12-2001

Seasonal isolation and genomic comparison of Discula destructiva, the causal agent of dogwood anthracnose, and an undescribed Discula species

Aimee Michele Sheer

To the Graduate Council:

I am submitting herewith a thesis written by Aimee Michele Sheer entitled "Seasonal isolation and genomic comparison of Discula destructiva, the causal agent of dogwood anthracnose, and an undescribed Discula species." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Mark T. Windham, Major Professor

We have read this thesis and recommend its acceptance:

Robert Trigiano, Alan Windham, Jerome Grant

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Seasonal Isolation and Genomic Comparison of *Discula destructiva,* the Causal Agent of Dogwood Anthracnose, and an Undescribed *Discula* species

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Aimee M. Sheer

December 2001

Acknowledgement

I am gratefully to many, but especially to my major advisor Dr. Mark Windham and to Dr. Robert Trigiano. They were very generous, helpful, and taught me valuable research techniques that will be essential in my future endeavors. I would also like to thank Dr. Alan Windham and Dr. Jerome Grant for their guidance and support on this project.

Special thanks to Missy Ament, Mary Dee, and Vernon Reich for their guidance and help with my project. They were an invaluable source of information.

Thanks to the entire Department of Entomology and Plant Pathology. It was a pleasure getting to know and work with everyone.

I would like to express my gratitude to my fellow students Kim Pickens, Leslie Chadwell, Amy Bartel and David Hyten who were a constant source of strength and encouragement. I would especially like to thank Diane Ashburn, without her help, patience, sense of humor, and constant source of friendship, I would have never made it through the hard times.

Lastly, I would like to thank my family and friends Heidi Hales and Jen Derkits. They were a constant source of strength and always encouraged me to do my best. I am truly lucky to have family and friends that are as special as mine.

Abstract

Forty-three isolates of *D. destructiva* and 28 isolates of an undescribed *Discula* sp. were collected from flowering dogwoods (*Cornus florida*) from 13 sites in Alabama, Georgia, North Carolina, Tennessee, and Virginia during 2000 and 2001. *Discula destructiva* and *Discula* sp. were identified using size and shape of conidia and presence of internal fat bodies. The fungi were differentiated into *D. destructiva* and *Discula* sp. using gallic acid medium. In the spring and summer, 13 more isolates of *D. destructiva* were obtained than *Discula* species. However, the frequency of isolating *Discula* sp. was higher than has been reported in previous studies. In the fall, *D. destructiva* was isolated once, and in the winter neither fungus was isolated. The ability to isolate fungi was not associated with aspect, proximity to water, elevation, and diameter breast height.

Genetic analysis was performed comparing DNA of 16 isolates of *Discula* sp. and two isolates of *D. destructiva*. DNA amplification fingerprinting (DAF) used five octomer primers that revealed polymorphisms were more common among isolates of *Discula* sp. than isolates of *D. destructiva*. Similarities in groups of *Discula* sp. isolates were mostly within the same site. These findings indicate that *Discula* sp. and *D.*

destructiva are most likely not the same species but are related fungi in the same family.

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Chapter I

Literature Review

Flowering dogwood (*Cornus florida* L.) is a native tree that is valued as food for wildlife and for its ornamental value throughout much of the eastern United States (Whitmore, 1992). Nutritional value of the ovid scarlet drupe fruit consists of 1.1% calcium, 16.7% fat, 5.5% protein, 24.6% fiber, 0.6% phosphorus, and 5.0% ash (Lay, 1961; Mitchell et al., 1988). Many animals, including white tail deer (*Odocoileus virginianus* Zimm.), eastern cottontail rabbit (*Sylvilagus floridanus* Al.), moose (*Alces alces* L.) and birds such as yellow-rumpled warbler (*Dendroica cornata* L.), northern cardinal (*Cardinalis cardinalis* L.) and the brown thrasher (*Toxostoma rufum* L.), consume dogwood fruit (Mitchell et al., 1988). Flowering dogwood is well suited for landscapes, because they are very attractive in all seasons and low maintenance. In Tennessee sales of dogwood gross an estimated 30 to 40 million dollars annually (Southards, 1995).

Leaf litter from flowering dogwood improves soil. The leaf litter decomposes faster than that of most tree species and has high calcium

content. Decomposition of dogwood leaf litter buffers the pH of forest humus (Hepting, 1971).

Dogwood anthracnose was first reported as "lower branch dieback" on Pacific dogwood (*Cornus nuttallii* Audbon) in Washington in 1976 and two years later in New York City on flowering dogwood (Byther and Davidson, 1979). By 1983, the disease had spread to CT, N.J., and PA and was known as dogwood anthracnose. The disease spread to states such as DE, MA, MD, VA, and W.VA. by 1987. One year later dogwood anthracnose was discovered in GA, N.C., and TN (Windham, 1989). From 1989 to 1994 dogwood anthracnose was confirmed in AL, D.C., IN, KN, KY, MN, MO, N.H., OH, R.I., and VT (Daughtrey and Hibben, 1994; Knighten and Anderson, 1993; Rane, 1993).

The pathogen associated with dogwood anthracnose has been difficult to classify. The disease was first discovered in 1976 on *C. nuttallii* in the Pacific northwest, and the pathogen was believed to be *Gloeosporium corni* Green (Byther and Davidson 1979). A year later dogwood anthracnose was then observed in the northeast and the causal agent was thought to be *Colletotrichum gloeosporioides* Penzig (Pirone, 1980). Smith-Salogga (1982) stated that *Discula* sp. was the causal agent of dogwood anthracnose producing disease in Pacific dogwood, flowering dogwood, and Japanese

dogwood (*C. kousa* Hans.). A year later, Daughtrey and Hibben (1983) confirmed *Discula* sp. as the pathogen of dogwood anthracnose in flowering dogwood. In 1986, Mielke and Langdon suggested that the species was likely *D. umbrinella* Berkeley & Broome. Scott Redlin officially named the pathogen in 1991 as *Discula destructiva* Redlin. In the same year an associated pathogen *Discula* sp. was observed with dogwood anthracnose.

Discula species are identified by the asexual stage. Conidia of both *D. destructiva* and *Discula* sp. are similar in size (7 to 12μm long; 2.5-4μm in width), hyaline, nonseptate, and have a truncate base with a smooth outer surface. Conidia of both fungi have one to several bipolar internal fat bodies (Redlin, 1991). A sexual stage has not been observed in either fungus. Redlin (1991) postulated that if the sexual stage existed, it might be similar to the genera *Apiogonomia* or *Gnomoniella* that are in the order Diaporthales. In 2001, a phylogenetic analysis of small subunits or ribosomal DNA (rDNA) was performed on *D. destructiva*, and *Discula* sp. along with 21 other diaporthalean species including anamorphs. *Discula destructiva* was located in the Diaporthales with a bootstrap of 97% while *Discula* sp. was also included but distantly related to other *Discula* sp. (Blackwell and Zhang, 2001).

Several researchers have described pathogen and disease development. Conidia are embedded in pinkish or beige cirrhi (protein matrices) that ooze from acervuli (Redlin, 1991; 1992). The protein matrix is nutrient rich and thought to aid in survival of conidia (Crozier et al., 1992). Conidial germ tubes penetrate leaf surfaces directly and infection results in necrosis of palisade and spongy parenchyma cells (Walkinshaw and Anderson, 1991). The disease will progress throughout the vascular tissue. Hyphae were found in the xylem, phloem and cambial tissues of inoculated leaves (Walkinshaw and Anderson, 1991).

Discula destructiva produces coarse mycelia whereas *Discula* sp. produces slimy mycelia that are oppressed to the culture surface (Trigiano et al., 1991). Trigiano et al. (1991) distinguished between the two *Discula* spp. using gallic acid media. Polyphenol oxidase was produced by *D. destructiva* when a mycelial plug was placed on agar containing gallic acid (3,4,5, trihydroxyl benzoic acid). The polyphenol in gallic acid was oxidized by the polyphenol oxidase, which cleaved the hydrogen bonds on the quinone leaving oxygen in an excited state. The oxygen was then able to bond with other quinones forming a chain. The chain of quinones produced a browning underneath the mycelial mat of *D. destructiva,* but not in *Discula* sp. (Trigiano et al., 1991).

Foliar symptoms on dogwood trees infected by *D. destructiva* and *Discula* sp. were similar (Trigiano et al., 1995b). Symptoms of dogwood anthracnose for both *D. destructiva* and *Discula* sp. included leaf spots, and blights, and twig dieback. Other symptoms associated with infections by *D. destructiva* included limb and annual trunk cankers and tree death. Usually the symptoms were prominent in the lower portion of the tree (Hibben and Daughtrey, 1988).

Discula conidia may be disseminated by insects, wind and/or rain (Daughtrey et al., 1996). Under laboratory conditions, convergent lady beetles (*Hippodamia convergens* Guerin-Ménèville) could disseminate *D. destructiva* to healthy dogwood trees (Colby et al., 1995). The slime matrix adhered to the dorsal and ventral surface of the insect. Lady beetles transferred viable conidia onto trichomes of healthy dogwood leaves (Colby et al., 1995). The depressions around the trichome base might provide a suitable environment for fungal invasion (Brown et al., 1994). Conidia are also transported internally by lady beetles and deposited in frass pellets. These conidia are viable for up to 96 hrs (Hed et al., 1999).

Arthropod dissemination may be associated to epidemics of dogwood anthracnose. In the Great Smoky Mountain National Park (GSMNP), peaks in arthropod densities on dogwood foliage occurred at the same time that

epidemics of dogwood anthracnose peaked (Neitch et al., 1994). Viable conidia of *D. destructiva* were detected on 7% of the arthropods collected within GSMNP (Holt et al., 1998). During June, a peak was found in the amount of dogwood anthracnose and conidial infestation of arthropods. In early July, dogwood anthracnose epidemics leveled off and the number of infested arthropods collected had declined (Holt et al., 1998).

Environmental parameters may affect dogwood anthracnose epidemics. Dogwood anthracnose epidemics were reported to be most severe when trees were subjected to drought stress and grown in deep shade (98% shade) (Erbaugh et al., 1995). Lesions on diseased foliage in full sunlight were observed to be smaller and had fewer conidiamata than lesions in full shade (Parham, 1992). Severity of dogwood anthracnose was highest in foliage on shaded trees and lowest in the exterior foliage of trees in full sun (Chellemi and Britton, 1992). In addition, Dowsett et al. (1992) observed that epicormic shoot dieback occurred after extremely cold periods.

Discula destructiva and *Discula* sp. isolates did not differ in virulence when conidial inoculum was used (Grand et al., 1994). However, when conidial inoculum was compared with mycelial inoculum, *Discula* sp. conidial inoculum led to higher disease ratings than did mycelial inoculum

of *D. destructiva* and *Discula* sp. isolates. Flowering dogwoods had more acervuli formation on leaves that were inoculated with conidia of *Discula* sp. than with mycelia of *D. destructiva* or *Discula* sp.

Temperature and relative humidity affected conidial survival of *D. destructiva* (Roncardori 1993). Conidial germination was 73-90% at 14 C regardless of humidity. At relative humidity levels of 51 and 60%, conidial germination was between 71-90%. When the relative humidity was increased to 70-90%, conidial germination dropped to 10-48%. Increasing the incubation temperature to 25 C with relative humidity at 51 and 60% resulted in a germination rate of 7-48%. Relative humidity at 70, 80, and 90% with temperature at 25 C decreased the germination rate to 0-27%. Germination did not occur when exposed to 32 C for 12 to 16 days (Roncardori, 1993).

Resistance of *C. kousa* to dogwood anthracnose was investigated by Ranney et al. (1994). Cultivars such as 'Wolf Eye', 'Moonbeam', and 'Autumn Rose' were equally susceptible to dogwood anthracnose. *Cornus kousa* seedlings and cultivars such as 'Steeple', and 'Milky Way' were highly resistant to the disease (Ranney et al., 1994).

Because of flowering dogwood's uniform susceptibility to dogwood anthracnose, resistance was believed to be nearly impossible to find

(Santamour and McArdle, 1985). In the early 1990s, several survivor trees were identified after a severe dogwood anthracnose epidemic in the Catoctin Mountain National Park, MD (Windham et al., 1998). Subsequent testing of dogwood clones by the Tennessee Agricultural Experiment Station and the U.S. Forest Service led to the release of *C. florida* 'Appalachian Spring' (Windham et al., 1998).

Several management strategies have been proposed for dogwood anthracnose. Cultural control methods, such as planting healthy trees, selecting a well-drained site with fertile soil, avoiding sites near water, pruning and removing dead or diseased limbs, and avoiding mechanical and chemical injury, have been developed for dogwoods in nursery and landscape settings. These strategies are not feasible for trees in forested areas (Anderson et al., 1994). If anthracnose is present, a fungicide foliar spray may be needed. Propiconazole (Banner MaXX®) is a systemic sterol inhibitor and produces no phytotoxic effects at recommended rates. Chlorothalonil (Daconil 2787®), a contact inhibitor, is an effective foliar spray except in extreme conditions (excessive heat or rain). Other fungicides, such as mancozeb (Dithane M-45®) and thiophanate-methyl

(Cleary's 3336®), have also proven effective against dogwood anthracnose (Windham, 1998).

In previous research, part of the genome of *Discula* species and *D. destructiva* were analyzed with DNA amplification fingerprinting (DAF) (Trigiano et al., 1995a). Many polymorphisms existed between the isolates of *Discula* sp. but not between isolates of *D. destructiva*. Based on their findings, Trigiano et al. (1995a) theorized that *Discula* sp. was native to North America and co-evolved with *Cornus* species. DNA fingerprints of *D. destructiva* exhibited few polymorphisms and suggested that *D. destructiva* was an exotic species. The low number of polymorphisms was an indication of a genetic bottleneck or founder's effect (Trigiano et al., 1995a). High genomic variation is usually consistent with endemic species in that the genome has changed from exposure to different environmental and geographical parameters. Whereas, conserved genomes are more likely exotic because a few species are usually brought into a new area resulting in a conserved genome.

In this study dogwood material was collected in every season to isolate *D. destructiva* and *Discula* sp. and genetic analysis was performed

indicating their taxonomic relationship. The objectives of this research were to:

1) compare seasonal isolation of *Discula* sp. and *D. destructiva* in each season, assess the environmental and geographical influences, and

2) determine if *Discula* sp. is genetically different than *D. destructiva*.

Chapter II

Seasonal Isolation and Identification of *Discula* species Associated with Dogwood Anthracnose in Five Southern States

i. Introduction

Dogwood anthracnose was first reported in 1978 and has caused wide spread decline of flowering dogwoods (Byther and Davidson, 1979). Symptoms of the disease included leaf spots and blights, twig blights, limb cankers, trunk cankers, and tree death (Windham et al., 1995). Diseased dogwoods were often less vital, had fewer blooms, and had poor ornamental value (Windham et al., 1995).

Two fungi, *D. destructiva* and an undescribed species of *Discula*, have been isolated from dogwood foliage symptomatic to dogwood anthracnose (Windham et al., 1995). In previous research the primary source for obtaining *Discula* was from the symptomatic foliage with a germination average of 91.4%, whereas, the woody foliage had a germination rate of 64.5% (Hibben and McArdle, 1993). *Discula* sp. and *D. destructiva* were identified by their absence of appressoria, internal fat bodies, spore size, and shape (Redlin, 1991). *Discula destructiva* had a coarse mycelium whereas *Discula* sp. produced a slimy, sticky, jelly-like

mycelium (Trigiano et al., 1991). In some cases, fungal cultures of the undescribed *Discula* sp. may have an intermediate appearance. *Discula* sp. and *D. destructiva* can be differentiated by gallic acid (polyphenol) medium. When *D. destructiva* is in contact with gallic acid an oxidation reaction occurs forming a quinone that leaves oxygen in an excited state. The excited state of the oxygen allows for the quinone to bond with other quinones forming a chain, which produces a dark diffusion surrounding the mycelial mat. The color change does not occur with *Discula* sp.; however, it will grow on the gallic acid medium whereas, no growth occurs with *D. destructiva* (Trigiano et al., 1991).

Discula destructiva and *Discula* sp. have been collected from areas with similar environmental and geographical aspects. Parameters that have contributed to disease are proximity to water and diameter breast height (DBH) (Windham, 1989). When dogwood trees were located 50 m away from a body of water, incidence of dogwood anthracnose decreased. As for DBH, larger trees (>3.0 cm) were less impacted by dogwood anthracnose than smaller trees (<2.0 cm) (Windham, 1989). Geographical features associated with disease incidence include elevation and aspect (Windham, 1989). Trees found at higher elevation (> 457 m) had increased incidence and severity of dogwood anthracnose. Dogwoods growing on a southern,

eastern, and western slope may be less susceptible due to drier conditions. A comparison of seasonal isolation of *Discula* sp. and *D. destructiva* has not been previously conducted.

The objectives of this study were to determine the seasonal isolation of *D. destructiva* and *Discula* sp. Furthermore, this study assessed environmental and geographical influences on the ability to isolate these fungi using parameters such as elevation, aspect, proximity to water, and DBH.

ii. Materials and Methods

Site Establishment

Sites were chosen in areas that were previously known to have a higher incidence of dogwood anthracnose. When assessing the areas some sites contained high levels of dogwood mortality and few live dogwoods. Nearby sites less conducive to dogwood anthracnose development due to lower elevation and/or with different aspects were chosen as alternative sites. Ten diseased dogwoods were selected at each site and marked with an aluminum tag that denoted the site and tree number. Site locations and geographical characteristics are given in Table 2.1.

Table 2.1. Location of sites and site characteristics where dogwood tissue was collected for isolation of fungi associated with symptoms of dogwood anthracnose in 2000-2001.

Site No.	Sites	Elevation (m)	Aspect[z]	River Distance[y]	No. of Trees
1	Oak Ridge, TN	1,080	1-5 = 190° 6-10= 236°	1	10
2	Sewanee, TN	1,010	190°	1	10
3	Lookout Mountain, TN	681	337°	5	10
4	Russell Cave, AL	1,105	82°	4	9
5	Lookout Mountain, GA	1,038	342°	5	5
6	Oswald Dome, TN	911	94°	5	10
7	Hiawassee Dam, NC	501	215°	5	6
8	Appalachian Trail, NC	474	260°	4	10
9	Bent Creek, NC	658	1-6= 159° 7-10= 220°	5	10
10	Ozone, TN	992	90°	1	10
11	Blue Ridge Parkway Tuggle Gap, VA 1	622	360°	5	10
12	Blue Ridge Parkway Tuggle Gap, VA 2	855	180°	5	10
13	Virginia Polytechnical Institute and State University, VA	975	223°	4	6

[z] The first number indicates the site and the second number indicates the tree sampled
[y] 1= 0-8, 2= 9-15, 3= 16-30, 4= 31-61, 5= over 62 m

Collection

Symptomatic dogwood leaves, twigs and epicormic shoots were collected from diseased trees at 13 sites once in spring (March/April), summer (June/July), fall (October/November), and winter (January/February) of 2000-2001. As much material as could be collected were placed in separate plastic bags (26.8 cm x 27.9 cm) that denoted the each site and tree.

Inoculum Production

Leaves, twigs, and epicormic shoots that were symptomatic for dogwood anthracnose were cut in 2 cm pieces and placed in moisture chambers, consisting of petri dishes (100 x 15 mm) containing wet filter paper damped with distilled water. Tissues were incubated at room temperature (approximately 21 C) inside plastic bags (26.8 cm x 27.9 cm). After two days, leaves, twigs and epicormic shoots were examined for presence of acervuli. Conidia of *D. destructiva* and *Discula* sp. were obtained by inserting a sterilized inoculation needle into an acervulus. The needle and conidia were then plunged into potato dextrose agar (PDA) in petri dishes that had been amended with 20 mg/l each of streptomycin sulfate and chlortetracycline HCl (PDA+). After one week of growth on the

PDA+ petri dishes (60 x 15 mm), spores were examined using a light microscope. Petri dishes were sealed with Parafilm (American National Can, Chicago, IL) and incubated at 20 C. Isolates were placed in vials of PDA+ for storage.

Sporulation was initiated by growing isolates on PDA+ agar dishes (100 x 15 mm) that contained dogwood leaves that had been sterilized by autoclaving for two hrs on two consecutive days. After sporulation occurred, cultures were incubated at 20 C.

Gallic Acid Test

Discula sp. and *D. destructiva* were transferred from PDA+ onto malt agar. Culture dishes (60 x 15 mm) were sealed with Parafilm and incubated at 20 C for one week. Agar plugs containing fungal mycelium were obtained with a 1.3 cm diameter cork borer for each isolate and were placed mycelium side down onto gallic acid ($C_6H_2(OH)3COOH{\cdot}H_2O$) agar (Davidson et al., 1938). The culture dishes were sealed with Parafilm and placed in darkness for 24 to 48 hrs. Plates were examined for a color change (browning) of gallic acid medium under the plug. Based on work by Trigiano et al. (1991), a positive reaction signified *D. destructiva,* whereas, no color change indicated the undescribed species of *Discula.*

Analysis

Data were analyzed to determine significance between environmental and geographical influences to the amount of *D. destructiva* and *Discula* sp. isolated in each seasons. The statistic program used to correlate the data was Proc Correlation (SAS/STAT User's Guide: Statistics, Ver. 6. SAS Institute, Cary, NC).

iii. Results and Discussion

Seventy-one isolates obtained in this study were consistent in conidia morphology for *D. destructiva*. Using the gallic acid assay, 43 of these isolates were confirmed to be *D. destructiva* and 28 isolates were determined to be *Discula* sp. (Fig. 2.1). Frequency of isolation of *D. destructiva* and *Discula* sp. was correlated ($R=0.24, 0.22$; $P<0.0001$) with time of year. Most isolates of *D. destructiva* were collected in March/April (n=23) and in June/July (n=19). Isolation of the undescribed *Discula* sp. was lower in March/April (n= 12) and similar to *D. destructiva* in June/July (n= 16)(Fig. 2.2). Spring and early summer temperatures and rainfall were the most conducive for sporulation of *Discula* and fresh leaf twigs and epicormic shoots were readily available for collection. *Discula* sp. was not isolated in

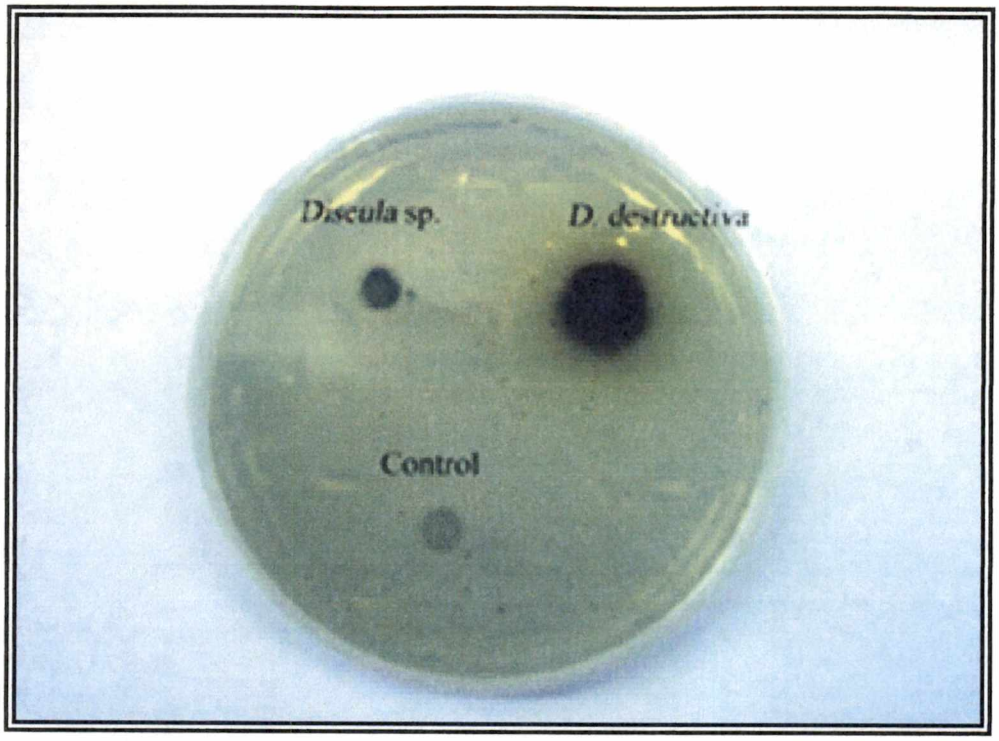

Fig. 2.1. Gallic acid assay of a mycelial plug of malt agar control (bottom center), *Discula* sp. (top left) and *D. destructiva* (top right). Browning of the media by *D. destructiva* indicates an oxidation reaction of the polyphenol into a chain of quinones.

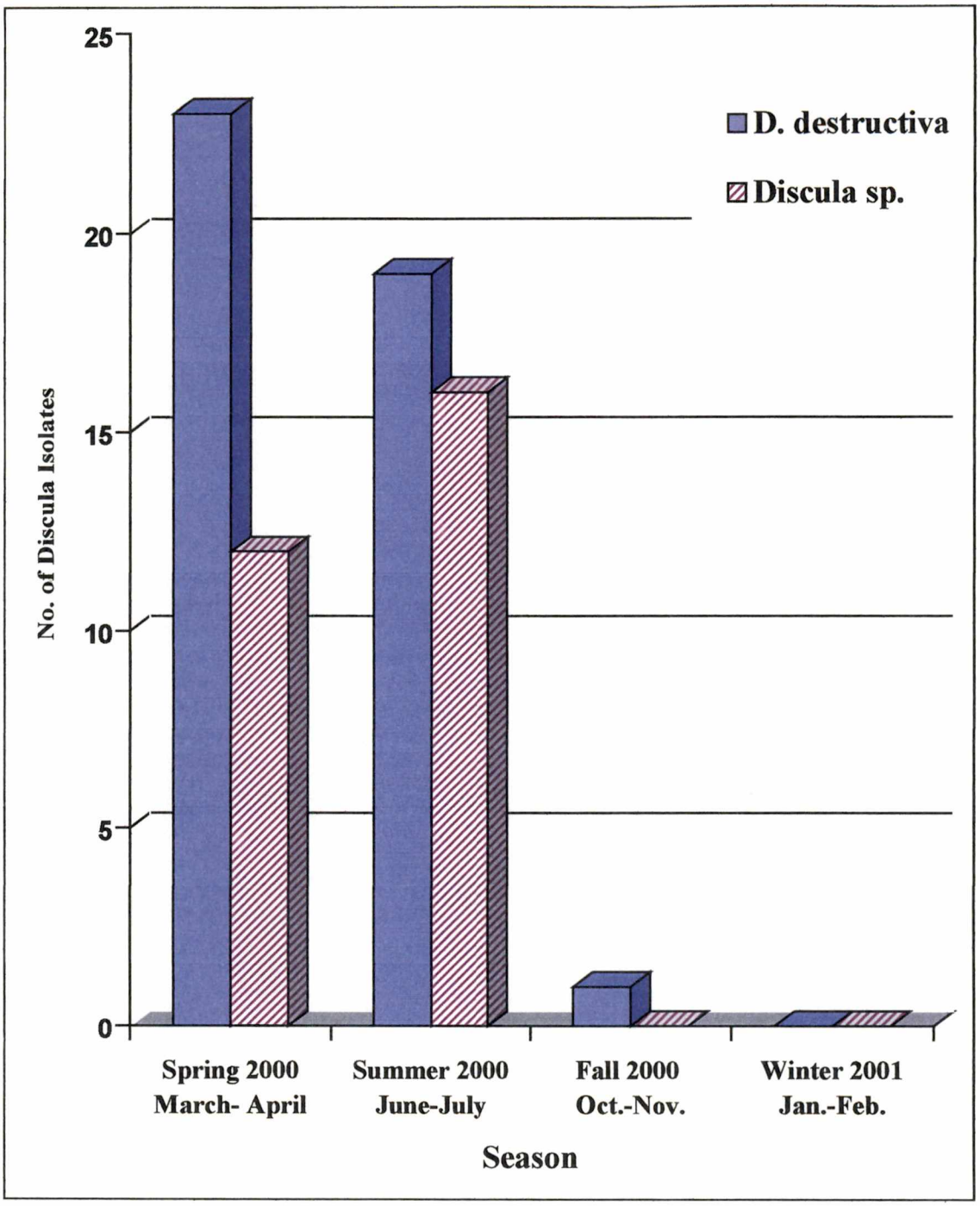

Figure 2.2. Frequency of isolates of *D. destructiva* and *Discula* sp. obtained in each season at all sites.

October/November (n= 0) and the number *D. destructiva* isolates were substantially less (n=1) than in the spring and summer (Table 2.2). The low number of fungal isolates could have been due to high temperatures in late summer, which could have hindered growth of *Discula* sp. and *D. destructiva*. Another factor that could have made isolation of *Discula* sp. more difficult was senescing foliage that had been invaded by other fast-growing fungi, including *Penicillium, Aspergillus,* and *Trichoderma*. In January and February, no isolates of *D. destructiva* or *Discula* sp. were collected. Lack of isolates in the winter may be caused by lack of fresh tissue for collection and isolation of fungi.

The sites with the highest amount of *D. destructiva* and *Discula* sp. isolates were Oak Ridge, TN (10 isolates), Sewanee, TN (15 isolates), Oswald Dome, TN (9 isolates), Bent Creek, NC (9 isolates), and two sites at the Blue Ridge Parkway, VA (8 isolates each) (Fig. 2.3). During the March/April and June/July collection periods, 23 and 27%, respectively, of isolates were of the undescribed *Discula* sp. Fungi in this study were isolated mostly from woody tissue whereas in previous work isolates were obtained from diseased foliage (Hibben and McArdle, 1993, Windham et al., 1994).

Table 2.2. List of *D. destructiva* and *Discula* sp. isolates found at each site on selected trees in the spring, summer, and fall of 2000.

Site	Isolates of *D. destructiva*[z]	Isolates of *Discula* sp.
Oak Ridge, TN	1-1, 1-4, 1-8, 1-9	1-4, 1-7, 1-8, 1-10
Sewanee, TN	2-1, 2-3, 2-6, 2-9, 2-10	2-1, 2-2, 2-3, 2-4, 2-5, 2-9, 2-10
Lookout Mountain, TN	3-1, 3-2	3-1
Russell Cave, AL	4-4	4-5
Lookout Mountain, GA	5-1	5-1
Oswald Dome, TN	6-2, 6-3, 6-4, 6-5, 6-10	6-3, 6-4, 6-5, 6-10
Hiawassee Dam, NC	7-3, 7-8	7-5
Appalachian Trail, NC	8-1, 8-4	None Collected
Bent Creek, NC	9-4, 9-5, 9-7, 9-8, 9-9, 9-10	9-4, 9-5, 9-8,
Ozone, TN	10-6, 10-7, 10-8	10-6
Blue Ridge Parkway Tuggle Gap 1, VA	11-1, 11-5, 11-7, 11-8, 11-9, 11-10	11-1
Blue Ridge Parkway Tuggle Gap 2, VA	12-1, 12-2, 12-6, 12-7, 12-10	12-2, 12-4, 12-5, 12-6,
Virginia Polytechnical Institute and State University, Blacksburg, VA	None Collected	None Collected

[z] The first number indicates the site and the second number indicates the tree sampled.

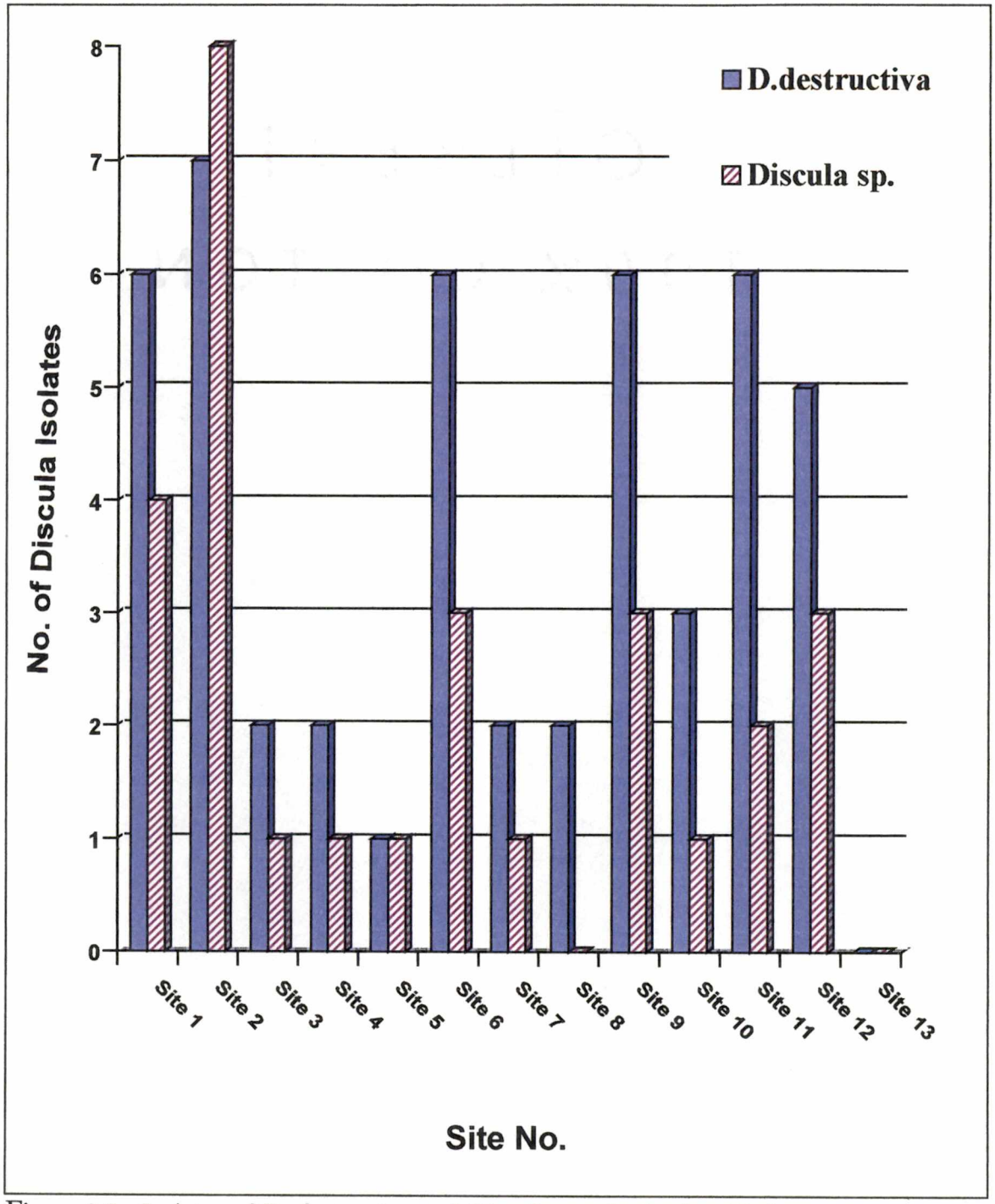

Figure 2.3. Isolates of *D. destructiva* and *Discula* sp. obtained at 13 sites.

No significant correlations were found between elevation, aspect, proximity to water, or DBH in relation to season and the ability to isolate the fungi. In previous studies, sites were chosen at random and researchers obtained few isolates of *Discula* sp. while isolates of *D. destructiva* were commonly obtained. In this study, sites were selected for high incidence of disease and severity of dogwood anthracnose. Because few living dogwoods were found in the initial sites, other sites had to be selected for this study. These newly selected sites differed in aspect and elevation from the original sites. The changes in site location may have confounded the ability to determine the effects of environmental factors on taxa population.

In areas selected for research sites in this study, many of the living trees that were used for collection purposes were near or scattered among numerous trees that had died. Most of the mortality could be attributed to dogwood anthracnose because of numerous elliptical cankers that were observed on trunks of dead trees. Dogwood trees used in this study had obviously survived intense disease pressure and may be more resistant to dogwood anthracnose due to a genetic resistance than the trees that had died. This differential in disease susceptibility may have made infection and colonization of survivor trees more difficult for *D. destructiva* than for *Discula* sp. Therefore differential in disease susceptibility may have

accounted for finding relatively more isolates of *Discula* sp. than found in previous studies, which were 7-8% (Trigiano et al., 1995a).

Elevation was not significant to seasonal isolation of *D. destructiva* and *Discula* sp. Elevation ranged from 474 m in Appalachian Trail, N.C., to 1,105 m in Russell Cave, AL. Windham (1989) and Chellemi et al. (1992) observed that as elevation of sites increased, dogwood anthracnose disease severity increased. In previous studies where elevation was correlated with disease severity, low elevation plots were 104 m lower than the lowest plot in this study. In this study Appalachian Trail was the lowest site used (elevation= 494 m) and was in the low range (<457 m) for disease severity. Lack of association between fungal isolation and elevation may be from a deficient number of higher elevation sites.

In previous studies, aspect was associated with incidence of dogwood anthracnose. Chellemi et al. (1992) observed that disease severity was greatest on dogwoods growing on the northeast-facing slopes and least on the southwest-facing slopes. Slopes with a northern aspect of 316° - 45° had a higher disease severity than did plots of eastern, western and southern aspects (Windham et al., 1993). In this study the aspect of sites ranged from 82° E to 360° N. Few of the site aspects ranged from northeast (<45°) to northwest (>315°) because of high dogwood mortality in those areas.

Dogwood anthracnose sites selected for this study were skewed to southern and western slopes due to extreme mortality rates of dogwoods on northern and eastern slopes and made determination of aspect on taxa frequency unattainable.

Stream proximity in association to seasonal isolation of *D. destructiva* and *Discula* sp. was not related. In previous studies dogwood trees were observed to decline more rapidly in high elevations located within 6.0 m of water than trees located more than 15.0 m away from water (Windham et al., 1992). An atmometer was used to determine that the evaporation potential in sites at high elevations was greater than those sites at low elevation (Windham et al., 1992). In addition, stream proximity was observed to have a greater evaporation potential when water was located less than 200 m from sites. In this study, only three sites (Oak Ridge, TN, Sewanee, TN, and Ozone, TN) had streams less than 7.6 m away from the dogwood plots. The other 10 plots had streams that were 30 to more than 61 m away. Choosing trees for this study after many trees had died from dogwood anthracnose made relationships between proximity to water and disease severity impossible to detect.

Size of trees was not associated with level of isolation frequency. In the GSMNP, smaller dogwood trees tended to have a higher increase in

severity than larger trees (Windham et al., 1991). In this study, DBH ranged from 2.0 to 25.0 cm (Table 2.3). One hundred and sixteen dogwood trees were sampled and 39% had a DBH between 2.0 and 5.0 cm, whereas 60% were above 5.1 cm. In most of the sites, smaller dogwood trees (<2.0 cm) had been killed, while larger trees (>3.0 cm) survived. Few, if any, seedlings were observed in or near any plot in this study.

iv. Summary

Isolates of *D. destructiva* and *Discula* sp. were most often obtained in spring (March/April) and summer (June/July) than at other times of year. The frequency of isolation of undescribed *Discula* sp. was much higher than in previous studies. The shift in isolation frequency may be due to a genetic shift in the host or isolating predominately from woody tissue instead of foliage tissue. There was no correlation between isolation of *D. destructiva* and *Discula* sp. and elevation, aspect, proximity to water, or DBH.

Table 2.3. Diameter breast height (DBH) (cm) of tagged dogwood tree at all 13 sites.

	Number of Trees									
Sites	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Oak Ridge, TN	9.0	6.0	6.0	6.0	4.0	3.0	6.0	4.0	2.0	6.0
Sewanee, TN	13.0	7.0	9.0	10.0	6.0	10.0	25.0	10.0	10.0	15.0
Lookout Mountain, TN	12.0	16.0	4.0	17.0	5.0	3.0	3.0	5.0	4.0	4.0
Russell Cave, AL	3.0	3.0	4.0	10.0	3.0	10.0	12.0	3.0	3.0	X
Lookout Mountain, GA	10.0	6.0	8.0	9.0	6.0	X[z]	X	X	X	X
Oswald Dome, TN	5.0	5.0	9.0	3.0	4.0	6.0	4.0	4.0	16.0	14.0
Hiawassee Dam, NC	8.0	13.0	4.0	9.0	4.0	4.0	X	X	X	X
Appalachian Trail, NC	3.0	7.0	7.0	8.0	11.0	4.0	5.0	4.0	4.0	4.0
Bent Creek, NC	16.0	3.0	5.0	4.0	2.0	8.0	8.0	3.0	3.0	7.0
Ozone, TN	5.0	9.0	7.0	7.0	3.0	7.0	3.0	2.0	16.0	7.0
Blue Ridge Parkway Tuggle Gap 1, VA	3.0	6.0	7.0	4.0	8.0	5.0	9.0	6.0	14.0	8.0
Blue Ridge Parkway Tuggle Gap 2, VA	4.0	6.0	7.0	8.0	4.0	3.0	5.0	3.0	5.0	4.0
Virginia Polytechnical Institute and State University, Blacksburg, VA	14.0	10.0	10.0	15.0	6.0	8.0	X	X	X	X

[z]In some sites not enough living dogwoods could be located.

Chapter III

Genomic Comparison of *Discula destructiva* and *Discula* species obtained from Flowering Dogwood in Five Southern States

i. Introduction

The causal agent of dogwood anthracnose was known by several different names until Redlin (1991) described the pathogen as *D. destructiva* Redlin and described an associated pathogen named *Discula* sp. When isolating fungi from diseased foliage, 7-8% of collected isolates were an undescribed *Discula* sp. (Windham et al., 1994). Even though, *D. destructiva* and *Discula* sp. produce analogous symptoms on dogwoods they can be distinguished from one another using morphological and physiological characters (Trigiano et al., 1995[a]).

Symptoms of *D. destructiva* and *Discula* sp. are similar on dogwood foliage. Indications of infection by *D. destructiva* and *Discula* sp. include leaf spots, and blights, and twig blights. Dogwoods infected with *D. destructiva* also may have symptoms such as limb cankers, and trunk cankers, which lead to tree death. The symptoms of dogwood anthracnose are usually prominent in the lower portion of the tree (Hibben and Daughtrey, 1988).

In culture *D. destructiva* and *Discula* sp. are morphologically different from one another. *Discula destructiva* produces a coarse mycelium that does not form abundant spore containing structures, whereas, *Discula* sp. produces a slimy, sticky, jelly-like mycelium and has abundant fruiting bodies. However, some isolates of *Discula* sp. can display cultural characters of both species (Trigiano et al., 1995a). Morphological characteristics do not provide enough information to discern genetic relationships between isolates of the same or different species (Trigiano et al., 1995a).

Taxonomical relationships can be accurately portrayed using genomic analysis because it is not affected by environmental conditions (Trigiano et al., 1995a). Complex DNA profiles can be generated using DNA amplification fingerprinting or DAF that uses relatively short primers in lengths of 7 or 8 nucleotides (Caetano-Anollés et al., 1991). DAF can be used to identify closely related organisms among viruses, bacteria, fungi, plants, and humans (Caetano-Anollés, 1993).

In previous research, DAF was used to compare three isolates of *Discula* sp. to 28 isolates of *D. destructiva* (Trigiano et al., 1995a). Genomic variations in *D. destructiva* were highly conserved (59 polymorphisms/302 loci), whereas genomic variations in *Discula* sp. were

high (114 polymorphisms/ 302 loci). However, only three isolates of *Discula* sp. were available for the study, and the low number of isolates may not have been a sufficient number to draw accurate inferences concerning genomic variations in *Discula* sp. In this study 16 isolates of *Discula* sp. were used to provide a more comprehensive analysis of the *Discula* sp. genome.

ii. Materials and Methods

Culture of Fungi

Sixteen isolates of *Discula* sp. and two isolates of *D. destructiva* were collected from diseased symptomatic leaf and twig tissues of *C. florida* from 13 forested sites in five southeastern states (Table 3.1). Isolates were grown on potato dextrose agar (PDA) with 20 mg/l each of streptomycin sulfate and chlortetracycline HCl (PDA+). Using a plastic straw (Kroger, Cincinnati, Ohio) eight plugs were cut and sliced into 4 sections. Two sliced plugs were placed into 4 Erlenmeyer 250 ml flasks containing 25ml of PDA broth and grown out for 1 week. Mycelium was harvested by vacuum filtration using a 250 ml Erlenmeyer flask, with a decanter placed on top. Seventy-millimeter filter paper was placed into the decanter in which the mycelium was poured onto, the liquid was vacuumed through the filter paper

Table 3.1. Origin of isolates of *Discula* sp. and *D. destructiva* used in genomic studies.

Sites	Fungus	Isolates
Oak Ridge, TN	*Discula* sp.	1-8, 1-9, 1-7
Sewanee, TN	*Discula* sp. *D. destructiva*	2-1, 2-4, 2-9, 2-2, 2-3, 2-5 2-10
Lookout Mountain, TN	*Discula* sp.	3-1
Russell Cave, AL	*Discula* sp.	4-5
Lookout Mountain, GA	*Discula* sp.	5-1
Oswald Dome, TN	N/A[z]	N/A
Hiawassee Dam, NC	N/A	N/A
Appalachian Trial, NC	N/A	N/A
Bent Creek, NC	*D. destructiva*	9-9
Ozone, TN	*Discula* sp.	10-6
Blue Ridge Parkway Tuggle Gap, VA 1	*Discula* sp.	11-7, 11-1
Blue Ridge Parkway Tuggle Gap, VA 2	*Discula* sp.	12-5, 12-6, 12-4
Virginia Polytechnical Institute and State University, Blacksburg, VA	N/A	N/A

[z] No isolates from these sites were used in DAF.

and the mycelium was colleted. Mycelium was placed in a plastic bag (.25 lb) and stored in a subzero freezer at –36 C.

DNA Isolation and Amplification

DNA was extracted from mycelia using a procedure by Yoon et al. (1991) and modified by Trigiano et al. (1995a). The reaction mixture (20µl) per isolate contained 0.88 µl of Stoffel fragment Amplitaq DNA polymerase (Perkins and Elmer, Norwalk, CT), 2 µl 10x Stoffel buffer, 5 mg/µl of template DNA, and 2 µl of dNTP, 100 mM $MgCl_2$, and 30 mM of primer. Primers had the following 5' to 3' prime sequences and arbitrary codes: GAGCCTGT (8.6A), GTAACGCC (8.6D), CCTGGTGG (8.7E), CCTGCTGG (8.7I), and AATGCAGC (8.7A). DNA was amplified in a thermocycler (Easy Cycler Twin Block System, Ericomp Inc., San Diego, CA) for 35 cycles. Each cycle consisting of 30, 72, and 96 C took approximately 5.5 min. Amplifications were done once for each template (DNA from an individual isolate/primer combination). DNA samples were prepared using the method of Yoon et al. (1991) modified by Trigiano et al. (1995a). Electrophoresis was stopped when the tracking dye was approximately 2 cm from the bottom of the gel. DNA was visualized using

a fast and sensitive silver staining procedure that detects picogram quantities of DNA (Bassam et al., 1991).

Data Analysis

Data analysis was done according to Trigiano et al. 1995a. All binary data were calculated using Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 2.0 (Exeter Software, Setauket, NY) (Trigiano et al., 1995a). A cladogram and a 3-D plot were performed using Unweighed Pair Group Cluster Analysis using Arithmetic Means (UPGMA).

iii. Results and Discussion

Polymorphisms were found on 13.4% of 215 loci of two isolates of *D. destructiva* and in 97.2% of 215 loci of 16 isolates of *Discula* sp. The combined polymorphisms for *D. destructiva* and *Discula* sp. isolates were a total of 238 polymorphic loci or 47.8 ± 4.55 polymorphisms/primer. The conserved genome in *D. destructiva* and variable genome in *Discula* sp. concurred with previous findings. Trigiano et al. (1995a) characterized 28 isolates of *D. destructiva* and three isolates of *Discula* sp. Polymorphisms were found on 19% of 302 loci of *D. destructiva* and 38% of 302 loci in

Discula sp. Although, the polymorphic percentage was higher in this study than in the study by Trigiano et al. (1995a) the trend is comparable.

Some primers [GTAACGGC (8.6M), CCTGTGAG (8.6B), and GCGAAGC-GCA (minihairpin 1)] used in this study resulted in poor amplification in many of the isolates. Each DAF gel used for analysis had sections or entire bands that were indecipherable. Reasons for poor banding patterns could be attributed to human error, inadequate amplification of the primer, or deterioration of the isolates' DNA. In this study, isolates were in culture for less than one year, and it is doubtful that deterioration of DNA is the cause of poor banding sites. According to Trigiano et al. (1995a) long term culture of fungi had no significant genetic variation on the primer sites.

Using a cluster analysis cladogram, 3-D plot and correlation indices, the relationship of the *Discula* isolates to one another could be determined (Fig. 3.1, 3.2). High frequency of monomorphic loci suggests a high sequence homology (Skroch et al., 1992) between isolates of *D. destructiva* Trigiano et al., 1995a) and a low sequence homology between isolates of *Discula* sp. Isolates 9-9 and 2-10 of *D. destructiva* had a coefficient of 80%. The lowest coefficient was 51%, which was isolate 11-7. Isolates 1-8, 2-1, 2-4, and 2-9 from Oak Ridge TN, and Sewanee, TN, were clustered due to

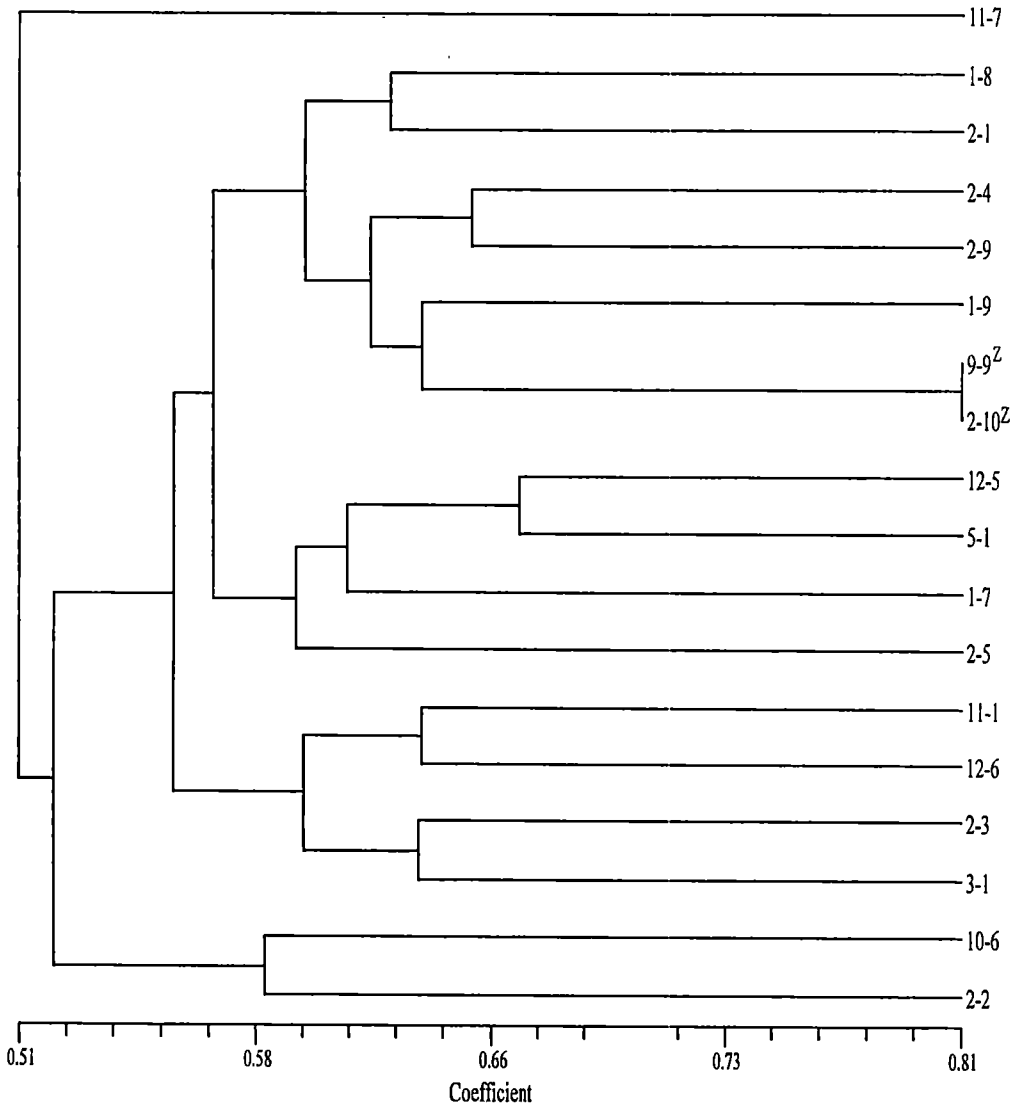

Figure 3.1. Graphic representation of NTSYS and cluster analysis. Similarities of genetic relationships between 16 isolates of *Discula* sp. and two isolates of *D. destructiva* were based on correlation coefficients of cluster groups. [Z] Indicates isolates of *D. destructiva* and *Discula* sp.

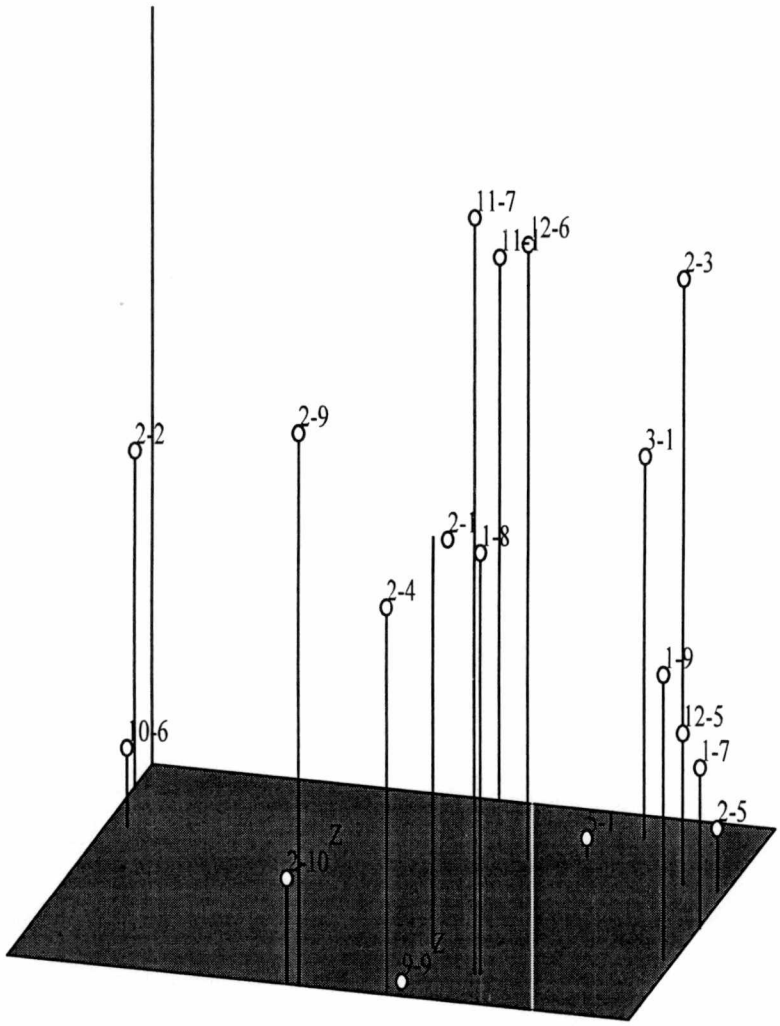

Figure 3.2. Graphic representation of NTSYS and a 3-D plot. Similarities of genetic relationships between 16 isolates of *Discula* sp. and two isolates of *D. destructiva* were based on distances from one another. [Z] Indicates isolates of *D.destructiva* and *Discula* sp.

genomic similarities with coefficients of 0.60, 0.60, 0.64 and 0.64, respectively. Other *Discula* sp. isolates that had genomes that were factioned together were 11-1 and 12-6 (coefficient of 0.66) from Blue Ridge Parkway, VA. Many of the groups of isolates with similar genomes were from locations with relatively close proximity. Correlation indices also indicated a closer relationship in the similar *Discula* sp. groups with the highest mean distance of 63% in association with 12-6 and 11-1 (Table 3.2). Since these fungal populations are from the same area, similar environmental conditions may cause the fungal genomes to be more conserved.

A phylogenic study was performed using two isolates of *D. destructiva* and 19 isolates of different *Discula* sp. (Caetano-Anollés et al., 1997). They used information from the rRNA gene interspacer region (ITS2) that spans between 5.8S rDNA and the 28S large nuclear rDNA. Many other species of *Discula*, such as *D. umbrinella* Berk. & Broome, *D. campestris* Cooke, *D. planti* Peck, and *D. fraxinea* Redlin & Stack were used for comparison with *D. destructiva* and *Discula* sp. *Discula destructiva* was shown to be related to the European tree pathogens *D. umbrinella* Berk. & Broome, *D. fraxinea* Redlin & Stack and *D. campestris* Cooke, whereas, *Discula* sp. was related more to the Oregon white oak

Table 3.2 Correlation indices that compare pairwise mean differences of 16 isolates of *Discula* sp. and two isolates of *D. destructiva*.

Isolate Number		Isolate Number								
		1	2	3	4	5	6	7	8	9
1	11-7	1.00								
2	1-8	0.55	1.00							
3	2-1	0.52	0.62	1.00						
4	2-4	0.52	0.55	0.64	1.00					
5	2-9	0.57	0.62	0.58	0.65	1.00				
6	1-9	0.56	0.60	0.57	0.59	0.59	1.00			
7	9-9[z]	0.54	0.64	0.58	0.64	0.57	0.66	1.00		
8	2-10[y]	0.53	0.59	0.61	0.63	0.68	0.60	0.81	1.00	
9	12-5	0.53	0.54	0.56	0.58	0.54	0.65	0.60	0.61	1.00
10	5-1	0.51	0.57	0.56	0.54	0.53	0.59	0.60	0.56	0.66
11	1-7	0.49	0.53	0.58	0.57	0.55	0.61	0.61	0.55	0.61
12	2-5	0.46	0.57	0.54	0.48	0.53	0.58	0.58	0.56	0.61
13	10-6	0.44	0.46	0.56	0.48	0.50	0.50	0.54	0.57	0.51
14	2-2	0.45	0.50	0.48	0.50	0.53	0.47	0.54	0.55	0.53
15	11-1	0.49	0.55	0.53	0.51	0.56	0.56	0.56	0.53	0.60
16	12-6	0.49	0.54	0.57	0.55	0.56	0.59	0.57	0.57	0.60
17	2-3	0.47	0.50	0.56	0.51	0.51	0.57	0.52	0.56	0.57
18	3-1	0.49	0.55	0.58	0.56	0.50	0.57	0.58	0.55	0.61

		10	11	12	13	14	15	16	17	18
10	5-1	1.00								
11	1-7	0.61	1.00							
12	2-5	0.56	0.60	1.00						
13	10-6	0.56	0.48	0.48	1.00					
14	2-2	0.57	0.49	0.48	0.58	1.00				
15	11-1	0.60	0.53	0.56	0.51	0.62	1.00			
16	12-6	0.52	0.55	0.55	0.51	0.54	0.63	1.00		
17	2-3	0.54	0.57	0.53	0.48	0.50	0.58	0.60	1.00	
18	3-1	0.62	0.55	0.56	0.52	0.52	0.62	0.58	0.63	1.00

[z] *Discula destructiva* isolate from site 9 and tree 9
[y] *Discula destructiva* isolate from site 2 and tree 10

pathogen *D. quercina* Berk. & Broome. The phylogeny of *D. destructiva* was found to be from a different origin than *Discula* sp. In a follow-up study, anthracnose fungi were analyzed using internal transcribed spaces of ribosomal DNA (rDNA) in order to understand their phylogeny. *Discula* sp. and *D. destructiva* were located in separate well-differentiated phylogenic groups. The different grouping of both fungi indicates that *D. destructiva* did not originate from an indigenous population of *Discula* sp. but more likely was an introduced pathogen (Caetano-Anollés et al., 2001). High genomic variability in *Discula* sp. and a conserved genome in *D. destructiva* are significant in that they are most likely not the same species. Since the genome in the undescribed *Discula* was highly variable, this species may actually be an aggregation of several fungal species that are similar in morphology and physiology. Also, it is possible that *Discula* sp. may not be in the genus *Discula* but related to another fungus in the same family (Melanconeacae).

iv. Summary

DNA amplification fingerprinting was used to analyze genomic DNA of 16 isolates of *Discula* sp. and two isolates of *D. destructiva*. Five octomer primers were used and the loci were scored and then analyzed using

NTSYS. *Discula* sp. may not be related to the genus *Discula* but to another genus in the same family. In addition, *Discula* sp. may have an aggregation of different species that are similar in morphology and physiology. Other findings included similarities within groups of *Discula* sp. that were mostly found in the same area. These analogous groups could stem from a population that is isolated and under similar environmental pressures.

Chapter IV

Conclusions

During 2000-2001, a study was initiated to compare the seasonal isolation and a portion of the genomes of *D. destructiva* and *Discula* sp. Experiments were conducted in all four seasons for detection of seasonal presence of *Discula* spp. in 13 sites in Alabama, Georgia, North Carolina, Tennessee, and Virginia. Genomic analysis was performed using DNA amplification fingerprinting (DAF), which incorporated the utilization of 10 different primers with 16 isolates of *Discula* sp. and two isolates of *D. destructiva.*

In the seasonal presence study, symptomatic dogwood tissue was collected at each site in every season and placed in moisture chambers for sporulation. Conidia from acervuli were then placed onto potato dextrose agar with antibiotics (PDA+) and examined. Light microscopy and gallic acid medium were used to distinguish between *Discula* sp. and *D. destructiva.* Most isolates of *D. destructiva* were collected in March and April (n= 23) and in June and July (n=19). The number of the undescribed *Discula* sp. isolates collected was lower in March/April (n= 12) and similar

to *D. destructiva* in June/July (n= 16). Only one isolate of *D. destructiva* was found in the fall, and no isolates were obtained in the winter.

An increase in undescribed *Discula* sp. may be due to a genetic shift in the host population, or isolation from predominately woody tissue instead of foliage tissue. No significance was found between the number of fungal isolates and stream proximity, elevation, aspect and diameter breast height. Lack of significance between these factors and isolation of *Discula* taxa could have been from a purposeful approach to site selection.

Genomic analysis (DAF) was performed to facilitate the understanding of the relationship between *Discula* sp. and *D. destructiva.* Banding patterns were scored and compared using Numerical Taxonomical and Multivariate Analysis System (NTSYS). *Discula* sp. was found to have a polymorphic genome, and the genome of *D. destructiva* was conserved. Previous researchers believed that the conserved genome of *D. destructiva* was evidence of an exotic species, whereas the polymorphic genome of *Discula* sp. may represent an endemic species (Trigiano et al., 1995a). Related groups of *Discula* sp. were identified and most groupings were delimited by geographical area. Genomic similarity within the group probably came from site isolation.

Isolation of *Discula* sp. occurred at a much higher rate than in previous research. Identification of *Discula* sp. is important to understand the relationship between it and related fungi. Because the genome of *Discula* sp. is so variable, the undescribed species may not be in the presumed genus or there may be an aggregation of morphologically and physiologically similar species.

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APPENDIX

Appendix 2.1 Scoring of all five primers used in DAF for 16 isloates of *Discula* sp. and two isolates of *D. destructiva*. Representation of numerical values were 0 = no loci, 1= loci present, and 9 = missing data.

Primer GAGCCTGT (8.6A)

Isolates								
11-7	1-8	2-1	2-4	2-9	1-9	9-9	2-10	12-5
0	0	1	9	0	0	0	9	1
0	0	1	1	1	0	1	1	0
1	0	0	0	9	9	1	1	1
1	0	1	0	0	1	1	1	1
0	1	1	1	9	1	1	1	1
1	1	1	1	1	1	1	1	1
9	1	1	1	1	1	1	1	1
9	0	1	1	9	1	1	0	1
9	1	0	0	0	0	1	1	0
9	1	1	1	1	1	1	1	1
9	1	1	9	0	1	9	1	1
1	1	1	1	0	0	1	1	0
0	1	1	1	9	9	1	1	0
0	1	1	0	0	9	1	1	0
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	9	1	1
1	1	1	1	1	1	1	1	0
0	1	1	1	1	1	1	1	9
1	1	1	1	1	1	1	1	1
0	1	0	1	1	0	1	1	1
1	1	0	0	0	1	1	0	1
1	0	0	0	0	1	1	0	0
1	1	1	1	9	1	1	1	1
1	1	1	1	0	1	1	1	1
0	1	1	1	1	1	1	1	1
0	0	1	1	1	9	1	1	0
0	1	1	1	1	0	1	1	1
1	1	1	1	1	1	1	1	1
1	0	0	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
0	1	1	1	1	1	1	1	1
1	1	0	0	1	1	1	1	1
0	1	1	1	1	1	1	1	1
1	1	1	0	1	1	1	1	0
0	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0
0	1	1	1	1	0	1	1	0
0	1	9	0	0	1	1	1	1
1	1	9	1	1	0	1	1	1
0	0	1	1	0	1	1	1	1
1	1	0	9	1	1	1	1	0

Primer GAGCCTGT (8.6A)

Isolates

5-1	1-7	2-5	10-6	2-2	11-1	12-6	2-3	3-1
9	1	1	1	1	1	1	1	1
0	1	0	0	0	0	0	0	0
1	1	1	1	1	0	1	0	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	0	9	0	1
1	1	1	1	1	1	1	1	1
0	1	1	0	0	0	1	1	1
1	1	1	1	1	1	1	1	1
0	0	1	0	0	0	0	0	0
1	1	9	0	0	9	1	1	0
1	0	1	0	0	9	1	1	1
0	0	0	1	1	1	1	0	0
0	9	0	1	1	9	1	0	1
1	1	1	1	1	9	0	0	0
1	1	1	1	1	0	0	1	0
1	0	1	1	1	0	0	0	0
1	1	0	1	1	1	1	1	1
1	1	0	1	1	1	1	1	1
9	1	0	0	0	0	0	0	0
9	1	0	1	1	1	1	0	9
1	1	1	1	1	0	0	0	0
1	1	1	1	1	0	0	9	1
1	1	0	0	0	0	0	0	0
1	1	1	0	0	9	0	0	0
0	1	1	0	0	1	1	1	0
0	1	1	0	0	1	1	1	1
1	1	1	1	1	0	1	1	1
1	1	1	1	1	1	1	1	1
1	1	9	1	1	1	1	1	1
1	1	0	0	0	1	1	1	0
1	1	0	1	1	1	1	1	0
1	1	1	1	1	1	1	1	1
1	0	1	0	0	1	0	1	1
1	1	1	1	1	1	1	1	0
0	1	1	1	1	1	1	1	0
0	1	1	9	9	1	1	1	0
1	1	1	0	0	1	0	1	0
1	0	1	1	1	1	0	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
0	0	1	0	0	0	0	1	0

Primer GTAACGCC (8.6D)

Isolates

11-7	1-8	2-1	2-4	2-9	1-9	9-9	2-10	12-5
0	1	1	1	0	0	0	0	0
1	9	9	9	1	1	1	1	1
9	9	9	9	1	0	9	9	9
9	9	9	9	1	9	9	9	1
9	9	9	9	0	9	9	1	1
1	1	1	1	1	0	0	1	0
1	0	1	9	1	0	0	1	0
0	0	0	0	0	1	0	0	1
1	0	1	1	1	0	1	1	0
0	0	1	9	1	0	9	1	0
0	0	1	0	0	0	0	0	0
1	0	1	1	1	1	1	1	0
0	0	0	0	0	1	0	0	1
1	9	1	1	1	1	9	1	1
1	1	9	0	1	1	0	1	1
1	0	9	0	1	9	0	1	0
0	1	1	0	0	1	1	1	1
1	1	0	0	1	1	1	1	1
0	0	0	0	0	1	0	0	1
0	1	0	1	0	0	1	1	0
1	1	1	0	1	0	0	1	1
0	0	1	0	1	0	0	1	1
1	1	9	9	1	0	1	1	1
1	0	1	9	1	1	0	0	1
1	1	1	1	1	1	0	0	0
1	1	1	1	1	1	1	1	0
1	1	0	0	1	1	1	1	1
1	0	0	1	1	1	1	1	1
0	0	1	1	1	0	0	1	0
0	1	1	0	1	1	1	1	0
1	1	1	1	1	0	1	1	1
1	1	9	0	0	1	0	0	1
1	1	0	1	1	1	1	1	1
0	0	1	1	0	0	1	1	0
1	1	9	1	1	1	1	1	1
0	0	9	0	0	1	0	0	1
1	1	9	0	0	1	0	9	1
1	0	0	1	9	9	1	0	0
0	1	9	9	9	1	1	0	0
1	0	9	9	9	0	1	0	1
1	0	9	1	0	1	1	9	1
1	0	9	1	9	1	0	0	1
0	1	1	0	0	1	1	9	0
0	0	0	0	0	0	0	0	0
0	0	1	1	9	0	1	9	1
1	9	0	0	9	1	1	1	0
0	1	1	1	9	1	1	0	1
0	0	1	0	0	1	0	0	1
1	0	1	0	9	1	0	0	0
1	0	1	1	1	1	0	1	0

Primer GTAACGCC (8.6D)

Isolates

5-1	1-7	2-5	10-6	2-2	11-1	12-6	2-3	3-1
0	1	1	9	9	1	1	1	0
1	1	0	9	1	0	9	9	9
1	1	9	1	1	1	0	1	1
0	0	1	0	0	1	1	0	0
0	0	0	0	1	0	1	1	1
0	1	0	0	0	0	1	1	0
0	0	1	0	0	0	9	9	9
0	0	9	1	1	1	0	9	9
1	1	1	0	9	1	1	1	9
0	9	1	1	9	0	1	9	9
0	0	9	1	1	1	9	1	9
0	0	1	1	9	0	1	0	0
1	1	1	0	1	1	1	0	0
1	1	1	1	1	1	1	9	9
0	0	0	1	1	0	1	1	0
9	9	1	1	0	1	1	1	0
1	0	0	0	0	1	1	1	1
1	1	1	1	1	1	0	1	1
1	1	1	0	0	1	1	0	1
1	0	0	1	1	1	1	1	1
1	0	1	1	1	1	9	0	9
1	0	0	1	1	1	1	1	1
0	0	1	0	1	0	1	0	9
1	1	1	1	9	1	1	0	0
0	1	0	1	9	1	1	0	1
0	0	1	1	0	1	1	1	1
1	1	1	0	0	1	9	0	1
1	1	1	0	1	1	1	0	1
0	0	0	1	1	0	0	0	1
1	0	1	1	1	1	1	0	0
1	1	0	0	1	1	1	1	1
1	9	1	0	0	1	1	1	1
1	1	1	0	1	0	1	0	1
1	0	1	1	1	1	0	0	1
9	0	1	1	1	1	1	1	0
0	0	1	0	0	1	1	1	1
1	0	1	0	0	1	0	0	1
0	0	0	9	1	1	1	0	0
0	1	1	0	0	1	1	1	0
1	1	1	0	0	1	0	0	1
0	1	0	1	0	0	1	0	0
0	0	1	0	0	1	0	1	1
1	1	1	0	0	1	0	0	1
1	1	1	1	0	0	0	1	1
1	1	0	1	0	1	1	0	1
1	0	0	1	1	1	1	1	1
1	1	1	1	0	0	1	0	1
1	0	1	0	0	1	1	0	0
0	0	0	1	1	1	1	1	1
0	0	0	0	0	0	1	1	1

Primer CCTGGTGG (8.7E)

Isolates

11-7	1-8	2-1	2-4	2-9	1-9	9-9	2-10	12-5
0	1	1	0	1	1	0	0	1
1	0	0	0	0	0	0	0	0
0	9	1	1	0	1	0	0	0
0	0	1	1	0	0	0	1	1
1	1	1	9	0	1	1	0	0
0	0	1	1	1	1	0	1	1
0	1	1	1	1	1	1	1	1
0	1	9	0	0	1	1	1	0
0	0	0	0	0	0	0	0	0
0	1	9	0	0	1	0	0	0
0	1	1	1	1	1	1	1	1
1	1	9	1	0	1	1	0	1
1	1	0	1	0	1	1	1	1
0	0	9	1	0	1	1	1	1
1	1	1	0	1	1	1	1	1
9	1	1	0	1	1	0	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	0	0	0	1	1	1	1
0	0	0	0	0	1	1	1	1
1	1	0	1	1	0	1	1	1
0	0	1	1	9	1	1	1	1
0	0	0	1	1	1	1	1	1
1	0	1	1	1	0	1	1	0
0	0	1	1	9	0	0	1	1
1	0	0	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0
1	0	0	0	1	1	1	1	1
1	1	1	1	1	0	1	1	1
1	0	0	1	1	1	9	1	1
0	1	1	1	0	1	1	0	1
1	0	1	1	0	1	1	1	1
1	0	1	1	0	1	1	1	1
0	1	0	1	1	1	1	1	1
1	1	0	1	1	1	1	0	0
0	0	0	1	0	1	1	1	1
0	1	1	1	1	1	1	9	1
1	1	1	0	9	1	1	0	1
0	0	0	1	0	1	1	1	1
1	0	0	0	0	0	0	0	1
0	0	0	1	0	0	0	0	0
1	0	1	1	1	1	0	0	1
1	1	1	1	1	1	1	1	0
1	1	1	1	1	1	1	1	1
9	0	1	9	9	1	9	1	0
1	1	1	0	1	1	1	1	1
0	1	0	1	1	1	0	1	9
1	1	1	1	0	0	1	0	0
0	1	0	0	0	1	1	1	1

Primer CCTGGTGG (8.7E)

Isolates

5-1	1-7	2-5	10-6	2-2	11-1	12-6	2-3	3-1
1	1	1	9	0	1	1	1	1
0	0	0	0	0	0	0	0	0
0	1	0	0	0	0	0	0	9
0	0	0	0	0	0	0	0	0
1	0	1	0	0	0	0	0	1
0	1	9	1	0	0	1	1	0
1	0	1	0	0	0	0	1	1
0	1	1	0	0	1	0	1	1
1	1	1	1	1	1	1	1	1
1	1	1	9	0	0	0	0	9
1	1	0	0	0	1	0	0	1
0	1	0	0	0	0	0	1	1
1	1	0	0	1	1	1	1	1
1	1	1	1	0	0	0	1	0
0	1	1	1	1	1	0	1	1
1	1	1	1	0	1	0	1	0
1	1	1	1	0	0	0	0	1
1	1	1	1	0	0	1	1	1
1	1	1	1	1	1	1	1	1
0	0	1	1	1	0	1	1	1
1	0	1	0	1	1	1	0	0
1	1	1	1	0	0	1	1	1
1	1	1	1	1	1	1	1	1
1	0	1	1	1	1	0	1	1
1	1	1	0	1	0	0	1	1
1	1	1	0	0	1	0	0	1
0	0	0	1	1	1	0	0	1
1	1	1	0	1	1	1	0	0
1	1	1	0	1	1	0	0	0
0	1	0	0	0	0	1	1	1
1	1	1	0	1	1	1	1	1
0	1	0	1	1	1	0	1	1
1	1	1	0	1	1	0	1	1
1	0	1	1	1	1	1	1	1
1	1	1	0	1	1	0	0	0
1	1	0	1	1	0	0	1	1
1	0	0	1	0	1	1	1	1
1	1	1	0	1	1	1	1	1
1	1	1	1	9	1	1	0	1
1	0	0	1	1	9	0	1	1
1	0	0	1	0	9	0	0	1
1	0	0	1	0	1	0	1	1
0	1	1	1	0	0	1	0	0
0	0	9	1	1	1	0	0	0
1	1	0	1	1	0	0	0	0
1	1	1	1	1	1	1	1	1
1	0	9	0	1	0	0	0	0
1	1	0	1	0	0	1	1	1
1	1	1	1	1	1	1	1	1

Primer CCTGCTGG (8.7I)

Isolates

11-7	1-8	2-1	2-4	2-9	1-9	9-9	2-10	12-5
0	0	1	1	1	1	1	1	1
0	9	0	1	1	1	1	1	0
1	1	1	1	1	1	0	0	1
1	1	0	1	1	1	0	0	1
1	1	1	1	0	1	1	0	1
0	1	9	9	1	1	1	1	0
1	1	1	1	1	0	1	1	1
0	1	0	0	1	1	1	1	0
1	9	1	1	0	0	1	1	1
1	1	1	1	1	1	9	9	1
1	1	1	1	1	1	1	1	1
0	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
9	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0
1	1	1	1	1	0	0	0	1
1	1	1	0	1	1	1	1	1
1	9	1	1	1	1	1	1	1
1	1	1	0	1	1	1	1	0
1	1	1	1	1	1	1	1	1
1	1	0	1	1	1	1	0	1
1	0	0	1	1	1	1	1	1
1	1	1	0	1	1	1	1	0
0	1	1	1	1	1	1	1	1
0	0	0	1	1	1	1	1	1
0	1	1	1	1	1	1	1	1
0	0	1	1	1	0	0	9	0
1	1	1	1	1	1	1	1	1
1	1	0	1	1	1	1	1	0
0	1	0	1	1	1	1	1	1
1	1	1	0	1	1	1	1	1
0	0	1	1	0	1	1	1	1
1	1	1	1	0	1	1	1	1
0	1	1	1	1	0	0	0	1
1	1	1	1	0	1	0	0	9
9	9	1	0	1	1	0	0	1
0	0	1	1	0	1	1	1	1
0	0	0	0	1	0	0	0	1
1	0	0	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0

Primer CCTGCTGG (8.7I)

Isolates

5-1	1-7	2-5	10-6	2-2	11-1	12-6	2-3	3-1
1	9	0	1	1	1	0	1	0
0	0	0	1	1	1	1	0	1
1	1	0	1	1	1	0	0	0
1	1	1	0	1	1	0	1	1
1	1	1	1	1	1	0	1	1
0	0	0	0	9	0	1	0	0
1	1	1	1	1	1	0	1	1
1	0	1	0	1	1	1	1	1
1	0	1	1	1	0	1	1	1
1	1	1	1	0	0	1	1	1
1	0	0	1	1	1	1	0	1
1	1	1	1	1	0	0	0	1
1	0	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0
1	0	1	1	0	1	1	0	1
1	1	0	0	1	0	0	1	0
1	0	1	0	1	1	1	0	1
0	1	0	1	1	1	1	1	1
1	1	1	0	1	1	1	1	1
0	1	1	1	0	0	1	1	1
0	1	1	0	0	0	1	1	1
1	0	1	1	0	0	1	0	0
1	1	1	1	0	0	0	1	0
1	9	0	1	1	1	1	1	1
9	9	1	1	1	1	1	0	1
0	1	1	1	1	1	1	1	0
9	0	1	1	0	1	1	0	1
0	1	1	0	0	0	0	1	1
1	0	1	1	0	0	1	0	1
0	1	9	0	1	1	1	0	1
1	1	9	0	1	1	1	1	1
1	1	1	1	0	1	1	1	1
1	1	0	0	0	0	1	1	1
1	0	1	1	1	1	1	1	1
1	1	1	0	1	1	1	1	1
1	1	1	9	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	0	1	1	1	1
1	1	0	0	1	1	1	1	0
0	1	1	1	1	1	1	0	0
1	1	0	1	0	9	0	0	1

Primer AATGCAGC (8.7A)

Isolates

11-7	1-8	2-1	2-4	2-9	1-9	9-9	2-10	12-5
0	1	1	0	1	0	9	9	1
0	9	9	1	1	1	1	1	1
1	1	1	1	1	1	0	0	0
1	1	1	1	1	1	1	1	1
0	1	1	1	0	1	1	0	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	0	0	1
0	0	0	0	1	1	1	1	0
1	1	1	1	1	1	0	0	1
0	1	0	1	1	0	1	1	1
0	1	0	1	1	1	0	0	0
1	1	1	0	0	1	0	0	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0
0	1	0	1	1	0	0	0	0
1	1	1	1	1	0	1	1	1
1	1	1	1	0	1	1	1	1
1	1	1	1	0	0	1	1	1
1	0	1	1	0	1	0	0	1
1	0	0	1	1	1	0	0	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	0	1	1	1
1	1	1	0	1	0	1	1	1
1	1	1	0	1	1	0	1	1
1	1	1	1	1	0	0	0	0
1	0	1	1	1	1	1	1	1
1	1	1	0	0	0	1	1	1
1	0	0	1	1	1	0	0	0
1	1	1	0	0	0	1	1	1
1	1	1	1	1	1	1	1	1
1	0	0	1	0	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	0	1	1	1	0	1	1

Primer AATGCAGC (8.7A)

Isolates

5-1	1-7	2-5	10-6	2-2	11-1	12-6	2-3	3-1
1	1	1	1	1	1	1	1	1
0	1	1	1	1	1	1	1	1
1	1	0	0	0	0	1	1	0
1	1	1	1	1	1	1	1	1
0	1	1	0	1	1	1	1	1
1	1	0	0	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0
1	1	0	1	1	1	1	1	1
1	1	0	1	1	1	1	0	0
1	0	1	0	1	1	1	1	1
1	1	1	1	1	0	1	0	0
1	1	9	1	1	1	1	1	1
0	1	1	0	1	0	1	1	1
0	0	1	0	1	1	1	1	1
1	1	1	1	1	1	1	0	1
1	1	1	1	0	0	1	1	1
1	0	0	1	1	1	1	1	1
1	0	1	0	1	0	1	1	0
0	1	1	0	0	1	1	1	1
0	1	1	0	0	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	0	1	1	1	1
1	1	1	1	1	1	1	1	1
0	1	0	0	1	1	1	0	1
1	1	0	0	1	1	1	1	1
1	1	1	1	9	1	1	1	1
1	1	0	1	9	1	1	1	1
1	1	1	0	9	1	1	1	1
1	1	1	1	9	1	1	1	1
0	0	0	0	9	1	1	1	1
1	1	0	1	9	0	0	1	1
1	0	1	1	9	1	0	1	1

VITA

Aimee Michele Sheer was born in Richmond, Virginia, on November 1, 1976. She participated in the science club and played on the junior varsity basketball team while attending Atlee High School from 1991 to 1995.

In 1999 Ms. Sheer graduated from Longwood College, at Farmville, Virginia, where she majored in biology with a concentration in environmental science. While attending Longwood College she worked as a history and biology tutor from 1996 to 1997 and as a computer lab assistant from 1998 to 1999. She was also actively involved in Alpha Sigma Tau and the chemistry club.

During her master's study at the University of Tennessee, Knoxville, Ms. Sheer was active in the Graduate Student Association in the Department of Entomology and Plant Pathology. She presented a paper entitled "Seasonal and genomic differences of *Discula destructiva* and *Discula* species" at the 2001 Southern Nursery Association in Atlanta, Georgia.

Ms. Sheer is a member of the Gamma Sigma Delta Honor Society and the American Phytopathological Society.