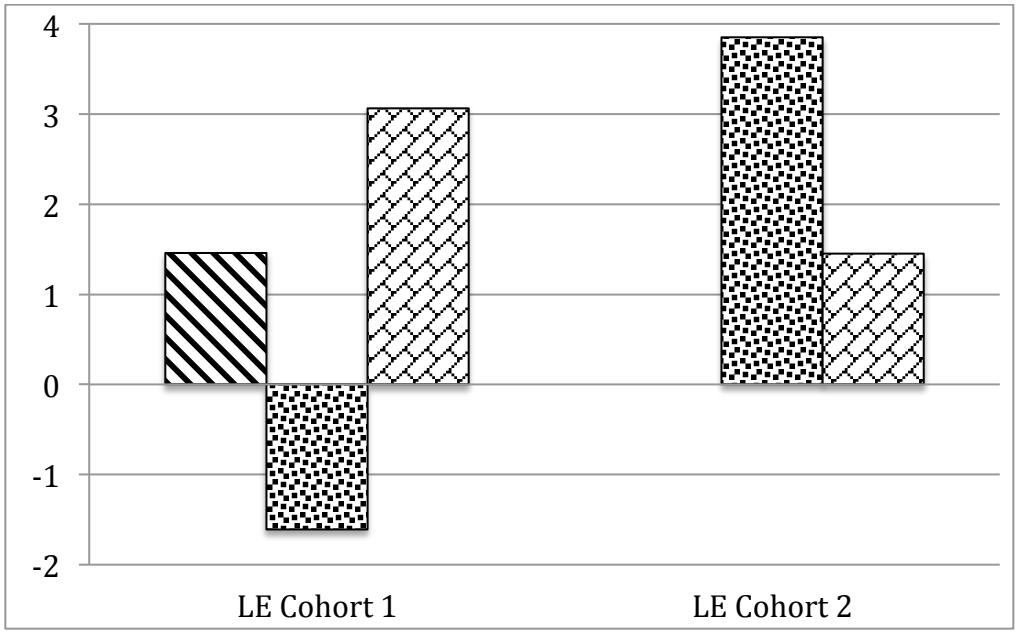

Figure 4-6. Percentage annual change in DBH for surviving trees at LE site. There is no control for the LE site. Diagonal bars are growth from 2009 to 2010, dotted bars are increase (or decrease) from 2010 to 2011, and brick bars represent the increase from 2011 to 2012. At LE Cohort 2, treatment did not begin until 2010.

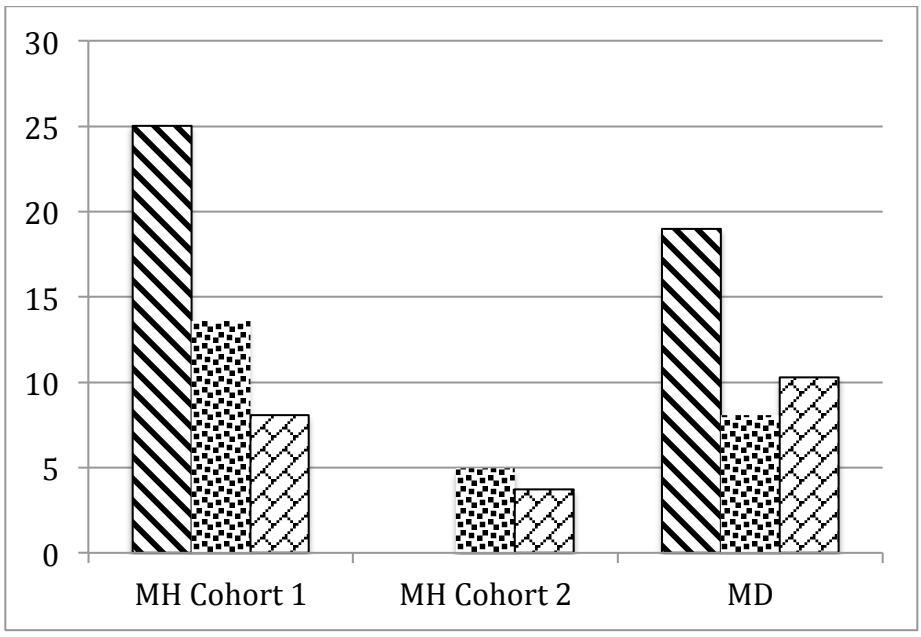

Figure 4-7. Percentage annual change in DBH for surviving trees at MH (treated) and MD (control) sites. Dark bars are growth from 2009 to 2010, dotted bars are increase from 2010 to 2011, and brick bars represent the increase from 2011 to 2012. At MH Cohort 2, treatment did not begin until 2010. Percentage change in DBH is calculated as the average increase in stem diameter over the previous year for Figures 6, 7, and 8.

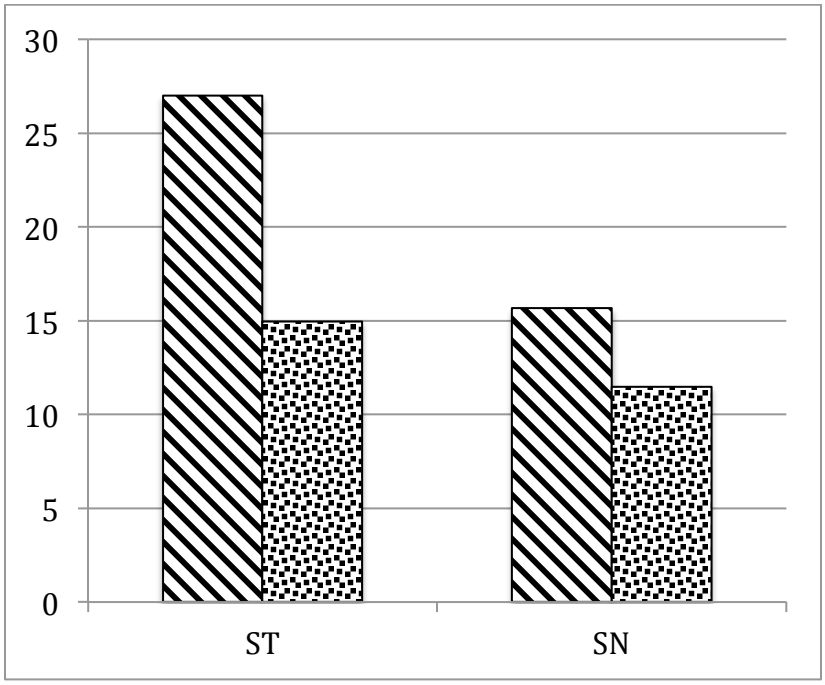

Figure 4-8. Percentage annual change in DBH for surviving trees at ST (treated) and SN (control) sites. Diagonal bars are growth from 2009 to 2010, dotted bars are increase from 2010 to 2011, Data for the final period are not available for either Stivers site.

DISCUSSION

Biological control of chestnut blight has received intense interest since mycoviruses were discovered in this system (Van Alfen 1982; Fulbright & Paul 1988; MacDonald & Fulbright 1991). However, the persistence and spread of mycoviruses has been difficult in North America, while mycovirus induced recovery in European chestnut populations has been more successful (Robin & Heiniger 2001; Milgroom & Cortesi 2004; Chapters 1 & 2). Failure in North America has been attributed to the high

VC diversity within *C. parasitica* populations (see Chapters 1 & 2) but may also be blamed on the fact that mycoviruses might not succeed in areas far beyond where they are found to be naturally occurring (MacDonald & Fulbright 1991), thus my choice to use GH2 for this study.

Survivorship may vary from year to year due to environmental conditions at individual sites. This is evident when cohorts that began treatment in different years perform similarly (*e.g.*, the LE site) across years. Differences among sites and among years may also be due to biotic interactions at individual sites. Performance at each treated site may be linked to community structure. Both ST and SN have little competition from other tree species and are nearly chestnut monocultures of small to medium sized trees. The MH site has few chestnut overstory trees and other tree species (Red Maple and American Beech) may be outcompeting infected American chestnuts. Where large overstory chestnuts have been harvested at the MH site, small chestnuts have begun to grow rapidly (JC Springer pers. obs.). The MD site has many large chestnut trees in the overstory that have died, allowing small, understory chestnuts to be released into the canopy at an increased rate probably due to increased light availability. The LE site is similar to the MH site in that many large non-chestnuts are in the overstory but there are some very large chestnuts succumbing to chestnut blight.

Data in this chapter should be interpreted as preliminary. The ultimate success or failure of using the native mycovirus, GH2, as a biological control may not be realized until a much longer time period has elapsed. Although trees were treated for three years, success of mycoviruses being transferred to cankers has been disappointingly

low. Future work should explore alternate methods of inoculating mycoviruses onto cankers that may prove to be more efficient at transferring the mycovirus

Based on stage-based demographic analyses, Davelos (1999) and Davelos & Jarosz (2004) proposed that treatment of American chestnuts in the size class of 1—10 centimeters DBH would be the most efficient at initiating population level recovery from blight. A clear trend of larger trees in the 1—10 cm DBH size class surviving better than smaller trees in that category was not found with my introduction of mycovirus GH2. It would be expected that treated trees above 3cm DBH would have better survivorship since smaller trees succumb to girdling cankers much faster than larger trees. Low survivorship of treated trees in the 1—10cm size class was not considered by Davelos & Jarosz (2004), although their data indicated the potential for poor performance for the smallest trees. The smallest trees in that category (1—2cm DBH) may actually perform most poorly since blight cankers may grow faster than trees expand even when mycoviruses are present. Davelos Baines et al. (*in press*) found similar results in their treatment of varying branch sizes with mycovirus; branches above 2cm diameter performed well while those less than 2cm diameter did not see enhanced growth. Jarosz et al. (*in press*) also found that larger trees outperformed smaller trees when mycovirus treatments were applied to main stems in West Salem, WI. More treatment may be needed at these Michigan sites and should focus on trees that have diameters greater than 3cm DBH. Ultimately however, more time is needed to determine how treated trees will perform at each treated sites. Additionally, it may be prudent to test success of mycovirus introduction in a managed forest situation where competing hardwoods are cut. Griffin et al. (1991) suggested that American

chestnut may grow rapidly when released from or competition from other species is lowered, especially on mesic sites.

Other important aspects of this interaction that should be studied in detail are: a) the influence of tree genotype on recovery since some trees that survive may be better able to tolerate infection by chestnut blight than their congeners, b) how individual chestnut blight VCGs and mycovirus GH2 interact may also be crucial to slow canker expansion, c) influence of the abiotic environment, timing of mycovirus application, and how often to apply mycoviruses, and d) how the mycovirus evolves may also be critical to mycovirus long-term persistence and spread. Additionally, the type of site may influence recovery, i.e., monoculture of chestnut versus a mixed forest situation. The LE and MH sites could be considered a mixed forest where a canopy of other species such as American Beech, Red Maple, and Sugar Maple may be suppressing the growth of understory American chestnuts. The Stivers sites are essentially a monoculture of American chestnut with other species rarely scattered throughout the community. Reduced competition may have allowed trees at the ST site to grow larger after mycovirus GH2 was introduced to chestnut blight cankers in that population.

Managing the chestnut blight fungus with mycoviruses and realizing the goal of broad-scale reintroduction of American chestnuts to our hardwood forests may require our persistence and patience. Many unanswered questions remain regarding our ability to use native mycoviruses as a biological control mechanism but progress is being made.

LITERATURE CITED

LITERATURE CITED

Anagnostakis SL. 1987. Chestnut Blight : The Classical Problem of an Introduced Pathogen. Mycological Society of America. 79:23–37.

Andrieu E, Fréville H, Besnard A, Vaudey V, Gauthier P, Thompson JD, and Debussche M. 2013. Forest-cutting Rapidly Improves the Demographic Status of *Paeonia officinalis*, a Species Threatened by Forest Closure. Population Ecology. 55:147–158.

Barton J, Fowler SV, Gianotti AF, Winks CJ, de Beurs M, Arnold GC, and Forrester G. Successful Biological Control of Mist Flower (*Ageratina riparia*) in New Zealand: Agent Establishment, Impact and Benefits to the Native Flora. Biological Control. 40:370–385.

Biraghi A. 1953. Possible active resistance to *Endothia parasitica* in *Castanea sativa*. Reports of 11th Congress of the International Union of Forest Research Organization, Rome, Italy. 643-645.

Burdon JJ, Groves RH, and Cullen JM. 1981. The Impact of Biological Control on the Distribution and Abundance of *Chondrilla juncea* in Southeastern Australia. Journal of Applied Ecology. 18:957–966.

Caswell H. 2010. Life Table Response Experiment Analysis of the Stochastic Growth Rate. Journal of Ecology. 98:324–333.

Chouvenc T, Su NY, and Grace JK. 2011. Fifty Years of Attempted Biological Control of Termites – Analysis of a Failure. Biological Control. 59:69–82.

Chua TH. 1978. A model of an aphid- parasite-hyperparasite system, with reference to timing of attack. Journal of Malaysian Agriculture. 51:375-86

Cooper TM, Frank JH, Cave RD, Burton MS, Dawson JS, and Smith BW. 2011. Release and Monitoring of a Potential Biological Control Agent, *Lixadmontia franki*, to Control an Invasive Bromeliad-eating Weevil, *Metamasius callizona*, in Florida. Biological Control. 59:319–325.

Crow WT. 2005. Alternatives to fenamiphos for management of plant-parasitic nematodes on bermudagrass. Journal of Nematology. 37:477-482.

Crowder LB, Crouse DT, Heppell SS, and Martin TH. 1994. Predicting the impact of turtle excluder devices on loggerhead sea-turtle populations. Ecological Applications. 4:437-445.

Crowder LB, Hopkins-Murphy SR, and Royle JA. 1995. Effects of turtle excluder devices (TEDs) on loggerhead sea turle strandings with implications for conservation. Copeia. 4:773-779.

Dawe AL and Nuss DL. 2001. Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. Annual Review of Genetics. 35:1–29.

Davelos AL. 1999. Double-stranded RNA mediated recovery of American chestnut populations: a demographic analysis. PhD thesis, Michigan State University, East Lansing, Michigan.

Davelos Baines A, Fulbright DW, and Jarosz AM. 2013. Effects of Branch Size and Pathogen Virulence on Canker Development and Branch Mortality. Acta Horticulturae. *In press*.

Davelos AL and Jarosz AM. 2004. Demography of American Chestnut Populations: Effects of a Pathogen and a Hyperparasite. Journal of Ecology. 92:675–685.

DeBach P. 1974. Biological control by natural enemies. Cambridge University Press, Cambridge, UK.

Elliston JE, Jaynes RA, Day PR, and Anagnostakis SL. 1977. A native American hypovirulent strain of *Endothia parasitica*. Proceedings of the American Phytopathological Society 4:83. (Abstract)

Enebak SA, Hillman BI, and MacDonald WL. 1994. A Hypovirulent Isolate of *Cryphonectria parasitica* with Multiple, Genetically Unique dsRNA Segments. Molecular Plant-Microbe Interactions. 7:590–595.

Fujiwara M and Caswell H. 2001. Demography of the Endangered North Atlantic Right Whale. Nature. 414:537–41.

Fulbright DW, Paul CP, and Garrod SW. 1988. Hypovirulence: a Natural Control of Chestnut Blight. In: Biocontrol of Plant Diseases Volume II [KG Mukerji and KL Garg]. pp 122–136.

Fulbright DW, Weidlich WH, Haufler KZ, Thomas CS, and Paul CP. 1983. Chestnut Blight and Recovering American Chestnut Trees in Michigan. Canadian Journal of Botany. 61:3164–3171.

Grente J. 1965. Les formes hypovirulentes d'Endothia parasitica et les espoirs de lutte contre le chancre du châtaignier. Comptes-rendus des Seances de l'Academie d'Agriculture de France. 51:1033-1037.

Grevstad FS. 2006. Ten-year Impacts of the Biological Control Agents *Galerucella pusilla* and *G. calmariensis* (Coleoptera: Chrysomelidae) on Purple Loosestrife (*Lythrum salicaria*) in Central New York State. Biological Control 39:1–8.

Griffin GJ, Smith HC, Dietz A, and Elkins JR. 1991. Importance of Hardwood Competition to American Chestnut Survival, Growth, and Blight Development in Forest Clearcuts. Canadian Journal of Botany. 69:1804–1809.

Havens K, Jolls CL, Marik JE, Vitt P, McEachern AK, and Kind D. 2012. Effects of a Non-native Biocontrol Weevil, *Larinus planus*, and Other Emerging Threats on Populations of the Federally Threatened Pitcher's Thistle, *Cirsium pitcheri*. Biological Conservation. 155:202–211.

Holt RD and Hochberg ME. 1997. When Is Biological Control Evolutionarily Stable (or Is It)? Ecology. 78:1673–1683.

Jarosz AM, Springer JC, Fulbright DW, Double ML, and MacDonald WL. 2013. Hypovirus influence on survivorship and growth of American chestnuts at West Salem, Wisconsin, USA. Acta Horticulturae. *In press*.

Jezorek H, Baker AJ, and Stiling P. 2012. Effects of *Cactoblastis cactorum* on the Survival and Growth of North American Opuntia. Biological Invasions. 14:2355–2367.

Kephart SR and Paladino C. 1997. Demographic change and microhabitat variability in a grassland endemic, *Silene douglasii* var. *oraria* (Caryophyllaceae). American Journal of Botany. 84:179–189.

Louda SM and O'Brien CW. 2002. Unexpected Ecological Effects of Distributing the Exotic Weevil, *Larinus Planus* Fabricius, for the Biological Control of Canada Thistle. Conservation Biology. 16:717–727.

MacDonald WL and Fulbright DW. 1991. Biological Control of Chestnut Blight: Use and Limitations of Transmissible Hypovirulence. Plant Disease. 75:656–661.

McEvoy PB and Coombs EM. 1999. Biological control of plant invaders: regional patterns, field experiments, and structured population models. Ecological Applications. 9:387–401.

Milgroom MG and Cortesi P. 2004. Biological Control of Chestnut Blight with Hypovirulence: a Critical Analysis. Annual Review of Phytopathology. 42:311–38.

Morin L, Reid AM, Sims-Chilton NM, Buckley YM, Dhileepan K, Hastwell GT, Nordblom TL, and Raghu S. 2009. Review of Approaches to Evaluate the Effectiveness of Weed Biological Control Agents. Biological Control. 51:1–15.

Murdoc WW, Chesson J, and Chesson PL. 1985. Biological Control in Theory and Practice. American Naturalist. 125:344–366.

Reilly JR and Hajek AE. 2012. Prey-processing by Avian Predators Enhances Virus Transmission in the Gypsy Moth. Oikos. 121:1311–1316.

Robin C and Heiniger U. 2001. Chestnut Blight in Europe: Diversity of *Cryphonectria parasitica*, Hypovirulence and Biocontrol. Forest Snow and Landscape Research. 367:361–367.

Shanmuganathan T, Pallister J, Doody S, McCallum H, Robinson T, Sheppard A, Hardy C. 2010. Biological Control of the Cane Toad in Australia: a Review. Animal Conservation. 13:16–23.

Silvertown J, Franco M, Pisanty I, and Mendoza A. 1993. Comparative plant demography: relative importance of life-cycle componenets to the finite rate of increase in woody and herbaceous perennials. Journal of Ecology. 81:465-476.

Simberloff D and Stiling P. 1996. How Risky Is Biological Control? Ecology. 77:1965–1974.

Smith SM. 1996. Biological Control with Trichogramma: Advances, Successes, and Potential of Their Use. Annual Review of Entomology. 41:375–406.

Van Alfen NK. 1982. Biology and potential for disease control of hypovirulence of *Endothia parasitica*. Annual Review of Phytopathology. 20:349–362.

Van Alfen NK, Jaynes RA, Anagnostakis SL, and Day PR, 1975. Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. Science. 189:890–891.

Yeates AG, Schooler SS, Garono RJ, and Buckley YM. 2011. Biological Control as an Invasion Process: Disturbance and Propagule Pressure Affect the Invasion Success of *Lythrum Salicaria* Biological Control Agents. Biological Invasions.14:255–271.

Zhang L, Baasiri RA, and Van Alfen NK. 1998. Viral suppression of fungal pheromone precursor gene expression. Molecular and Cellular Biology. 18:953- 959.

CHAPTER 5

CONCLUSIONS

When dsRNA mycovirus hyperparasites invade populations of the chestnut blight pathogen, *Cryphonectria parasitica*, they appear to change the structure of the blight population. Where mycoviruses are present, they prevent sexual reproduction of *C. parasitica* and reduce blight pathogen virulence on American chestnut, allowing American chestnuts, *Castanea dentata,* to tolerate infection. Since sexual reproduction is likely not occurring in populations of *C. parasitica* where mycoviruses are present, fungal reproduction is limited to asexual, splash-dispersed conidia, which limits dispersal and by extension migration relative the more mobile, wind-dispersed, sexually produced ascospores. Further, as presented in Chapter 2, vegetative compatibility group (VCG) diversity of *C. parasitica* is low in populations where mycoviruses are present. Populations with mycovirus presence generally have a unique VCG signature while populations of *C. parasitica* without mycoviruses have higher VCG diversity and those VCGs are often shared between populations. Mating type analyses presented in Chapter 2 lent further support that mycovirus infected blight populations are clonal since mating type allele frequencies were skewed for the presence of one mating type.

Natural selection may play a role in generating the unique VCG signatures found at mycovirus-infected *C. parasitica* populations in Michigan; populations are clonal and selection may act on all possible combinations of mycovirus and fungal genotype to find the best combination for a particular location, thus reducing VCG diversity over time. It has been hypothesized that mycoviruses would only invade populations of *C. parasitica*

if VCG diversity is low, since closely related VCGs may allow easy spread of mycoviruses through populations. I suggest that mycoviruses may invade all VCGs in a blight population and natural selection will operate to reduce VCG diversity and potentially reduce genome-wide diversity within a local population. This may be one of the reasons why we find a unique VCG signature AND unique mycoviruses at each Michigan site studied in Chapters 2 and 3.

Microsatellite analyses from Chapter 3 provided some support to the hypothesis that mycovirus presence in populations of *C. parasitica* would reduce overall genomic diversity. In populations where dsRNA mycoviruses have been present for a long time (CL and RC), genomic diversity does appear to be much lower relative to *C. parasitica* populations not infected with mycoviruses (LE and MD). FR, although infected with mycoviruses has high VCG and also high genomic diversity indicating that sexual reproduction may be occurring there. Conversely, ST a mycovirus free site exhibits low diversity but high VCG relative to other sites in Michigan; it is unclear why diversity at that site may be low. Sexual reproduction at mycovirus free sites may likely increase diversity and allow migration of ascospores between these sites; there is evidence of high migration rates between mycovirus free sites relative to mycovirus infected sites. Together with higher number of migrants predicted for epidemic populations and low migration observed between recovering populations these generalizations makes sense given the biology of the system. The influence of mycoviruses on the whole genome of *C. parasitica* is only partially understood at this point. More work should be done in this area to develop a full dataset of genomic diversity by optimizing primers developed for

C. parasitica populations in Europe or by developing new molecular markers unique to populations in North America.

Mycovirus introduction into *C. parasitica* populations has been shown to help American chestnut trees recover or at least tolerate blight infection. A test of a hypothesis developed by Davelos (1999) and Davelos & Jarosz (2004) was conducted to understand whether mycovirus-mediated biological control of *C. parasitica* would be most successful on American chestnut trees in the 1—10cm diameter at breast height (DBH) size class rather than focusing on the largest trees in a population. My preliminary results for this long-term study show that the smallest trees in that size class may not be the best choice for treatment and should instead focus on the trees between 3—15cm DBH. Annual survivorship of treated trees varied from 87% to 65%. Cohorts that began treatment in different years responded similarly to mycovirus treatment, suggesting that tree survivorship may vary from year to year due to environmental conditions. Competition from other tree species may also play a role in mycovirus success; trees at ST responded better to mycovirus treatment, possibly because this site is a monoculture of chestnuts with little competition or overstory compared to the LE and MH sites. Mycovirus GH2 may hold some promise for allowing recovery of American chestnuts in Michigan: it has a moderate virulence on the chestnut blight pathogen relative to other mycoviruses while still allowing mycovirus-containing asexual spores of *C. parasitica* to be produced that could ideally spread naturally throughout a local blight population. One important issue not to be ignored when introducing mycoviruses for biological control is their persistence in *C. parasitica* cankers over time. Treated blight cankers in my study were examined one year after

each of three successive treatments which revealed an average of on 50% of cankers containing mycoviruses. Repeated introductions of mycoviruses into blight cankers may be necessary to achieve recovery in American chestnut populations. Future work in this area should also explore whether tree genotype may play a role in the success or failure of biological control of *C. parasitica* with mycoviruses. Individuals or populations of American chestnut that are less susceptible may be predisposed to tolerating blight infection, thus work that unites moderate virulence mycoviruses and known genotypic effects of American chestnuts would be a great addition our understanding of this tri-trophic interaction. I suggest testing other mycoviruses from Michigan for their effectiveness as biological control agents. It is possible that effective biological control will involve matching a specific mycovirus with the *C. parasitica* and American chestnut populations found at a given sites.

The long-term storage of isolates of *C. parasitica*, especially those infected by mycoviruses is important for future work that considers how mycoviruses have evolved over short time period with regard to their effect on the fitness of the fungal host. Work in Appendix A examined effectiveness of resurrecting isolates from sterile water long-term storage. However, isolates that were infected when stored in the mid to late 1990s were not found to be harboring mycoviruses when they began growing again for this study; only two of 119 isolates, originally identified at mycovirus-infected could be confirmed to still contain dsRNA. A stable, reliable storage method for mycovirus infected isolates needs to be found. I have initiated an experiment that will test mycovirus retention under two different storage methods.

My studies on VCG and microsatellite diversity suggest that *C. parasitica* population structure will change as mycoviruses invade. Monitoring the LE and MH sites where I have been introducing mycoviruses since 2009 may provide an opportunity to follow this expected structural change. Studies that try to understand how VCG diversity change over time in these populations would be important to understand my hypothesis presented at the end of Chapter 2 stating that VCG diversity may actually decrease as natural selection works to find the best adapted combination of *C. parasitica* genotype by mycovirus for a local population. Moreover, expansion of microsatellite work in Chapter 3 should include further comparisons of populations of *C. parasitica* long infected by mycoviruses (such as those treated multiple times with mycoviruses as part of a biological control scheme, i.e., the West Salem, WI site) and those where mycoviruses have not been successful (i.e., the natural range of American chestnut in the eastern United States). A comparison of C. parasitica genomic diversity in Europe and the U.S. to that in Asia, the purported native range of the blight pathogen, would lead to a better understanding of expansion of pathogen diversity after colonization to its non-native ranges.

LITERATURE CITED

LITERATURE CITED

Davelos AL and Jarosz AM. 2004. Demography of American chestnut populations: effects of a pathogen and a hyperparasite. Journal of Ecology 92:675-685.

Davelos AL. 1999. Double-stranded RNA mediated recovery of American chestnut populations: a demographic analysis. PhD thesis, Michigan State University, East Lansing, Michigan.

APPENDIX

EVALUATING THE LONG-TERM STORAGE OF CRYPHONECTRIA PARASITICA

ABSTRACT

Isolates of the Chestnut blight pathogen, *Cryphonectria parasitica*, from six populations in Michigan, were stored in the late 1990s as agar plugs of mycelium in vials of sterile water held at room temperature. Approximately 29% of the fungal isolates were infected with mycoviruses at the time of storage. Each isolate was tested for revivification effectiveness by taking aliquots from vials filled with agar plugs of *C. parasitica* and sterile water and plating onto potato dextrose agar. Average revivification success was 70.5% across populations with a range of 33—84% within populations . In situations where vials had dried out during storage, success was low (4%), while success for vials that retained sterile water averaged 90%. Most importantly however, is the loss of mycoviruses from stored isolates; only 2 of 119 stored mycovirus infected isolates still contained mycoviruses after storage, suggesting that the double-stranded RNA mycoviruses are degraded during storage.

INTRODUCTION

A well-known example of long-term storage of living organisms is the work of Richard Lenski and colleagues (Lenski & Travisano 1994). In their work, aliquots of *Escherichia coli* bacteria have been stored every 500 generations for greater than 20 years, creating a frozen, revivable equivalent to a fossil record (Woods et al. 2011; Cooper & Lenski 2000). In this way, baseline, ancestral clones have been available for direct comparison with evolved lines at any point their evolutionary history (Lenski et al. 1991) especially to determine average changes in fitness over time (Woods et al. 2011).

Collecting and storing living isolates from diverse taxa can allow temporal changes in both phenotype or genotype to be readily assessed. Storage of fungal cultures, for example, in a viable and stable state is important for future studies that relate to pathogen identification, disease control, quarantine, and breeding resistant plants (Abd-Elsalam et al. 2010) and for detecting any changes in virulence.

Fungal isolates are commonly stored for short or long time periods by sub-culturing onto new media filled petri plates, silica gel, or water suspension at $5^{o}C$ (Richter and Bruhn 1989), or the use of organic substrates such as wood chips or filter paper, or freezing (Nakasone et al 2004). Isolates were stored originally using a method developed by Jones et al. (1991) for use in storing taxonomically diverse fungal species. The method is simple, economical, and does not require freezing or refrigeration. It has not been tested previously with *C. parasitica*, the pathogen

responsible for chestnut blight disease in American chestnuts, *Castanea dentata* (Marsh) Borkh.. Cultures for this study have been in storage since being collected from six populations in Michigan between 1996 and 2000 (Davelos 1999; AL Davelos, unpublished data). This study reports on my attempts to revive these samples for use in a temporal study of vegetative compatibility group patterns across *C. parasitica* populations in Michigan (Chapter 2).

MATERIALS AND METHODS

INITITAL STORAGE

Storage in one-dram vials followed the technique of Jones et al. (1991). After growing for about two weeks on Potato Dextrose agar (PDA, Difco: Becton, Dickinson and Company. Sparks, MD). two to three plugs of *C. parasitica* mycelia made using a 3-millimeter cork borer or sterilized end of a glass pipette and placed into 15 x 45 millimeter 1 dram glass vials (Kimble Glass, Inc.), filled with approximately 2mL of sterile water and then sealed with parafilm. The isolates were originally collected from six Michigan populations of *C. parasitica*: Roscommon (RC), Stivers (ST), County Line (CL), Frankfort (FT), Missaukee Diseased (MD) and Missaukee Healthy (MH). All populations are located in the northern half of Michigan's lower peninsula (see Davelos and Jarosz 2004 for detailed locations and see Chapter 2 for a map). Numbers of samples saved and year of storage were variable for each population (Table 1). Isolates from three populations (RC, CL, and FT) were infected with double-stranded RNA (dsRNA) mycoviruses, which reduce both growth rates and conidia production of

infected *C. parasitica* cultures. Mycoviruses were not present in isolates from the remaining three sites (ST, MD, and MH).

REVIVIFICATION TESTS

In 2010, vials were inspected for water content and filled with sterile water if they had dried out. Sterile water was added to dry vials along the vial shoulder in order to attempt to rehydrate spores or mycelia. All vials were vortexed to homogenize the sample and allowed to rest for approximately one hour before 200μL aliquots were removed and spread onto PDA plates with a sterile glass hockey stick. Growth of *C. parasitica* or contaminants such as bacteria or other fungal species was noted after approximately two weeks; successfully revived isolates were placed back into long-term storage in new vials using initial storage methods. Samples were only tested once.

RESULTS

Stored cultures were considered revived if *C. parasitica* grew from the plated sample. Lack of growth or, growth of bacterial or fungal colonies other than *C. parasitica* were noted as unsuccessful or contaminated, respectively. In rare cases, tiny sub-samples or hyphal tips of contaminated *C. parasitica* could be taken from a contaminated plate and successfully plated onto fresh PDA media. *Cryphonectria parasitica* was successfully revived from 70.5% of the stored vials, but populations differed in their success rate ranging from a low of 33.3% for MD samples to 84.3% for CL samples (Table 1). The success rate for vials that had dried out was very low (3 of 73 = 4%), while 89.7% of the samples with water remaining were successfully revived. Additionally, there was no significant difference in the revivification success for isolates

Table A-1. Overview of isolates tested for resurrection success from six Michigan chestnut blight populations. Totals are whole numbers, percentages or averages.

Population	Total tested	wet alive	dry alive	% alive	Wet dead	dry dead	% dead	Contaminated	% contaminated
Roscommon	140	111	1	80.0%	8	18	18.6%	2	1.4%
Frankfort	86	52	0	60.5%	2	30	37.2%	2	2.3%
County Line	89	73	2	84.3%	2	11	14.6%	1	1.1%
Stivers	32	18	0	56.3%	8	4	37.5%	2	6.3%
Missaukee Diseased	36	12	0	33.3%	12	2	38.9%	10	27.8%
Missaukee Healthy	27	20	0	74.1%	1	5	22.2%	1	3.7%
Totals/Averages	410	286	3	70.5%	33	70	25.1%	18	4.4%

infected with mycoviruses (71.8%) and isolates not infected with mycovirus (81.1%). Most importantly however, mycoviruses were recovered from only two of 119 stored cultures known to be infected with mycoviruses at the time of storage. Finally, only 4.4% of the vials were contaminated with other microbes.

DISCUSSION

Storing of biological samples for long time periods is an important aspect of scientific record keeping. Voucher samples of fungi should be stored in a viable state , so they can be used for future use in pathogen identification, disease control, quarantine, and breeding resistance. Work by Richter and Bruhn (1989) has shown that viability among fungal species, even after a few months, can vary from 0 to 100% for their 5^{o}C refrigerated water storage technique. Additionally, Borman et al. (2006) have shown that revival of fungal isolates averages 90% for isolates stored since 1983 but that a species effect is seen (Hartung de Capriles et al. 1989). Techniques, such as mineral oil and silica gel were a less successful and freezing at -80^{o}C was the worst (Pumpyanskaya 1964). My work reviving isolates of *Cryphonectria parasitica* indicates that there are differences in revival success even among populations. For instance, at the Missaukee Diseased population, stored isolates that remained wet were still unable to be resurrected 50% of the time. The County Line population however, had very good resurrection success: 97% of revivable isolates were in tubes that still contained sterile water.

Reviving stored *Cryphonectria parasitica* samples was effective as long as some distilled water remained in the glass vial. If the water evaporated entirely, allowing spores

and mycelia to desiccate, re-growth of the culture could not be obtained even when dry material in the tubes was immersed in fresh sterile. Thus, a tight seal of the cap and wrapping with parafilm are vital for water retention in the vials.

Most important from this study, however, may be the fact that only two isolates out of 119 isolates infected with mycoviruses maintained their mycovirus until 2010. This has important implications for the long-term storage of isolates that are used for biological control of *C. parasitica,* especially if isolates are stored from year to year and are revived to continue treatment of blight cankers during subsequent field seasons.

If presence of mycovirus in *C. parasitica* mycelia is not important, periodic inspection of water levels in glass vials must be done to determine if additional sterile water should be added to maintain isolate viability. Alternatively, if water has begun to evaporate from tubes, cultures can be grown again and stored immediately in a new water-filled tube. Duplicate or triplicate tubes of isolates should be kept so that the chances of future resurrection are increased, especially if there are inter-tube differences.

Although it is not my intent to completely review techniques for storing and reviving fungal cultures, these data on the chestnut blight fungus, *C. parasitica,* can be added to the list of studies documenting sample storage methods and success of reviving after a relatively long time period. This simple method of storing *C. parasitica* requires minimal effort, is economical, and easy to maintain with no input of electricity and therefore avoids the potential hazard of an electrical outage destroying isolates.

The ability to effectively store and revive a fungal isolate is important for comparative purposes such as confirming the identification of a unknown quarantine isolate, using isolates for comparative purposes in taxonomic studies, determining,

determining changes in virulence over time (if the culture stored has remained static during storage), and simply good record-keeping of biological samples.

At present, a test of long-term persistence of mycoviruses is being done. Chestnut blight isolates known to contain mycoviruses have been stored in sterile water and PDA slants at ambient temperature and on filter paper at -20 degrees Celsius. This long-term experiment will test mycovirus persistence over 30 years tested at designated intervals.

LITERATURE CITED

LITERATURE CITED

Abd-Elsalam, KA Yassin MA, Moslem MA, Bahkali AH, de Wit PJGM, McKenzie EHC, Stephenson SL, Cai L, and Hyde KD. 2010. Culture Collections, the New Herbaria for Fungal Pathogens. Fungal Diversity 45:21–32.

Borman AM, Szekely A, Campbell CK, and Johnson EM. 2006. Evaluation of the Viability of Pathogenic Filamentous Fungi After Prolonged Storage in Sterile Water and Review of Recent Published Studies on Storage Methods. Mycopathologia 161:361–368.

Cooper VS and Lenski RE. 2000. The population genetics of ecological specialization in evolving E. coli populations. Nature. 407:736-739.

Davelos AL. 1999. Double-stranded RNA mediated recovery of American chestnut populations: a demographic analysis. PhD thesis, Michigan State University, East Lansing, Michigan.

Hartung de Capriles C, Mata S, and Middelveen M. 1989. Preservation of fungi in water (Castellani): 20 years. Mycopathologia. 106:73—79.

Jones RJ, Sizmur KJ, and Wildman HG. 1991. A Miniaturised System for Storage of Fungal Cultures in Water. Mycologist. 5:184–186.

Lenski RE, Rose MR, Simpson SC, and Tadler SC. 1991. Long-term Experimental Evolution in Escherichia Coli. I. Adaptation and Divergence During 2,000 Generations. American Naturalist .138:1315–1341.

Lenski RE. and Travisano M. 1994. Dynamics of adaptation and diversification. A 10000 generations experiment with bacterial populations. Proceedings of the National Academy of Science. USA. 91:6808–6814.

Nakasone KK, Peterson SW, Jong SC. 2004. Preservation and distribution of fungal cultures. In: Foster MS, Bills GF, Mueller GM (eds) Biodiversity of fungi: Inventory and monitoring methods. Elsevier Academic, USA, pp 37–47

Pumpyanskaya LY 1964. Storage of microorganisms under oil. Mikrobiologiya. 33: 1065–1070.

Richter DL and Bruhn JN. 1989. Revival of saprotrophic and mycorrhizal basidiomycete cultures from cold storage in sterile water. Canadian Journal of Microbiology. 35:1055—1060.

Woods RJ, Barrick JE, Cooper TF, Shrestha U, Kauth MR, and Lenski RE. 2011. Second-Order Selection for Evolvability in a Large Escherichia Coli Population. Science 331: 1433–1436.