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GENETIC DIVERSITY AND VARIATION IN MICHIGAN ISOLATES OF *MONILINIA FRUCTICOLA* AND *MONILINIA LAXA*

By

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GENETIC DIVERSITY AND VARIATION IN MICHIGAN ISOLATES OF *MONILINIA FRUCTICOLA* AND *MONILINIA LAXA*

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One hundred and seventeen vegetative compatibility groups (VCGs) were identified among 169 isolates of *M. fructicola* collected from Michigan cherry orchards. Compatibility varied between three individual orchards with 70 isolates forming 25 VCGs, 61 isolates forming 54 VCGs, and 38 isolates forming 38 VCGs. PCR-mediated analysis of DNA from *M. fructicola* and *M. laxa* revealed internal transcribed spacer 1 (ITS1) regions that were 146 bp in length and 98% similar. A base pair change at position 105 created a *Mse* I restriction site in *M. fructicola*. A 421-bp region present in the SSU rDNA of 32 isolates of *M. fructicola* but absent from eight isolates of *M. laxa* was identified as a group I intron. PCR amplification of the intron-containing region yielded a product of approximately 940 bp from *M. fructicola* and 520 bp from *M. laxa*. Arbitrarily primed-PCR with primers $(GACA)_4$ and $(GTG)_5$ each yielded distinct multiple banding patterns between the species.

For my parents, George and Yvonne Snyder,
for all of their love and support

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INTRODUCTION

Brown rot, caused by *Monilinia fructicola* (Winter) Honey, is the most economically important disease of stone fruits in the United States. Within Michigan orchards, *M. fructicola* is a serious concern for the cherry industry, causing blossom blight, twig cankers and significant pre- and post-harvest fruit rot (Jones and Sutton, 1996). A second brown rot fungus, *M. laxa* Aderhold and Ruhland, is common along the Pacific coastal regions and has caused minor outbreaks in Wisconsin, Michigan, and New York (Cation *et al.*, 1949; Kable and Parker, 1963; Keitt *et al.*, 1943; Jones and Sutton, 1996).

The impact of brown rot on stone fruit industries indicates the need for study of the exact nature of the disease cycle and the genetic diversity that exists within field populations of the pathogen. While limited study of the genetics and diversity of *M. fructicola* has been performed in California populations of the pathogen (Sonoda *et al.*, 1990; Free *et al.*, 1996), little has been done to examine the diversity that exists within Michigan populations. The level of diversity that exists within populations of the pathogen and the role of sexual sporulation in the disease cycle are all important factors in understanding the spread of the disease and implementing the most effective and economical control measures. With the occurrence of *M. laxa* and *M. fructicola* together in some regions, it is also important to understand the relationship between these two

species and to develop methods for distinguishing between hard to identify isolates of the two species.

The purpose of this study was to began an assessment of the level of diversity which exists within populations of *M. fructicola* and *M. laxa*. This study focused specifically on diversity within Michigan populations of these fungi, and the results found from these local populations may not indicate the diversity of the global population. However, this study had the additional purpose of identifying techniques useful for revealing diversity within the species and which can be applied to future studies on a broader geographic scale.

Vegetative compatibility and a variety of molecular techniques have been used by mycologists and plant pathologists to examine diversity within species as well as to determine the relationship between different species. Many of these studies have also uncovered rapid means of distinguishing between species and important sub-specific groups.

This study of Michigan field populations utilized vegetative compatibility to initiate an examination of the diversity of *M. fructicola* in Michigan. As will be discussed in chapter 1, the frequency of vegetative compatibility may directly reflect the level of genetic diversity within a population. Molecular methodology was employed to obtain a more accurate view of the genetic diversity that exists within *M. fructicola*, and to develop an understanding of the relationship between *M. fructicola* and *M. laxa*. Nucleotide sequence analysis of the internal transcribed spacer 1 (ITS1) region provided evidence of the diversity within *M. fructicola* and also revealed the apparent close

relationship between *M. fructicola* and *M. laxa*. Examination of the small subunit (SSU) ribosomal DNA for the presence of an intron and random amplification of microsatellite DNA provided data regarding the apparent level of diversity within *M. fructicola* and *M. laxa*. Each of the these three methods also identified differences which may serve as useful markers for the rapid differentiation of Michigan isolates of *M. fructicola* and *M. laxa*.

REVIEW OF LITERATURE

Brown Rot

Brown rot is one of the most economically important diseases of stone fruit throughout the temperate regions of the world (Byrde and Willletts, 1977). The brown rot fungi include three species of Discomycetes within the Family Sclerotiniaceae: *Monilinia fructicola* (Winter) Honey, *M. laxa* Aderhold & Ruhland, and *M. fructigena* Honey. These fungi cause symptoms ranging from fruit rot to blossom blight, stem and leaf blight, and stem cankers. Economic losses result primarily from the rotting of fruit in the orchard before harvest, but losses also occur during transport of harvested fruit and from yield reduction by destruction of blossoms early in the season (Jones and Sutton, 1996).

The earliest description of a brown rot fungus was Persoon's 1796 description of a European fungus with buff-colored conidial pustules (then *Turola fructigena*). In 1818, Ehrenberg described a second brown rot fungus with grey conidial pustules on apricot (*Oidiium laxum*). Both species were eventually transferred to the genus *Monilia*, and a third name, *M. cinerea*, was proposed for the brown rot on cherries. In 1886 Saccardo and Voglino listed the three brown rot species of Europe as *Monilia fructigena, M. laxa,* and *M. cinerea*, but the latter two were eventually found to be conspecific. In 1893, Schröter transferred the brown rot fungi to the genus *Sclerotinia* based on the morphology of their conidial stages.

The first description of a brown rot fungus in America was Peck's 1881 description of a fungus which he identified as *Monilia fructigena*. Two years later, Winter made the first known record of the perfect stage of any brown rot fungus. He named this fungus, found on mummified peaches in Pennsylvania, *Ciboria fructicola* (later changed to *Sclerotinia fructicola*). In 1902, Norton made a description of the perfect stage of a second brown rot fungus in North America which he called *S. fructigena*. However, Reade later determined that *S. fructicola* and *S. fructigena* were synonymous when referring to the common American brown rot fungus and Pollack suggested the use of the species epithet *fructicola.* In 1928, Honey proposed the generic name *Monilinia* to include those members of the Sclerotiniaceae which produce moniloid conidia.

M. fructicola, commonly called the American brown rot fungus, is widespread throughout stone fruit growing regions of the United States. A European brown rot fungus, later identified as *M. laxa,* was discovered in Oregon in 1915 and since its discovery has become common along the Pacific coastal regions, and also appears to be endemic on sour cherry (*Prunus cerasus* L.) in Michigan (Cation et al., 1949), New York (Kable and Parker, 1963), and Wisconsin (Keitt *et al.*, 1943). However, *M. fructicola* remains the species of greatest economic importance throughout the United States. The third brown rot pathogen, *M. fructigena* is widely distributed in Europe, but is currently not found in North America.

The primary role of *M. fructicola* is that of a fruit rot pathogen. Fruit is rotted in the orchard, but significant post-harvest fruit rot also occurs during transportation and

storage. *M. fructicola* infects a wide range of stone fruits, including apricot, peach, plum, nectarine, and cherry. Within Michigan orchards, *M. fructicola* is the primary, and often only, brown rot fungus evident, causing significant pre- and post harvest fruit rot on both sweet and sour cherry. *M. laxa*, unlike *M. fructicola,* is a blossom and twig blight pathogen and rarely causes fruit rot. The destruction of blossom and fruit spurs results in a reduced fruit yield latter in the season. Within the Great Lakes region, including Michigan, *M. laxa* rarely causes significant levels of disease, although a serious outbreak occurred in Michigan and Wisconsin in 1993 (Jones and Sutton, 1996). When *M. laxa* does occur in Michigan it is only found on sour cherry.

The brown rot fungi overwinter as mycelium in infected peduncles, twig cankers, and mummified fruit on the tree, or as pseudosclerotia in mummified fruit on the ground. Primary inoculum for infection of blossoms in the spring are conidia produced on infected peduncles, cankers, and mummies on the tree, and, for *M. fructicola*, ascospores produced within apothecia that arise from mummies buried in the ground. However, while apothecia have been noted in California orchards (Hewitt and Leach, 1939, Free *et al.*, 1996), they are rarely evident in Michigan orchards, and no apothecia were observed in New York orchards over a three year period (Wilcox, 1989). Because of the apparent absence of apothecia from orchards of the Midwest and East coast, the importance of the sexual stage of *M. fructicola* to spread of the disease in these regions remains unclear and conidia are considered the most important inoculum for fruit infection and spread of the disease throughout the growing season (Byrde and Willets, 1977).

A few studies regarding the genetics of *M. fructicola* have been conducted. Ezekial (1924) and Harada (1977) reported that *M. fructicola* is homothallic and additional studies have described the fungus as heterokaryotic and capable of out-crossing (Byrde and Willetts, 1977; Sanoamuang *et al.*, 1995; Thind and Keitt, 1949; Sonoda *et al.*, 1982). However, a recent study suggests that *M. fructicola* may actually be heterothallic (Free *et al.*, 1996). The study of single ascospore progeny revealed the segregation of fungicide resistance genes (Sanoamuang *et al.*, 1995) and that the genes for vegetative compatibility segregate independent of one another (Free *et al.*, 1996).

Vegetative Compatibility

Fungal vegetative compatibility refers to the formation of stable heterokaryons via anastomosis (fusion) of the vegetative hyphae of fungi. Isolates which anastomose to form stable heterokaryons are considered to be vegetatively compatible and are assigned to a single vegetative compatibility group (VCG). Hyphal fusion between some isolates cannot occur or results in the death of the cells involved. These isolates, between which stable heterokaryons cannot form, are vegetatively incompatible and are placed in different VCGs.

Several methods have been developed to test for compatibility, including auxotrophic mutants, morphological mutants, and barrage formation (Anagnostakis, 1977; Puhalla, 1979; Sonoda *et al.*, 1982a; Puhalla, 1985; Correll *et al.*, 1987). However, the method employed typically depends on the growth habits of the fungus being studied and must also be carefully evaluated to ensure that the chosen method offers the most

accurate results. Studies of *Verticillium dahliae* using microsclerotial color mutants revealed sixteen VCGs among 86 isolates (Puhalla, 1979; Puhalla and Hummel, 1983). However, in later work, representatives from fifteen of the known VCGs were found to form only four VCGs when tested using nitrate-nonutilizing (nit) mutants (Joaquim and Rowe, 1990). The disparity between the results from these two methods may reflect the occurrence of weakly compatible reactions (Joaquim and Rowe, 1990). While some interactions were compatible, the interaction was too weak to generate the expected microsclerotial color, but strong enough to be detected with nit mutant testing. The conflicting results from the studies of *V. dahliae* also indicate the need to exercise caution when interpreting VCG data.

Within many species of fungi, vegetative hyphae will undergo anastomosis regardless of their natural compatibility or incompatibility. Anastomosis of vegetative hyphae which contain vegetatively incompatible nuclei results in a killing reaction which ultimately leads to the death of the newly formed heterokaryotic cell. The killing reaction leads to the formation of a region of dead and dying cells between the isolates, and a layer of dark pigment is often deposited between the isolates. The region of dead and dying cells and dark pigment is commonly referred to as the barrage zone. Barrage zone formation has become one to the primary means by which vegetative incompatibility is scored in ascomycetes.

Vegetative compatibility testing within *M. fructicola* has utilized the formation of barrage zones between incompatible isolates (Sonoda *et al.*, 1982a; Sonoda *et al.*, 1990; Free *et al.*, 1996). The production of dark lines between some isolates of *M. fructicola*

when grown together in culture was first described by Thind and Keitt (1949). This barrage formation has since been identified as characteristic of vegetative incompatibility in Ascomycetes. Sonoda *et al.* determined that these interactions could be used to genetically group isolates of *M. fructicola* (1982a). Vegetative compatibility studies involving both *M. fructicola* and *M. laxa* also suggested that these interactions might be useful in distinguishing cultures of *M. laxa* from *M. fructicola* (Sonoda *et al.*, 1982b). Vegetative compatibility has been examined in California populations of *M. fructicola* (Sonoda *et al.*, 1990; Free *et al.*, 1996), but has never been examined in Michigan populations.

Vegetative compatibility reactions have been used for a number of purposes in the study of other fungi. Vegetative compatibility has been described as a potential diagnostic tool on that basis that strains of the same sub-specific group, such as a pathogenicity group, may be members of only one or a few VCGs (Puhalla, 1985). The pathogen could then be identified rapidly by placement into a VCG rather than the more time consuming method of pathogenicity testing. However, this use of vegetative compatibility as a diagnostic tool has displayed only limited success (Elias and Schneider, 1991; Jacobson and Gordon, 1988; Ploetz and Correll, 1988).

Vegetative compatibility has also been used in the study of diversity and epidemiology in a variety of plant pathogens (Puhalla, 1986; Kohn *et al.*, 1990; Chacko *et al.*, 1994; Daayf *et al.*, 1995). As will be discussed in chapter 1, the frequency of VCG diversity may reflect the level of genetic diversity within a population (Leslie, 1993). Vegetative compatibility may also provide insight into the mode of reproduction and

spread of a pathogen (Nalim *et al.*, 1995). The occurrence of VCGs, their location relative to one another within a field, and the location of isolates of the same VCG may establish a pattern which would provide insight into the role of ascospores verus conidia in the spread of disease as well as revealing the general pattern of spread (Adams *et al.*, 1990; Sonoda *et al.*, 1990; Nalim *et al*, 1995).

Molecular Methods in Mycology

While vegetative compatibility may be used as an initial step to identify the potential level of diversity that exists within populations, molecular methodology must be employed to obtain the most accurate view of genetic diversity. Molecular methodology and DNA analysis have provided useful tools for phylogenetic and taxonomic studies, as well as studies of population genetics and the analysis of genetic variability within species. Studies of variability within species have also often revealed methods for distinguishing important sub-specific groups, such as pathogenicity groups, and have revealed the nature of mutations causing a variety of phenomena, such as fungicide resistance.

One of the commonly used techniques in both population and phylogenetic studies is sequence analysis of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA. The ITS regions are non-coding sequences and are thought to be less subject to selection and may therefore display random genetic drift (Morton *et al.*, 1995). The ITS regions are generally conserved at the species level but are variable at higher taxonomic levels (Bruns *et al.*, 1991). ITS analysis has therefore become a useful tool for

species differentiation and phylogenetic studies (Chen *et al.*, 1992; Lee and Taylor, 1992; Carbone and Kohn, 1993). However, the ITS regions of several fungi have been shown to be highly variable at the species level, providing a means to identify subspecific variation and also aiding in phylogenetic analysis (O'Donnell, 1992; Morton *et al.*, 1995; Kusaba and Tsuge, 1995).

The ITS1 region of *M. fructicola* and three related species, *M. fructigena, M. megalospora* (Woronin) Whetzel, and *M. oxycocci* (Woronin) Honey, were examined in a study of diversity within the Sclerotiniaceae (Carbone and Kohn, 1993). The results of the study suggested that sequence variation within the ITS1 region could be used to distinguish *M. fructicola* from other *Monilinia* species. The study also revealed that *M. fructicola* and *M. fructigena* appear to be more closely related to one another and to various *Sclerotinia* species, than either is to the other *Monilinia* species examined. However, the ITS1 sequence of *M. laxa* was not included in this study, and its relationship to the other *Monilinia* species is uncertain.

The small subunit (SSU) ribosomal DNA is a conserved region that has also been utilized in various phylogenetic and taxonomic studies (Hendriks *et al.*, 1992; Wilmotte *et al.*, 1993; Liu *et al.*, 1995; Morales *et al.*, 1995). The SSU gene is highly conserved at the species levels and is primarily usefulness for phylogenetic studies of higher taxonomic levels, such as Family and Order. In recent years, several studies have revealed the presence of group I introns within the SSU gene of *Ustilago maydis* (De Watcher *et al.*, 1992), *Pneumocystis carinii* (Sogin and Edman, 1989), *Protomyces inouyei* (Nishida *et al.*, 1993), and several other fungal species. The presence of introns

in the SSU gene of widely divergent species is thought to be due to lateral gene transfer (Sogin *et al.*, 1986; Dujon, 1989). Introns present in pathogenic species may also provide a means of identifying an intron-containing organism within host tissue (Gargas *et al.*, 1995).

Sclerotinia sclerotiorum, a close relative of *M. fructicola* and *M. laxa*, was shown to possess group I introns in both the nuclear SSU and the mitochondrial SSU (Wilmotte *et al.*, 1993; Carbone *et al.*, 1995). The SSU genes of *M. fructicola* and *M. laxa* have not been examined, but isolates of one or both of these species may possess one or more introns. The identification of an intron within either species, and the location of the intron, may provide further insight into the relationship between these species and may also provide a rapid means of distinguishing isolates of the two species.

A third molecular method used in mycology involves the detection of microsatellite DNA. Microsatellites, or short tandem repeats, are stretches of tandem one to four nucleotide repeats, of varying length. These repeats are widespread in the genome of eukaryotes, including fungi (Bruford and Wayne, 1993; Rosewich and McDonald, 1994). The loci containing microsatellites may be useful for studies of genetic variation because they are typically highly polymorphic and usually undergo Mendelian inheritance (Groppe *et al.*, 1995).

Different alleles of a microsatellite-containing locus have been identified by size variation when amplified with oligonucleotides corresponding to the sequences flanking the microsatellite. A study of *Epichloë* species identified five alleles of a single microsatellite locus from a field of 91 isolates (Groppe *et al.*, 1995). Arbitrarily primed-

PCR (ap-PCR), using oligonucleotides corresponding directly to the microsatellites, has also been used to identify polymorphisms between species and among individuals within a species (Freeman and Rodrigez, 1995; Groppe *et al.*, 1995; Freeman *et al.*, 1996; Longato and Bonfante, 1997). However, success in identifying intraspecies polymorphisms has varied. Ap-PCR of *Colletotrichum gloeosporioides* isolates revealed no polymorphisms between 57 Israeli almond isolates, while 57 Israeli avocado isolates revealed eight distinct phenotypes (Freeman *et al.*, 1996).

Each of the three techniques described above may be useful to identify diversity within populations of *M. fructicola* and *M. laxa*. These techniques might also reveal differences between the two species which will provide a rapid means of distinguishing the two species from one another.

Chapter 1

VEGETATIVE COMPATIBILITY IN MICHIGAN POPULATIONS OF *MONILINIA FRUCTICOLA*

Introduction

Monilinia fructicola is an ascomycetous fungus with a known teleomorph. Apothecia arise from mummified fruit on the orchard floor early in the spring and the ascospores may serve as inoculum for initial blossom infection. Conidia are considered the most important inoculum for fruit infection and spread of the disease throughout the growing season (Byrde and Willetts, 1977). While apothecia have been noted in California orchards (Hewitt and Leach, 1939; Free *et al.*, 1996), they are rarely evident in Michigan orchards, and no apothecia were observed in New York orchards over a 3 year period (Wilcox, 1989). Because of the apparent absence of apothecia from orchards of the Midwest and the East coast, the importance of the sexual stage of *M. fructicola* to spread of the disease in these regions remains unclear.

The level of genetic diversity that exists within field populations of *M. fructicola* is also uncertain. Studies have been made of the spread and persistence of fungicide resistance in field populations of *M. fructicola*, but little has been done to examine the general diversity that exists within populations of this pathogen. Knowledge of the level of genetic diversity within populations of a species can provide insight into the

population structure, adaptability, and potential migratory history of the species. One approach to the study of genetic diversity in fungal populations utilizes the phenomenon of vegetative compatibility. Thind and Keitt (1949) first described the production of dark lines between some isolates of *M. fructicola* when these isolates were grown together in culture. This barrage formation has since been identified as a common characteristic of vegetative compatibility in Ascomycetes. Vegetative compatibility has been used in the study of diversity and epidemiology in a variety of plant pathogens (Chacko *et al.*, 1994; Kohn *et al.*, 1990; Puhalla, 1986; Daayf *et al.*, 1995). Sonoda *et al.* (1982a) determined that these interactions could be used to genetically group isolates of *M. fructicola.*

Examination of vegetative compatibility among California isolates of *M. fructicola* has been conducted (Sonoda *et al.*, 1990; Free *et al.*, 1996). Seventy four isolates collected from individual lesions on nectarines formed 38 VCGs, revealing 51% incompatibility (Sonoda *et al.*, 1990). Fifty four of the 74 isolates came from three trees, while the remaining twenty were collected one each from twenty additional trees. The same study revealed that while individual lesions were colonized by only one VCG, neighboring lesions may contain different VCGs. The study of single ascospore progeny from 82 apothecia, found that individual apothecia typically produced progeny which fell into eight or more VCGs (Free *et al.*, 1996). The study further revealed that the genes for mycelial compatibility segregated independent of one another.

The purpose of this study was to examine the occurrence of vegetative incompatibility within Michigan field populations of *M. fructicola.* These data may then be used to begin an evaluation of the genetic diversity of Michigan field populations of the fungus.

Material and Methods

One-hundred sixty nine isolates of *M. fructicola* were collected from two cherry orchards (designated JB and KK) near Traverse City, Michigan, and one orchard (SC) near Paw Paw, Michigan, in summer 1993. Seventy and 61 isolates from orchards JB and KK, respectively, were collected at random throughout the orchard from individual sweet cherry fruit. Thirty-eight isolates from orchard SC were similarly collected from individual sour cherry fruit. Cultures from the fruit were then isolated by hyphal tipping.

Methodology for compatibility testing was adapted from Sonoda *et al.* (1982) and Kohn *et al.* (1990). Hyphal tipped Isolates were cultured on Difco potato-dextrose agar (PDA) at 25°C. After five days, 4 mm plugs were taken from the growing margin of cultures. To test for compatibility, mycelial plugs of selected isolates were placed 3 cm apart on PDA agar in 9 cm diameter plates. Compatibility test plates were incubated in the dark at 25°C. Each isolate was tested against itself and against all other isolates.

Tests were initially performed on half strength PDA amended with six drops/L of McCormick's red food color (Kohn *et al.*, 1990), but growth was very slow. The media was therefore amended to full strength PDA with six drops/L of red food color. Previous study of vegetative incompatibility in *Sclerotinia sclerotiorum* (Kohn *et al.*, 1990) revealed that red pigment from the food color was deposited in the barrage zone between incompatible isolates, thus providing an addition character for scoring interactions. However, deposition of red pigment was not detected in this study of *M. fructicola*. Replicates of each test were performed on full strength PDA without red food color.

Cultures were examined at 7, 10, 14 and 21 days to identify compatible versus

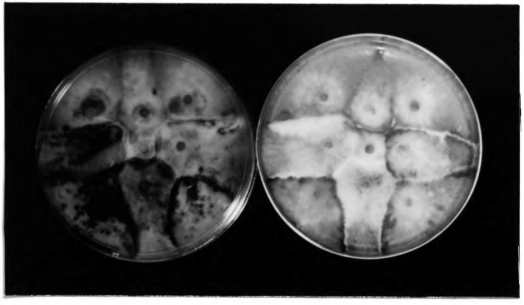


Figure 1. Cultural interactions of isolates of *Monilinia fructicola*. Interactions were more easily scored as compatible or incompatible when viewed from the underside of the plate (left). The top three isolates which grew together with no distinct interaction zone were scored as compatible. The remaining isolates between which dark barrage zones formed were scored as compatible.

incompatible reactions. Pairings were scored as incompatible if a dark barrage zone formed between neighboring isolates, or as compatible when isolates grew together with no distinct interaction zone (Figure 1), based upon the criterion outlined by Kohn *et al.* (1990).

Results

All isolates of *M. fructicola* examined in this study were found to be self-compatible. Vegetative compatibility was identified among the isolates collected from orchard JB and among the isolates collected from orchard KK, but no vegetative compatibility was identified among the 38 isolates collected from orchard SC (Table 1).

The 70 isolates from orchard JB formed 25 VCGs. Three of these groups contained 28, 16, and 4 isolates, respectively. The remaining 22 groups each contained one isolate. The 61 isolates from orchard KK formed 54 VCGs. Three of these groups contained 5, 3, and 2 isolates, respectively. The remaining 51 groups each contained one isolate. No compatibility was identified among the isolates from orchard SC. These results show 36, 89, and 100 % vegetative incompatibility within orchards JB, KK, and SC respectively (Table 1). Vegetative compatibility groups containing more than one isolate are given in Appendix A.

No compatibility was identified between isolates collected from different orchards. The 169 isolates examined formed 117 VCGs, 111 of which contained only one isolate each. This revealed 69 % incompatibility for all isolates examined (Table 1).

Table 1. Frequency of vegetative incompatibility among isolates of *Monilinia fructicola* collected from Michigan cherry orchards.

Orchard	Location	Number of Isolates	Number of VCGs[a]	Percent Incompatibility[b]
SC	Paw Paw	38	38	100
KK	Kewadin	61	54	89
JB	Empire	70	25	36
All isolates		169	117	69

[a] Vegetative compatibility groups
[b] Number of VCGs/ Number of isolates X 100

Discussion

The examination of vegetative compatibility among Michigan isolates of *M. fructicola* revealed an 69 % incompatibility among all field isolates collected. However, this value may underestimate the actual level of incompatibility. All isolates from orchard SC were collected from different trees and it is unlikely that the sample included any fruit infected through contact with adjacent fruit. However, infected fruit from orchards JB and KK were collected by local extension personnel who may have taken less care in obtaining single fruit from different trees in the orchard. While isolations were made from a random sample of infected fruit received from these orchards, it is possible that isolations were made from two or more fruit infected with the same strain. In such an instance, the reactions recorded as compatible may reflect self-compatibility.

The frequency of vegetative compatibility varies widely among fungi. *Podospora anserina* has 17 identified genetic loci affecting vegetative compatibility (Glass and Kuldau, 1992). This would suggest a minimum of 2^{17} possible VCGs. *Verticillium dahliae*, however, has only four identified VCGs (Joaquim and Rowe, 1990). Previous study of *M. fructicola* (Sonoda *et al.*, 1990) identified 38 VCGs among 74 isolates of *M. fructicola* collected from within a single nectarine orchard in California.

The frequency of vegetative compatibility within a population may correlate directly with the degree of genetic variability within the population and may provide insight into the relative significance of sexual and asexual reproduction (Leslie, 1993). In a predominantly asexually reproducing population, only a small number of VCGs would be expected. Offspring of a given parent would share a common genotype, including

compatibility genes, and would therefore be compatible with one another. Alternatively, the presence of a large number of vegetative compatibility groups within a population suggests the predominance of sexual reproduction. With each generation a variety of genotypes, and of compatibility gene combinations, would be generated thus increasing the likelihood of incompatibility. The increased genetic variability associated with sexual versus asexual reproduction would be translated to a higher number of VCGs.

The frequency of vegetative incompatibility revealed in this study of *M. fructicola* collected from Michigan cherry orchards suggests a high degree of genetic variability within populations of *M. fructicola*. This also suggests that the parasexual phenomenon is an inadequate model to account for the frequency of genetic recombination. The large number of VCGs revealed here would inhibit genetic exchange and recombination via fusion of vegetative hyphae, indicating that genetic variability results primarily from sexual reproduction.

The predominance of sexual reproduction may play a crucial role in the spread of fungicide resistance within populations of *M. fructicola*. In an asexual population, resistance genes must correlate with other fitness characters in order to be maintained in the population (Sanoamuang et al., 1995). However, in a sexually reproducing population resistance genes may be spread, via recombination, regardless of VCG or other characteristics. The segregation of carbendazim resistance and benomyl resistance has been shown to occur among sexual progeny of *M. fructicola* (Sanoamuang *et al.*, 1995; Free *et al.*, 1996). Free *et al.* (1996) also showed that the segregation of benomyl resistance is independent of the segregation of vegetative compatibility.

However, vegetative compatibility alone does not accurately reflect the genetic diversity within a population, and cannot identify a predominantly sexual verus asexual population. A study of a population of *Aspergillus flavus*, a fungus known to only reproduce asexually, was found to have a large number of VCGs relative to the other local field populations of *Aspergillus* (Baymen and Cotty, 1991). The authors of the study suggested that the source of the high number of VCGs may be the influx of spores from external sites. Similarly, the VCG diversity revealed in this study of *M. fructicola* may reflect massive migration of isolates into the orchards: VCGs would be introduced to the orchards from external populations, rather than arising independently within the orchards.

Alternatively, VCG diversity may be favored by selection. In a population in which VCG is under frequency dependent selection, VCG diversity could be maintained in the absence of diversity in other, non-selected portions of the genome. Therefore, VCG diversity may not give an accurate representation of the genetic diversity within a population or of the predominance of sexual versus asexual reproduction.

While the vegetative compatibility data presented here appear to suggest that field populations of *M. fructicola* possess a high level of genetic diversity. The examination of regions of the nuclear rDNA will clarify the true nature of diversity within this species.

Chapter 2

VARIATION IN INTERNAL TRANSCRIBED SPACER SEQUENCES, ARBITRARILY PRIMED-PCR PRODUCTS, AND GROUP I INTRON PRESENCE BETWEEN *MONILINIA FRUCTICOLA* AND *M. LAXA*

Introduction

Brown rot, caused by various *Monilinia* species, is an economically important disease of stone fruit crops worldwide. In North America the brown rot fungus *Monilinia fructicola* (Wint.) Honey is common in all areas where stone fruits are grown while the European brown rot fungus *M. laxa* Aderhold & Ruhland is common primarily on stone fruit crops in the West. Minor outbreaks of *M. laxa* have been reported on sour cherry (*Prunus cerasus* L.) from Michigan (Cation *et al.*, 1949), New York (Kable and Parker, 1963), and Wisconsin (Keitt *et al.*, 1943). After the 1960s the disease was quiescent in the Great Lakes region of eastern North America until 1993 when an outbreak occurred on sour cherry in Michigan and Wisconsin (Jones and Sutton, 1996). A third brown rot pathogen, *M. fructigena* Honey, is widely distributed in Europe but eradicated from North America (Batra, 1979).

An examination of cultural characteristics on potato dextrose agar (PDA) is the primary means of distinguishing isolates of *M. laxa* from those of *M. fructicola*. *M. laxa* generally produces lobed, slow growing colonies with poor conidial production, while *M. fructicola* generally produces colonies with smooth margins and abundant conidial

production (Hewitt and Leach, 1939). The similarity in colony morphology of some isolates of *M. fructicola* and *M. laxa* often requires the use of other characteristics to distinguish hard to identify isolates. Electrophoretic patterns (Penrose *et al.*, 1976), germ tube branching (Jenkins, 1965), and vegetative compatibility interactions (Sonoda *et al.*, 1982) have been used to distinguish *M. laxa* from *M. fructicola* where identifications based on cultural characteristics are uncertain. A study of the internal transcribed spacer (ITS) region in several species within the Sclerotiniaceae by Kohn and Carbone (1993) suggested that sequence variation in the ITS1 region could be used to distinguish *M. fructicola* from other *Monilinia* species. However, no *M. laxa* and only a single isolate of *M. fructicola* were included in their study and no sequence data for the ITS1 region of *M. laxa* or additional sequences for *M. fructicola* were found in a search of GenBank.

Other potential methods of distinguishing the two species may include length variation of the small subunit (SSU) rRNA gene directly preceding the ITS1 region and arbitrarily primed PCR (ap-PCR) of genomic DNA. Wilmotte *et al.* (1993) identified a group I intron in the SSU rDNA of *Sclerotinia sclerotiorum,* however, neither this intron nor any other has been reported in *Monilinia*. Arbitrarily primed-PCR (ap-PCR) analysis may be used to distinguish isolates of a single species (Freeman *et al.*, 1995; Sreenivasaprasad *et al.*, 1992). Ap-PCR analysis of isolates of *Colletotrichum gloeosporioides* from avocado with four primers led to the identification of eight distinct phenotypes (Freeman *et al.*, 1996). Ap-PCR analysis may also be useful for distinguishing *M. laxa* from *M. fructicola.*

The objective of this study was to examine the genetic diversity within

populations of *M. fructicola* and *M. laxa*. An additional objective was to determine if isolates of *M. fructicola* could be distinguished from isolates of *M. laxa* on the basis of sequence differences in their ITS1 region. In addition, the length of the SSU rRNA gene which directly precedes the ITS1 region and the pattern of ap-PCR products were examined to determined whether there were differences between *M. laxa* and *M. fructicola*

Materials and Methods

Isolates, growth conditions, and DNA extraction. All isolates of *M. fructicola* were collected from sweet and sour cherry orchards in Michigan (Table 2). One isolate was collected in 1975 (Jones and Ehret, 1976), the others were collected in 1993 from three geographically isolated orchards. These isolates were a subgroup of 50 to 75 isolates per orchard, each from a separate fruit. Isolates used in this study were selected to include members of common vegetative compatibility groups (VCGs) as well as members of different VCGs within an orchard. The isolates of *M. laxa* were from infected spurs of sour cherry collected at three sites in Michigan and one site in Wisconsin in either 1993 or 1994.

Each isolate was grown in 125 ml of potato dextrose broth (Difco Laboratories, Detroit, MI) at room temperature for 7-10 days. Using sterile forceps, mycelium was transferred from liquid culture to a 1.5 ml microcentrifuge tube and centrifuged for 1 minute. After the supernatant was poured off, additional mycelium was transferred to the tube and it was centrifuged again. This process was repeated until the tube contained

approximately 500 μl of mycelium. The mycelium was washed with 600 μl of TE buffer, centrifuged for 5 min, and the supernatant poured off (Cenis, 1992). Then 600 μl of extraction buffer (200 mM Tris-HCl, pH 8.5; 200 mM NaCl; 25 mM EDTA; 0.5% SDS; Reader and Broder, 1985) was added and the mycelium was macerated using a disposable pellet pestle. DNA was purified by phenol/chloroform extraction, precipitated with two volumes ethanol and 1/10 volume 3M sodium acetate overnight at -20°C, pelleted, rinsed twice with 70% ethanol, vacuum dried, and suspended in 100 μl of TE buffer (Maniatas *et al.*, 1982). DNA was stored at -20°C.

Amplification and Sequencing. All primers were synthesized by the Macromolecular Structure Facility at Michigan State University. Primers for the ITS1 region (ITS1F and ITS2) and for the 3' end of the SSU rDNA (NS7 and NS8) were taken from Gardes and Bruns (1993) and White *et al.* (1990). Primers AJ139 and AJ140 were developed using Oligo primer analysis software (National Biosciences, Inc., Plymouth, MN) based on the SSU rDNA sequence of *S. sclerotiorum* (GenBank accession number X69850). These primers were used to amplify the part of the SSU rDNA just upstream from primer NS7. The sequences and positions of these primers in the rDNA are given in Appendix B. Microsatellite primer $(GACA)_4$ for arbitrarily primed-amplification of genomic DNA was taken from Freeman *et al.* (1995) and microsatellite primer $(GTG)_5$ was taken from Longato and Bonfante (1997). All polymerase chain reactions (PCR) were performed in MJ Research thermocycler model PTC-150 (MJ Research, Inc., Watertown, MA).

PCR reactions for amplification of the ITS1 region and the SSU rRNA gene were carried out in a 50 μl volume containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM

$MgCl_2$, 0.1% Triton X-100, 160 μM each dNTP, 50 pmols of each primer, 2 U *Taq* DNA polymerase, and 10-100 ng of template DNA and overlaid with two drops of mineral oil (Kohn *et al.*, 1991). A negative control containing no template was also included. The thermal program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 3 min, followed with a final extension at 72°C for 7 min. PCR amplifications were performed two or three times from each isolate.

PCR products were observed by electrophoresis on a 1% agarose gel (SeaKem LE, FMC BioProducts, Rockland, ME) in 1×TAE buffer. Products were purified from the PCR reaction mix using a DNA binding resin (Promega Wizard PCR Preps DNA purification system, Promega, Madison, WI). Automated flourescent sequencing of PCR amplification products from each primer set was performed by the MSU-DOE-PRL Plant Biochemistry Facility using the ABI Catalyst 800 for Taq cycle sequencing and the ABI 373A Sequencer for the analysis of products. All products were sequenced from both strands. Sequences of the two strands were used to generate a consensus strand for each isolate. Ambiguities revealed by alignment of the two strand sequences were resolved by sequencing the region of DNA of the given isolates again. Sequences obtained from additional sequencing were added to the original two to generate a consensus. Identity of the ITS1 sequence was confirmed by aligning the consensus sequence from PCR products with ITS1 sequences retrieved from GenBank: *M. fructicola* (Accession number U21815), *M. fructigena* (U21825), *M. oxycocci* (U21833), and *M. megalospora* (U21834), *Sclerotinia sclerotiorum* (U21810), and *S. minor* (U21818). The SSU

sequence was confirmed by aligning the consensus sequence from PCR products with the SSU sequence for *Sclerotinia sclerotiorum* (X69850) retrieved from GenBank. Sequence comparison was performed by MegAlign (DNASTAR, Inc., Madison, WI) using the Clustal V method to examine sequence distances for all sequence pairs and then align sequence groups (Higgins and Sharp, 1989).

Ap-PCR amplification of genomic DNA. Random amplifications using microsatellite primers $(GACA)_4$ and $(GTG)_5$ were performed on 36 isolates following the protocol of Freeman *et al.* (1995). Each reaction was performed in a 50 μl volume containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 μM each dNTP, 50 pmols of primer, 2 U *Taq* DNA polymerase, and 10-100 ng of template DNA and overlaid with two drops of mineral oil. A negative control containing no template was also included. The thermal program consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 48°C for 30 sec, and extension at 72°C, for 1.5 min, with a final extension at 72°C for 4 min. PCR amplifications were performed two or three times per isolate. PCR products were separated on a 1.5% TAE agarose gel and electrophoresed at 80 V for 2 h.

Restriction digestion. The ITS1 regions from twelve isolates of *M. fructicola* and eight isolates of *M. laxa* were amplified and purified as described above. The purified product from each reaction was digested with 5 U of *Mse* I (ICN Pharmaceuticals Inc., Costa Mesa, CA) at 37°C for 3 h. Restriction fragments were separated on a 2% TAE agarose gel.

Nucleotide sequence accession numbers. The nucleotide sequences of the ITS1 regions

of *M. laxa* and *M. fructicola* are available in Genbank under accession numbers AF10503 and AF10500, respectively. ITS sequences showing sequence divergence are available under accession number AF10540 for *M. laxa* and AF10501 and AF10502 for *M. fructicola.* The nucleotide sequence of the group I intron of *M. fructicola* including the flanking SSU rRNA gene sequences is available under number AF10505. The sequence of the 3' end of the SSU rRNA gene of *M. laxa* is available under number AF010506.

Results

Sequence analysis of the ITS1 region. PCR with primers ITS1F and ITS2 yielded a product of approximately 250 bp from each of 30 isolates of *M. fructicola* and eight isolates of *M. laxa* (Table 2). The nucleotide sequence of the 250-bp PCR fragment from each of 12 isolates of *M. fructicola* and four isolates of *M. laxa* revealed the 3' end of the SSU rRNA gene, a 146-bp ITS1 region, and the 5' end of the 5.8S rRNA gene (Figure 2). The partial SSU rDNA and 5.8S rDNA sequences of all 16 isolates were identical. These flanking sequences also exhibited sequence identity with sequences surrounding the ITS1 region in *M. fructicola, M. fructigena, M. oxycocci, M. megalospora, S. sclerotiorum,* and *S. minor* obtained from GenBank.

Alignment of the ITS1 sequences from the 12 isolates of *M. fructicola* revealed only minor nucleotide differences among the isolates (Figure 2). No correlation was found between ITS1 sequence variation and VCG. The consensus ITS1 sequence for isolates of *M. fructicola* from Michigan matched the *M. fructicola* U21815 ITS1 sequence. Alignment of the ITS1 sequences from the four isolates of *M. laxa* revealed

Table 2. Isolates of *Monilinia fructicola* and *M. laxa* used in polymerase chain reaction (PCR)-mediated analysis and the results of analysis.

Isolate	Year of isolation	Locality	Host	PCR-ITS1 fragment (bp)[a]	PCR-SSU fragment (bp)[b]	AP-PCR band patterns[c]
Monilinia fructicola						
SC-5	1993	Paw Paw, MI	Montmorency sour cherry	250	nd	2, 4
SC-41	1993	Paw Paw, MI	Montmorency sour cherry	250 (146)	940(421)[d]	nd
SC-44	1993	Paw Paw, MI	Montmorency sour cherry	250 (146)	940	nd
SC-49	1993	Paw Paw, MI	Montmorency sour cherry	250 (146)	940	nd
SC-55	1993	Paw Paw, MI	Montmorency sour cherry	250	940	nd
SC-57	1993	Paw Paw, MI	Montmorency sour cherry	250 (146)	940	2, 4
SC-59	1993	Paw Paw, MI	Montmorency sour cherry	nd	940	2, 4
SC-70	1993	Paw Paw, MI	Montmorency sour cherry	250	940	2, 4
SC-75	1993	Paw Paw, MI	Montmorency sour cherry	nd	940	2, 4
KK-11	1993	Kewadin, MI	Sweet cherry	250	940	2, 4
KK-15	1993	Kewadin, MI	Sweet cherry	250	940	2, 4
KK-20	1993	Kewadin, MI	Sweet cherry	250 (146)	940 (421)[d]	2, 4
KK-22	1993	Kewadin, MI	Sweet cherry	250	940	2, 4
KK-27	1993	Kewadin, MI	Sweet cherry	nd	940	nd
KK-29	1993	Kewadin, MI	Sweet cherry	nd	nd	2, 4
KK-31	1993	Kewadin, MI	Sweet cherry	250	940	2, 4
KK-33	1993	Kewadin, MI	Sweet cherry	nd	940 (421)[d]	nd
KK-45	1993	Kewadin, MI	Sweet cherry	250	940	2, 4
KK-48	1993	Kewadin, MI	Sweet cherry	250 (146)	940	2, 4
KK-49	1993	Kewadin, MI	Sweet cherry	250	nd	2, 4
KK-50	1993	Kewadin, MI	Sweet cherry	nd	nd	2, 4
KK-54	1993	Kewadin, MI	Sweet cherry	250 (146)	940	2, 4
JB-2	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-4	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-6	1993	Empire, MI	Sweet cherry	250	940	nd
JB-8	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-9	1993	Empire, MI	Sweet cherry	250 (146)	940	2, 4
JB-10	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-11	1993	Empire, MI	Sweet cherry	250 (146)	940	2, 4
JB-12	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-13	1993	Empire, MI	Sweet cherry	250 (146)	940	2, 4
JB-14	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-26	1993	Empire, MI	Sweet cherry	250 (146)	940 (421)[d]	nd
JB-62	1993	Empire, MI	Sweet cherry	250	940	2, 4
JB-65	1993	Empire, MI	Sweet cherry	250	940	2, 4
CB-2	1975	Hart, MI	Montmorency sour cherry	250 (146)	940	3, 4

Table 2. Continued

Monilinia laxa						
DG-7	1993	Suttons Bay, MI	Meteor sour cherry	250	520	1, 5
DG-8	1993	Suttons Bay, MI	Meteor sour cherry	250 (146)	520	1, 5
NP-1	1993	Northport, MI	Montmorency sour cherry	250	520	1, 5
NP-2	1993	Northport, MI	Montmorency sour cherry	250 (146)	520(460)[e]	1, 5
WI-2	1993	Sturgeon Bay, WI	Montmorency sour cherry	250 (146)	520(460)[e]	1, 5
WI-3	1993	Sturgeon Bay, WI	Montmorency sour cherry	250	520	1, 5
Grants	1994	Shelby, MI	Érdi Botermö sour cherry	250 (146)	520	1, 5
Meteor	1994	Suttons Bay, MI	Meteor sour cherry	250	520	1, 5

[a] Length of the amplification fragments obtained with primers ITS1F and ITS2. Number in parentheses indicates the actual length of the ITS1 region alone, determined by sequence analysis and confirmed by comparison to sequences retrieved from GenBank.
[b] Length of the SSU DNA amplified with primers AJ139 and AJ140.
[c] Band patterns 1,2, and 3 were obtained using microsatellite primer $(GACA)_4$. Pattern 3 shares six bands in common with pattern 2 and one band in common with pattern 1. Band patterns 4 and 5, obtained with primer $(GTG)_5$, share no bands in common.
[d] Number in parentheses indicates the length of the intron determined by sequence analysis.
[e] Number in parentheses indicates the region of the SSU rDNA confirmed by sequence analysis.
nd = not done

Figure 2.

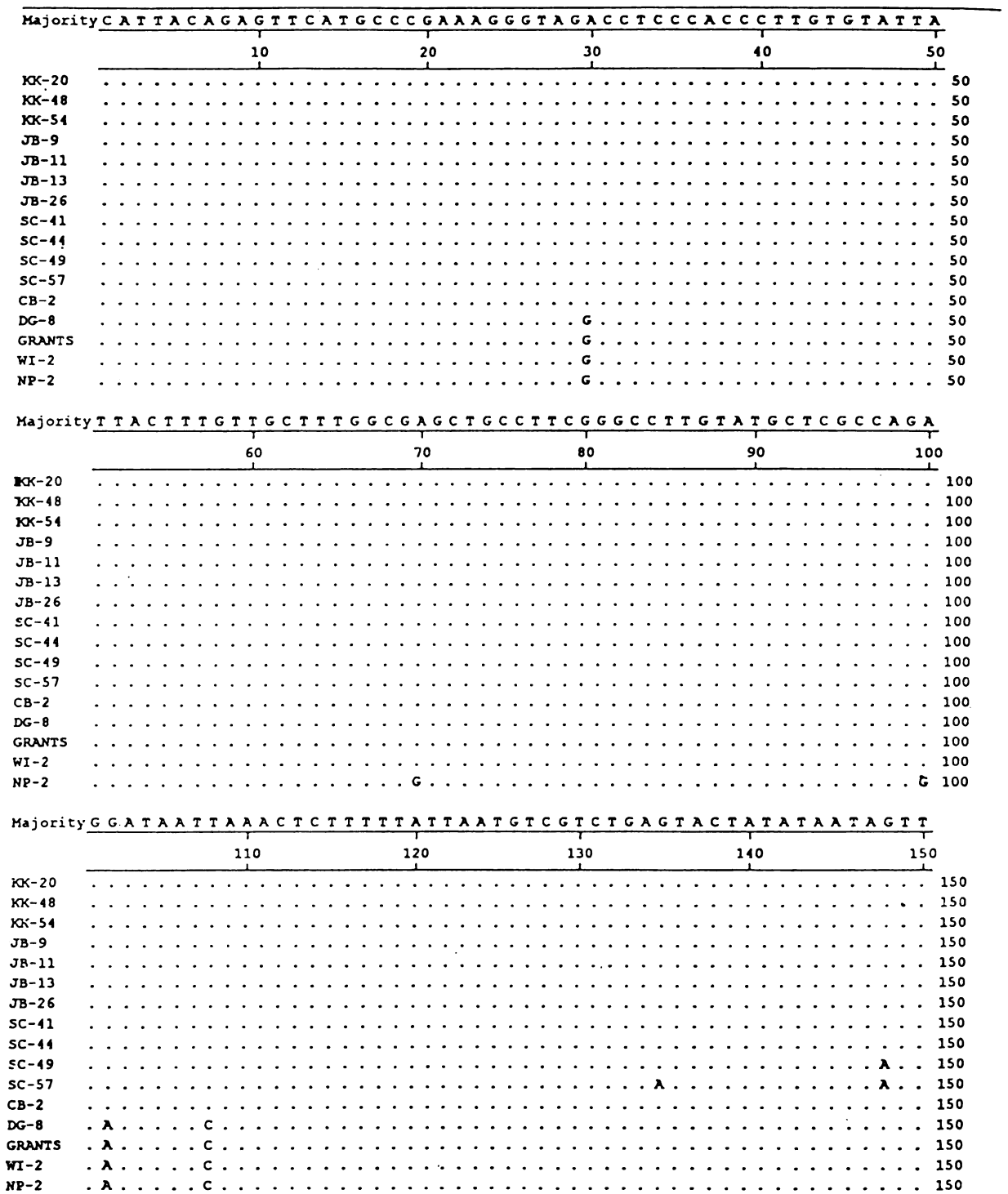
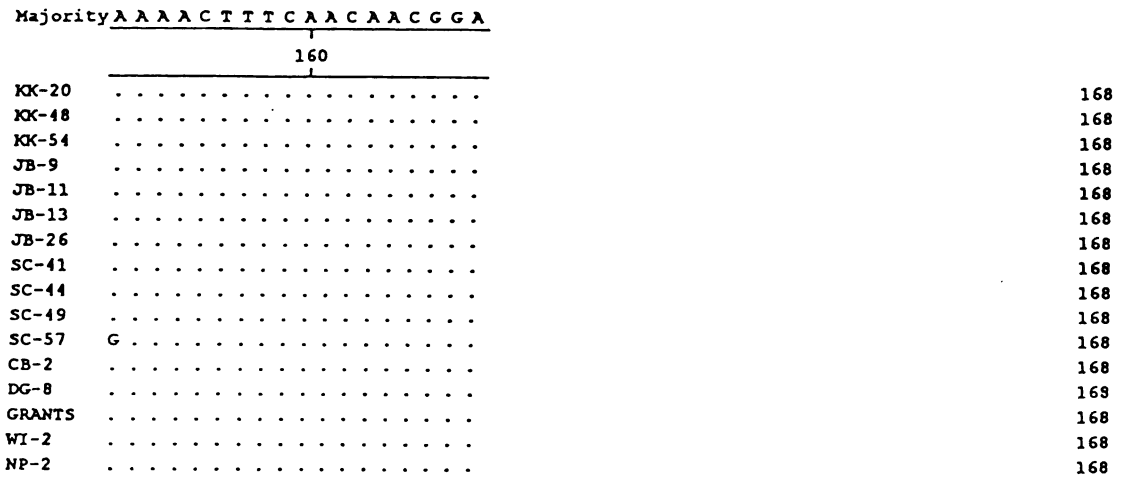

Majority CATTACAGAGTTCATGCCCGAAAGGGTAGACCTCCCACCCTTGTGTATTA
10 20 30 40 50
KK-20 50
KK-48 50
KK-54 50
JB-9 50
JB-11 50
JB-13 50
JB-26 50
SC-41 50
SC-44 50
SC-49 50
SC-57 50
CB-2 50
DG-8 . . . . . G 50
GRANTS . . . . . G 50
WI-2 . . . . . G 50
NP-2 . . . . . G 50
Majority TTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGA
60 70 80 90 100
KK-20 100
KK-48 100
KK-54 100
JB-9 100
JB-11 100
JB-13 100
JB-26 100
SC-41 100
SC-44 100
SC-49 100
SC-57 100
CB-2 100
DG-8 100
GRANTS 100
WI-2 100
NP-2 . . . . . G . . . . . G 100
Majority GGATAATTAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT
110 120 130 140 150
KK-20 150
KK-48 150
KK-54 150
JB-9 150
JB-11 150
JB-13 150
JB-26 150
SC-41 150
SC-44 150
SC-49 . . . . . A . . 150
SC-57 . . . . . A . . . . . A . . 150
CB-2 150
DG-8 . A . . . . . C 150
GRANTS . A . . . . . C 150
WI-2 . A . . . . . C 150
NP-2 . A . . . . . C 150

Figure 2 (cont'd).



Alignment of the ITS1 sequences of isolates of *Monilinia fructicola* and *M. laxa* determined in this study. Positions 1-5 represent the 3' end of the 18S (SSU) rRNA gene. Positions 151-168 represent the 5' end of the 5.8S rRNA gene. A dot (.) indicates a match and a letter indicates a mismatch. Letters and numbers to the left of sequences indicate the isolate from which DNA was purified for PCR amplification. The first twelve sequences are from isolates of *M. fructicola*. The last four isolates, DG-8, Grants, WI-2, and NP-2, are from isolates of *M. laxa*.

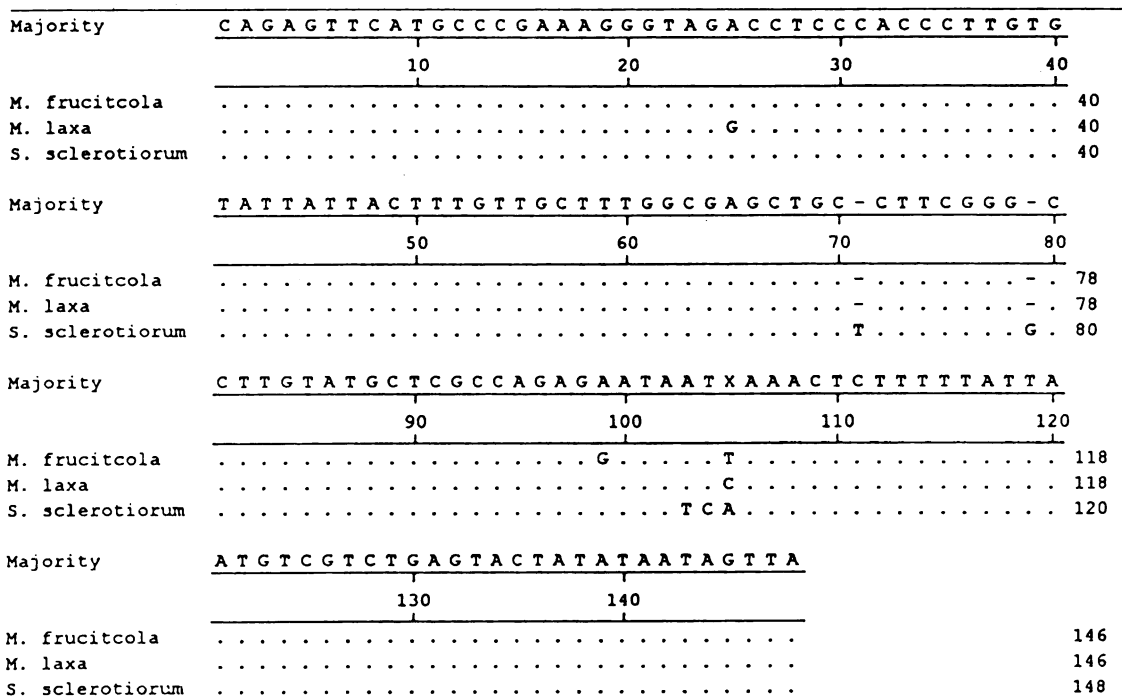


Figure 3. Alignment of the consensus sequences of the ITS1 region of *Monilinia fructicola* and *M. laxa* determined in this study with *Sclerotinia sclerotiorum* (U21810). A dot (.) Indicates a match, a dash (-) indicates an insertion/deletion event, and a letter indicates a mismatch.

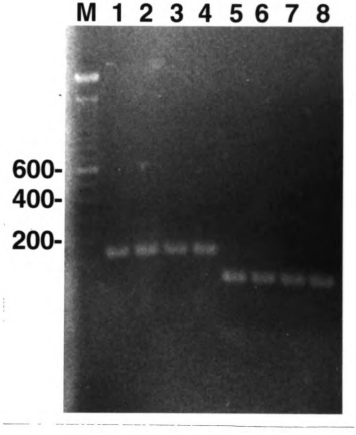


Figure 4. Restriction fragment length polymorphism in *Mse* I-digested PCR-amplified ITS1 DNA from *Monilinia laxa* isolates DG-8, NP-2, WI-2, and Grants (lanes 1-4) and *M. fructicola* isolates SC-41, KK-20, JB-13, and CB-2 (lanes 5-8). Lane M: 100 bp size marker.

only minor sequence variation among isolates of *M. laxa* (Figure 2.). Alignment of the consensus ITS1 sequences for each species revealed three nucleotide differences between *M. laxa* and *M. fructicola* (Figure 3). *M. fructicola* showed transition mutations at positions 25, 99, and 105 (Figure 3). The base pair change at position 105 created a *Mse* I restriction site in *M. fructicola*, creating a restriction fragment 14 bp smaller than the corresponding fragment in *M. laxa* (Figure 4). Comparison of the consensus nucleotide sequences for the ITS1 region of *M. laxa* and of *M. fructicola*, *M. fructigena* U21825, *M. megalospora* U21834, *M. oxycocci* U21833, *S. sclerotiorum* U21810, and *S. minor* U21818 showed 98, 96, 83, 74, 97, and 97 % similarity, respectively.

Identification of an intron in M. fructicola. PCR amplification of the 3' end of the SSU rDNA with primers NS7 and NS8 yielded a product of approximately 380 bp from both *M. fructicola* and *M. laxa*. Amplification using primers AJ139 and AJ140 yielded a product of approximately 940 bp from 32 isolates of *M. fructicola* and of 520 bp from eight isolates of *M. laxa* (Table 2, Figure 5). Sequence analysis of the PCR products from four isolates of *M. fructicola* and two of *M. laxa* revealed the presence of a 421 bp region in *M. fructicola* that was absent in *M. laxa* (Figure 6). However, the flanking sequences (460) from both species exhibited 99 % similarity. Given the large size of the SSU rRNA fragment, the entire region could not be accurately sequenced from one strand. Approximately 30 bp, including the primers and a few adjacent nucleotides, could not be obtained for either end. Alignment of the SSU rDNA sequences for *M. fructicola* and *S. sclerotiorum* (X69850) showed that the insert in *M. fructicola* was located at the same position as a group I intron in *S. sclerotiorum*. The flanking regions of the SSU

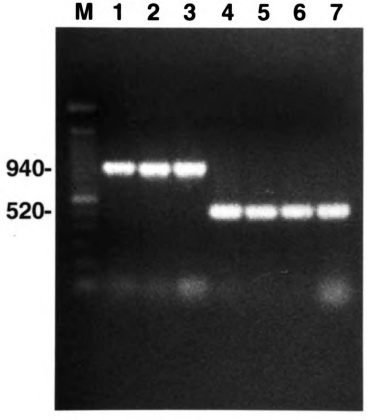


Figure 5. Products of PCR amplification of a region of the SSU rRNA gene of *Monilinia fructicola* and *M. laxa* using primers AJ139 and AJ140. Lane M, 100-bp ladder; lanes 1-3, *M. fructicola* isolates SC-41, KK-20, JB-13; lanes 4-7, *M. laxa* isolates DG-8, NP-2, WI-2, Grants.

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M. fructicola CATTAATCAGTGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAA 60
M. laxa       CATTAATCAGTGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAA 60

M. fructicola CCATAAACTATGCCGACTAGGGATCGGGCGATGTTATCTTTTTGACTCGCTCGGCACCTC 120
M. laxa       CCATAAACTATGCCGACTAGGGATCGGGCGATGTTATCTTTTTGACTCGCTCGGCACCTC 120

M. fructicola ACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAG 180
M. laxa       ACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAG 180

M. fructicola AAATTGACGGAAAGGCACCACCAGGCGTTAACTGCAGTAACTCTGCGCCGAAAAGCAGCC 240
M. laxa       AAATTGACGGAAAGGCACCACCAGGCGT------------------------------ 208

M. fructicola CGTAAGGGTGAGGTGGTTCGCCTTCAACTTAAATGCTAGTCTATTATAAGGCTACATTCC 300
M. laxa       ------------------------------------------------------------ 208

M. fructicola CAAATTGCGGGAACACCCTAATGCTCTCACTTCCAAGCTGACATTTGAAAGAATGCAGTG 360
M. laxa       ------------------------------------------------------------ 208

M. fructicola GCCAGGCTAATCACCTGGGTTATGGACATAACGTTCAAGAGATGATACAATGGGCTATCC 420
M. laxa       ------------------------------------------------------------ 208

M. fructicola GCATCCTTTCCCTTCATAACGCATAGTATATGGAAAAGGTTCAGAGACTAAATGGGAATG 480
M. laxa       ------------------------------------------------------------ 208

M. fructicola GCTAGTTTATTAAATTCAACGTTTTAGATGCGTACTGAAGTTAATGATACTAGTTAAGAT 540
M. laxa       ------------------------------------------------------------ 208

M. fructicola ATAGTCCGTCGGTAGGTGAAAACTTACGGTTCAAAGCTTGAAGCTTGACCCTTGGAAGCA 600
M. laxa       ------------------------------------------------------------ 208

M. fructicola ACTCAATGCTTTCGAGTGAATATAAAACGGGAGCCTGCGGCTTAATTTGACTCAACACGG 660
M. laxa       ----------------------------GGAGCCTGCGGCTTAATTTGACTCAACACGG 239

M. fructicola GGAAACTCACCAGGTCCAGACACAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATTT 720
M. laxa       GGAAACTCACCAGGTCCAGACACAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATTT 299

M. fructicola TGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGA 780
M. laxa       TGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGA 359

M. fructicola TAACGAACGAGACCTTAACCTGCTAAATAGCCCGGCTAGCTTTGGCTGGTCGCTGGCT   839
M. laxa       TAACGAACGAGACCTTAACCTGCTAAATAGCCCGGCTAGCTTTGGCTGGTCGCTGGCT   417
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Figure 6. Alignment of the SSU rDNA sequences amplified with primers AJ139 and AJ140 from *Monilinia fructicola* strain KK-20 and *M. laxa* strain DG-7. The *M. fructicola* sequence contained an 421 bp insertion (indicated by dashes(-)) relative to the sequence of *M. laxa*.

rDNA of *M. fructicola* and *S. sclerotiorum* showed 99% similarity, however, the intron from *M. fructicola* showed only 54% similarity to the intron from *S. sclerotiorum*.

Analysis of the sequence and secondary structure of the 421-bp insertion sequence from *M. fructicola* demonstrated that it possessed the characteristic features known to be conserved among group I introns (Cech, 1988). The conserved GU immediately preceding the 5' splice site and the G at the 3' splice site were present. The sequence also contained the four conserved sequence elements P, Q, R, and S and the order of their occurrence in the sequence (5'- P-Q-R-S-3') necessary for the formation of the group I intron core structure. Conserved sequence elements P and Q base-paired to form helix P4, and elements R and S paired to form helix P7. Also, the 3' end of element Q was base-paired with the 5' end of element R to begin helix P6. Helices P1 to P9, including a pseudoknot formed by P3 and P7, were also identified. Five fungi containing introns in the SSU rDNA at the same location as the insert in *M. fructicola* were selected from GenBank for comparison of the intron region (Figure 7). All contained the conserved sequence elements characteristic of group I introns, but the sequences varied in length from 310 to 453 bp and showed little sequence similarity outside of the conserved sequence elements.

Comparison of M. fructicola *and* M. laxa *by ap-PCR.* From *M. fructicola* DNA the microsatellite primer $(GACA)_4$ generated six PCR products that ranged in length from 200 to 1500 bp (Figure 8). Isolate CB-2 showed a distinct seventh band of approximately 1500 bp that was not observed for any of the other 27 isolates of *M. fructicola*. Ap-PCR amplification of DNA from eight isolates of *M. laxa* generated three PCR products of

	Intron size	SSU gene	5' splice site ↓	P	Q
Mf	420	5'---ACCACCAGGCGU----		AAUUGCGGG–AA----	UAUCCGCAUC----
Ss	310	5'---ACCACCAGGCGU----		AACUGCGGGGAA----	AAUCCGCAUC----
Um	410	5'---ACCACCAGGAGU----		AAUUGCGGGAAA----	AAUCCGCAGC----
By	453	5'---ACCACCAGGUGU----		AACUGCGGGAAA----	UAUCCGCAUC----
Pi	339	5'---ACCACCAGGAGU----		AAUUGCGGGAAA----	AAUCCGCAGC----
Pl	339	5'---ACCACCAGGAGU----		AAUUGCGGGAAA----	AAUCCGCAGC----

	R	S	3' splice site ↓	SSU gene
Mf	---GUUCAGAGACUAAA----	AAGAUAUAGUCC----	ACG	GGAGCCUGCG---3'
Ss	---GUUCAGAGACUAAA----	AAGAUAUAGUCC----	AUG	GGAGCCUGCG---3'
Um	---GUUCAGAGACUAAA----	AAGAUAUAGUCC----	UCG	GGAGCCUGCG---3'
By	---GUUCAGAGACUAGA----	AAGGUAUAGUCC----	AAG	GGAGCCUGCG---3'
Pi	---GUUCAGAGACUAGA----	AAGAUAUAGUCC----	AUG	GGAGCCUGCG---3'
Pl	---GUUCAGAGACUAGA----	AAGAUAUAGUCC----	AUG	GGAGCCUGCG---3'

Figure 7. Alignment of the conserved sequence elements of group I intron within the nuclear SSU rDNA of *Monilinia fructicola* with introns from five other fungi. The conserved GU immediately preceding the 5' splice site and the conserved G preceding the 3' splice site are underlined. Fungi (and GenBank accession number) are abbreviated: Mf, *Monilinia fructicola*; Ss, *Sclerotinia sclerotiorum* (X69850); Um, *Ustilago maydis*(X62396); By, *Bensingtonia yamatoana* (D38239); Pi, *Protomyces inouyei* (D11377), and Pl, *P. lactucae-debilis* (D14164).

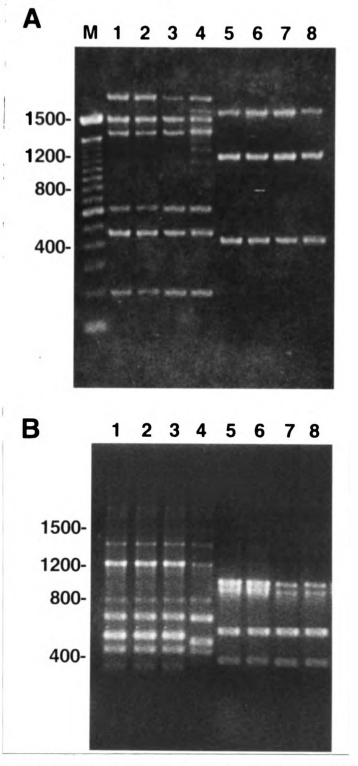

Figure 8. Arbitrarily primed-PCR amplification of genomic DNA from *Monilinia fructicola* and *M. laxa* using microsatellite primers $(GACA)_4$ in **A** and $(GTG)_5$ in **B**. Lane M, 100-bp ladder of empty; lanes 1-4, *M. fructicola* isolates SC-41, KK-20, JB-13, CB-2; and lanes 5-8, *M. laxa* isolates DG-8, NP-2, WI-2, Grants. PCR products in **A** and **B** were separated by electrophoresis through 1.5% agarose gels.

approximately 410, 1150, and 1500 bp in length. The patterns for isolates of the two species did not share any bands in common except the 1500 bp product in *M. laxa* which was shared by *M. fructicola* isolate CB-2.

Amplification of *M. fructicola* DNA with the microsatellite primer $(GTG)_5$ generated six PCR products that ranged in length from 300 to 1500 bp (Figure 8). Amplification of DNA from eight isolates of *M. laxa* generated four PCR products of approximately 350, 500, 900, and 1000 bp in length. No common bands were revealed between the two species.

Discussion

M. fructicola and *M. laxa* both cause brown rot of stone fruit and are of considerable economic importance in the United States. Traditionally, *M. fructicola* was considered the American brown rot fungus and *M. laxa* the European brown rot fungus. With the occurrence of *M. laxa* in stone fruit growing regions of the United States it has become necessary to develop an understanding of these two pathogens and the relationship between them. The examination of the ITS1 region from a large number of isolates of *M. fructicola* and *M. laxa* from different geographic areas of Michigan and Wisconsin suggests that each species has a highly conserved ITS1 region. Sequence analysis of the ITS1 region also revealed only three nucleotide differences between the two species, suggesting that *M. laxa* is more closely related to *M. fructicola* than to other *Monilinia* species. Comparison of the *M. laxa* ITS1 sequence with the previously reported sequence of *M. fructigena* (U21825) suggests that *M. fructigena* is also more closely related to *M.*

laxa than to those *Monilinia* species which do not cause brown rot. These results are consistent with the report that several species in the Sclerotiniaceae have highly conserved ITS1 regions and that the number of nucleotide differences between some members of the Sclerotiniaceae is quite limited (Kohn and Carbone, 1993). The limited number of nucleotide differences between the *M. laxa* and *M. fructicola,* and the limited diversity within each species, suggests the relatively recent divergence of these species.

While *M. laxa* and *M. fructicola* both occur on stone fruit crops, they are often identified based on colony morphology when grown on PDA (Hewitt and Leach, 1939) and a few other criteria (Jenkins *et al.*, 1965; Penrose *et al.*, 1976; Sonoda, 1982). However, accurate identification based on colony morphology is not always possible given the similarity in morphology of some isolates. The occurrence of morphologically similar and therefore hard to identify isolates requires the development of alternative means of distinguishing the species. Although the ITS1 sequences for *M. laxa* and *M. fructicola* are very closely related, it was possible to differentiate them based on *Mse* I restriction digestion of PCR amplification products of the ITS1 region. The apparent highly conserved nature of the ITS1 region supports the efficacy of this method to distinguish the species. Restriction digestion relies on single base pair changes that in more variable species may change quickly. The highly conserved nature of the ITS1 regions of *M. laxa* and *M. fructicola* indicate that the sequence is relatively static in these species.

The detection of the same group I intron in the SSU rRNA gene of all isolates of *M. fructicola* regardless of their geographic origin in Michigan indicates that this is also a

highly conserved character of *M. fructicola*. Presence of the intron in an isolate collected in 1975 further suggests that this intron is a stable character within Michigan populations. It was possible to differentiate *M. laxa* from *M. fructicola* on the basis of length variation in PCR-amplifications products of the intron-containing region of the SSU rRNA gene. As with the ITS1 region, the highly conserved nature of the intron indicates that it is a stable character for differentiation of *M. laxa* from *M. fructicola*.

Ap-PCR of genomic DNA also provided a rapid means of distinguishing between the species. Ap-PCR arbitrarily amplifies genomic DNA and the source of DNA in each band of the pattern is unknown. Patterns based on DNA of unknown origin are consequently of unknown stability within the populations. However, with primer $(GACA)_4$ all but one isolate of *M. fructicola* yielded the same band pattern and all isolates of *M. laxa* yielded a common pattern. With primer $(GTG)_5$ all isolates of *M. fructicola* yielded the same band pattern and all isolates of *M. laxa* yielded a common band pattern. This consistency of banding patterns within each species suggests that this is also a stable character for differentiation of the two species.

Data from chapter 1 indicates a relatively high level of vegetative compatibility group (VCG) diversity among Michigan field populations of *M. fructicola*. Such diversity was interpreted as a possible indication of a corresponding high level of genetic variability. However, the data presented here indicates the *M. fructicola* possesses little intra-species genetic variability. If the VCG loci are under frequency dependent selection, VCG diversity could be maintained even in the absence of diversity in unselected regions of the genome. The results of this study, combined with the data

acquired from the study of VCG diversity among the same isolates, indicate that VCG diversity is highly selected for in *M. fructicola*.

The results of this study also demonstrate that while *M. laxa* and *M. fructicola* are closely related they can be separated by the restriction digestion of the ITS1 sequence, length variation in the SSU rRNA gene due to the presence of an intron, and in ap-PCR band patterns. Isolates that are difficult to identify on the basis of colony morphology can be positively identified with these PCR-mediated techniques. However, this study utilized only isolates from Michigan and a few from Wisconsin. Further research is necessary to determine if these techniques are applicable to the global populations of *M. laxa* and *M. fructicola*.

Chapter 3

FINAL CONCLUSIONS

Molecular data from Chapter 2 identified three PCR-mediated techniques for rapidly distinguishing between isolates of *M. fructicola* and *M. laxa*. Isolates of *M. fructicola* used in this study were collected from three geographically isolated orchards, and while most isolates were collected in 1993, one isolates was originally collected in 1975. Isolates of *M. laxa* were collected from three geographically isolated orchards in Michigan and from one orchard in Wisconsin. All isolates within a species possessed the same molecular characteristics regardless of geographic origin or the year of collection. All isolates of *M. fructicola* possessed an *Mse* I restriction site in the ITS1 region which was not present in the ITS1 region of *M. laxa*. Amplification of the intron containing region of the SSU gene resulted in a product of approximately 940 bp from *M. fructicola*, but only 520 bp from *M. laxa*. All isolates within a species also shared a common ap-PCR band pattern. The occurrence of these common characters across geography and time suggests the stability of these characters and indicates that they will be useful characteristics for distinguishing between Michigan isolates of the two species.

While providing tools for distinguishing between the two species, sequence analysis of the ITS1 region, the presence of an intron in the SSU rDNA, and ap-PCR patterns also revealed limited genetic diversity within *M. fructicola* and *M. laxa*.

However, the high frequency of vegetative incompatibility revealed in Chapter 1 suggested that *M. fructicola* populations are very genetically diverse. The high frequency of VCG diversity was also interpreted to indicate that sexual reproduction may occur more frequently in Michigan than was previously believed. The data from the two previous chapters appear to be in direct conflict with one another, but there are possible resolutions to this apparent conflict. Two points must be kept in mind when interpreting the data presented here: 1) VCG diversity may not reflect genetic diversity within *M. fructicola* and 2) the regions of the genome utilized in this study may not be the most appropriate regions for study of genetic diversity within *M. fructicola*.

In Chapter 1, it was stated the VCG diversity reflects genetic diversity within a species. However, this statement is an oversimplification of the actual events involved in establishing and maintaining diversity within a population. The suggestion that VCG diversity reflects genetic diversity may be a generalization to which *M. fructicola* is an exception. Clearly, from the data presented here, VCG diversity reflects genetic diversity in neither nuclear ribosomal DNA nor in microsatellite regions with the GACA or GTG motif. It is, therefore, also possible that VCG diversity in *M. fructicola* does not reflect diversity in other region of the genome.

Furthermore, vegetative compatibility is governed by multiple loci (referred to here are vegetative incompatibility, or *vic*, loci) within the genome. Only a small change at a single *vic* locus is required to generate an incompatible reaction. A single amino acid change at one locus has been shown to be sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina* (Deleu *et al.*, 1993). The ability of a small change at any

one of several loci to elicit vegetative incompatibility indicates the relative ease with which new VCGs could arise in a population. With various regions of the genome under different selection pressures, it is not unreasonable to consider that *vic* loci might be under higher selection pressure than other regions of the genome. Frequency dependent selection, for example, acting on *vic* loci could select for the maintenance of a large number of different VCGs. A high level of VCG diversity could be maintained in the absence of an equivalent amount of diversity in other regions of the genome, and regardless of the role of sexual or asexual reproduction in the population.

While VCG diversity may not reflect genetic diversity within *M. fructicola*, it is also possible that the regions of the genome selected for study may not be the most appropriate regions for the intended purpose. This study utilized only nuclear rDNA and microsatellite regions with GACA or GTG motif. As with diversity at the *vic* loci, diversity in these portions of the genome may not accurately reflect diversity in other regions of the genome. The examination of different regions or the use of different molecular techniques may have revealed greater diversity than that which was found in this study of rDNA and microsatellites.

A final analysis of this research, keeping the above points in mind, suggests that *M. fructicola* is a species of limited genetic diversity, but that VCG diversity is highly selected for in Michigan populations of this pathogen.

The data revealed in this study has implications beyond an assessment of genetic diversity within *M. fructicola* and *M. laxa*. Sonoda *et al.* (1982b) suggested the use of vegetative compatibility reactions to distinguish between isolates of *M. fructicola* and *M.*

laxa: The formation of barrage zones (incompatible reactions) between isolates of the two species could serve as a means of separating *M. laxa* from *M. fructicola*. However, this study revealed a large number of incompatible reactions among isolates *M. fructicola* alone. While an incompatible reaction may indicate isolates of different species, as suggested by Sonoda *et al.* (1982b), it may also indicate incompatibility between isolates of one species. Incompatibility reactions will therefore be inadequate for distinguishing *M. laxa* from *M. fructicola*.

Molecular data clearly indicate high conservation of the ITS1 regions of Michigan isolates of *M. fructicola* and *M. laxa*, suggesting that the ITS1 sequences determined in this study have become the fixed ITS1 types for these populations. Concerted evolution during meiotic recombination would act to homogenize the ITS region (Dover, 1982), leading to this fixation of ITS types within the population (O'Donnell, 1992).

Previous studies of the ITS sequences of *Monilinia* species also suggested the highly conserved nature of the ITS sequences within the Sclerotiniaceae, including the *Monilinia* species (Carbone and Kohn, 1993; Holst-Jensen *et al.*, 1997). Comparison of ITS1 sequences determined in this study with those from previous studies further indicated the high conservation of the ITS1 region within the two species. Two previously examined isolates of *M. fructicola* from Ontario, Canada, (Carbone and Kohn, 1993; Holst-Jensen *et al.*, 1997) showed 100% nucleotide sequence similarity to the Michigan isolates examined here. Three isolates of *M. laxa* from Norway and one isolate from Italy (Holst-Jensen *et al.*, 1997) showed greater than 99% nucleotide sequence similarity (one bp difference) to the Michigan isolates studied here. This

comparison clearly indicates the highly conserved nature of the ITS1 region of *M. fructicola* and *M. laxa*, and further indicates that ITS1 sequence variation will not be a useful technique for identifying diversity within the global population.

The vegetative compatibility data and the molecular data also have implications for control of the disease. A high number of VCGs present within an orchard will limited the ability of advantageous characters, such a fungicide resistance, to be spread throughout the population. The apparent insignificant level of sexual reproduction within Michigan populations limits genetic recombination during meiosis as a source of genetic variation and spread of advantageous characters. The high number of different VCGs within an orchard also limits genetic exchange via vegetative hyphal fusion, and therefore inhibits the spread of characters.

The limited genetic diversity suggested by the molecular data also has implications for control. Limited genetic diversity within any species restricts the ability of that species to rapidly adapt to a changing environment. A more genetically diverse population is more able to rapidly adapt to changes. If *M. fructicola* is as limited in genetic diversity as the data suggest, then the species is also less able to rapidly adapt to changing environmental conditions, such as the application of fungicides. The high frequency of VCG diversity and the limited genetic diversity within *M. fructicola*, therefore, suggest that the spread of characters such as fungicide resistance will occur at a slower rate than if the species had fewer VCGs and more genetic diversity.

As indicated above, there are several implications of the data presented in this thesis. However, the basic findings of the research remain: 1) *M. fructicola* possesses a

group I intron in the SSU rDNA, 2) both *M. fructicola* and *M. laxa* possess limited intraspecies diversity, and 3) VCG diversity is highly selected for in *M. fructicola*. However, further research is needed to develop a more accurate view of the genetic diversity within *M. fructicola.*

RECOMMENDATIONS FOR FUTURE STUDY

The data presented in this thesis clearly indicate the limited nuclear rDNA and microsatellite DNA diversity of Michigan populations of *M. fructicola* and *M. laxa*. Further research is necessary to identify a marker more suited to the identification of genetic diversity within these species, and to determine if the results found among Michigan populations reflect the characteristics of the global population.

Future research must first expand the geographic range of sampling. Isolate collection should be made from locations throughout the world where *M. fructicola* is a significant pathogen. These locations should include California, Canada, South America, South Africa, and Japan. A sampling of approximately twelve to fifteen isolates from each of these locations will provide a sufficient set of isolates to began analysis of diversity within the global population. In regions where *M. fructicola* and *M. laxa* coexist, a similar number of isolates of *M. laxa* should also be collected.

To determine if the tools for distinguishing Michigan isolates of *M. laxa* from *M. fructicola* can be applied to isolates throughout the world, DNA from isolates of both species should undergo the same molecular analysis as the Michigan isolates in this study. However, as these characters are to be used simply to distinguish between isolates of the two species, it is unnecessary to sequence the ITS1 and SSU rRNA gene regions for this purpose.

The ITS1 region and the group I intron of the SSU rRNA gene of one to two isolates from each location should be sequenced. This will verify the high conservation of these regions as suggested by this, and previous, study (Carbone and Kohn, 1993; Holst-Jensen *et al.*, 1997). Given the highly conserved nature of these regions, the examination of genetic diversity within the species does not require the sequencing of all isolates collected.

A potentially useful tool for analyzing genetic diversity within *M. fructicola* is that of amplified fragment length polymorphism (AFLP). This technique involves the selective PCR amplification of restriction fragments of genomic DNA, and has been shown to be a useful tool for identifying genetic diversity within a variety of organisms (Vos *et al.*, 1995; Janssen *et al.*, 1996; Majer *et al.*, 1996). AFLP analysis is capable of detecting polymorphisms within species where little diversity was revealed by RFLP analysis (Majer *et al.*, 1996).

Analysis of genomic DNA of *M. fructicola* and *M. laxa* via AFLP analysis may be a useful method by which to identify diversity within these species. All isolates collected from each area, including Michigan, should be examined via AFLP analysis.

An additional PCR-mediated technique would employ the intergenic spacer (IGS) region of the nuclear ribosomal DNA. The IGS region, unlike the ITS regions, is not transcribed and is the most rapidly evolving of the rDNA spacers (Hillis and Dixon, 1991). This region may therefore present more variability than other regions of the rDNA. Sub-repeat elements occur in the IGS region of many species, and these sub-repeats may vary in sequence and the number of repeat elements between individuals of a

species (Morton *et al.*, 1995). Difference in the number of repeat elements creates length variation which may be detected by simply PCR amplification and gel analysis. Primers for amplification of the IGS region are available in the literature (Anderson and Stasovski, 1992; Appel and Gordon, 1996).

Attempts to PCR-amplify the IGS region in this study were unsuccessful. The development of better primers in the future and improved long-distance PCR may resolve some of the difficulties found in initial attempts to amplify the IGS region of *M. fructicola* and *M. laxa*. While other regions of the rDNA show very limited diversity, the IGS region may display greater diversity given its more rapid rate of evolution. All isolates should be examined for length variation in the PCR product. If length variation is revealed, at least two products of each length should be sequenced for further analysis. In the absence of length variation, one to two isolates from each region could be examined for sequence variation.

In addition to increasing the geographic rang from which isolates are collected, it may also be beneficial to include isolates collected in previous years. The inclusion of isolates from other years, such as isolate CB-2 collected in 1975, may provide insight into changes in population structure and diversity over time.

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APPENDICES

APPENDIX A

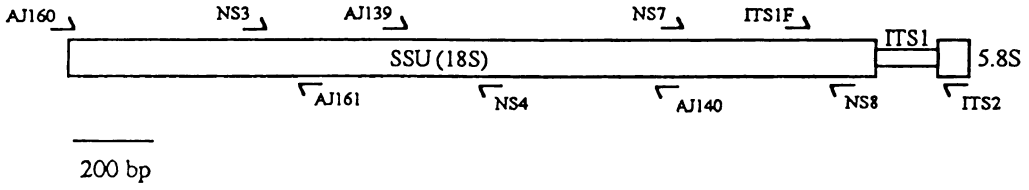
Vegetative compatibility groups (VCGs) containing more than one isolate of *Monilinia fructicola.*

VCG	Number of Isolates	Isolates within VCG[a]
1	5	KK-25, KK-44, KK-48, KK-52, KK-54
2	2	KK-49, KK-50
3	3	KK-15, KK-35, KK-40
4	4	JB-16, JB-22, JB-25, JB-35
5	28	JB-1, JB-2, JB-10, JB-13, JB-14, JB-17, JB-19, JB-20, JB-24, JB-26, JB-29, JB-30, JB-31, JB-32, JB-34, JB-50, JB-51, JB-52, JB-54, JB-56, JB-58, JB-60, JB-63, JB-64, JB-65, JB-66, JB-67, JB-68
6	16	JB-8, JB-9, JB-11, JB-15, JB-18, JB-21, JB-27, JB-33, JB-36, JB-37, JB-38, JB-44, JB-47, JB-61, JB-69, JB-70

[a] KK isolates were collected from Kewadin, MI. JB isolates were collected from Empire, MI. Isolates within each group did not form mycelial interaction zones with members of the same group, but did form lines of interaction with members of all other groups.

APPENDIX B

Sequences and positions of oligonucleotide primers in the rDNA.



Primer	sequence	approximate product length *M. fructicola*	*M. laxa*
ITS1F	CTT GGT CAT TTA GAG GAA GTA A		
ITS2	GCT GCG TTC TTC ATC GAT GC	250	250
NS3	GCA AGT CTG GTG CCA GCA GCC		
NS4	CTT CCG TCA ATT CCT TTA AG	600	600
NS7	GAG GCA ATA ACA GGT CTG TGA TGC		
NS8	TCC GCA GGT TCA CCT ACG GA	380	380
AJ139	GAA GAC TAA CTA CTG CGA AAG C		
AJ140	ACC TGT TAT TGC CTC AAA CTT	930	510
AJ160	GTC ATA TGC TTG TCT CAA AGA T		
AJ161	CCA AGG TTC AAC TAC GAG	620	620