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ZACHARY JAMES BLANKENHEIM

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M.S. degree in PLANT PATHOLOGY


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**THE IDENTIFICATION, DISTRIBUTION AND SPREAD OF DOGWOOD
ANTHRACNOSE IN MICHIGAN**

By

Zachary James Blankenheim

A THESIS

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

MASTER OF SCIENCE

Department of Plant Pathology

2003

ABSTRACT

THE IDENTIFICATION, DISTRIBUTION AND SPREAD OF DOGWOOD ANTHRACNOSE IN MICHIGAN

By

Zachary James Blankenheim

Dogwood anthracnose, caused by the fungal pathogen *Discula destructiva*, has led to wide spread mortality of the native flowering dogwood tree, *Cornus florida*. Since its initial identification in the 1970s, *D. destructiva* has spread throughout much of the range of *C. florida*, but only one county in Michigan was identified as infected in 1993. The objectives of this study were to determine the distribution of dogwood anthracnose in Michigan forests, design species-specific PCR primers for detection of *D. destructiva*, and survey imported flowering dogwood stock in Michigan nurseries by microscopic and PCR methods. A total of 52 long-term monitoring plots recorded using global positioning systems (GPS) were developed during surveys for dogwood anthracnose in Michigan. Dogwood anthracnose was found at 13 sites in 7 counties in Michigan. PCR primers DdF and DdR targeted the internal transcribed spacer (ITS) region of rDNA, amplifying a 460 bp fragment of DNA. PCR primers for DdF and DdR were found to amplify DNA from both *D. destructiva* and *D. fraxinae*. A survey of five Michigan nurseries in 2001 examined 342 newly arrived *C. florida* and *C. kousa* trees from ten out-of-state nurseries. Analysis of twig samples from nursery trees by microscopic methods yielded an infection rate of 10.23%, with infections occurring in 8 of 10 out-of-state nurseries and all five Michigan nurseries. PCR of nursery samples using DdF and DdR detected infected trees in 2 of 10 out-of-state nurseries and 2 of 5 Michigan nurseries, for a 4.39% infection rate.

DEDICATION

I dedicate this to my grandparents Ralph and Beatrice Blankenheim, and Albert and Helen Itzin. Your love throughout my life and support of my college career allowed me to follow my dreams. Thank you.

ACKNOWLEDGMENTS

I would like to thank those people that contributed to the successful completion of these studies. Dr. Gerard Adams, my major professor, you provided me the opportunity to work under your guidance in plant pathology and introduced me to the world of molecular genetics. Dr. Janice Glime, your teaching made the realm of plants truly interesting to me. Dr. John Adler, your support as my mentor and friend throughout my college career has been inspiring.

To my mom, dad and brother – your love and support over the years is more than any son/brother could have asked for. You've always been behind my career choices without hesitation and that means the world to me, thank you.

Tina your enthusiasm for the outdoors, interest in my work, love and friendship throughout the years has kept me going during good and bad times. I love you.

TABLE OF CONTENTS

LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
LITERATURE REVIEW	1
Introduction	2
Symptoms and Signs	4
Culture Morphology and Sporulation	6
Dissemination of Dogwood Anthracnose	6
Spread and Impact	7
Use of PCR for Detection of Fungal Plant Pathogens	9
Genetics of <i>Discula destructiva</i>	11
Literature Cited	13
CHAPTER I. THE INTRODUCTION, DISTRIBUTION AND SPREAD OF DOGWOOD ANTHRACNOSE IN MICHIGAN.....	19
Introduction	20
Materials and Methods	25
Results	34
Discussion	49
Literature Cited	57

LIST OF TABLES

<u>Table</u>	<u>Page</u>
CHAPTER I. THE INTRODUCTION, DISTRIBUTION AND SPREAD OF DOGWOOD ANTHRACNOSE IN MICHIGAN	
1. Fungal cultures used for development, specificity and sensitivity testing of <i>Discula destructiva</i> PCR primers DdF and DdR.....	30
2. Location of dogwood health monitoring plots in Michigan by county and geographic coordinates developed using global positioning systems, and the presence or absence of dogwood anthracnose.....	36
3. Inspection of <i>Cornus florida</i> and <i>C. kousa</i> nursery trees exported to Michigan nurseries in 2001, for <i>Discula destructiva</i> , using microscopy and PCR primer detection.....	38
4. Michigan forest sites identified as infected with <i>Discula destructiva</i> from symptomatic twigs, using microscopy and PCR primers DdF and DdR.....	45

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
CHAPTER I. THE INTRODUCTION, DISTRIBUTION AND SPREAD OF DOGWOOD ANTHRACNOSE IN MICHIGAN	
1. Map of Lower Michigan showing counties containing <i>Cornus florida</i> trees, location of all 52 health monitoring transects including the 11 transects infected with <i>Discula destructiva</i> , and one infected site without a transect.....	28
2. Results showing the number of imported <i>Cornus florida</i> nursery trees in 2001, identified microscopically as infected with <i>Discula destructiva</i> , based on the presence, morphology and size of phialides and conidia.....	39
3. PCR amplification of nuclear rDNA from total DNA isolated from pure cultures of various <i>Discula</i> species and a unknown <i>Phoma</i> sp. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. The two outer lanes contain 1KB plus molecular markers (Invitrogen). Odd numbered lanes contain samples amplified using universal primers ITS1F and ITS4, and even numbered lanes contain samples amplified using primers DdF and DdR. Lanes 1 and 2, <i>Discula destructiva</i> #23; lanes 3 and 4, <i>Discula destructiva</i> #100; lanes 5 and 6, <i>Discula campestris</i> Williams Hollow; lanes 7 and 8, <i>Discula campestris</i> Blessed Mountain; lanes 9 and 10, <i>Discula fraxinae</i> 96001; lanes 11 and 12, <i>Discula fraxinae</i> 96003; lanes 13 and 14, <i>Phoma</i> sp. B10-t; lanes 15 and 16, <i>Phoma</i> sp. GR1; lanes 17 and 18, no template DNA (i.e. negative controls).....	42
4. PCR amplification of rDNA from total DNA extracted from Michigan cultures of <i>Discula destructiva</i> and woody twig extracts, both from symptomatic forest samples. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. The two outer lanes contain 1KB plus molecular markers (Invitrogen). Lanes 1 to 18 contain samples amplified using primers DdF and DdR. Lanes 1 to 5, cultured <i>Discula destructiva</i> ; lanes 6 to 16, woody extracts. Lane 1, T3; lane 2, T26; lane 3, T5; lane 4, T44; lane 5, T52; lane 6, T3; lane 7, T26; lane 8, T5; lane 9, T44; lane 10, T52; lane 11, T24; lane 12, T48; lane 13, T47; lane 14, FCTC 25; lane 15, FCTC 40; lane 16, T22. Lane 17, <i>Discula destructiva</i> #23 (i.e. positive control); lane 18, no DNA template (i.e. negative control).....	43

LIST OF FIGURES (con't)

<u>Figure</u>	<u>Page</u>
5. PCR products of rDNA, testing sensitivity of primers DdF and DdR against DNA extraction of infected and uninfected woody material. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. The two outer lanes contain 1KB plus (Invitrogen) molecular marker. Lanes 1 to 3, samples amplified with universal primers NS7 and NS8. Lane 1 and 2 contain uninfected live and dead twig samples, respectively; lane 3, no DNA template (i.e. negative control). Lanes 4 to 18 contain samples amplified with primers DdF and DdR. Lanes 4 and 5, live and dead uninfected twig samples, respectively; lane 6 to 13, extraction of decreasing number of acervuli; lane 6, 110 acervuli; lane 7, 80 acervuli; lane 8, 60 acervuli; lane 9, 40 acervuli; lane 10, 30 acervuli; lane 11, 20 acervuli; lane 12, 15 acervuli; lane 13, 10 acervuli. Lanes 14 to 17, progressive ten-fold dilutions (100ng, 10ng, 1ng and 100pg) of woody extract containing <i>Discula destructiva</i>. Lane 18, no DNA template (i.e. negative control).....	44
6. Results showing the number of imported <i>Cornus florida</i> nursery trees in 2001, identified as infected with <i>Discula destructiva</i> using PCR primers DdF and DdR, based on the presence of <i>Discula</i> nuclear DNA at a concentration greater than or equal to 10ng.....	46
7. PCR amplification of dogwood woody extracts from nursery samples. Nursery sample set: <i>Cornus florida</i> var. cloud 9, from nursery #5, Oregon, individual trees 1 to 8 tested. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. Amplification using primer combinations DdF and DdR (A), and NS7 and NS8 (B). The two outer lanes contain 1KB plus (Invitrogen) molecular markers. For both gels, odd numbered lanes contain undiluted samples and even numbered lanes contain 10 fold dilutions. For both gels (A & B) lanes 1 and 2, tree 1; lanes 3 and 4, tree 2; lanes 5 and 6, tree 3; lanes 7 and 8, tree 4; lanes 9 and 10, tree 5; lanes 11 and 12, tree 6; lanes 13 and 14, tree 7; lanes 15 and 16, tree 8; lane 17, <i>Discula destructiva</i> #23 DNA template (i.e. positive control); lane 18, no template DNA (i.e. negative control).....	47
8. Comparison of microscopic and PCR results positive for the presence of <i>Discula destructiva</i>, from imported <i>Cornus florida</i> nursery trees in 2001.....	48

LITERATURE REVIEW

Introduction

The native eastern flowering dogwood, *Cornus florida* Link, is a popular ornamental tree in the United States and an important forest sub-canopy species with a wide geographical range. The natural range of flowering dogwood extends from the Atlantic coast in the east, to mid-Michigan and southern Maine in the north, to Missouri, eastern Oklahoma and Texas in the west, and to northern Florida and southern Alabama in the south (McLemore 1990). *C. florida* is found growing in well drained, moderately moist to dry, light textured soils with a pH of 6 to 7, with a shallow root system, which makes this tree susceptible to drought (Vimmerstedt 1965, McLemore 1990). Due to the broad geographical range and tolerance of soil conditions, the flowering dogwood has been recorded in 22 forest cover types. However, it is most commonly associated with oak-hickory forests and southern pine forests (Eyre 1980, McLemore 1990). Flowering dogwood is tolerant to shade and reacts quickly to increased levels of sunlight, acting as a gap closer in forest communities (Wallace and Dunn 1980, McGee 1986).

Flowering occurs between mid-March in the south to late May in the north, often before leaf-out of most associated species (McLemore 1990). Inflorescences contain approximately 30 perfect flowers, consisting of very large, white, showy bracts and yield of about 1-5 fruit (Carr and Banas 2000). Fruit consist of thin, fleshy, red drupes that contain a two-seeded stone (McLemore 1990).

Besides having an important role in gap dynamics, *C. florida* has other important ecological roles. Stiles (1980) and Rossell et al. (2001) reported that fruit from the flowering dogwood tree are high in lipid content (14-17%), and Servello and Kirkpatrick (1987) also reported crude protein to be 10%. This makes dogwood drupes a high quality

fruit in the fall for many mammals, as well as nearly 40 species of overwintering and migratory birds (Stiles 1980, Mitchell et al. 1988, Rossell et al. 2001). Leaf litter plays an important role in calcium cycling as it contains 2.0 – 3.5% calcium by dry weight (Thomas 1969). *C. florida* leaves decompose quickly returning calcium to the soil and is an important pH buffering agent in acidic soils (Hiers and Evans 1997). Studies in other temperate forests have shown that calcium cycling in acidic soils is critical for birds, which depend on calcium obtained through invertebrates for proper eggshell formation (Graveland et al. 1994).

In addition to the ecological significance, *C. florida* is economically important as well. Flowering dogwood is an important ornamental tree due to its size and flowering ability, with an estimated 20 to 30 million in sales in Tennessee in 1989 (Windham 1990). In the southeastern United States one acre of flowering dogwoods can generate up to \$60,000 in gross revenue (Badenhop et al. 1985).

During the mid 1970s, the health of *C. florida* on the east coast and *Cornus nuttallii* Audubon, the Pacific dogwood, on the west coast declined. A new mysterious disease named ‘dogwood anthracnose’ was reported in Washington State in 1976 and 1977 on *C. nuttallii*, and *C. florida* trees (Byther and Davidson 1979). In southeastern New York and southwestern Connecticut in 1978 and 1979, Pirone (1980) reported a massive decline of flowering dogwood, which was attributed to the fungus *Colletotrichum gloeosporioides* (Penz.) Sacc., in conjunction with wet springs. In 1983, this new disease affecting flowering dogwood in the east was named ‘lower branch dieback’ (Daughtrey and Hibben 1983) and was caused by an unknown species of *Discula* (Salogga 1983). Hibben and Daughtrey (1988) concluded that ‘lower branch

dieback' in the east and 'dogwood anthracnose' in the west, were caused by the same *Discula* sp. and agreed on the common name 'dogwood anthracnose.' Redlin (1991) described the pathogen as a new species, *Discula destructiva* Redlin, and named *C. florida*, *C. nuttallii* and *C. kousa* as the hosts.

A second undescribed species of *Discula* isolated from 7-8% of the symptomatic lesions was identified as causing dogwood anthracnose of forest trees (Windham et al. 1994). *Discula destructiva* (type 1) and the unknown *Discula* sp. (type 2) are similar in colony morphology and cause similar symptoms on dogwood trees, however only *D. destructiva* can grow on gallic acid medium (Trigiano et al. 1993a, Daughtrey and Hibben 1994). Type 1 and 2 isolates can also be distinguished by DNA amplification fingerprinting (DAF; Trigiano et al. 1993b).

Symptoms and signs

Leaf spots, usually found on the lower canopy, are the first symptoms of dogwood anthracnose. Leaf spots consist of spots, necrotic blotches, and blight (Hibben and Daughtrey 1988). Leaf spots are first observed as small (<7mm diameter) purple/red rimmed lesions that expand slowly, and can take on a shot-hole appearance when the necrotic tissue in the center breaks apart. Brown-olive leaf blotches are larger and usually form along the edge of the blade, sometimes with a reddish-purple border, but are not observed in Michigan. Infection progressing through the petiole leads to leaf blight, most often of the terminal pair of leaves and especially on terminal leaves of epicormic shoots. Leaf abscission can occur when infections resulting in leaf blight occur early in the growing season as reported by Britton (1993). Otherwise blighted leaves will remain attached until the following spring (Hibben and Daughtrey 1988). Rainy periods in the

spring can lead to red-brown spots and necrotic blotches on flowers. Progression of the fungus through the petiole leads to tan-white colored twig cankers, with brown discolored areas beneath the bark at the point of leaf attachment (Hibben and Daughtrey 1988). Sunken lesions (<3mm diameter) varying from orange-red to black often form on the small shoots. High incidence of twig dieback on the lower branches is what originally led to the early disease name of 'lower branch dieback' (Daughtrey and Hibben 1983). Epicormic shoots often form on the larger branches and trunk of diseased trees. Infection occurs rapidly in the epicormic shoots and provides an infection avenue into the main branches and trunk. Brown elliptical cankers are sometimes present underneath the bark where infected epicormic shoots were attached. These annual cankers are often delimited by callus beneath areas of sunken, cracked bark and can expand, hastening the decline of the tree (Daughtrey et al. 1988, Hibben and Daughtrey 1988).

Signs of *D. destructiva* include production of conidia from conidiomata.

Conidiomata are found abundantly in the necrotic tissue on the abaxial side of leaves and in the dead bark of twigs and epicormic shoots (Hibben and Daughtrey 1988, Daughtrey et al. 1988). Conidiomata on leaves are reddish brown to black, acervular, subcuticular, 30-135µm wide and are often found below trichomes (Hibben and Daughtrey 1988, Redlin 1991). On twigs, conidiomata are irregularly elliptical acervuli, 90-340µm wide and orange-black in color (Hibben and Daughtrey 1988, Redlin 1991). Conidia are released in gelatinous masses or cirrhi, and are white-gray to orange-pink in color (Hibben and Daughtrey 1988). Conidia are hyaline, single celled, elliptical-fusiform with polar guttules, 6-12 x 2.5-4µm in size, and formed from enteroblastic, phialidic cells 10-18 x 2.5-4.5µm in size (Redlin 1991). To date, no known sexual state has been identified,

however a teleomorph may be found in the genera *Apiognomonia* based on phylogenetic analysis of rDNA (Redlin 1991, Zhang and Blackwell 2001).

Culture morphology and sporulation

D. destructiva colonies on potato dextrose agar (PDA) or malt agar (MA) are initially white but turn gray-green to black with age. Colonies are appressed with an undulate margin, sparse aerial mycelium, and form droplets of exudate near the center of the colony, which upon drying give the colony a pitted appearance (Hibben and Daughtrey 1988, Redlin 1991). Variances in colony morphology have been reported including sectoring, pigmentation and growth rate, which is believed to be due to the presence of double stranded RNA (Yao et al. 1993, McElreath and Tainter 1993). Conidiomata formation on PDA or MA is sparse and typically forms toward the outer edges of the colony, with white-pink masses of conidia being formed (Hibben and Daughtrey 1988, Redlin 1991). Sporulation in culture is enhanced by adding dogwood tissue (Hibben and Daughtrey 1988), ground up oak or maple leaves (McElreath and Tainter 1993), or by using oatmeal agar (Redlin 1991). Optimal conidia production is enhanced by light (McElreath and Tainter 1993) and germination occurs at 20-24°C (Britton 1989), with no growth at 27°C (Salogga 1982).

Dissemination of dogwood anthracnose

Primary spread of *D. destructiva* over short distances occurs via wind and rain splash of conidia during weather events (Daughtrey et al. 1988). Studies have also suggested that birds may contribute to the spread of dogwood anthracnose. *D. destructiva* has been successfully cultured from infected dogwood seeds. Birds may contribute by the

spread by consuming infected fruits and excreting the seeds, which they cannot digest (Britton et al. 1993).

Insect mediated transportation of *D. destructiva* spores is possible in a laboratory setting. The convergent lady beetle, *Hippodamia convergens* Guerin-Meneville can pick up spores on infected leaves of *C. florida*. These spores remained viable for up to 16 days when the temperature was close to 20°C with 51-60% relative humidity (Colby 1993, Colby et al. 1995, Colby et al. 1996). More importantly, lady beetles carrying *D. destructiva* spores were able to infect healthy flowering dogwood leaves in the laboratory (Colby 1993). Research has shown that *D. destructiva* spores that are ingested by *H. convergens* remain viable when excreted as frass, with a range of viability from 80% at 12 hours to 12.5% at 96 hours (Hed et al. 1999).

Several researchers have alluded to the possibility of dissemination of dogwood anthracnose through infected nursery stock (Daughtrey et al. 1996) or by propagation with infested seeds (Britton et al. 1993). However, at this time there are no reports documenting any infected material being shipped within and among states.

Spread and impact

After the initial 1983 report of dogwood anthracnose as a new disease in New York, Pennsylvania, New Jersey and Connecticut, the disease expanded through much of *C. florida*'s natural range (Daughtrey and Hibben 1983). By 1987, Maryland, Delaware, Massachusetts, Georgia, Virginia and West Virginia had been added to the growing list of states with dogwood anthracnose (Anderson 1990). In 1988, the disease was reported in North Carolina, South Carolina and Tennessee, with Kentucky and Alabama being added to the list in 1989 (Knighten and Anderson 1992, Daughtrey and Hibben 1994).

Other states that were confirmed as having infected trees included Ohio and the District of Columbia (1990) (Daughtrey and Hibben 1994), New Hampshire (1991), Rhode Island (1992) (Daughtrey et al. 1996), Vermont, Michigan (Daughtrey et al. 1996) and Indiana (1993) (Rane 1993), and Kansas and Missouri (1994) (Daughtrey et al. 1996).

Early surveys in 1984 at Catocin Mountain National Park, Maryland, revealed that only 3% of flowering dogwoods were free of dogwood anthracnose symptoms and nearly 33% were dead (Mielke and Langdon 1986). Schneeberger and Jackson (1989) followed up this survey in 1988 and reported that mortality had increased to 89%, with 100% disease incidence, and little regeneration. A final survey of the same impact plots in 1994 showed a 94% loss of flowering dogwood (Sherald et al. 1996). Another long-term study by Knighten and Anderson (1992, 1993) reported that the number of hectares of diseased dogwoods in 210 impact assessment plots increased from 0.2 million hectares in 1988 to 7.0 million hectares in 1993, with an increase in mortality from 0% (1988) to 23% (1993). Hiers and Evans (1997) conducted a study at the Cumberland Plateau, Tennessee, on dogwood stem density and disease incidence. Two sites were studied and showed a 98% and 87% reduction in live stems, and 87% and 86% disease incidence, respectively. Despite *D. destructiva* causing a high incidence of mortality, McEwan et al. (2000), reported that long-term mortality trends were difficult to quantify. It has been suggested that not all areas exhibiting dogwood decline suffer from dogwood anthracnose. In a study conducted in Kentucky, old-growth forest plots had a 36% stem reduction from 1989-1998, which corresponded to anthracnose infection. However, the percent of decline was similar for the same sites during 1979-1989 when dogwood anthracnose was not present.

Use of PCR for detection of fungal plant pathogens

Methods for detecting and identifying plant pathogens are not always quick or reliable (Henson and French 1993). The polymerase chain reaction (PCR) has expanded pathologists' ability to detect disease by amplification of specific DNA sequences, which allows for the identification and comparison of species on a molecular level, instead of solely relying on traditional identification methods (Mullis and Faloona 1987). One particular area of the fungal genome that has been targeted for both phylogenetic and diagnostic studies has been the internal transcribed spacer (ITS) region, which contains two variable non-coding segments that lie between the highly conserved ribosomal DNA (rDNA) 18S, 5.8S and 28S subunits (White et al. 1990). The ITS region is a useful target for molecular identification because of 'universal primers' that will semi-selectively amplify the fungal ITS region, which is only 500-800 bp in length (White et al. 1990, Gardes and Bruns 1993). The rDNA region is present in high quantity allowing for amplification from small or degraded samples (Russel et al. 1984). Also the ITS is useful for identification because the base sequence can be quite variable among morphologically distinct species (Anderson and Stasovski 1992, Chen et al. 1992, Gardes et al. 1991) but shows little intraspecific variation (Lee and Taylor 1992, Anderson and Stasovski 1992). This allows for possible identification to the species level with the right oligonucleotide primers. Species-specific oligonucleotide primers are often designed for diagnostic purposes for use with diseased plant samples. These species-specific primers have allowed for identification to the species level of several pathogenic fungi including *Serpula lacrymans* (Schmidt and Moreth 2000), *Ophiostoma picea* and *O. quercus* (Kim

et al. 1999), *Pythium ultimum* (Kageyama et al.1997), and *Verticillium albo-atrum* and *V. dahliae* (Nazar et al. 1991).

Using species-specific primers and PCR technology has allowed researchers to identify fungi to the species level and has been used to overcome various difficulties. For example *Plasmodiophora brassicae*, the causal agent of clubroot (Faggian et al.1999), and various ectomycorrhizal basidiomycetes that serve as symbionts to many plants (Gardes and Bruns 1993). PCR can be used to identify these fungi that would otherwise be difficult to identify since they cannot be grown in culture. The use of PCR with species-specific primers has also reduced the time it takes to identify wood rot and stain fungi, from months to hours, through bypassing tedious culturing (Kim et al. 1999, Schmidt and Moreth 2000). PCR technology saves time, money and may help prevent the movement of quarantined pathogens. Powdery scab of potato (*Spongospora subterranea*) is a quarantined pathogen that despite all efforts has spread worldwide due to its ability to exist in an asymptomatic state. The development of species-specific primers has improved detection capabilities, which may allow for better containment and reduced economic loss (Bulman and Marchall 1998). PCR is also effective for assaying for pathogens when other methods fail due to plant inhibitory compounds. Enzyme-linked immunosorbent assay (ELISA) is a sensitive method for detection of wood decay fungi but is often inhibited by compounds found in the wood. With the right DNA extraction procedure and PCR species-specific primers, reliable detection has been achieved (Jasalavich et al. 2000).

Genetics of *D. destructiva*

Dogwood anthracnose appears to be an exotic fungus introduced during the 1970s. This long-supported hypothesis (Salogga and Ammirati 1983, Hibben and Daughtrey 1988), originates from the rapid appearance of *D. destructiva* at two coastal port cities, its unchecked spread over much of the geographic range of *C. florida* (Daughtrey et al. 1996), and lack of host resistance (Mielke and Langdon 1986, Brown et al. 1996).

Recent research supports the concept of *D. destructiva* as an introduced pathogen. Early molecular analysis using DNA amplification fingerprinting (DAF) on pooled isolates from the western versus the eastern United States shows that the genome is highly conserved (Trigiano et al. 1995). Further work utilizing arbitrary signatures from amplification profiles (ASAP) distinguished fine population differences between western and eastern isolates (Caetano-Anolles et al. 1996). Additional evidence for *D. destructiva* being an introduced pathogen has come from the discovery of double-stranded RNA, present in many isolates (McElreath et al. 1994, Yao et al. 1997). Studies show dsRNA are present in all eastern isolates (McElreath et al. 1994), but only 25% of western isolates from the United States. Generation of agarose-banding profiles show differences between the western and eastern isolates (Yao et al. 1997, Rong et al. 2001). While genetic diversity of *D. destructiva* is low, especially when compared to other fungal species (Caetano-Anolles et al. 2001), there are two main populations of isolates from the western and the eastern United States. In addition, these latest findings used amplified fragment length polymorphisms and sequencing of the intergenic spacer (IGS), β -tubulin genes, and translation elongation factor-1 α , which showed that out of 72 isolates there

were 20 genotypes present (Zhang and Blackwell 2002). However, of these 20 genotypes, 17 were found in the eastern United States, suggesting that the eastern population is more diverse than the western population.

Since *D. destructiva* has limited genetic diversity, it may be adventitious to develop species-specific primers. Species-specific PCR primers, coupled with a DNA extraction procedure that avoids PCR inhibition when diagnosing *D. destructiva* from *C. florida*, could be useful for diagnostic clinics and state nursery inspectors.

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CHAPTER I.
THE IDENTIFICATION, DISTRIBUTION AND SPREAD OF DOGWOOD
ANTHRACNOSE IN MICHIGAN

INTRODUCTION

The native flowering dogwood, *Cornus florida* Link, has a large natural range covering nearly the eastern third of the United States. Flowering dogwoods are in highest abundance in the Appalachian mountains, but also reach as far north as Michigan and Maine, west to Missouri, Arkansas and east Texas, and as far south as northern Florida (McLemore 1990). During the late 1970s *C. florida* on the east coast and the Pacific dogwood, *Cornus nuttallii* Audubon, on the northwest coast, began declining at epidemic proportions (Byther and Davidson 1979, Pirone 1980). This decline was initially termed 'lower branch dieback of flowering dogwood' (Daughtrey and Hibben 1983) and was attributed to an undescribed *Discula* species (Salogga 1983). Lower branch dieback was renamed 'dogwood anthracnose' in 1988 (Hibben and Daughtrey 1988) and was described as a new species, *Discula destructiva* Redlin in 1991 (Redlin).

Symptoms and signs of dogwood anthracnose first appear on the foliage of the lower branches. Leaf symptoms include brown, necrotic, leaf spots and can include large blotches or blight during severe outbreaks (Hibben and Daughtrey 1988). However, purple margins are not readily apparent in leaf spots in Michigan. Light tan twig cankers result from fungal penetration through the petiole or open wounds. Black fruiting bodies (acervuli) are visible on cankered twigs and within leaf spots. These fruiting bodies produce masses of white-gray to orange-pink conidia in a gelatinous cirrhi. Acervuli also permits secondary infection to occur in wet seasons and allows the fungus to over winter, acting as an inoculum reservoir for the following spring (Daughtrey et al. 1988, Hibben and Daughtrey 1988). Limb dieback occurs as the disease progresses, often accompanied by the production of epicormic shoots. Infection of epicormic shoots often results in the

formation of brown-black elliptical trunk cankers underneath the bark, which eventually leads to girdling of the tree.

Dogwood anthracnose is spread in several ways. Rain splash is the primary method of spread, allowing the conidia to disperse over short distances through forest stands (Daughtrey et al. 1988). The spread of infected dogwood seeds by wildlife, more specifically birds, has been suggested (Britton et al. 1993). *D. destructiva* has been isolated from infected seeds; therefore it is possible that birds feeding on infected fruits could spread the disease since they do not digest the seeds. Insect mediated transport of conidia is another possibility as it has been shown in a laboratory setting (Colby 1993). *Hippodamia convergens* Guerin-Meneville, the convergent lady beetle, was able to pick up conidia from infected leaves of *C. florida*, and deposit them on healthy leaves causing new infection (Colby 1993). These conidia remained viable for up to 16 days when the temperature was approximately 20°C with 51-60% relative humidity (Colby 1993, Colby et al. 1995, Colby et al. 1996). Additional studies showed spores of *D. destructiva* that were ingested by *H. convergens* remained viable (12.5%) when excreted as frass up to 96 hours later (Hed et al. 1999). The possibility of spreading dogwood anthracnose through propagation of infected seeds (Britton et al. 1993) or by state-to-state movement of infected nursery stock has also been suggested (Daughtrey et al. 1996). At this time however, no studies have been conducted to determine if infected material is being shipped within and among states.

The impact of dogwood anthracnose on native *C. florida* mortality has been significant over the past 25 years. Studies done at Catoctin Mountain National Park, Maryland, showed an increase in mortality from 33% to 89% between 1983 (Mielke and

Langdon 1986) and 1988 (Schneeberger and Jackson 1989), with little regeneration occurring. Knighten and Anderson (1992, 1993) reported that 210 impact plots surveyed between 1988 and 1993 showed the number of dead dogwoods to have increased from 0.2 million hectares to 7.0 million, respectively.

The destruction of the flowering dogwood is important ecologically for several reasons. One aspect is that *C. florida* grows in a wide geographical range of soils and forest cover types, from upland oak-hickory forests to southern pine forests (Eyre 1980, McLemore 1990). Flowering dogwood constitutes an important component of these different forest ecosystems for several reasons. First, while never acting as a dominant canopy tree, flowering dogwood acts as a gap-closer when exposed to increased levels of sunlight (Wallace and Dunn 1980, McGee 1986). Second, *C. florida* possesses a high ecological value from a wildlife perspective. Fruit from the flowering dogwood have a high lipid content of 14-17% (Rossell et al. 2001) and contain 10% crude protein (Servello and Kirkpatrick 1987). Due to their nutritional value dogwood fruits are an important food sources for many mammals and nearly 40 species of migratory and overwintering birds (Stiles 1980, Mitchell et al. 1988, Rossell et al. 2001). Finally, the leaves of the flowering dogwood play an important role in cycling of calcium, with a reported dry-weight of 2.0-3.5% (Thomas 1969). Dogwood leaves, which are high in calcium, decompose readily, returning calcium to the soil, while acting as a pH buffer in acidic soils (Heirs and Evans 1997). Studies have shown that calcium cycling is important as invertebrates consume calcium rich debris and are in turn consumed by various species of birds that depend on the calcium for proper eggshell formation (Graveland et al. 1994).

The flowering dogwood is also valuable from an economic perspective. *C. florida* is one of the first forest trees to bloom in the spring, and its large showy white bracts, make it aesthetically pleasing to the public (McLemore 1990). This unique characteristic, along with its size has led to its utilization by the ornamental tree industry as a widely planted landscape tree. One acre of flowering dogwood can bring up to \$60,000 in gross revenue and many dogwoods are shipped between states for retail nurseries (Badenhop et al. 1985, Daughtrey et al. 1996).

Methods for identifying *D. destructiva* currently include culturing and microscopic identification, both of which are especially difficult with this species. Improvements in disease detection through the use of the polymerase chain reaction (PCR), has allowed for accurate and quick diagnosis of many other plant pathogens that are difficult to identify by traditional methods (Henson and French 1993, Gardes and Bruns 1993, Dan et al. 2001). PCR primers have been designed to detect specific fungal species from various diseased sources such as *Plasmodiophora brassicae* in soil (Faggian et al. 1999), *Xanthomonas campestris* pv. *citri* on leaves (Hartung et al. 1993), and *Ophiostoma* from wood (Kim et al. 1999). The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is particularly useful for designing species-specific primers for two reasons. First the ITS region lies between the highly conserved 18S, 5.8S and 28S ribosomal subunits, which can be selected for with 'universal primers' (White et al. 1990, Gardes and Bruns 1993). Second, the ITS region shows variability between morphologically distinct species (Anderson and Stasovski 1992, Chen et al. 1992) but also shows minimal intraspecific variation (Lee and Taylor 1992, Anderson and Stasovski 1992). PCR using species-specific primers could prove useful for a disease

such as dogwood anthracnose, where culturing frequently fails and diagnosis based on microscopic examination is unusually difficult.

In Michigan only one site in Kalamazoo County was identified as infected with dogwood anthracnose by 1993, and no subsequent studies on its spread were pursued (Daughtrey et al. 1996). Unlike the Appalachian Mountains where flowering dogwood grows in abundance, Michigan's populations of flowering dogwood may be protected from infection as they are fragmented due to large areas of land utilized by agriculture. However, many nurseries in Michigan import dogwoods from other heavily infected states. Nursery importation of infected dogwood trees has been suggested as a means of spread of dogwood anthracnose to states on the edge of its natural range, or to isolated stands separated by large areas of agriculture (Daughtrey et al. 1996). To date, no studies have been done to assess the amount of *D. destructiva* that may be entering Michigan via the nursery trade.

Based on the lack of knowledge of dogwood anthracnose in Michigan forests and nurseries, several research objectives were addressed, including: (i) determination of the extent of spread of *D. destructiva* in Michigan since 1993 by surveying areas of *C. florida* throughout its natural growing range; (ii) establishment of forest health monitoring plots which would allow for future monitoring of dogwood anthracnose spread; (iii) determining what level, if any, of dogwood anthracnose was being imported into Michigan by inspecting Michigan nurseries and collecting samples of flowering dogwood for microscopic examination; (iv) development of PCR based detection methods utilizing species-specific primers to both verify and expedite detection of the pathogen in plant tissues.

MATERIALS AND METHODS

Survey for *C. florida* in Michigan

In 1997, surveys were initiated to determine areas with sufficient numbers of flowering dogwood trees for disease inspection and to establish GPS (global positioning system) health-monitoring plots. Letters explaining the importance of flowering dogwoods, the impact of dogwood anthracnose and the research that was to be conducted on the disease, were sent out to people in Michigan who were presumed to have interest and knowledge of native trees and who might recognize *C. florida* in a natural setting. Recipients included in the survey were members on mailing lists of the Michigan Nursery and Landscape Association (MNLA), Michigan Christmas Tree Association, Michigan Department of Natural Resources forestry division (MDNR), Michigan Department of Agriculture (MDA), Michigan State University county extension agents and master gardeners. In addition to the letter, a self-addressed stamped envelope and form consisting of the survey with a map of their county was included. The survey form requested whether they might indicate and describe locations of flowering dogwoods, whether they had noticed any possible decline, dieback, death or obvious disease symptoms, and their phone numbers for future reference. In addition to letter surveys, phone surveys were conducted at nature centers, gardens, and headquarters in state park and recreation areas. When possible, homeowners near sites showing disease symptoms were asked if they had ever planted a dogwood tree purchased from a nursery in the area. Based on survey information, state and private landowners that indicated knowledge of locations having sufficient amounts of dogwoods present, diseased or healthy were contacted for permission to enter their property. Suitable sites containing high numbers of

C. florida (dead or alive) were investigated and sampled, as described below. The establishment of three widely-spaced sample plots per county within the native range of *C. florida* was desired but this was not always possible due to lack of dense dogwood stands in some counties.

Monitoring tree disease using GPS

Using a compass and hip chain, a 100m transect was laid out in suitably dense stands of dogwood trees for establishment of long term GPS plots. The first ten trees within one meter of either side of the transect having a minimum DBH (diameter at breast height) of 3.8cm, were selected for disease monitoring. Preference was given to live trees but recently killed trees in stands where mortality was occurring were also utilized. A GeoExplorer II Global Positioning System (GPS) unit by Trimble (Trimble, Sunnyvale, CA, USA) was used for marking the position of each tree in the transect. A data dictionary was also created for the GPS system, which allowed for recording of features of the target trees. Features included DBH, noting the presence or absence of limb dieback, epicormic shoots, cankers and leaf spots. For each tree a minimum of 30 satellite positions were recorded for accuracy. Leaf and twig samples showing disease symptoms were collected and placed in marked envelopes from transect trees, in addition to several other dogwood trees showing the best disease symptoms at the site. Transects in forests with considerable canopy cover often contained a tree of a different species as the first marked tree. This was done to allow the GPS unit to calibrate its location in order to minimize error. Transect data was differentially corrected using Pathfinder Office software (Trimble, Sunnyvale, CA, USA) and databases provided by the Basic Science and Remote Sensing Institute (BSRSI), Michigan State University, East Lansing,

Michigan. A map showing transect placement was developed using ArcView GIS software (ESRI, Redlands, CA, USA) (Figure 1).

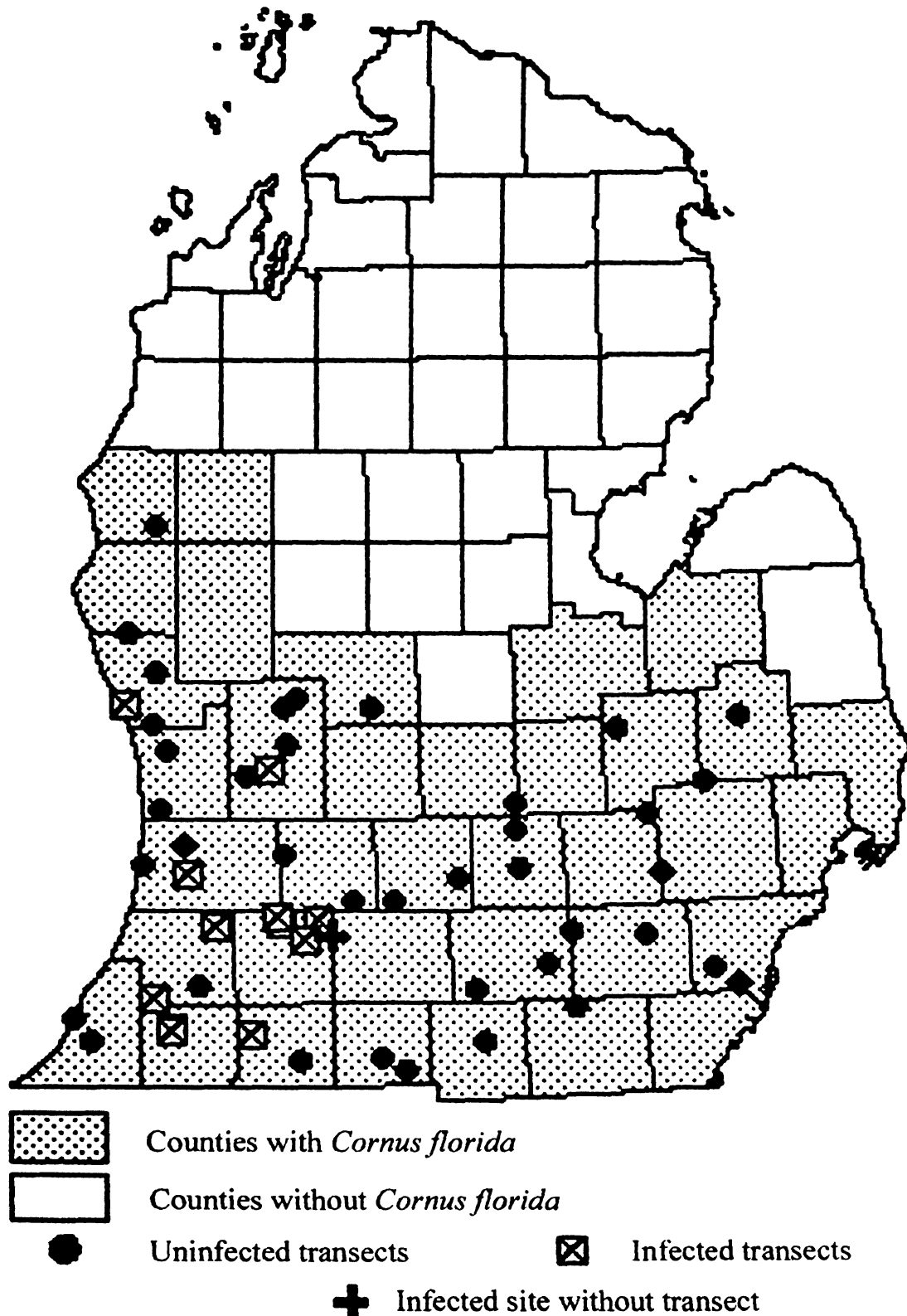
Microscopic identification

Samples collected from infected sites were viewed under a dissecting microscope to first determine the presence or absence of fungal fruiting bodies. When fruiting bodies were present they were hand-sectioned and stained with an equal mixture of 95% ethanol and 95% lactophenol cotton blue stain (Tuite 1969). Slides were examined at 1000X, using phase contrast and oil immersion optics. This allowed for confirmation or rejection of dogwood anthracnose based on conidia (6-12 x 2.5-4µm) and conidiophore (10-18 x 2.5-4.5µm) size and morphology, as previously described by Redlin (1991).

Nursery inspection for *D. destructiva*

In late April through early May in 2001, five Michigan nurseries were inspected for dogwood anthracnose. Dormant trees without leaves, being shipped from nurseries in Oregon, North Carolina, Alabama, Tennessee and Ohio were examined. *C. florida* and *Cornus kousa* Hance were inspected, however hybrids (*C. florida* x *C. kousa*) were not included in the survey. *C. kousa* was included as it is partially resistant to *D. destructiva* but is believed to be a carrier. Trees were inspected for dead twigs showing symptomatic cankers and/or fruiting bodies, often with the aid of a 10X hand lens. Distinct groups of dogwoods designated first by cultivar type and second by the nursery size class, shipped from one location, were sampled. These groups were utilized in order to look at trends of tree size and origin, in relation to the amount of disease that might be imported from states and nurseries. An average of ten trees were sampled per group, though this was dictated by what was being ordered by the Michigan nurseries. Twigs were placed into

Figure 1. Map of Lower Michigan showing counties containing *Cornus florida* trees, location of all 52 health monitoring transects including the 11 transects infected with *Discula destructiva*, and one infected site without a transect.



envelopes for each individual tree, allowing a tree-by-tree diagnosis in the lab. Samples were examined microscopically in the lab as previously described and infection totals were calculated.

Inspection of remaining unsold *C. florida* trees in nurseries were carried out in July through August of 1997 to check for leaf spots in order to observe if *D. destructiva* was spreading throughout the nursery.

Fungal culture

Twigs with fruiting bodies from forest plots were placed on a glass slide in moist chambers at approximately 22-25°C, with 10/14 hrs light/dark, for 2-3 days (Britton 1989, Hibben and Daughtrey 1988). Moist masses of conidia that formed on individual acervuli in the moist chamber were picked up using the tip of a sterile scalpel blade and transferred to potato dextrose agar containing 150ppm streptomycin sulfate. *Discula* cultures received from other sources and isolates recovered from Michigan forest samples are given in Table 1.

DNA isolation

Genomic DNA was isolated from cultured mycelia, in addition to twigs collected from both nursery and forest plots. Extraction was performed using a cetyltrimethylammonium bromide (CTAB) extraction buffer following the methods of Jasalavich et al. (2000), Taylor et al. (1993), and Wilson et al. (1987). For extraction from mycelial cultures, mycelia was scraped from petri plates and ground in liquid nitrogen with a mortar and pestle. Approximately 200 to 300µl of ground mycelium was then transferred to a sterile 1.5ml microfuge tube.

Table 1. Fungal cultures used in the development and testing of PCR primers DdF and DdR for *Discula destructiva* specificity.

Species	Isolate	Location/Supplier	Transect Number	DdF x DdR +/-
<i>Discula destructiva</i>	#23	David Wedge ¹	N/A	+
<i>Discula destructiva</i>	#100	David Wedge ¹	N/A	+
<i>Discula campestris</i>	Williams Hollow	David Wedge ¹	N/A	-
<i>Discula campestris</i>	Blessed Mountain	David Wedge ¹	N/A	-
<i>Discula fraxinae</i>	96001	David Wedge ¹	N/A	+
<i>Discula fraxinae</i>	96003	David Wedge ¹	N/A	+
<i>Discula destructiva</i>	Kal Haven	Van Buren Co., MI	T3	+
<i>Discula destructiva</i>	St. Joesph	St. Joesph Co., MI	T26	+
<i>Discula destructiva</i>	Allegan	Allegan Co., MI	T5	+
<i>Discula destructiva</i>	Van Buren	Van Buren Co., MI	T44	+
<i>Discula destructiva</i>	Galesburg	Kalamazoo Co., MI	T52	+
<i>Phoma</i> sp.	B10-t	Muskegon Co., MI	T48	-
<i>Phoma</i> sp.	GR1	Kent Co., MI	T47	-

¹ Dr. David Wedge, USDA-ARS Natural Products Utilization Research Unit, University of Mississippi.

For extraction from twigs, samples with fruiting bodies were observed under a dissecting scope at 10X magnification and a length of twig, approximately 1 to 2cm in length, was selected to be ground in a mortar and pestle using liquid nitrogen. Approximately 200 to 300µl of ground twig material was added to a 1.5ml microfuge tube.

For fungal extraction, 2X CTAB extraction buffer was used, as compared to 1X extraction buffer for woody material (Jasalavich et al. 2000). Extraction of DNA then proceeded according to Jasalavich et al. (2000). After alcohol precipitation, samples were incubated at -20°C for 30 minutes to an hour, before centrifugation. DNA pellets were brought up in 50µl of PCR-grade sterile H₂O. A single ten-fold dilution was made of all DNA samples prior to PCR.

PCR reaction conditions

PCR was performed using 25µl reactions containing various prepackaged buffers and polymerases. Primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') (Gardes and Bruns 1993) and ITS4 (3' CGTATAGTTATTCGCCTCCT 5') (White et al. 1990) were used to amplify the internal transcribed spacer (ITSI, ITSII and the 5.8S ribosomal gene) for use in sequencing. Approximately 10 to 50ng of DNA template were used per PCR reaction with ITS1F and ITS4 primers. For amplification using species-specific primers, 1µl of the extracted genomic DNA solution was added to the reaction mixture, with both undiluted and diluted extractions being tested for the presence of *D. destructiva*. PCR reactions were conducted using a DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA). Thermal cycling conditions consisted of an initial denaturation (94°C, 4 min), 35 cycles of denaturation (94°C, 1 min), annealing (54°C for ITS1F and ITS4, 57°C for

species-specific primers, 1 min), and extension (72°C, 1 min 30 s), followed by one final cycle of extension (72°C, 10 min), then storage at 4°C. All extracted samples were tested twice using species-specific PCR primers, and the appropriate negative and positive controls were included. PCR products were resolved on 1.5% agarose gels containing 100ng/ml of ethidium bromide and 1KB plus molecular markers (Invitrogen, Carlsbad, CA, USA), which were viewed under UV light.

Sequencing and primer design

The ITS1 and ITS2 region of *D. destructiva* isolate #23 was amplified using ITS1F and ITS4, as noted above. Dye terminated capillary electrophoresis on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Forest City, CA, USA) at the Michigan State University Genomics Technology Support Facility was used to sequence PCR products. Sequence results were confirmed as *D. destructiva* by alignment to other *D. destructiva* ITS sequences published on GenBank using BLAST (Altschul et al. 1990). From the sequence of *D. destructiva* isolate #23, species-specific forward primer DdF (5' GGTGCTACCCAGAAACCCATTG 3') and reverse primer DdR (3' CTGATCACCTGAAGAACGGCA 5') were generated using the PrimerSelect 3.05 module of DNASTar (DNASTar, Madison, WI, USA). Primers DdF and DdR were then used to amplify a region of *D. destructiva* DNA, which was then sequenced. This PCR product was also run through the BLAST search engine to confirm it was a match to existing *D. destructiva* sequences.

Primer specificity

Primer specificity was tested against DNA extracted from other species of *Discula* and an unidentified species of *Phoma* that was routinely cultured from infected

plant material (Table 1). In addition, primers DdF and DdR were tested against *D. destructiva* cultured from suspected infection sites in Michigan (Table 1). To ensure that primers DdF and DdR were not selectively amplifying extracted plant DNA from minipreps, woody extracts from both live and dead uninfected dogwood twigs were tested using universal nuclear small primers NS7 (5' GAGGCAATAACAGGTCTGTGATGC 3') and NS8 (3' AGGCATCCACTTGGACGCCT 5') (White et al. 1990, Gardes and Bruns 1993).

Primer sensitivity

Sensitivity of primers DdF and DdR to *D. destructiva* was evaluated by two methods. The first test conducted examined the amount of acervuli growing on woody twigs that could allow a positive PCR reaction. Fruiting bodies were miniprepped in varying quantities from twigs collected from transect 5 in Allegan County, Michigan. These samples were positively identified by microscopic means and cultures as *D. destructiva*. Quantities of acervuli tested included: 110, 80, 60, 40, 30, 20, 15 and 10 per miniprep.

The second test for primer sensitivity was performed by making a progressive 10-fold dilution of DNA extracted from woody twigs. Dilution concentrations tested ranged from 100ng down to 100pg and were quantified with a Beckman DU 7400 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Testing Michigan forest and nursery samples with *Discula* primers

Twig samples from 13 forest sites in Michigan with fruiting bodies were miniprepped as described above. A ten-fold dilution of the extracted DNA was made, and both undiluted and diluted DNA was tested using primers DdF and DdR.

Nursery samples collected in 2001, which were examined microscopically were also used in a comparative study utilizing the PCR primers DdF and DdR. As described above, DNA was extracted from twigs of individual tree samples and a single 10-fold dilution of the extracted DNA was performed. Both diluted and undiluted extraction products were tested by PCR. In addition, rDNA universal primers NS7 and NS8 were tested to check for DNA extract viability (White et al. 1990, Gardes and Bruns 1993).

RESULTS

Development of forest health monitoring plots in Michigan

The responses gathered from the dogwood questionnaires enabled the scouting and location of stands containing significant densities of flowering dogwood. A total of 36 counties in Michigan were identified as containing *C. florida* based on our observations and Voss (Voss 1985) (Figure 1). The northern most stands of *C. florida* located in this survey were in Mason County, although individual plants may survive further north. In addition, several dogwood stands that were believed to be infected were identified. Health monitoring plots containing 100m GPS generated transects were set up throughout the range of flowering dogwood in Michigan at sites meeting transect criteria. A total of 52 GPS health monitoring transects, covering 26 counties, were established (Table 2, Figure 1).

Inspection of Michigan nurseries for dogwood anthracnose

The inspection of imported *C. florida* and *C. kousa* from five Michigan nurseries in 2001 showed that trees were being imported from a total of ten different out of state nurseries. Our method of sample collection produced a total of 17 sample sets, consisting of 15 sets of various *C. florida* cultivars and two sets of *C. kousa*. A total of 342 trees

were examined, with samples being collected from 209 of the trees. The other 133 trees examined did not possess dead or diseased twigs for collection (Table 3).

Inspections in 1997 confirmed that dogwood anthracnose was spreading within nurseries to unsold *C. florida* and *C. kousa* trees. Four nurseries were found to have both infected *C. florida* and *C. kousa* trees showing leaf spot symptoms and acervuli of *D. destructiva*.

Microscopic analysis of nursery samples

Microscopic analysis of twigs from all 209 trees containing samples yielded a total of 35 trees, which were identified as infected with *D. destructiva* based on morphology of conidia and conidiophores. This nursery stock gave a 10.23% rate of infection for all 342 trees examined. Of the 35 trees diagnosed as infected, 33 were various cultivars of *C. florida*, and two were *C. kousa*. Both groups of *C. kousa* had trees identified as infected. Fourteen of the 17 sample sets were found to have at least one tree identified as having *D. destructiva*. All five states included in this study were exporting infected flowering dogwood trees to Michigan. Eight of the 10 nurseries from the five states were transporting infected dogwoods to Michigan (Table 3, Figure 2).

Species-specific PCR primer design and specificity

Amplification of the ITS1, 5.8S and ITSII region of *D. destructiva* isolate #23 for initial sequencing gave a product 579 bp in length, which was confirmed as the correct species through matches with existing ITS sequences of *D. destructiva* available on GenBank. Primers DdF and DdR produced a 460 bp fragment consisting of part of the ITS1, 5.8S and ITSII region of DNA amplified from *D. destructiva* Michigan isolate Galesburg, which also was a 100% match against *D. destructiva* sequences on GenBank.

Table 2. Location of dogwood health monitoring plots in Michigan by county and geographic coordinates developed using global positioning systems, and the presence or absence of dogwood anthracnose.

Location (County)	Transect number	Latitude	Longitude	<i>Discula destructiva</i> positive (+/-)
Berrien	T1	42°00'23.4"	86°32'05.2"	-
Berrien	T2	41°54'58.5"	86°27'15.0"	-
Van Buren	T3	42°21'21.6"	85°51'20.9"	+
Van Buren	T4	42°07'46.0"	85°56'42.0"	-
Allegan	T5	42°33'04.4"	85°59'41.3"	+
Allegan	T6	42°39'18.7"	86°00'26.1"	-
Allegan	T7	42°35'15.0"	86°12'44.5"	-
Kent	T8	42°55'16.9"	85°42'30.1"	-
Ottawa	T9	43°00'59.0"	86°05'35.5"	-
Kent	T10	43°10'50.6"	85°30'42.1"	-
Ottawa	T11	42°48'02.3"	86°07'36.4"	-
Kent	T12	43°13'07.6"	85°26'06.2"	-
Ottawa	T13	43°05'57.1"	86°10'38.4"	-
Muskegon	T14	43°17'50.2"	86°07'34.7"	-
Muskegon	T15	43°27'38.6"	86°16'53.2"	-
Eaton	T16	42°31'19.6"	84°39'49.7"	-
Montcalm	T17	43°10'20.7"	85°05'00.5"	-
Montcalm	T18	43°10'20.4"	85°04'59.9"	-
Eaton	T19	42°26'22.4"	84°58'46.7"	-
Barry	T20	42°37'31.4"	85°30'44.7"	-
Calhoun	T21	42°26'27.8"	85°10'52.5"	-
Kalamazoo	T22	42°22'01.9"	85°31'53.1"	+
Kalamazoo	T23	42°26'26.9"	85°34'15.7"	+
Kalamazoo	T24	42°22'06.8"	85°21'08.0"	+
Jackson	T25	42°12'19.7"	84°14'51.5"	-
St. Joseph	T26	41°56'27.8"	85°41'02.5"	+
Branch	T27	41°48'05.0"	84°56'56.7"	-
Branch	T28	41°51'24.5"	85°03'30.0"	-
Branch	T29	41°51'23.5"	85°03'31.2"	-
St. Joseph	T30	41°50'50.9"	85°27'18.5"	-
Ingham	T31	42°42'50.6"	84°22'25.8"	-
Ingham	T32	42°33'23.3"	84°22'02.7"	-
Jackson	T33	42°19'28.3"	84°07'41.5"	-
Washtenaw	T34	42°17'56.1"	83°45'38.4"	-
Jackson	T35	42°06'41.8"	84°35'36.9"	-
Genesee	T36	43°05'15.3"	83°51'36.1"	-
Oakland	T37	42°31'49.0"	83°39'57.6"	-

Table 2 (con't).

Livingston	T38	42°45'22.5"	83°43'00.5"	-
Wayne	T39	42°10'22.6"	83°25'24.9"	-
Wayne	T40	42°06'21.2"	83°18'42.6"	-
Lapeer	T41	43°07'27.8"	83°14'59.0"	-
Oakland	T42	42°52'17.2"	83°26'30.1"	-
Lenawee	T43	42°02'04.5"	84°06'49.9"	-
Van Buren	T44	42°04'29.7"	86°08'59.8"	+
Cass	T45	41°57'40.4"	86°03'52.3"	+
Hillsdale	T46	41°54'21.5"	84°34'19.2"	-
Kent	T47	42°56'33.5"	85°34'39.6"	+
Muskegon	T48	43°11'04.5"	86°16'57.6"	+
Kent	T49	43°02'21.9"	85°29'30.6"	-
Mason	T50	43°52'02.3"	86°08'24.4"	-
Kalamazoo	T51	42°18'00.4"	85°25'04.8"	+
Clinton	T52	42°48'45.2"	84°22'38.7"	-

Table 3. Inspection of *Cornus florida* and *Cornus kousa* nursery trees exported to Michigan in 2001, for *Discula destructiva*, using microscopy and PCR primer detection.

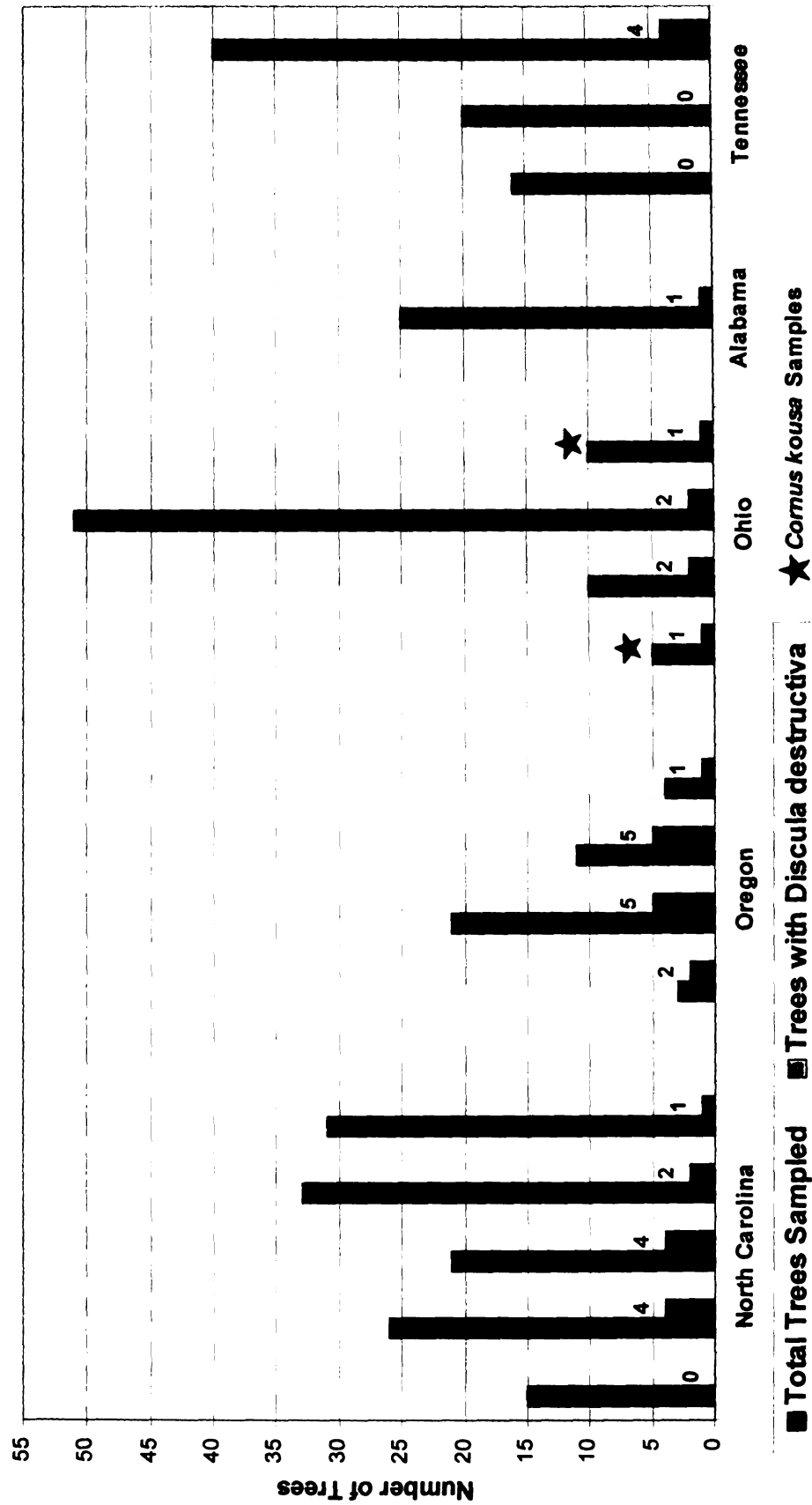
Nursery #	State	Species	Cultivar	Size	Total trees examined	Trees with samples collected and DNA extracted		Trees positive ¹ by microscopic diagnosis		Trees positive ² by PCR with primers NS7 and DdR		Trees positive ³ by PCR with primers DdF and DdR
						Size	examined	collected and DNA extracted	diagnosis	NS8		
1	NC	<i>C. florida</i>	cherokee chief	6-7'	15		15	12	0	12		0
2	NC	<i>C. florida</i>	cherokee princess	8-10'	26		26	25	4	19		0
3	NC	<i>C. florida</i>	rubra	10-12'	21		21	19	4	14		0
4	NC	<i>C. florida</i>	cloud 9	8-10'	33		33	27	2	20		0
		<i>C. florida</i>	rubra	8-10'	31		31	25	1	21		0
5	OR	<i>C. florida</i>	cherokee daybreak	6-7'	3		3	3	2	3		3
		<i>C. florida</i>	rubra	6-8'	21		21	20	5	19		1
		<i>C. florida</i>	cloud 9	6-7'	11		11	11	5	9		5
		<i>C. florida</i>	cherokee chief	8-10'	4		4	4	1	4		4
6	OH	<i>C. kousa</i>	chinensis	5-6'	5		5	5	1	3		0
7	OH	<i>C. florida</i>	rubra	7-9'	10		10	5	2	5		0
		<i>C. florida</i>	native	6-7'	51		51	10	2	10		0
		<i>C. kousa</i>	bush form	6-7'	10		10	6	1	5		2
8	AL	<i>C. florida</i>	rubra	4-5'	25		25	21	1	12		0
9	TN	<i>C. florida</i>	cherokee chief	8-10'	16		16	9	0	7		0
		<i>C. florida</i>	cherokee chief	5-6'	20		20	1	0	1		0
10	TN	<i>C. florida</i>	cherokee princess	6-9'	40		40	6	4	6		0
Totals					342		209	35	10.23	170		15
% Infection										81.34		4.39

¹ Positive samples having phialides and conidia of appropriate size and morphology for *D. destructiva*.

² Positive results indicate the presence of eucaryote nuclear DNA and lack of PCR inhibitory compounds.

³ Positive results indicate the presence of *Discula* nuclear DNA at a concentration of greater than or equal to 10ng.

Figure 2. Results showing the number of imported *Cornus florida* nursery trees in 2001, identified microscopically as infected with *Discula destructiva*, based on the presence, morphology and size of phialids and conidia.



Specificity of PCR primers DdF and DdR were tested against extracts of hyphae of *D. destructiva* isolated in Michigan, other species of *Discula*, and an unidentified *Phoma* sp. that was ubiquitous in the twigs (Table 1). Primers DdF and DdR did not amplify *Discula campestris* (Pass) Anx. and the unknown *Phoma* sp. (Figure 3). Amplification of a 460 bp fragment of DNA did occur for all *D. destructiva* isolates (Figure 3 and 4), in addition to both isolates of *Discula fraxinae* (Peck) Redlin and Stack (Figure 3).

Testing of PCR primer set NS7 and NS8 against plant DNA from uninfected live and dead twig extracts gave positive bands approximately 375 bp in length. No amplification of plant DNA from the same uninfected live and dead twig extracts occurred in the presence of primer set DdF and DdR (Figure 5).

PCR primer sensitivity

PCR amplification using DdF and DdR with DNA extracted from decreasing quantities of acervuli produced bright bands visible down to 40 acervuli. Faint bands showed detection of *D. destructiva* as low as 15 acervuli but no detection occurred at 10 acervuli (Figure 5).

A ten-fold serial dilution of DNA from a woody twig infected with dogwood anthracnose, amplified with PCR primers DdF and DdR, produced visible bands. Amplification of fungal DNA was visible at the 100ng and 10ng concentration, however no bands were detected at the 1ng and 100pg level (Figure 5).

PCR analysis Michigan forest twig samples and nursery samples

Symptomatic twig samples from a total of 13 Michigan forest sites from 7 different counties were collected (Table 4, Figure 1). Only 11 of these sites contained

transects as the other two located at Fort Custer Training Center were only noted as infected (Figure 1). Testing of total extracted DNA from symptomatic forest twigs using primers DdF and DdR yielded visible bands for all samples (Figure 4). PCR amplification bands were of the same size as those amplified from cultures obtained from the same forest samples and *D. destructiva* isolate #23 (Figure 4).

DNA was extracted from twig samples of all 209 nursery trees analyzed microscopically and was used in PCR-based detection using the species-specific primers DdF and DdR. Positive PCR results, indicating the presence of *D. destructiva*, were obtained for 15 trees of the 209 possible (Table 3, Figure 6). This yielded an overall infection rate of 4.39% for all 342 nursery trees examined. PCR diagnosis showed that two of the 10 nurseries, one each in Oregon and Ohio, were found to be exporting infected dogwood trees to Michigan (Table 3). Of the 15 trees that tested positive for *D. destructiva*, 13 were from *C. florida* received from nursery #5 in Oregon. Two *C. kousa* from nursery #7 in Ohio also tested positive for *D. destructiva* (Table 3). Figure 7A shows the PCR test results of *C. florida* cultivar Cloud 9, trees 1 to 8 from nursery #5, Oregon. Trees 1, 2, 4 and 5 all tested positive for *D. destructiva* by primers DdF and DdR. Figure 7B shows amplification of all minipreps of the same sample set using universal primers NS7 and NS8. Overall success of DNA extraction from twigs was 81.34% when checked with NS7 and NS8 (Table 3).

Ten of the 15 trees yielding positive PCR results coincided with the diagnosis from the microscopic study (Figure 8). The other five trees that expressed a positive PCR reaction were not identified as having *D. destructiva* based on initial microscopic examination of fruiting bodies.

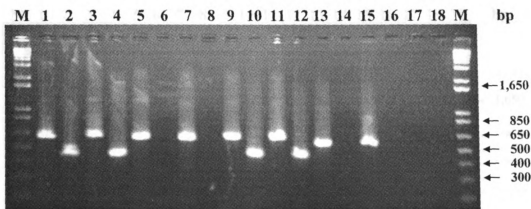


Figure 3. PCR amplification of nuclear rDNA from total DNA isolated from pure cultures of various *Discula* species and a unknown *Phoma* sp. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. The two outer lanes contain 1KB plus molecular markers (Invitrogen). Odd numbered lanes contain samples amplified using universal primers ITS1F and ITS4, and even numbered lanes contain samples amplified using primers DdF and DdR. Lanes 1 and 2, *Discula destructiva* #23; lanes 3 and 4, *Discula destructiva* #100; lanes 5 and 6, *Discula campestris* Williams Hollow; lanes 7 and 8, *Discula campestris* Blessed Mountain; lanes 9 and 10, *Discula fraxinae* 96001; lanes 11 and 12, *Discula fraxinae* 96003; lanes 13 and 14, *Phoma* sp. B10-t; lanes 15 and 16, *Phoma* sp. GR1; lanes 17 and 18, no template DNA (i.e. negative controls).

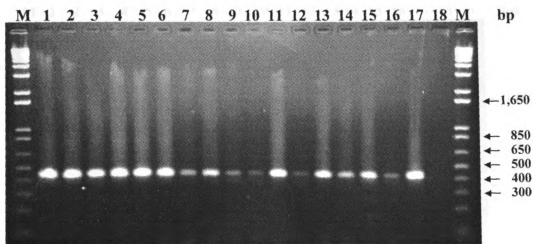


Figure 4. PCR amplification of rDNA from total DNA extracted from Michigan cultures of *Discula destructiva* and woody twig extracts, both from symptomatic forest samples. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. The two outer lanes contain 1KB plus molecular markers (Invitrogen). Lanes 1 to 18 contain samples amplified using primers DdF and DdR. Lanes 1 to 5, cultured *Discula destructiva*; lanes 6 to 16, woody extracts. Lane 1, T3; lane 2, T26; lane 3, T5; lane 4, T44; lane 5, T52; lane 6, T3; lane 7, T26; lane 8, T5; lane 9, T44; lane 10, T52; lane 11, T24; lane 12, T48; lane 13, T47; lane 14, FCTC 25; lane 15, FCTC 40; lane 16, T22. Lane 17, *Discula destructiva* #23 (i.e. positive control); lane 18, no DNA template (i.e. negative control).

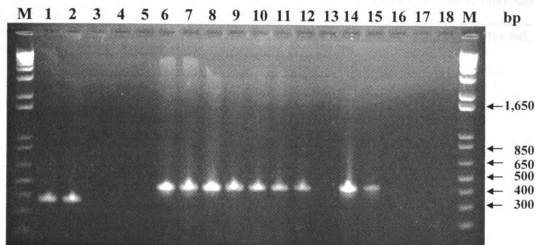
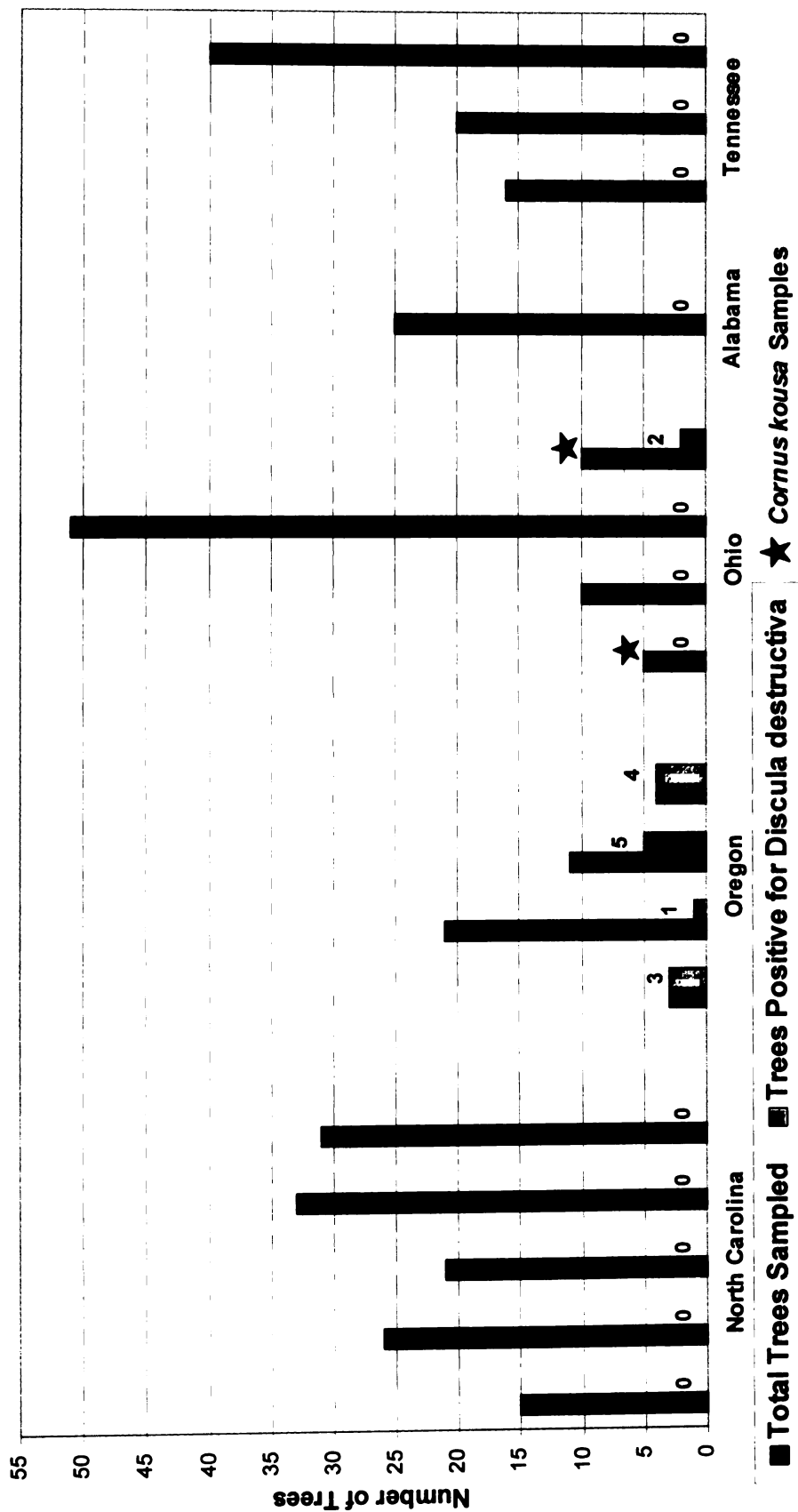


Figure 5. PCR products of rDNA, testing sensitivity of primers DdF and DdR against DNA extraction of infected and uninfected woody material. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. The two outer lanes contain 1KB plus (Invitrogen) molecular marker. Lanes 1 to 3, samples amplified with universal primers NS7 and NS8. Lane 1 and 2 contain uninfected live and dead twig samples, respectively; lane 3, no DNA template (i.e. negative control). Lanes 4 to 18 contain samples amplified with primers DdF and DdR. Lanes 4 and 5, live and dead uninfected twig samples, respectively; lane 6 to 13, extraction of decreasing number of acervuli; lane 6, 110 acervuli; lane 7, 80 acervuli; lane 8, 60 acervuli; lane 9, 40 acervuli; lane 10, 30 acervuli; lane 11, 20 acervuli; lane 12, 15 acervuli; lane 13, 10 acervuli. Lanes 14 to 17, progressive ten-fold dilutions (100ng, 10ng, 1ng and 100pg) of woody extract containing *Discula destructiva*. Lane 18, no DNA template (i.e. negative control).

Table 4. Michigan forest sites identified as infected with *Discula destructiva* from symptomatic twigs, using microscopy and PCR primers DdF and DdR.

Isolate	Location by County	Transect Number	Year Infected
Allegan	Allegan	T5	2000
C45	Cass	T45	1997
Area 51	Kalamazoo	T24	1998
Benner	Kalamazoo	T22	1993
FCTC 25	Kalamazoo	none	1999
FCTC 40	Kalamazoo	none	1999
Galesburg	Kalamazoo	T52	1999
Kal23	Kalamazoo	T23	1995
Grand Rapids	Kent	T47	1995
Muskegon	Muskegon	T48	1995
St. Joseph	St. Joseph	T26	1999
Kal Haven	Van Buren	T3	1997
Van Buren	Van Buren	T44	1995

Figure 6. Results showing the number of imported *Cornus florida* nursery trees in 2001, identified as infected with *Discula destructiva* using PCR primers DdF and DdR, based on the presence of *Discula* nuclear DNA at a concentration greater than or equal to 10ng.



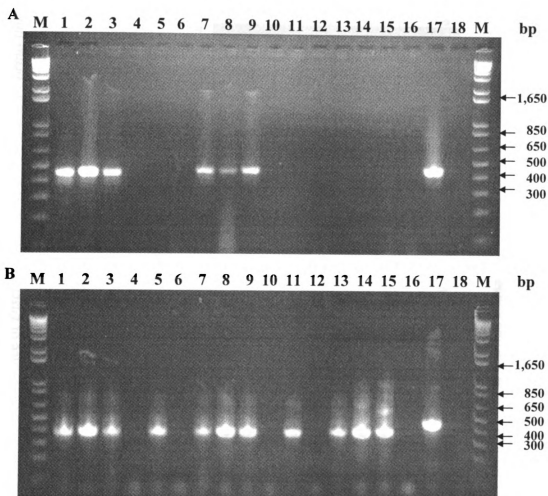
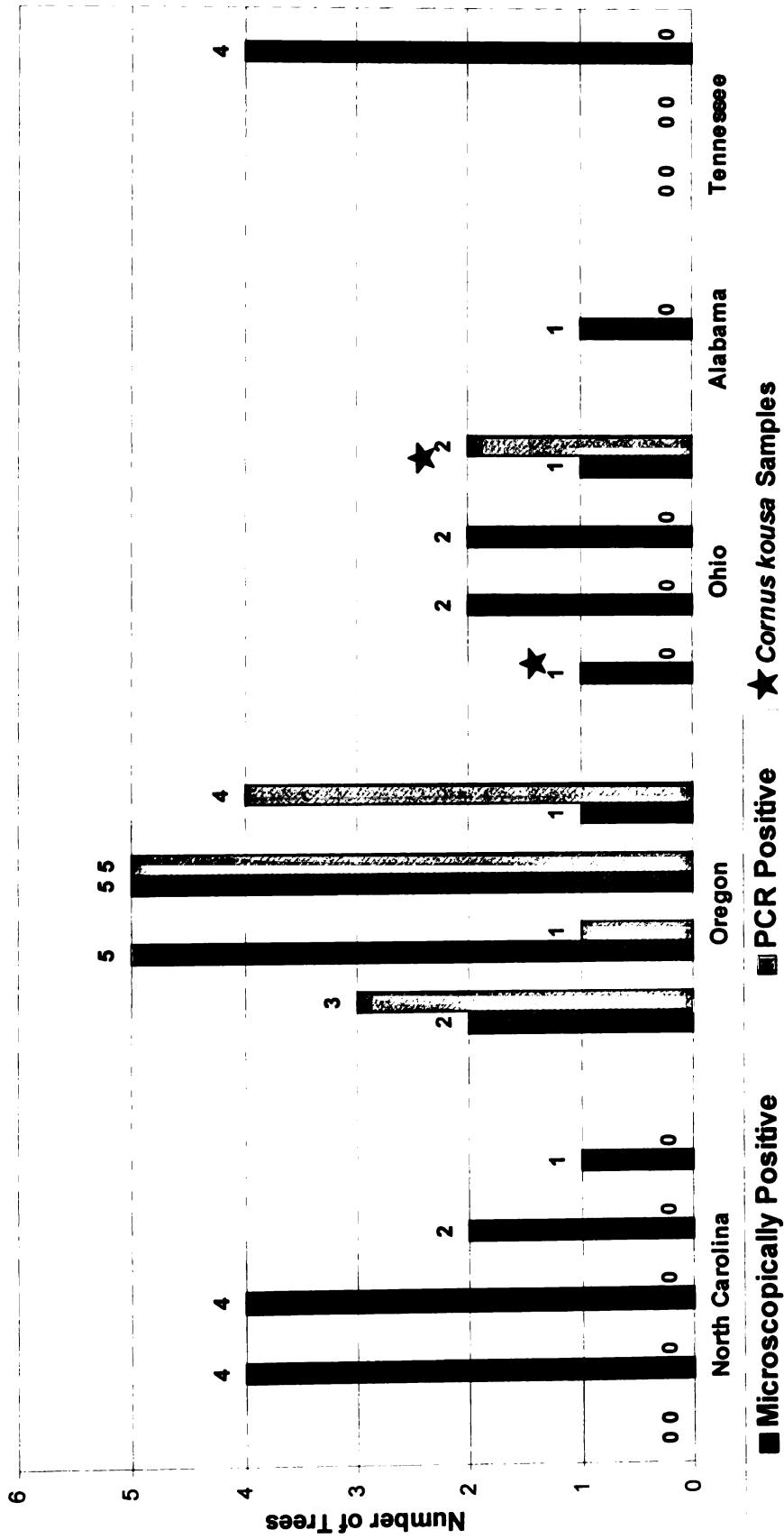


Figure 7. PCR amplification of dogwood woody extracts from nursery samples. Nursery sample set: *Cornus florida* var. cloud 9, from nursery #5, Oregon, individual trees 1 to 8 tested. Electrophoresis in 1.5% (wt/vol) agarose in 1x TAE. Amplification using primer combinations DdF and DdR (A), and NS7 and NS8 (B). The two outer lanes contain 1KB plus (Invitrogen) molecular markers. For both gels, odd numbered lanes contain undiluted samples and even numbered lanes contain 10 fold dilutions. For both gels (A & B) lanes 1 and 2, tree 1; lanes 3 and 4, tree 2; lanes 5 and 6, tree 3; lanes 7 and 8, tree 4; lanes 9 and 10, tree 5; lanes 11 and 12, tree 6; lanes 13 and 14, tree 7; lanes 15 and 16, tree 8; lane 17, *Discula destructiva* #23 DNA template (i.e. positive control); lane 18, no template DNA (i.e. negative control).

Figure 8. Comparison of microscopic and PCR results positive for the presence of *Discula destructiva*, from imported *Cornus florida* nursery trees in 2001.



DISCUSSION

The use of species-specific PCR primers for diagnosing plant disease has been well documented (Langrell 2002, Faggian et al. 1999, Kageyama 1997). In particular, this method of detection is valuable when dealing with highly destructive pathogens, such as *Spongospora subterranea* (powdery scab of potato) and *Phytophthora ramorum* (sudden oak death) that are at risk of spreading further through their host range, either by natural means or commercial movement (Bulman and Marshall 1998, Kox et al. 2002).

With the eradication of flowering dogwood by *D. destructiva* occurring throughout much of its native range, species-specific PCR primers may be useful in aiding identification. In Michigan there has been only one county identified in 1993 as infected (Daughtrey et al. 1996). The identification of new infection sites and development of plans to protect the remaining stands of flowering dogwood could be enhanced by the development of these primers. One particular application would be improved screening of imported dogwood nursery stock from infected states, which are sold in Michigan for landscaping.

The internal transcribed spacer (ITS) region of the rDNA has been utilized extensively by other researchers for developing species-specific PCR primers, due to it often being conserved within species but not between species (White et al. 1990, Gardes and Burns 1993). In this study the fungal specific forward primer ITS1F (Gardes and Burns 1993) and the reverse primer ITS4 (White et al. 1990), were used to successfully amplify the ITS1, 5.8S and ITSII regions of *D. destructiva* isolate #23 and Michigan isolate Galesburg. The sequences of both isolates matched *D. destructiva* ITS sequences from GenBank. The design of species-specific primers from these sequences yielded two

optimum primers, DdF and DdR. Primer DdF is downstream of the 18S rDNA and DdR is upstream of the 28S rDNA, resulting in a PCR product 460 bp in length.

Testing against *D. campestris* and *D. fraxinae* revealed that primers DdF and DdR were not truly 'species-specific,' although they did not amplify DNA of *D. campestris*, they did amplify a fragment of *D. fraxinae* DNA, producing a product similar in size to that of *D. destructiva*. The absence of species-specificity within genera can be attributed to a lack of heterogeneity of the ITS region being used to develop the primers. This has been the case for pathogens such as the beech bark disease causing complex, where early primers were unable to distinguish between *Neonectria coccinea*, *N. coccinea* var. *faginata* and *N. galligena* (Brown et al. 1993, Langrell 2002). In the case of dogwood anthracnose where there is only one primary pathogen, the amplification of *D. fraxinae* should not be cause for concern due to host specificity. Primer specificity against an ubiquitous *Phoma* sp. that routinely was isolated from diseased dogwood samples in Michigan was deemed more important. In addition, tests of DdF and DdR in conjunction with extracted uninfected dogwood DNA confirmed that amplification of plant DNA did not occur. This is an important consideration with any species-specific primer being used in disease diagnosis from plant material in order to prevent false-positive reactions (Zhang et al. 1997). The positive amplification of uninfected dogwood trees by targeting the nuclear small (18S) region of rDNA from uninfected dogwood extracts, using primers NS7 and NS8, was a necessary check to ensure that DNA extractions from twigs yielded quality DNA without contaminating substances that inhibit PCR amplification reactions (Kim et al. 1999, Gardes and Bruns 1993). PCR-inhibiting substances were present in DNA extracts of dogwood twigs when several standard extraction methods were used

(Taylor et al. 1993, Catal et al. 2001) and only the Jasalavich procedure (2000) was effective in this study.

The ability to quantify primer sensitivity is another important aspect when using species-specific primers for plant diagnostics. Researchers have gone about quantifying primer sensitivity in various ways. One method that has been utilized is to simply make a dilution series of template DNA from the targeted fungal pathogen (Glen et al. 2001, Faggian et al. 1999). Another method utilized has been extracting various amounts of infected plant material in order to determine the minimum amount needed to generate a positive PCR reaction (Jasalavich et al. 2000, Faggian et al. 1999). Both of these methods were employed for this study. DNA template extracted from heavily infected dogwood twigs was quantified with a spectrophotometer, and then subjected to a progressive 10-fold dilution series. This method allowed for detection in the range of 100ng to 10ng of DNA. The number of acervuli needed to elicit a positive reaction was also determined. Primers DdF and DdR produced bright bands from 110 acervuli to 40 acervuli, and fainter bands down to 15 acervuli. Despite not knowing the exact amount of fungal material being tested for, this is still seen as a valid test since fruiting bodies of *D. destructiva* are being sought in nursery and forest inspections in the absence of trunk cankers or leaf symptoms.

Dogwood anthracnose has moved largely unhindered through much of the eastern states with devastating consequences (Daughtrey et al. 1996). For the states in the western range of *C. florida*, patches of dogwood trees are isolated due to large expanses of agricultural land and suburban development. In Michigan only one site in Kalamazoo County was initially identified in 1993 as being infected with *D. destructiva* (Daughtrey

et al. 1996). No further studies were conducted to determine if the pathogen was spreading to the various isolated stands of native dogwood around the state until 1997. The importance of flowering dogwood in the ecosystem and for Michigan's ornamental nursery industry cannot be understated. A total of 52 permanent health-monitoring plots were placed in 26 counties throughout the range of *C. florida* in Michigan utilizing GPS technology, for the future monitoring and studies of disease impacts. For this study a total of three forest transects were sought per county. However in many counties this was not possible due to the lack of dogwood stands with densities sufficient for a minimum of ten trees intersecting the 100m transect because of the fragmented nature of Michigan's forests. More extensive surveys and transects were established in Kalamazoo County due to disease incidence and spread. This also allowed us to observe the initial infection site (Transect 22) as an infection epicenter, in order to estimate the rate in which the pathogen was spreading outward. The timeline as to when other 12 infections in the state occurred is somewhat arbitrary as it was necessary to rely on property owner memories and impressions, and our own estimate following inspections. Assuming that transect 22 in Kalamazoo County was a single and isolated introduction of the pathogen, then disease spread from the initial site to the Fort Custer Training Center could be estimated at 0.53km per year. Transects T47 and T48 are important to note as communication with the homeowners at both sites strongly suggested that *C. florida* trees from nurseries planted in close proximity to native dogwood stands initiated infections, as opposed to natural spread of the pathogen.

Much of the spread of dogwood anthracnose in the eastern United States has been through natural means, such as short distance rain splash (Daughtrey et al. 1988).

Dissemination of dogwood anthracnose into native stands by the movement of infected nursery stock has been suggested (Daughtrey et al. 1996). Until this study no inspections of newly arrived dogwood stock from infected states had been done to determine if the nursery trade was escalating the spread of this disease through Michigan forests.

The inspection of five Michigan nurseries was the first attempting to quantify the number of infected trees that were being imported from other states. For this study *C. florida* and *C. kousa* were examined since they are the primary cultivars being sold for landscaping. Hybrid cultivars of *C. florida* were not used for the study as some show varying levels of resistance to *D. destructiva*, which could prove difficult to detect if the morphology of the symptoms and signs changed as a result of the crossing (Ranney et al. 1995, Windham et al. 1998). However, it is important to keep in mind that while hybrids and the Chinese flowering dogwood *C. kousa* may not be as susceptible to dogwood anthracnose, they may act as carriers of the disease. *C. kousa* was examined despite this as it possible that *D. destructiva* originated with it, and was heralded as a replacement tree for *C. florida* in landscaping (Brown et al. 1996).

Another important aspect taken into account for this study was the timing of the inspections. Examination of imported trees was done as soon as they arrived at the nursery, and only newly imported trees were inspected. This was important since excessive movement of the dogwood trees may have broken off the dead twigs that were being collected. In addition, trees were sampled immediately incase the nursery contained *D. destructiva* infected holdovers from the previous season that could have spread the pathogen to the newly imported trees once they were placed in the same growing beds.

Many of the trees examined from the smaller size classes were devoid of twigs, either alive or dead, which created sample sets with very few twigs for examination.

Microscopic diagnosis established that eight of the 10 out-of-state nurseries were exporting infected dogwood trees to Michigan and that 10.23% of all trees examined were infected. Discula-specific PCR primers established that at least 4.39% of trees were infected. All five states included in the survey are infected with dogwood anthracnose but no quarantine exists for controlling dogwood anthracnose and regular inspections are limited (Daughtrey et al. 1996). The infection rates are relatively low, but it only takes one infected tree to spread the pathogen to other healthy trees within the nursery.

Suburban sprawl continues to spread into forested areas, bringing with it the possibility of planting an infected dogwood tree in close proximity of native stands. This type of action has quite likely occurred in Michigan in at least three sites already (T22, T47 and T48), where homeowners planted nursery flowering dogwood trees. Within a couple of years of planting the imported dogwood, homeowners reported to us that they noticed a marked decline in tree vigor, followed by a similar decline in the native dogwoods in the adjacent woods.

Only 10 of the 15 trees that yielded positive results by PCR primer detection coincided with the microscopic diagnosis. The differences between microscopic and PCR diagnosis could have several explanations. From a microscopic perspective the five samples that yielded a positive PCR reaction but were diagnosed as not being *D. destructiva* could have been misidentified. Perhaps the wrong fruiting bodies were selected for observation because many of the twigs collected had other fruiting bodies on

them from secondary pathogens. Also the *D. destructiva* acervuli could have already exuded its spores, making accurate identification difficult.

There are several reasons from a molecular standpoint as to why the infection percentages differed between microscopic and molecular diagnosis. The first possibility is there may not have been enough fungal material (fruiting bodies) present on the samples to elicit a positive reaction. With a minimum number of 15 acervuli needed for detection, it is likely that some samples did not possess enough fungal material, yet could allow for the spread of the pathogen. Additionally, the dead twigs may have been on the tree for more than one year, allowing degradation of fungal DNA to occur.

One last possibility as to why primers DdF and DdR were not as sensitive as microscopic methods could be due to genetic variation between isolates. While no studies have been done on the ITS region of *D. destructiva*, there are studies that suggest some genetic diversity overall is present in the population (Caetano-Anolles et al. 2001, Zhang and Blackwell 2001, Zhang and Blackwell 2002). If genetic changes have occurred in the ITS region of populations of *D. destructiva* over the past 30 years, it might have prevented full detection by ITS primers DdF and DdR in this study.

While the sensitivity of primers DdF and DdR are somewhat low, PCR diagnosis was valuable when used with suspect forest samples taken from transects during periods of dry weather when other symptoms were difficult to spot. Other PCR protocols used in disease diagnosis that may improve on this study include nested PCR (Hamelin 1996) and magnetic capture hybridization (Jacobson 1995, Langrell and Barbara 2001).

The survey of Michigan's flowering dogwood population shows that dogwood anthracnose is a continued threat to the majority of the natural stands. Given enough time,

dogwood anthracnose will likely result in the high levels of mortality throughout the state of Michigan, as observed in the eastern United States (Daughtrey et al. 1996, Hiers and Evans 1997). Our study has shown that dogwood anthracnose has been spreading over the last ten years in Michigan. How *D. destructiva* is spreading is difficult to determine. One method could be from the movement of spores by insect vectors as seen with ladybird beetles in a laboratory setting (Colby 1993, Colby et al. 1995, Colby et al. 1996, Hed et al. 1999). However, since most stands of *C. florida* are highly fragmented in Michigan, the likely scenario is the introduction of *D. destructiva* into new areas with infected nursery plantings, followed by natural spread to contiguous stands. Spread of *D. destructiva* within nurseries during the summer months to all *C. florida* and *C. kousa* trees will allow for a continued increase in the number of infected trees that are being planted throughout Michigan. Hopefully future studies utilizing the permanent GPS forest health monitoring plots developed in this project will allow for continual monitoring and research on the spread of dogwood anthracnose in Michigan in order to conserve the flowering dogwood tree, *C. florida*.

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