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DISTRIBUTION, IDENTIFICATION, AND POPULATION DIVERSITY OF *ARMILLARIA* SPP. IN MICHIGAN CHERRY ORCHARDS

presented by

MARGARET LEE ELLIS

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DISTRIBUTION, IDENTIFICATION, AND POPULATION DIVERSITY OF *ARMILLARIA* SPP. IN MICHIGAN CHERRY ORCHARDS

BY

MARGARET LEE ELLIS

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ABSTRACT

DISTRIBUTION, IDENTIFICATION, AND POPULATION DYNAMICS OF *ARMILLARIA* SPP. IN MICHIGAN CHERRY ORCHARDS

BY

MARGARET LEE ELLIS

Species of *Armillaria* (Fr.:Fr.) Staude cause shoestring root rot, an important disease of Montmorency tart cherry trees in Michigan. The first objective of this study was to evaluate the current distribution of *Armillaria* spp. in the cherry producing regions. Isolates were collected from 14 counties in Michigan, and identified to species using PCR amplification of the IGS-1 region and RFLP analysis. Sexual compatibility tests were used to distinguish between species with the same DNA banding patterns. Isolates were identified as *A. ostoyae*, *A. gemina*, *A. mellea*, or *A. gallica*. *Armillaria ostoyae* was the predominant species found on cherry, corresponding to the previous survey by Proffer et al. (1987). *Armillaria gemina* and *A. gallica* were found for the first time on orchard grown *Prunus* spp. The second objective was to examine the population and strain diversity of *Armillaria* within two orchards using somatic incompatibility tests and microsatellites. In 2006, two tart cherry orchards in the northwest region of Michigan were intensively surveyed. Six individual clones of *A. ostoyae* were detected from 22 isolates from the first orchard. From the second orchard four different species were detected from 81 isolates. The isolates included 11 clones of *A. ostoyae*, four clones of *A. gemina*, four clones of *A. mellea*, and three clones of *A. gallica*. These findings expand available information on population diversity of *Armillaria*, providing growers with valuable information on whether a site is suitable for orchards or vineyards.

DEDICATION

I would like to dedicate this thesis to my loving family and closest friends, especially my father Mark Lee Ellis, mother Deborah Lynn Ellis, and my sister Elizabeth Stewart Ellis. They have been a great source of support and guidance throughout my life.

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KEY TO ABBREVIATIONS

BS: Biological species

LSU region: nuclear large ribosomal subunit

IGS region: Intergenic spacer region

ITS region: Internal transcribed spacer region

mtDNA: Mitochondrial DNA

NABS: North American biological species

PCR: Polymerase chain reaction

rDNA: Ribosomal DNA

RAPD: random amplified polymorphic DNA

RFLP: Restriction fragment length polymorphisms

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SI: Somatic incompatibility

SSR's: Simple sequence repeats also known as microsatellites

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION

The genus *Armillaria* (Fr.:Fr.) Staude is classified within the fungal class homobasidiomycetes. *Armillaria* spp. produce gilled pileate basidiomes, typically with an annulus around the central stipe, and grow in caespitose clumps around the base of dead trees or stumps (reviewed in Watling et al. 1991). The common names for this edible fungus, honey mushroom or honey agaric, come from the pileus or cap of the mushrooms that are tan/amber/honey colored. The other common name given to *Armillaria* is the oak fungus because of the regular occurrence of one of the major species (*Armillaria mellea* (Vahl:Fr.) Kummer) on this forest host. *Armillaria* spp. are among the few fungi known to produce rhizomorphs. These shoestring-sized, black or brown root-like strands can frequently be found under the bark of dead forest trees, along decorticated logs on the forest floor, and in the soil around infested trees (IPM: Landscape and Turf: Armillaria Root Rot of Trees and Shrubs 2000).

In forest ecosystems, *Armillaria* generally functions as a saprophyte and decomposer of fallen logs. In this role, *Armillaria* is a white rot fungus, breaking down both the lignin and cellulose components of wood (reviewed in Kile et al. 1991). However, *Armillaria* can also act as an opportunistic pathogen on stressed or declining trees, or as a primary and virulent pathogen (Shaw and Roth 1978). As a pathogen, *Armillaria* causes a soilborne disease, using rhizomorph growth from an established food source to spread the fungus through the soil from tree to tree (Jones and Sutton 1996). Once the rhizomorphs penetrate through the root surface, the mycelium of the fungus spreads out across the vascular cambium and phloem forming white mycelial fans, a diagnostic feature of *Armillaria* infection. The destruction of phloem in the cambium

causes tree death (reviewed in Morrison, Williams, and Whitney 1991). The role of *Armillaria* and its pathogenicity depends on the host, environment, and species of *Armillaria* present (reviewed in Gregory et al. 1991).

As a pathogen, species of *Armillaria* infect a broad range of trees, shrubs, vines, and some herbaceous plants. The disease caused by *Armillaria* is often referred to as Armillaria root rot or shoestring root rot because it produces rhizomorphs (IPM: Landscape and Turf: Armillaria Root Rot of Trees and Shrubs 2000). In Michigan, *Armillaria* spp. are the causal agents of a serious localized disease in tart (sour) cherry (*Prunus cerasus*) orchards. These pathogens infect other fruit orchards, forest, tree stands, landscapes, and Christmas tree plantations (Banik et al. 1995; Proffer et al. 1987; Smith et. al. 1990, 1992, 1994).

In 1987, Proffer et al. surveyed the distribution of species of *Armillaria* in tart cherry orchards in Michigan and found that *Armillaria ostoyae* (Romagn.) Herink was the predominant species in the Northwestern orchards, with *A. mellea* becoming more prevalent further south in the state. Most of the orchards surveyed by Proffer et al. (1987) are now out of cherry production because there is no consistently effective control for Armillaria root rot disease. According to Louvet (1979) fungicide injections are not successful because the movement of systemic fungicides is typically acropetal. In order for fungicides to be affective in controlling *Armillaria*, translocation must be basipetal. Kissler et al. (1973) found soil fumigation effective in destroying root fragments infected with *Armillaria* in orchards, vineyards, and floriculture operations (reviewed in Hagle and Shaw 1991). Although fumigation practices have been able to show improvement in infected sites, they do not provide complete control due to the depth that *Armillaria* can

be found in the soil (Bliss 1951) and also because the bark acts as a protective layer preventing penetration of chemicals into infected tissue that remains in the soil (reviewed in Hagle and Shaw 1991). Another restriction to using fumigants is that they can not be applied in established orchards or forest stands. Not growing susceptible crops, such as grasses, can reduce inoculum; however, infected roots can remain in the soil for several years, even decades according to James Nugent, 2005, personal communication, (District Horticulturist and coordinator of the Northwest Michigan Horticultural Research Station, Traverse City, MI 49684) and Munnecke et al. 1981. Unless all of the infected roots are removed from the field there is still a high potential for later infection. *Trichoderma viride* Pers.:Fr., *Scytalidium lignicola* Pesante, *Peniophora gigantea* (Fr.) Massee, *Rhizoctonia lamellifera* Small, *Pleurotus ostreatus* (Jacq.:Fr.) P. Kumm., *Coriolus veriscolor* (L.:Fr.) Quél, *Stereum hisutum* (Willd.:Fr.) S.F. Gray, *Xylaria hypoxylon* (L.:Fr.) Grev., and *Hypholoma fascicular* (Huds.:Fr.) Kumm. are just some of the fungal species shown to be antagonistic against *Armillaria* and have been tested as potential biological controls. One nematode, *Aphelenchus avenae*, was found to reduce fungal growth and vigor in a French vineyard. The genus *Trichoderma* Pers.:Fr. has been the most studied group of fungi for biological control since they are common soil inhabitants (reviewed in Hagle and Shaw 1991). However, according to Munnecke et al. (1981) biological controls are hard to establish because the host and the biological control agent interaction must happen in a relatively short time under rather specific environmental conditions in order to be successful.

Because *Armillaria* continues to be a problem for Michigan cherry growers and new sites have been reported to be infected that were previously not surveyed by Proffer

et al. (1987) a reevaluation of current orchards for the distribution of species of *Armillaria* is needed. The technique Proffer et al. (1987) used relied on sexual compatibility tests first used by Hintikka (1973), using single basidiospore isolates collected from basidiomes. Since the 1987 survey, new DNA-based techniques allowing identification using fungal tissue from rhizomorphs from roots and soil, or mycelia from fans found under the bark of diseased trees have been published (Harrington and Wingfield 1995). Since the methodology used by Proffer et al. (1987) relied on basidiomes, species not producing basidiomes during their survey period would have been missed.

The next step after determining the current species of *Armillaria* in orchards would be to examine the clonal spread with in an orchard site. The information gathered from this study would help in the understanding of populations of *Armillaria* in Michigan affecting the cherry orchards today. Somatic incompatibility tests and microsatellite markers or simple sequence repeats (SSRs) are currently used to identify individual clones of *Armillaria*. Somatic incompatibility tests involve pairing test isolates on a culture medium and examining the visible interaction of the expanding colonies as they make contact. The formation of a barrage zone along the line of contact indicates the isolates are different clones. Microsatellite markers are used to confirm genetic variation between individual isolates from different clones.

Many cherry orchard sites in Michigan are being converted into grape vineyards due to the higher value of the crop per acre. New growers, not knowing the history of the site and some established growers converting their orchard lands are often unaware that *Armillaria* has been reported on grapes (*Vitis* spp.) in Europe, Australia, Brazil, and

California (Hood et al. 1991). Michigan's grape industry is much younger than those of both Europe and California, and the effects or presence of infection with *Armillaria* have not yet been reported. Michigan does have one of the species of *Armillaria* reported on grapes, *A. mellea* and another pathogenic species *A. ostoyae* that could be a potential problem. In the Northwestern cherry-producing region where many of the vineyards are currently being established, *A. ostoyae* tends to be the predominant species and it is known as a virulent pathogen with a wide host range. While most reports of *A. ostoyae* are on conifers, it has proven to be an aggressive pathogen of cherry in Northwest Michigan, as seen by Proffer et al. in the 1987 survey. With the increase of viticulture in the state of Michigan it is important to assess *A. ostoyae* and *A. mellea* for their ability to infect grape rootstocks used in Michigan.

The hypotheses of this research were: (1) that new species of *Armillaria* would be identified on cherry using molecular identification techniques not reliant on basidiomes as in the 1987 survey by Proffer et al. (1987); (2) the diversity of species in an orchard may be much greater than previously reported; and (3) that the current *Armillaria* species known to infecting orchard grown *Prunus* will move to grape when orchards are converted to vineyards. To test these hypotheses a broad distribution survey of orchards in the cherry producing region of Michigan was done, as well as a clonal mapping of *Armillaria* within two Montmorency tart cherry orchards using somatic incompatibility tests and microsatellite markers. The third hypothesis was tested by beginning greenhouse and field trial inoculations of grape rootstocks grown in Michigan for the future assessment of susceptibility to *A. ostoyae* and *A. mellea*.

LITERATURE REVIEW:
TAXONOMY

The taxonomic and nomenclatural status of the genus *Armillaria* (Fr.:Fr.) Staude has gone through many changes over the last century. The first reports of mushrooms that would now be classified as *Armillaria* are believed to be from either 1729 by Micheli or 1755 by Battarra. Fries (1819) first established the genus *Armillaria* as a tribe of *Agaricus*, which included *Agaricus melleus* Vahl:Fr., now classified as *Armillaria mellea*, and later treated it in the *Systema Mycologicum* (Fries 1821). In 1825 Fries (1825) placed *Agaricus melleus* in the tribe *Lepiota*, abandoning the tribe *Armillaria* but later reversed himself in 1938 (Fries 1838) by replacing it into the tribe *Armillaria*. In 1957 Staude raised *Armillaria* to generic rank, including four species; *Agaricus mucidus*, *Ag. melleus*, *Ag. aurantius*, and *Ag. robustus*. Today *Ag. aurantius* and *Ag. robustus* are considered to be in the genus *Tricholoma* (Fr.) Staude, and *Ag. mucidus* is placed in the genus *Oudemansiella* Spegazzini (or *Mucidula* Pat.). However, Singer (1951, 1955, 1986) believed that Staude's entry was inadmissible according to the International Code of Botanical Nomenclature. Singer argued that it was Kummer (1871) who should be given credit as the author. In reviewing the nomenclatural trail, however, both Donk (1949, 1962) and Watling et al. (1982) supported Staude as the valid author for the genus *Armillaria* (reviewed in Volk and Burdsall; Watling et al. 1991).

The nomenclatural confusion was compounded by Karsten (1881) who created the genus *Armillariella* in 1881. Some authors, following the lead of Singer, continued to use this genus name into the 1970's. Karsten, however used *Ag. melleus* as the type species which was within Fries' tribe *Armillaria*. The use of the genus name *Armillariella* was ended after the taxonomic review by Watling, Kile, and Gregory in

1982, which established *Armillariella* as an obligate synonym of *Armillaria* (reviewed in Volk and Burdsall; Watling et al. 1991).

Prior to the mid-seventies, Armillaria root rot was considered to be caused by a single polymorphic species, *Armillaria mellea sensu lato*. Studies examining the bifactorial sexual incompatibility system in *Armillaria* changed this view. Hintikka (1973) developed a technique of pairing single basidiospore isolates on Petri plates to demonstrate sexual compatibility. Additional work done by Korhonen (1978) helped to distinguish five biological species (BS) of *Armillaria* in Europe (BS A = *A. borealis* Marxmüller & Korhonen, BS B = *A. cepistipes* Velenovsky, BS C = *A. ostoyae,* BS D = *A. mellea*, BS E = *A. gallica* Marxmüller & Romagnesi (a.k.a. *A. bulbosa* (Barla) Kile & Watling and *A. lutea* Gillet). Today, *Armillaria* is divided into around 40 distinct biological species and is found on every continent, with the exception of Antarctica, infecting a huge range of hosts (reviewed in Volk and Burdsall).

In North America, the genus *Armillaria* was divided into 10 North American Biological Species (NABS) by Anderson and Ullrich (1979) using the Hintikka (1973) technique as expanded by Korhonen (1978). Most of the North American biological species (NABS) of *Armillaria*, identified using the sexual compatibility system, have been formally equated to European species or described as new species. Assigned NABS Roman numerals designate *Armillaria* species with the exception of *A. tabescens* (Scop.) Emel which has not yet been assigned a number (Table 1-1).

Table 1-1: Species of *Armillaria* in North America with assigned NABS:

Modified from Volk and Burdsall

NABS	Species	Author	Synonyms	Author of Synonyms	Distribution	Ecological Role/ Pathogencity
I	*Armillaria ostoyae*	(Romagn.) Herink	*Armillaria obscura*	(Schaeff.) Herink [nomen ambiguum]	Pacific Northwest and Northern United States found most often on conifers in the northern conifer zone	Pathogenic
II	*Armillaria gemina*	Bérubé & Dessureault			Limited to the Eastern United States and Southeastern Canada on hardwoods	Generally not considered pathogenic
III	*Armillaria calvescens*	Bérubé & Dessureault			Northeast of the United States and the Southeast in Canada on maples and hardwoods	Saprophyte in the soil but can act as an opportunistic pathogen
V	*Armillaria sinapina*	Bérubé & Dessureault			Northeast United States mostly on hardwoods and in the Pacific Northwest on conifers	Pathogenic
VI	*Armillaria mellea*	(Vahl:Fr.) Kummer			Eastern United States and Canada, and Northern California on hardwoods and occasionally conifers in mixed forests	Pathogenic
VII	*Armillaria gallica*	Marxmüller & Romagn	*Armillaria lutea*	Gillet [nomen ambiguum]	Eastern and Southern United States and Midwest on hardwoods	Saprophyte found in the forest soil
			Armillaria bulbosa	(Barla) Kile & Watling [misapplied name]		

Continuation of Table 1-1: Species of *Armillaria* in North America with assigned NABS:

Modified from Volk and Burdsall

NABS	*Species*	Author	Synonyms	Author of Synonyms	Distribution	Ecological Role/ Pathogencity
IX	*Armillaria nabsnona*	Volk & Burdsall			Pacific Northwest on hardwoods	
X	Unnamed				Found only in Idaho and British Columbia	
XI	probably = *Armillaria cepistipes*	Velenovsky	Species F	Morrison *et al.* 1985	Pacific Northwest	
	Armillaria tabescens	(Scop.) Emel			Eastern United States, Midwest, and in the West in Texas and Oklahoma. Common on Oak.	Pathogenic

MORPHOLOGICAL FEATURES

The basidiomes of *Armillaria* are generally found in caespitose clumps (*A. ostoyae*, *A. gemina*, *A. mellea*, and *A. tabescens*) at the base of a tree or stump, in gregarious clusters (*A. calvescens*, *A. gallica*, and *A. nabsnona*), in clumps of two to three or single (*A. sinapina*). The pileus is fleshy and the color of the pileus can be honey colored (*A. mellea*), brown to brownish-yellow (*A. ostoyae*, *A. gemina*, *A. sinapina*, *A. calvescens*, and *A. gallica*), tan (*A. calvescens*, and *A. gallica*), pinkish-brown (*A. calvescens*, and *A. gallica*), ivory (*A. tabescens*), or orange brown (A. nabsnona). The pileus is usually smooth (*A. calvescens*, *A. mellea*, *A. sinapina*, *A. gallica*, *A. nabsnona*, and NAABS XI) but can be scaly (*A. ostoyae*, and *A. gemina*). The flesh of the pileus is pale, while the stipe is white at first but then can turn, brown, tan, or yellow. The stipe is central and typically annulate, with the exception of *A. tabescens*, with floccose-membranous to arachnoid veil. The annulus can be thick and wooly (*A. mellea*), thick (*A. ostoyae*, and *A. gemina*), or cortinaceous (arachnoid, cobwebby) (*A. calvescens*, *A. sinapina*, *A. gallica*, and NABS XI). The gills are often decurrent, but can be sinuate, adnexed, or subdecurrent depending on the species. The basidia are usually 4-spored but can be 2-spored and thin-walled, often with a basal clamp-connection. The spores are often thick-walled and hyaline to cream colored and produce a white to cream-colored spore print (reviewed in Volk; Watling et al. 1991).

Armillaria was first physically described as a gilled mushroom or basidiome in Fries Systema Mycologicum (1821). The ontogeny of *Armillaria* and other agaricus fungi was first described by Hoffman (1861). Basidiome development of *Armillaria* spp. was also studied by Hartig (1874), Beer (1911), and Atkinson (1914). Production of a

basidiome in culture was first described by Molisch (1904) where he used autoclaved bread to grow the fungus. *Armillaria mellea* was grown from basidiospores to basidiome by Falck (1907). Falck reported that light was necessary for basidiome development. Since Molisch and Falck there have been many subsequent studies reporting development of basidiomes *in vitro* (reviewed in Garraway et al. 1991). Although many reports show success in development of basidiomes *in vitro*, the techniques are not reliable and there are many limitations as described by Ullrich and Anderson (1978). The *in vitro* development of basidiomes is also variable depending on the species (reviewed in Garraway et al. 1991).

Rhizomorphs are another morphological structure that characterizes the genus *Armillaria*. Rhizomorphs are defined as discrete, filamentous aggregations that are highly differentiated, fully autonomous, and grow apically. They are more complexly organized than mycelial cords, often produced by fungi which grow on the forest floor. Mycelial cords are aggregations of parallel, undifferentiated hyphae. Rhizomorphs are so distinctive that they were first described as a separate species, *Rhizomorpha fragilis* Roth. Schmitz (1848) published the description of different forms of *R. fragilis*, including *R. subterranean* found in the forest soil and *R. subcorticalis* found growing beneath the bark of trees. Robert Hartig (1874) later provided proof that rhizomorphs belonged to the mushrooms of the genus *Armillaria* (reviewed in Garraway et al. 1991).

Rhizomorph organization is described in detail by Hartig (1870, 1874). In 1877, the apical growth of rhizomorphs was described by Brefeld, and in 1969 Motta improved the descriptions with the use of electron microscopy. Hartig described the thallus organization of rhizomorphs as having three layers including; a cortex, subcortex, and

medulla layer. The three layers can be characterized by three different types of hyphae: skeletal, binding, and generative. The mucilaginous nature of the tip of the rhizomorph and the differences in the cell wall layers were also described by Hartig. The cortex layer is composed of melanized densely packed cells. Bloomfield and Alexander (1967), and Khuo and Alexander (1967) found that the melanin content of the cortex protects the inner layers of rhizomorphs from potential hazards in the environment. The subcortex is a transition layer from the cortex to the medulla where the hypha becomes wider in diameter and looser. The medulla layer is composed of loose hyphae and forms a central canal. Jenning (1984) described how the medulla layer is responsible for both water and nutrient transport and Smith and Griffin (1971) described how the canal is responsible for oxygen translocation (reviewed in Garraway et al. 1991).

The nutrient uptake, transport, and requirements of rhizomorphs have been studied. Falck (1912) was the first to propose the idea that rhizomorphs were used for transport of nutrients and water. Chlorides, phosphates, and ammonium ions were all shown to transport through rhizomorphs by Morrison (1975), later confirmed by Anderson and Ullrich (1982) using radioactive labeling. Eamus and Jennings (1984) found a considerable gradient of water and turgor potential from the tip to the base of rhizomorphs, and hypothesized that long-distance transport occurred predominantly by solutes moving along vessel hyphae of the medulla. This view was later supported when Eamus and Jennings (1985) measured the internal structure and hydraulic conductivity of rhizomorphs (reviewed in Garraway et al. 1991).

There have been many *in vitro* studies on the nutrient requirements of *Armillaria*. The molecules: carbohydrates, lipids, phenols, and alcohols have been examined.

Addition of low molecular weight alcohols to glucose media were shown to increase the growth rate of *A. mellea* (Vance and Garraway 1973). Bell (1970), Cruickshank and Perrin (1971), and Masuko et al. (1970) have shown phenols to be inhibitory to many fungi (reviewed in Vance and Garraway 1973). However, when low molecular weight alcohols such as ethanol, propanol, butanol, or isobutanol were added to media, the inhibitory affect of phenols on the growth of *Armillaria* isolates decreased (Vance and Garraway 1973). Studies have suggested many reasons why phenols inhibit growth. Smith and Griffin (1971) suggested that in *A. luteobubalina* Watling & Kile (previously reported as *Armillariella elegans*) polymers formed by phenols which oxidize to melanins and lignins can be incorporated into cell walls of rhizomorphs causing increased rigidity and thus prevent cell wall expansion. It has also been suggested that phenols inhibit enzymes that could be important in mycelial growth and rhizomorph morphogenesis. Phosphorylases (Schwimmer 1958), cellulases (Kosuge 1969), transaminases (Braunstein 1947), and decarboxylases (Hartman et al. 1955) are all known to be inhibited by phenols (reviewed Vance and Garraway 1973).

In pathogenic species of *Armillaria*, rhizomorphs are used as a mode of infection. The growth habit of rhizomorphs in different species of *Armillaria* is correlated with pathogenicity (Morrison 1989, 2004). Morrison examined 15 species of *Armillaria* from Europe, North America, Australia, and New Zealand; species whose rhizomorphs branched dichotomously were more virulent then those that branched monopodially (Morrison 2004). Rhizomorphs infect their host by sending branches into their host as they grow epiphytically along the host (Garrett 1956). Once infected, the root acts as the inoculum or food-source for further spread. Such infected sources are important in the

success of progressively establishing infections. Garrett, using woody inoculum of varying sizes with potato (*Solanum tuberosum* L.) tubers as the potential host at different distances from the inoculum demonstrated that the successful establishment of new infections depended upon factors such as the size of the food-base and the distance from the food-base (Garrett 1956).

LIFE CYCLE

Armillaria has a unique life cycle compared to many other basidiomycetes. The initial interactions of a compatible mating in most basidiomycetes result in cell fusion (plasmogamy) with delayed nuclear fusion (karyogamy). A dikaryotic stage (n+n) is the predominant nuclear condition in the vegetative cells of most basidiomycetes (Peabody et al. 2000). Clamp connections, a characteristic found in most basidiomycetes, allow for nuclear migration where a dikaryotic cell donates one nucleus to a new monokaryotic cell. This process of nuclear migration and mating of many basidiomycetes was first examined by Buller (1931) with *Coprinus* Pres. and is now known as the Buller Phenomenon. This process can be used to distinguish biological species, and involves pairing of a dikaryotic and monokaryotic isolate. If the cells from the dikaryotic isolate are able to successfully transfer nuclei to the cells of the monokaryotic isolate, which is "dikaryotized', the isolates are said to be from the same biological species and are compatible. Today this unique phenomenon is used for many basidiomycetes to distinguish biological species within a genus (reviewed in Callac et al. 2006; Rizzo and May 1994).

In contrast to most basidiomycetes, the predominant nuclear stage of *Armillaria* is diploid. The basidiomes, mycelial fans found under the bark of trees, and rhizomorphs tend to be diploid. Haploid mycelium is rare but has been seen in nature (Rishbeth 1985). The dikaryotic stage of *Armillaria* is limited to a short period right after the haploid mycelium of two different mating types mates fuse. Karyogamy occurs immediately and the hyphae become diploid. However, some species of *Armillaria* appear to have a second dikaryotic stage in the subhymenial cells that form clamp connections with young basidia. This was first observed by Korhonen (1980) in *Armillaria ostoyae*, which helped to explain why clamp connections were often present in fruiting bodies of some species of *Armillaria*. Korhonen (1980) observed that fruiting bodies of *Armillaria ostoyae* produced in the lab were clampless unlike ones seen in nature where the basidia have a basal clamp. From this initial work, two hypotheses were developed to explain how this could happen. The first hypothesis is that *Armillaria* goes through two diploidization and two haploidization cycles prior to formation of the basidia (Grillo et al. 2000). Several earlier works favored this hypothesis, and fluorescence microspectrophotometric work supports this theory (Korhonen 1980; Peabody et al. 1978; Peabody and Peabody 1984, 1985, 1986, 1987; Tommerup and Broadbent 1975). Another theory is that *Armillaria* is a genetic mosaic. This theory can be supported by Peabody et al. (2000) who first found haploid, monokaryotic fruiting bodies occurring in nature and were later able to demonstrate that the basidiomes of *A. gallica* were a genetic mosaic, by comparing genotypes of different cells within the same fruiting body. Furthermore, Rizzo and May (1994) were able to produce 2N+ N mycelium using *A. ostoyae* strains in the lab, and suggested that this could be a stable phase in nature. If this stage was stable in nature and

basidiomes were produced from this mycelium it could explain diversity of allelic combinations, thus supporting the genetic mosaic theory. Peabody and Peabody (2003) support both hypotheses, stating that because of genetic mosaicism in the basidiome, *A. gallica* has a life cycle with two diploidizations and two haploidizations. Like other basidiomycetes, *Armillaria* retains the ability to mate diploid mycelium with compatible haploid mycelium to produce new diploid cells (Anderson and Ullrich 1982; Korhonen 1978; Rizzo and Harrington 1992). Diploid-haploid mating along with haploid-haploid mating is used today to identify biological species.

Armillaria root rot infections involve a source of inoculum, such as colonized stumps or roots from a previously infected host. From the pre-established inoculum source, rhizomorphs can grow through the soil or along a root, thus acting as the primary mode of dispersal. Rhizomorphs are not the only means of infection; rhizomorph infection does not occur in some species such as *A. tabescens*. *Armillaria tabescens* infection occurs via root to root contact or interlocking root systems. Basidiospores can also act as a source of inoculum by colonizing a stump. Stump colonization by basidiospores is believed to be rare but can play an important role in establishing new foci or infection centers of disease (Rishbeth 1970). Genotype identification studies have shown some indirect evidence of spore infections. Many researchers have tried to replicate this process and have had either no success or limited success infecting stumps with basidiospores (reviewed in Redfern and Filip 1991).

Once in contact with a new host invasion of this host can happen one of three ways: from pre-exiting lesions in which the pathogen was formerly held in check by host resistance, from an epiphytic position on a root, or from outside by newly arrived

rhizomorphs (reviewed in Redfern and Filip 1991). Rhizomorph infection begins with its attachment to a root and the hardening of the mucilaginous substance at its tip. Once this occurs a single hypha develops from the rhizomorph's tip, which penetrates the outer layer of cork cells by mechanical force. After initial penetration, the rhizomorph branches spread out laterally and radially into the bark. Toxins are also believed to be produced by the fungus, helping host colonization. Shallow brown spots on the bark's outer parenchyma and cork layers are sloughed off and the fungus eventually reaches the cambium and cankers develop. Once the fungus reaches the cambium, mycelial fans can form penetrating the cambium. Bark tissue becomes necrotic (reviewed in Morrison et al. 1991).

Disease and symptom development upon infection depend greatly on the host and species of *Armillaria*. Symptoms of *Armillaria* infection include: reduction in shoot growth, defoliation, crown dieback, stress-induced reproduction, canker development at or above the root collar, and resin exudation. Because many of the symptoms are non-specific to *Armillaria*, the presence of basidiomes, rhizomorphs, mycelial fans, and pseudosclerotial plate (zones line) formation are important indicators to confirm *Armillaria* as the cause of disease. Dead trees in a circular pattern with new growth coming back up in the center are another indicator of Armillaria root disease, both in the field and in forests. The circular pattern is due to the radial expansion of the pathogen, with the most recent death at the edges of the pathogens growth.

How the disease and corresponding symptoms develop is not well understood. Two possible mechanisms have been proposed. One is that the disruption of the host's vascular system by the fungus and the host's response to infection is the cause of the

observed symptoms. The explanation is supported on the observations of shoot decline and changes in foliage seen over time due to infection by *Armillaria* and the destruction of the host's vascular system. A second proposed mechanism is that toxins produced by *Armillaria* cause symptoms to develop (reviewed in Morrison et al. 1991). This has been observed in conifers, infected with root rot pathogens *Armillaria* and *Heterobasidion annosum* (Fr.) Bref.(Rishbeth 1985).

CONTROL

Control of Armillaria root rot has been proved difficult. Many cultural and chemical controls have been investigated and none have been highly successful or easy to implement (Turner and Fox 1988). The control of Armillaria root rot depends on the host and the environment in which one is trying to control the disease, so before control measures can be taken these factors need to be assessed to try and find the most effective control.

In forests and orchards, the use of resistant species or rootstocks, avoidance of hazardous sites, cultural manipulations, chemical applications, and biological methods have all been assessed for control of Armillaria root rot. Currently the best method of management seems to be a combination of methods: avoiding areas with high disease hazard, removal of infected stumps, and the reduction of other inoculum sources such as roots (reviewed Shaw and Roth 1978; and Hagle and Shaw 1991).

In California orchards and vineyards, fumigation or heating, along with biological controls have provided temporary control (Munnecke 1973, 1976, 1981). The control is temporary because the fumigant does not penetrate deeply enough into the soil (Bliss

1951). This has been observed in vineyards where following fumigation the newly established vines are healthy for several years, but once the vine's roots grew deep enough they came in contact with inoculum that escaped fumigation and were subsequently infected (Gubler 1992).

Host resistance is an option that has been found to be somewhat effective in managing Armillaria root rot in both forests and orchards. Morrison (1981) and Hadfield et al. (1986) found that ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) is much more resistant than true firs (*Abies* spp.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.)) in Western North America. Greig and Strouts (1983) found Douglas-fir was more resistant than Scots pine in Great Britain. Resistance of fruit rootstocks to *Armillaria* species has also been tested. Variation in citrus (*Citrus* spp.) rootstock susceptibility to Armillaria root rot was observed by Thomas et al. (1948). Guillaumin et al. (1989) observed plum (*Prunus* spp.) to be more resistant than almond (*Prunus amygdalus* Batsch.), apricot (*Prunus armeniaca* L.), cherry (*Prunus* spp.), and peach (*Prunus persica* Sieb. & Zucc.) which were highly susceptible to *Armillaria mellea* in France (reviewed in Hagle and Shaw 1991). In Michigan, cherry rootstocks were tested in cold frames; *P. mahaleb* L. (mahaleb) rootstock, commonly used with tart cherry, was more susceptible than *P. avium* L. (mazzard) rootstock, commonly used with sweet cherry *Prunus avium* L. (Proffer et al 1988). However, this resistance may not hold up in the field, as seen by James Nugent. According to Nugent, the death of trees grafted on mazzard rootstock does not occur as quickly, but the final mortality appears to be the same. In other words, trees grafted on mahaleb rootstock planted into inoculated soil often die in two to four

years, whereas trees on mazzard rootstock die in four to seven years (Nugent, 2005, personal communication).

Reduction of inoculum is another control option. This involves the physical process of turning the soil to a considerable depth and removing all the stumps and roots of infected trees, and then cropping the land for several years with non-woody plants (reviewed Hagle and Shaw 1991). In orchards in Michigan, this method is time consuming and requires many years of abandonment of that orchard site. It also does not ensure all of the removal of inoculum due to the depth of the root systems. It only takes one piece of infected root tissue to begin new infection, and the inoculum can survive for several years. For these reasons, this control method is often not feasible or reliable to cherry growers with heavily infected orchards (Nugent, 2005, personal communication).

Other cultural controls, such as root collar excavation, and digging trenches to reduce spread have also been tested. In New Zealand, trenches were dug and lined with plastic and then back filled to serve as mechanical barriers to rhizomorph and root spread in kiwifruit (*Actinidia* spp.) orchards. However, this method was not cost effective due to the lack of profitability of kiwifruit (reviewed in Hagle and Shaw 1991). The efficacy of root collar excavation of grape, as a post-infection control method, was examined by Baumgartner (2004). In this study, root collars were exposed by removing the soil in an area of approximately 0.5 m in diameter around the root collar, and 0.3 m in depth. The goal was to determine if over time the mycelial fans would recede before severe disease occurred resulting in decay of vascular tissue. Baumgartner (2004) found that in one grape vineyard, excavation significantly increased yield and cluster weight of symptomatic grapevines. Also from this study it was revealed that mycelial fans had

receded from root collars of symptomatic-excavated grapevines when reexamined the following year (Baumgartner 2004).

Chemical fumigants, such as methyl bromide, carbon disulphide, and chloropicrin have been investigated as a means of control (reviewed in Hagle and Shaw 1991). Methyl bromide has been recommended as a control for Armillaria root rot (Filip and Roth 1977; and Munnecke et al. 1973, 1981). The use of carbon disulfide as a soil fumigant was first employed in Europe by Girard (1894) and Oberlin (1984) and was later recommended by Horne (1914) for destruction of *A. mellea* in California (reviewed in Bliss 1951). Bliss (1951) looked at nine different soil fumigants including carbon disulfide, formalin, tetrachlorethane, chloropicrin, chlorine, sulfur dioxide, ammonium hydroxide, ethylene oxide, and dichlorethyl ether. Bliss (1951) extensively examined carbon disulfide as a fumigant in citrus soils and found that injections at regular intervals over infected sites after the removal of stumps protected fruit crops in California from infection by *Armillaria* (Bliss 1951). The use of methyl bromide and carbon disulfide has been shown to predispose the pathogen to attack by antagonistic soilborne fungi, such as *Trichoderma viride* Pers.:Fr., helping to eradicate mycelium in buried wood segments. However, this method is not effective in killing all of the *Armillaria* in infected roots because the fumigants do not penetrate deeply enough into the soil (reviewed in Baumgartner 2004). Chemical treatments including ammonium sulphamate, 2,4,6-T (2,4,5-trichlorophenoxyacetic acid), Vorlex (80% chlorinated hydrocarbons, 20% methyl isothiocyanate), Picfume (99% chloropicrin), carbon disulfide, Dowfume (98% methyl bromide), and Vapam (32.7% sodium *N*-methyldithiocarbamate) have also been tested to reduce inoculum in forests by fumigation of infected stumps (Filip and Roth 1977; and

Rishbeth 1976). More recently Aguín et al. (2006) studied the *in vitro* capability of four sterol demethylation inhibitors including cyproconazole, hexaconazole, propiconazole, and tetraconazole, and another six downwardly mobile systemic chemicals including, azoxystrobin, cubiet (copper bis(ethoxy-dihydroxy-diethylamino)sulfate), fosetyl-Al, potassium phosphite, sodium tetrathiocarbonate, and 2-(thiocyanomethylthio) benzothiazole for possible control of *A. mellea*. Their results indicated that the four sterol demethylation inhibitor fungicides were the best at inhibiting fungal growth, especially cyproconazole and hexaconazole which showed a reduction of 67-72% at an EC_{50} dose of only 1 mg AI litre^{-1} (Aguín et al. 2006).

Fungicides have not provided long term control in the field. Delivering the fungicide to the infected tissue is a difficult task because the movement of systemic fungicides is typically acropetal, and when it does move into the root system it may not reach all of the infection (reviewed in Hagle and Shaw 1991). Adaskaveg et al. (1999) observed that the fungicide propiconazole when injected into almond trees was effective at increasing life of the trees for about two years before tree death, whereas most of the control trees at similar stages of decline at the time of treatment died within four months (Adaskaveg et al. 1999). An earlier study by Turner and Fox (1988) looked at the potential of ergosterol-biosythesis inhibitors (EBI's) *in vitro*, including: hexaconazole, flutriafol, and fenpropidin and also at a guanide, a phenolic, and a mixture of cresylic acids for control to *A. mellea*, *A. ostoyae*, and *A. gallica* (published under the synonym *A. bulbosa*). At a concentration of 500 mg ai/ml, all of the fungicides were effective in controlling Armillaria root rot. The EBI's as well as the gaunide showed effectiveness at lower concentrations in controlling of Armillaria root rot (Turner and Fox 1988).

Another fungicide, Armillatox, a phenolic emulsion, has been specifically marketed for control of Armillaria root rot. This fungicide was developed after successful control of *Armillaria* with the compound creosote (Bray 1970). However, Pawsey (1973) found creosote to be phytotoxic, and doubted its ability to control *Armillaria* (reviewed in Hagle and Shaw 1991). Redfern (1971) applied Armillatox to both forest and agricultural soils and found that the fungicide reduced rhizomorph growth but only at the highest concentration in agricultural soils. In results he concluded that there were no beneficial effects from using the fungicide (Redfern 1971).

Biological controls have also been found to be partially successful, especially when implemented with other control methods such as fumigation or heat as observed by Bliss (1951) and Munnecke (1976, 1981). The most studied biological controls for *Armillaria* are species of *Trichoderma*. Two fungitoxic compounds from *Trichoderma* species have been isolated including trichodermin and an unidentified compound by Ishikawa et al. (1976). Both *in vitro* tests and field trials have demonstrated the antagonistic effects of *Trichoderma* species, especially *T. viride*. Other fungi have also been looked at for possible antagonistic effects to *Armillaria* such as *Penicillium* Link:Fr., *Peniophora* Cooke, *Scytalidium* Pesante, *Rhizoctonia lamellifera*, *Pleurotus ostreatus*, *Coriolus veriscolor*, *Stereum hisutum*, *Xylaria hypoxylon*, and *Hypholoma* Fr. Quél. Sokolov (1964) showed that *Penicillium* and *Peniophora* were antagonistic in *in vitro* tests when plated with *Armillaria*. Cusson and LaChance (1974) were able to show that *Scytalidium lignicola* Pesante produced a toxin, syctalidin, with antifungal properties toward *Armillaria*. Fedorov and Bobko (1989) found that *Peniophora gigantae* and *Pleurotus ostreatus* were effective in preventing *Armillaria* from colonizing fresh cut

stumps. Redfern (1969) found *Hypholoma*, a cord-forming fungus, to be competitive with Armillaria in colonizing stumps. One nematode, *Aphelenchus avenae* was found to reduce fungal growth and vigor in a French vineyard. (reviewed in Hagle and Shaw 1991). However, although many possible biological controls such as *Trichoderma* have been shown to be antagonistic toward *Armillaria*, they are hard to establish in natural situations because certain factors must interact in a relatively short time under a set of specific environmental conditions (Munnecke 1981).

MOLECULAR METHODS USED FOR SPECIES IDENTIFCATION

Before the development of molecular techniques, *Armillaria* species were distinguished using sexual compatibility (infertility) tests. These tests were described by Buller (1931) and were first used to distinguish *Armillaria* species by Hintikka (1973), and later expanded by Korhonen (1978). Serological studies have shown potential for distinguishing *Armillaria* species as shown by Lung-Escarmant et al. (1978, 1985) and Lung-Escarmant and Dunez (1979, 1980) by using an antiserum produced in rabbits to a partially purified antigen of an *A. mellea* isolate. Using serology, Lung-Escarmant et al. were able to distinguish between six European species of *Armillaria* (reviewed in Burdsall et al.1990). Burdsall et al. (1990) used serology to distinguish between three North American species of *Armillaria* using chicken eggs as a source of antibodies. In recent years, many techniques have been developed for more rapid diagnosis of *Armillaria* species targeting structural elements such as proteins, polysaccharides, glycoproteins, and nucleic acids (Schulze and Bahnweg 1998). Methods have varied

from SDS-PAGE analysis of whole-cell proteins, isoenzyme pattern analysis, and the restriction fragment patterns of mtDNA or nuclear rDNA.

Whole-cell protein SDS-polyacylamide electrophoresis pattern analysis was attempted by Morrison et al. (1984), Poon (1988), and Lin et al. (1989), who found this technique ineffective at distinguishing between *Armillaria* species. Lung-Escarmant et al. (1985) used this technique with European species were only able to distinguish *A. gallica* from other European species (reviewed in Schulze and Bahnweg 1998).

Isozyme analysis is another tool that can differentiate morphologically similar or closely related species, varieties, and formae speciales. It can also be used to analyse genetic variability, trace pathogen spread, follow the segregation of genetic loci, and identify ploidy level of fungi and other pathogens (reviewed in Schulze and Bahnweg 1998). Harries and Hopkinson (1976) discuss how isoenzyme variation arises from three phenomena: different alleles at a single locus (termed allozymes), multiple loci coding for a single enzyme, and post-translational processing and formation of secondary isoenzymes. Using isoenzyme analysis, six *Armillaria* intersterility groups in British Columbia were distinguished using esterase and polyphenol oxidase by Morrison et al. (1985). Lin et al. (1989) attempted to distinguish four NABS's using isoenzyme analysis of 20 different enzymes. Of the 20 enzymes tested, only alcohol dehydrogenase, esterase, and polyphenol oxidase showed activity and only the esterase was able to distinguish between species. Wahlström et al. (1991) used pectin esterase and polygalacturonase to differentiate between five European species (reviewed in Schulze and Bahnweg 1998).

Anderson and Stasovski (1992) sequenced a portion of the Intergenic Spacer (IGS) of the ribosomal RNA operon and suggested that restriction enzymes digests of this region could be used to discriminate species. Harrington and Wingfield (1995) used the primers developed by Anderson and Stasovski (1992) for the IGS-1 region and tested their suggestion using restriction fragment length polymorphisms (RFLPs). The restriction enzyme AluI produced patterns that could distinguish 6 of 11 *Armillaria* species from European and American sources. Species not distinguishable with AluI could usually be separated using other restriction enzymes (Harrington and Wingfield 1995). North American isolates of *A. gallica* and *A. calvescens* could not be distinguished using this technique. Since Harrington and Wingfield (1995), other authors have used this protocol to help characterize and distinguish species of *Armillaria* in North America (Banik et al. 1996; Kim et al. 2000; Sierra et al. 1999; and White et al. 1998). Banik et al. (1996) found that isolates of *A. sinapina* and *A. gallica* from the state of Washington could not be distinguished from one another using banding patterns from restriction digests of the IGS-1 region (Banik et al. 1996). White et al. (1998) were able to use the IGS-2 region and restriction enzyme AluI to differentiate between *A. sinapina* and *A. gallica* isolates with the same IGS-1 patterns. The use of restriction enzyme digestion of the IGS region is currently the most used system to distinguish species of *Armillaria* (White et al. 1998).

Sequencing of the Internal Transcribed Spacer (ITS) region has also been used to show species differentiation. Chillali et al. (1998a,b) used the ITS region to differentiate between European species and found that the ITS-2 region showed more variability between the species than in previous work looking at the ITS-1 region. Lochman et al.

(2004) used PCR-RFLP of the ITS rRNA genes to analyze six *Armillaria* species from the Czech Republic (Lochman et al. 2004). They found that restriction enzyme HinfI was able to discriminate between all six species. The attempt to design species-specific primers for six European species was partially successful using variation within the IGS and ITS regions of *Armillaria* isolates. However, due to high similarity between *A. ostoyae* and *A. borealis* and between *A. cepistipes* and *A. gallica*, these species pairs could not be distinguished (Sicoli et al. 2003).

Molecular phylogeny has also been used to examine relatedness of *Armillaria* species. Phylogenetics is an approach that allows one to recognize fungal species based on concordance of multiple gene genealogies and compare them to other methods such as morphology and reproductive behavior. The concept of species in fungi depends heavily on the method that was employed to distinguish the species. Mayden (1997) characterized different species concepts as either operational or theoretical. The only true theoretical species concept was the Evolutionary Species Concept, which defines a species as a single lineage of ancestor-descendent populations that maintain identity from other lineages, thus having its own evolutionary tendencies and historical fate (Wiley 1978). Other species concepts are considered secondary to the Evolutionary Species Concept by Mayden (1997) which include the Morphological Species Concept (based on variation in morphology), Biological Species Concept (based on mating capability), and the Phylogenetic Species Concept (reviewed in Taylor et al. 2000). To date, phylogenetic studies of *Armillaria* species agree with classifications based on the Biological Species Concept. The most studied sequences for *Armillaria* include the ITS and IGS region (Anderson and Stasovski 1992; Chillali et al. 1998; Coetzee et al. 2003, 2005ab; Hanna et

al. 2003, 2004; and Piercey-Normore et al. 1997). From these studies the authors were able to infer a historical framework of species divergence. According to Hanna et al. (2003), the ITS region along with another region [the nuclear large ribosomal subunit (LSU)] were the most promising for evaluating evolutionary relationships among *Armillaria* species, while the IGS-1 region was found to be more useful for revealing intra-specific relations. Hanna et al. (2004) were also able to show genetic variability among western *A. ostoyae* isolates from direct sequencing of these three regions with the strongest variations in the ITS and IGS-1 regions. The next step is to examine other regions and genes within the genome for variation between species. Maphosa et al. (2006) has begun some of this work by studying the relationship of 42 isolates of *Armillaria*, representing the majority of the species, using the elongation factor 1-alpha DNA sequence and found the results similar to previous comparisons using sequences of the ITS and IGS-1 region (Maphosa et al. 2006).

SOMATIC INCOMPATIBILITY

Somatic incompatibility (SI) is referred to by Worrall (1997) as "the prevention of effective fusion and integration following allorecognition (recognition of nonself) between genetically distinct, conspecific tissues when isogenic (self) contacts result in such fusion. 'Somatic' specifies a nonreproductive domain, distinguished the system from sexual incompatibility." This phenomenon is widespread among various organisms including animals, slime molds, plants, and fungi. Somatic incompatibility in fungi can be defined as the mycelial rejection between genetically distinct individuals within the same species, and is used to maintain individuality of mated or secondary mycelium from

genetic exchange. In *Armillaria*, as well as other fungi, when two isolates of secondary mycelium come in contact they are said to be incompatible if anastomose fail and a barrage zone (zone of inhibition) forms between them. The isolates are said to be compatible if the two mycelia merge and anastomoses persist between them. This technique has been used among *Armillaria* and other fungi to identify clonal isolates (reviewed in Worrall 1997).

Before molecular technology, the only other test, besides somatic incompatibility reactions, for identifying genetic individuality were mating-type allele tests. Mating-type allele tests involve four steps as described by Guillaumin et al. (1996): collection of fruiting bodies, single spore isolation of all mating types, identification of the four mating types from single spore isolates, and pairing of mating types with each other, involving 16 different haploid-haploid matings (Guillaumin et al. 1996). These two methods were first used by Korhonen (1978) to look at clonal spread, and later others followed with similar studies using either one or both of the methods (Anderson et. al. 1979b; Berthelay and Guillaumin 1985; Guillaumin and Berthelay 1990; Kile 1983, 1986; Rizzo and Harrington 1993; Thompson 1984; Ullrich and Anderson 1978). Smith et. al. (1990) were among the first to use molecular markers to compare *Armillaria* genets (clones). They compared mating-type allele tests to mitochondrial DNA restriction fragment patterns and found perfect correspondence between the two methods, despite the fact that mating types are encoded by nuclear DNA. Later studies found that correspondence between these two methods is not always the case and that the number of mtDNA types often tends to be lower than the number of genets found by the previous study (Guillaumin et. al. 1996; Smith et al. 1994). Other studies found that using DNA

fingerprinting or analysis of nuclear DNA using random amplified polymorphic DNA (RADP) analysis and microsatellites tend to correspond much better to somatic incompatibility reactions and mating-type allele tests.

Today the fastest and most convenient method, if available, to distinguish between SI groups is through the use of microsatellites, also known as simple sequence repeats (SSRs). These are short repeats of DNA nucleotides usually 20-60 bp in length with tandem repetition of 1-5 base pairs such as $(GT)_n$, $(CA)_n$, $(CAA)_n$, or $(GACA)_n$. These repeats are a common feature in all eukaryotes occurring in as many as 10^5 different locations. However, basidiomycetes rarely contain more than 5% repetitive DNA, unlike the older division Zygomycota which usually contains 30% or more repetitive DNA. Regions with highly repetitive DNA have a high frequency of mutation allowing these regions to be used to differentiate between taxa or an individual within a single species (reviewed in Wöstemeyer and Kreibich 2002). In studying 19 isolates of *A. ostoyae* belonging to six SI groups, Langrell et al. (2001) observed 12 distinct loci harboring a repetitive motif and were able to develop a set of 12 primers to distinguish SI groups. Langrell et. al. (2001) also observed that with the 12 primers they developed there was a high level of cross-species amplification with closely related *Armillaria* species, and suggests this set of primers may be able to explore SI groups across the genus. Lefrancois et al. (2002) later explored this idea with *A. gallica* using six of the original primers developed for *A. ostoyae*, and were able to improve a set of five novel primer pairs for *A. gallica*. Worrall et al. (2004) used two of the di-nucleotide primer sets developed by Langrell (2001) and also developed two primer sets for tri-nucleotide repeats to distinguish clones of *A. ostoyae* in a Colorado campground. Although SSRs

are a fast and easy way to identify individual genets, at this time they still should be backed up with previous methods. With more exploration within species and also across the genus the use of SSRs can be better utilized.

DISTRIBUTION STUDIES

There were few early distribution studies which examined the spread of *Armillaria mellea senso lato* in orchards. In 1925, Hendrickson published a paper on the spread of *Armillaria* in a plum and apricot orchard in California over a period of 23 years. Nearly 66% of the trees died over the course of the study. Due to multiple sources of infection, Hendrickson was unable to determine a linear rate of spread of an individual clone (reviewed in Marsh 1952). In 1952, Marsh published a study of the spread of *Armillaria* in apple (*Malus* spp.) orchards and in a black currant (*Ribes nigrum* L.) planting, concluding that the pattern of spread was primarily due to root to root contact. He also found that the trees killed in the orchard tended to be over 15 years old, half of them were over 30 years old. Marsh also found that the rate of spread tended to be higher in black currants than apples at about 1.8 m per year (Marsh 1952). Rishbeth (1968) observed the rate of spread from various locations in England to be 1.1 m to 1.6 m per year in ash, Norway spruce (*Picea abies* (L.) Karst.), and Douglas-fir (reviewed in Kable 1974). Kable (1974) studied the natural spread of *Armillaria* (*Armillariella*) from a single source in an irrigated peach orchard in Australia. This study suggested that peach is highly susceptible and that the age of the tree did not influence its susceptibility. Spread was found to be at a rate of 0.8 m to 3.2 m per year from growth of rhizomorphs and was found to be more rapid in soil receiving direct furrow irrigation (Kable 1974).

The radial rate of spread for *A. ostoyae* has been estimated by Shaw and Roth (1976) and Peet et al. (1996) at approximately 1 m per year in young conifer stands and 0.22 m per year in a 110 year old Douglas-fir stand.

After *Armillaria mellea senso lato* was divided into a number of new biological species, many distribution surveys were published. Guillamin et al. (1993) compiled over 4000 records of the six European species of *Armillaria* to develop distribution maps. Qualities such as geographical and altitudinal distribution, host range, pathogenicity, dissemination, and ecological roles were also compiled and discussed (Guillamin et al. 1993). In North America, species distribution studies have mostly been conducted in forested areas (Blogdett and Worrall 1992; Harrington and Rizzo 1993; McLaughlin 2001). In New York, Blodgett and Worrall (1992) collected samples of *Armillaria* from 273 forested sites, and examined the host and species relationship for six North American biological species of *Armillaria*. Similar studies conducted by Harrington and Rizzo (1993) identified six species of *Armillaria* from the state of New Hampshire, and in Southern Ontario McLaughlin identified six species of *Armillaria*. The host range in all three studies found that *A. gemina*, *A. calvescens*, *A. mellea*, *A. gallica*, and *A. sinapina* were almost always found on hardwood species, while *A. ostoyae* was the only species predominantly found on conifers. Proffer et al. (1987) assessed the distribution of *Armillaria* species in Michigan tart cherry orchards. In this study, three species of *Armillaria* were found to be vigorous pathogens to orchard grown *Prunus*, including *A. ostoyae*, *A. mellea*, and *A. calvescens*. Although *A. ostoyae* is a principal host to conifer, Proffer et al. (1987) found that it is the predominant pathogen to tart cherry in Leelanau,

Grand Traverse, and Benzie counties where cherry production is greatest and the pathogen is widespread.

Currently many studies are looking at the spread and distribution of species of *Armillaria* within distinct areas. These studies have focused largely on clonal spread in forests and some within orchards (Anderson et al. 1979b; Bendel 2006; Ferguson et al. 2003; Klein-Gebbinck et al. 1991; Lung-Escarmant and Gayon 2004; Prospero et al. 2003; Rizzo et al. 1998; Shaw and Roth 1976, Smith et al.1992; Smith et al. 1994; Worrall 2004). Most of these studies focus on the clonal spread of *A. ostoyae* in coniferous forests or plantations. Klein-Gebbinck et al. (1991) determined the spread of *A. ostoyae* clones in juvenile lodgepole pine (*Pinus contorta* Dougl. ex Loud.) in Alberta Canada. From the two study sites they found nine genets at one site and five genets at another site. The infected trees were aggregated, which was determined using a variance mean ratio of 2.72. They also found that many of the discontinuous patches of trees were infected by a single genet of *Armillaria*. Another study in northern Michigan, examined the clonal spread of *A. ostoyae* in infected red pine (*Pinus resinosa* Aiton) seedlings (Smith et al. 1994). The plantation was planted within a 1.2-ha clearing in a hardwood forest. From this study 22 genets of *A. ostoyae* were found within the plantation and around the plantation vicinity. Smith et al. (1994) concluded that based on the estimates of size and shape, that some of the genets were established at the time of stand conversion, and that the largest clone may predate the existing hardwood forest, which replaced a forest of mostly pine. Rizzo et al. (1998) examined the clonal spread of *A. mellea* in two pear (*Pyrus* spp.) orchards, previously hardwood forests, in California. In this study, Rizzo et al. (1998) discovered four genets at one site with three of the genets

being over 100 m in length. At the second site five genets were found, however these genets were smaller, ranging in sizes from 20 to 60 m in length. The conclusions of this study suggest that based on the size of many of these genets, they were established prior to planting of the orchards, however the size indicates that expansion has occurred since the pears were planted. Another study examined the delineation and biology of genets of three *A. ostoyae*, *A. gemina*, and *A. calvescens* at a mixed conifer-hardwood site and at a spruce-fir site in New Hampshire (Rizzo and Harrington 1993). The results from this study found six genets of *A. ostoyae* at the spruce-fir site, colonizing 34% of the conifers and 13% of the hardwoods. At the mixed conifer-hardwood site *A. ostoyae* and *A. calvescens* were found colonizing both conifers and hardwood hosts, while *A. gemina* was only found colonizing only hardwood hosts. Six *A. ostoyae* genets, two *A. calvescens*, and two *A. gemina* genets were identified using somatic incompatibility tests and isoenzyme analysis. A study, which examined the clonal spread of *A. gallica* (*A. bulbosa*) in the Upper Peninsula of Michigan, found that *Armillaria* was among the world's largest and oldest living organisms. This study found a clone or genet of *A. gallica* to be a minimum of 15 hectares with a growth rate at approximately 0.2 m per year and that had remained genetically stable for over 1,500 years (Smith et al. 1992). The finding from this publication by Smith et al. (1992) was picked up by the popular press and *Armillaria* was referred to as the "humongous fungus" (Volk 2003). Since then a genet of *A. ostoyae* has been found to extend over 37 hectares and was estimated to be 1000 to 2000 years old (Bendel 2006) and in Oregon a genet of *A. ostoyae* was found to be up 965 hectares (Ferguson et al. 2003).

THE HOST RANGE

Armillaria has a broad host range both as a saprophyte and as a pathogen. *Armillaria* species have been reported on conifer and hardwood hosts through out North America, Europe, Asia, South America, Australiasia and Africa (Hood et al. 1991). In North America, *Armillaria* is primarily known to act as a virulent pathogen on conifers in the west, and as an opportunistic pathogen of already stressed hardwood species in the east (Shaw and Roth 1978). Studies on Armillaria root rot in the United States have focused on hardwood species, conifer species, and on fruit trees. In fruit crops grown in the United States, *Armillaria* spp. have been reported on stone fruits (*Prunus* spp.), pome fruits (*Malus* spp. and *Pyrus* spp.), kiwifruit (*Actinidia* spp.), avocado (*Persea* spp.), citrus fruits (*Citrus* spp.), berryfruits (*Ribes* spp. and *Rubus* spp.), fig (*Ficus carica*), guava (*Psidium* spp.), and strawberry (*Fragaria* spp.) (Hood et al. 1991). Within the state of Michigan, saprophytic species and pathogenic species of *Armillaria* have been reported on a variety of forest hosts and crops (Table 1-2).

Cherries, one of the major crops of Michigan agriculture, are severely impacted by Armillaria root rot. Michigan leads the nation in tart cherry production, producing about 80 % of the nation's tart cherries, with 30,800 acres devoted to tart cherry production. In 2001, 297 million pounds of tart cherries were produced, with a total value of production at approximately $44 million (Crop Profile for Tart Cherries in Michigan 2003). Michigan also produces approximately 17% of the nation's sweet cherries (7,400 acres). Leelanau County in Michigan contains approximately 51% of Northwest Michigan's cherry acreage. Michigan has both favorable soil and climatic

Table 1-2: Reported host range of both saprophytic and pathogenic species of *Armillaria* in Michigan: Modified by Kromroy (2004)

Species	NABS[1]	Host	Location Found	Reference
A. calvescens	III	*Prunus cerasus*	Lower Peninsula	Proffer et al. 1987
		Acer saccharum *Betula papyrifera* *Lonicera* spp. *Populus tremuloides* *Quercus rubra* *Quercus veluntina* *Tilia Americana* *Ulmus* spp.	Upper Peninsula	Banik et al. 1995
A. gallica	VII	*Pinus resinosa*	Lower Peninsula, Northern Michigan	Smith et al. 1992
		Abies balsamea *Pinus resinosa* *Pinus strobus* *Acer rubrum* *Acer saccharinum* *Acer saccharum* *Betula papyrifera* *Carya ovata* *Fraxinus pennsylvanica* *Populus tremuloides* *Prunus serotina* *Quercus alba* *Quercus ellipoidalis* *Quercus macrocarpa* *Quercus rubra* *Quercus veluntina* *Ulmus* spp.	Upper Peninsula	Banik et al. 1995
A. mellea	VI	*Prunus cerasus* *Quercus* spp.	Lower Peninsula	Proffer et al. 1987
		Acer saccharum *Quercus* spp.	Upper Peninsula	Banik et al. 1995
A. ostoyae	I	*Prunus cerasus* *Prunus avium* *Prunus persica* *Malus pumila*	Lower Peninsula	Proffer et al. 1987
		Pinus resinosa	Lower Peninsula, Northern Michigan	Smith et al. 1990

Continuation of Table 1-2: Reported host range of both saprophytic and pathogenic species of *Armillaria* in Michigan: Modified by Kromroy (2004)

Species	NABS[1]	Host	Location Found	Reference
A. ostoyae	I	*Acer rubrum* *Betula papyrifera* *Pinus resinosa* *Populus* sp.	Lower Peninsula, Northern Michigna	Smith et al. 1994
		Abies balsamea *Acer rubrum* *Acer saccharum* *Betula alleghaniensis* *Betula papyrifera* *Fraxinus nigra* *Picea banksiana* *Picea glauca* *Pinus resinosa* *Pinus strobus* *Populus tremuloides* *Quercus rubra* *Quercus veluntina* *Thuja occidentalis* *Tsuga Canadensis* *Ulmus* spp.	Upper Peninsula	Banik et. al. 1995
A. sinapina	V	*Populus* sp.	Lower Peninsula, Northern Michigan	Smith et al. 1994
		Abies balsamea *Thuja occidentalis* *Tsuga Canadensis* *Acer rubrum* *Betula alleghaniensis* *Fraxinus nigra* *Populus tremuloides* *Quercus rubra*	Upper Peninsula	Banik et al. 1995

1. NABS = North American Biological Species. See Table 1 for more information on NABS.

conditions for growing cherries. Lake Michigan provides moderate temperatures with frost-free autumns and a delayed bloom period in the spring (Cherries 2006).

Cherries, along with other stone fruits, belong to the genus *Prunus* in the family *Rosaceae* (Zomlefer 1994). There are two species of cultivated cherry: the sweet cherry, *Prunus avium* L., and the sour cherry, *Prunus cerasus* L. Both species originated fromEurope and Southwest Asia. In the United States, the tart cherry industry is virtually a monoculture with 'Montmorency' being the dominant variety grown. However in recent years Morello varieties are also being grown in Michigan. The variety 'Montmorency' is a 400 year old variety from France (Iezzoni 2006).

All commercially grown cherries are grafted. A rootstock makes up the healthy root system to which the upper part of the tree, or scion, is grafted. The rootstock and scion can be different species but must be closely related to be compatible. Rootstocks influence many aspects of the scion's growth and vigor, including productivity, precocity, tree size, tree architecture, fruit size, and fruit quality, and also horticultural decisions such as pruning, training, tree support, and labor management. Cherry rootstocks in Michigan have been selected for precocity, productivity, vigor control, disease tolerance, and adaptability to different soils or climates (Lang 2007). In Michigan, *P. mahaleb* L. (mahaleb) rootstock is the primary rootstock used with tart cherry. Mahaleb rootstock does well in the sandy and acidic soil conditions in which tart cherry trees are usually grown and is also known to keep the trees smaller and easier to manage. Sweet cherry in Michigan is usually grafted onto *P. avium* L. (mazzard) rootstock.

Cherry rootstocks play a role in susceptibility to Armillaria root rot. Proffer et al. (1988) tested a variety of *Prunus* rootstocks including mahaleb, mazzard, and 17 hybrids.

In that inoculation study mahaleb rootstock was more susceptible than mazzard rootstock. Mazzard is however found to be infected with *Armillaria* in the field (Nugent 2005). The tested hybrids were found to be neither immune nor highly resistant to infection (Proffer et al. 1988).

The possible susceptibility of grapevine (*Vitis* spp.) to Armillaria root rot is a new concern of Michigan growers. Michigan is the fourth largest grape producer in the United States, with approximately 14,400 bearing acres. In 2005 over 102,000 tons of grapes were produced. Michigan produces mostly juice grapes, with 'Concord' and 'Niagara' the dominant grape varieties. Although Michigan mostly produces juice grapes, its production of wine grapes is growing, especially in the cherry regions where *Armillaria* is often a problem (Creighton 2007). Leelanau County has 275 acres devoted to grapes (MDA-Grape & Wine 2007) and the Leelanau Peninsula and the Old Mission Peninsula produce 51% of Michigan's wine grapes, while another 45% of wine grapes are grown along the southwest Michigan shoreline and in Fennville (Michigan Wineries 2007). Because of the growing wine industry in Michigan, *Armillaria* may soon be found to be a problem for many Michigan growers who are converting cherry orchards into vineyards.

Although this may be a new concern to Michigan growers, Armillaria root rot has been reported on grape in Australia, Brazil, Central and Eastern Europe, Southeast California, and the West coast of California of the United States. In 1880, Millardet first reported a root rot disease on samples of *Vitis* species from Belgium, Bulgaria, England, France, Germany, Greece, Hungary, Italy, Scotland, Spain, and Switzerland. The causative disease described by Millardet was later identified as *A. mellea* (reviewed in

Aguín-Casal 2004). More recently, Aguín-Casal et al. (2004) reported *A. gallica* and *A. cepistipes* for the first time on *Vitis* spp. in Spain. In California, Baumgartner et al. (2006ab) have begun to test grape rootstock susceptibility to *A. mellea*. Baumgartner tested eight different rootstocks finding rootstocks 3309C and Riparia Gloire, commonly used in Michigan, most susceptible to *A. mellea*, while Freedom (not commonly used in Michigan) was most resistant. Other rootstocks that commonly do well in Michigan soils, that were not tested by Baumgartner, include 5-BB, SO4, 101-14 Mgt (Howell et al. 1999). Eventually these rootstocks as well as the rootstocks tested by Baumgartner should be tested for susceptibility to Michigan isolates of *Armillaria*.

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CHAPTER II

DISTRIBUTION, IDENTIFICATION, AND POPULATION DIVERSITY OF *ARMILLARIA* SPP. IN MICHIGAN CHERRY ORCHARDS

INTRODUCTION

Armillaria root rot is an important, localized disease affecting Montmorency tart cherry and other stone fruit in Michigan. In 1987, Proffer et al. surveyed cherry orchards in the western cherry-producing region of Michigan for *Armillaria* infection. In that study, haploid single basidospore isolates were established, cultured, and identified to species using sexual mating tests with known tester isolates. Using this method, species of *Armillaria* were identified that infected tart cherries in Michigan (Proffer et al. 1987). A new *Armillaria* distribution survey is pertinent because many of the orchards are now out of production, new orchards have been established, and the methodology used was limited by the need for sporulating basidiomes to obtain tester isolates. Modern methods, such as PCR and restriction fragment length polymorphism (RFLP) analysis of the intergenic spacer 1 (IGS-1) region (Banik et al. 1996; Harrington and Wingfield 1995; Kim et al. 2000; Sierra et al. 1999; White et al. 1998), have removed the reliance on basidiomes, and isolates can now be identified to species with mycelium, rhizomorphs, and basidiomes. This is important because some species of *Armillaria* causing infection may have been missed in the 1987 survey if they did not produce mushrooms that year. The increased flexibility of newer PCR based identification methods and the need to examine orchard sites currently reporting losses to *Armillaria* support the need for a follow up survey.

Many studies in other regions have looked at the distribution of species and the clonal spread of *Armillaria* both in orchards and in forests stands (Anderson et. al. 1979; Banik et al. 1995, 1996; Bendel 2006; Berthelay and Guillaumin 1985; Blogdett and Worrall 1992; Guillamin et al. 1993, 1996; Guillaumin and Berthelay 1990; Harrington

and Rizzo 1993; Kable 1974; Kile 1983, 1986; Klein-Gebbinck et al. 1991; Kromroy 2004; Lung-Escarmant and Gayon 2004; Marsh 1952; McLaughlin 2001; Proffer et al. 1987; Prospero et al. 2003; Harrington and Rizzo 1993; Rizzo et al. 1998; Shaw and Roth 1976; Smith et al. 1992, 1994; Thompson 1984; Ullrich and Anderson 1978; and Worrall 2004). In North America, species distribution studies have mostly been conducted in forested areas (Blogdett and Worrall 1992; Harrington and Rizzo 1993; McLaughlin 2001). The host range in all three studies found that *A. gemina*, *A. calvescens*, *A. mellea*, *A. gallica*, and *A. sinapina* were almost always found on hardwood species, while *A. ostoyae* was the only species predominantly found on conifers. Proffer et al. (1987) assessed the distribution of *Armillaria* species in Michigan tart cherry orchards. In this study, three species of *Armillaria* were found to be vigorous pathogens to orchard grown *Prunus*, including *A. ostoyae*, *A. mellea*, and *A. calvescens*. Although *A. ostoyae* is a principal host to conifer, Proffer et al. (1987) found that it is the predominant pathogen to tart cherry in Leelanau, Grand Traverse, and Benzie counties where cherry production is greatest and the pathogen is widespread. Many of the clonal studies have focused on the spread of *A. ostoyae* in coniferous forests or pine plantations, finding the genets at a platation site often pre-date the plantation and were established from the existing forest before conversion (Klein-Gebbinck et al. 1991; Lung-Escarmant and Gayon 2004; Prospero et al. 2003; Smith et al. 1994; Worrall 2004). The study by Smith et al. (1994) examining infected red pine seedlings in Michigan, concluded that based on the estimates of size and shape, some of the genets were established prior to the time of stand conversion, and that the largest clone may predate the existing hardwood forest, which replaced a forest of mostly pine. Rizzo et al. (1998) examined the clonal spread of *A.*

mellea in two pear orchards, previously hardwood forests, in California. In this study, Rizzo et al. (1998) discovered four genets at one site with three of the genets being over 100 m in length. At the second site five genets were found, however these genets were smaller, ranging in sizes from 20 to 60 m in length. The conclusions of this study suggest that based on the size of many of these genets, they were established prior to planting of the orchards, however the size indicates that expansion has occurred since the pears were planted. Another study examined the delineation and biology of genets of three *A. ostoyae*, *A. gemina*, and *A. calvescens* at a mixed conifer-hardwood site and at a spruce-fir site in New Hampshire (Rizzo and Harrington 1993). The results from this study found six genets of *A. ostoyae* at the spruce-fir site, colonizing 34% of the conifers and 13% of the hardwoods. At the mixed conifer-hardwood site *A. ostoyae* and *A. calvescens* were found colonizing both conifers and hardwood hosts, while *A. gemina* was only found colonizing only hardwood hosts. Six *A. ostoyae* genets, two *A. calvescens*, and two *A. gemina* genets were identified using somatic incompatibility tests and isoenzyme analysis. Revisiting species distribution and examining clonal spread would be the next step to understanding the Michigan populations of *Armillaria* currently in the cherry orchards. Somatic incompatibility tests are the most used method to identify individual clones of *Armillaria*, as seen in the many publications (Anderson et al. 1979b; Bendel 2006; Ferguson et al. 2003; Klein-Gebbinck et al. 1991; Lung-Escarmant and Gayon 2004; Prospero et al. 2003; Rizzo et al. 1998; Shaw and Roth 1976, Smith et al.1992; Smith et al. 1994; Worrall 2004). This method involves pairing test isolates on culture media and examining the visible interaction of the expanding clones as they make contact. The formation of a barrage zone along the line of contact between isolates

indicates the isolates are different clones. Mating-type allele tests may also be used to distinguish between individual clones. However, this method may only be used with the single spore isolates of all mating types. Thus, unless all of the individual clones at the site produce mushroom, this technique is not useful. Molecular techniques have also been used to identify clones. Analysis of nuclear DNA using random amplified polymorphic DNA (RADP) analysis and microsatellites also known as simple sequence repeats (SSRs) correspond well with somatic incompatibility reactions and mating-type allele tests to show genetic distinction between clones. Langrell et al. (2001) developed a set of 12 primers for di-nucleotide repeats that could be used to distinguish different somatic incompatibility groups of *Armillaria ostoyae* isolates. Worrall et al. (2004) used two of the di-nucleotide primer sets developed by Langrell (2001) and also developed two primer sets for tri-nucleotide repeats to distinguish clones of *A. ostoyae* in a Colorado campground. Although the use of SSRs provides a fast and easy way to identify individual clones, they still should be backed up with methods such as somatic incompatibility tests.

The hypotheses of this research were: (1) that new species of *Armillaria* would be identified on cherry using molecular identification techniques not reliant on basidiomes as in the 1987 survey by Proffer et al. (1987); and (2) the diversity of species in an orchard may be much greater than previously reported. To test these hypotheses a broad distribution survey of orchards in the cherry producing region of Michigan was done, as well as a clonal mapping of *Armillaria* within two Montmorency tart cherry orchards using somatic incompatibility tests and microsatellite markers.

MATERIAL AND METHODS:
SURVEY AND FIELD SAMPLING

Fruit orchards and hardwood deciduous forests in 14 Michigan counties were examined for the presence of *Armillaria* spp. in 2005 and 2006 (Table 2-1). In forested areas, fallen logs and stumps were examined for signs of rhizomorphs and mycelium. In orchards, the early defoliation of trees, circular pockets of dead and declining trees in the orchard, and mushrooms characteristic of *Armillaria* at the base of a tree or stump were used to identify potential *Armillaria* sites. Trees or stumps suspected to be infected with *Armillaria* were examined by digging approximately 0.5 m around the base. When present, samples of mycelium and/or rhizomorphs under bark were collected by breaking off infected tissue with a shovel. The samples were transferred to plastic bags, placed on ice, and taken back to the laboratory for processing. The type of trees samples were collected from at the site and the GPS coordinates were recorded when possible.

Samples of unexposed mycelium under the bark were removed by cutting into the bark with a sterile scalpel to expose mycelium and then small pieces, approximately 2-4 mm in diameter of the freshly exposed mycelium were removed using sterile forceps for plating on agar medium. Rhizomorphs were cut into 1-2 cm pieces and surface disinfected in a wash of 10% bleach for 3 min, followed by 30% hydrogen peroxide for 2 min, and a final rinse in sterile deionized water for three min. The samples were blotted on sterile filter paper before being plated. Samples were plated on 2% malt extract agar (MEA) (20g/l of malt extract and 20g/l of agar) or 2% water agar. The cultures were kept in the dark at 25° C. The culture plates were checked daily for signs of contamination from other microbes. If plates were contaminated, transfers were made from the *Armillaria* samples that appeared non-contaminated to a clean 2% MEA plate.

Table 2-1: Location, date, and description of sites surveyed for Armillaria root rot

Site #	County	GPS	Date collected	Host
1	Oceana	N/A	05-19-05	Tart Cherry
2	Ingham	N 42° 43 W 084° 28	06-02-05 06-08-05 06-16-05 06-23-05	Hardwood Forest (Beech, Oak, Maple, and Black Cherry)
3	Leelanau	N 44° 50 W 085° 39	09-09-05	Peach
4	Van Buren	N 42° 19 W 086° 02	10-07-05	Plum
5	Berrien	N 42° 08 W 086° 15	10-07-05	Tart Cherry
6	Berrien	N 42° 06 W 086° 17	10-07-05	Apple
7	Leelanau	N 44° 55 W 085° 39	10-14-05	Tart Cherry
8	Leelanau	N 44° 56 W 085° 39	10-14-05	Tart Cherry
9	Leelanau	N 44° 56 W 085° 37	10-14-05	Tart Cherry
10	Leelanau	N 44° 56 W 085° 36	10-14-05	Tart Cherry
11	Leelanau	N 44° 57 W 085° 36	10-14-05	Tart Cherry
12	Leelanau	N 45° 03 W 085° 34	10-14-05	Tart Cherry
13	Leelanau	N 44° 59 W 085° 36	10-14-05	Tart Cherry
14	Grand Traverse	N 44° 46 W 085° 44	10-18-05	Tart Cherry
15	Leelanau	N 44° 46 W 085° 44	10-18-05	Tart Cherry
16	Benzie	N 44° 39 W 086° 04	10-18-05	Tart Cherry
17	Benzie	N 44° 31 W 086° 07	10-18-05	Peach Tart Cherry Sweet Cherry

Continuation of Table 2-1: Location, date, and description of sites surveyed for Armillaria root rot

Site #	County	GPS	Date collected	Host
18	Leelanau	N 45° 01 W 085° 38	10-18-05	Apple Tart Cherry
19	Kent	N 43° 09 W 085° 46	10-25-05	Apple
20	Kent	N 43° 07 W 085° 48	10-25-05	Peach
21	Kent	N 43° 15 W 085° 46	10-25-05	Tart Cherry
22	Clinton	N/A	11-01-05	Maple, Oak, and Elm
23	Ionia	N/A	11-01-05	Hardwood Forest (Beech, Elm, Maple)
24	Grand Traverse	N 44° 40 W 085° 35	11-11-05	Tart Cherry
25	Antrim	N 44° 52 W 085° 25	11-11-05	Tart Cherry
26	Antrim	N 44° 56 W 085° 21	11-11-05	Tart Cherry
27	Antrim	N 44° 56 W 085° 21	11-11-05	Tart Cherry
28	Antrim	N 45° 00 W 085° 21	11-11-05	Tart Cherry
29	Leelanau	N 44° 57 W 085° 36	11-11-05	Tart Cherry
30	Manistee	N 44° 19 W 086° 14	06-07-06	Apple Sweet Cherry
31	Oceana	N/A	06-22-06	Tart Cherry Plum
32	Oceana	N/A	11-04-06	Tart Cherry
33	Montcalm	N/A	08-04-06	Frasier Fir
34	Oceana	N/A	11-04-06	Tart Cherry
35	Oceana	N/A	11-04-06	Sweet Cherry
36	Tuscola	N/A	10-08-06	Hardwood Forest

This process was repeated until pure cultures were obtained. Pure cultures were transferred to 3% MEA and were maintained in an incubator at 25° C in the dark.

During the summer of 2006, intensive sampling was completed in two orchards in Grand Traverse and Leelanau counties. This was done to examine clonal spread within Michigan orchards. The two orchards were chosen because they were cleared forested areas that were planted to Montmorency tart cherry grafted on mahaleb rootstock. The first orchard (site # 14 from Table 2-1) was approximately 30 years old and was located along the Leelanau and Grand Traverse county line. The trees in the orchard were planted 6x6 m apart. The orchard was split into four sections by a single row of poplar (*Populus*) trees, with 13 -15 rows per section. Within each section there were circular pockets where trees had been removed due to infection from *Armillaria*. There was a mixed maple (*Acer*) forest along the south side of the orchard. All 913 trees in the orchard were examined for the presence of infection from *Armillaria*. Trees 5 m into the surrounding forest were also examined for the presence of infection from *Armillaria*. Samples were collected and processed as described above.

The second orchard (site # 29 from Table 2-1) surveyed was 30 years old and located on the Old Mission Peninsula. The trees were planted 3 x 5.4 m apart and the last 14 rows were planted 2.7 x 5.4 m apart. The orchard was divided into four sections, which was divided by a main gravel road running east to west and the two sections were divided by a smaller grass path running north to south. The first two sections to the south of the main gravel road had 16 rows, while the second two sections to the north had 28 rows. There were approximately 50 to 150 trees per row depending on where the row started and on how many trees had been removed due to Armillaria root rot or other

causes such as injury. Forest containing mostly beech (*Fagus grandifolia* Ehrh.) and maple surrounded the south and west sides of the orchard. The second orchard also had circular pockets where trees had been removed due to *Armillaria* infection. Approximately 50 to 60 trees were lost annually in this orchard to *Armillaria* infection according to the grower. All symptomatic trees, trees adjacent to infected trees, and some asymptomatic trees, totaling approximately 25% of the 4,339 trees in the orchard, were examined for the presence of *Armillaria*. Trees 5 m into the surrounding forest were also examined for the presence of infection by *Armillaria*. Samples were collected and processed as described above for the survey.

SPECIES IDENTIFICATION

DNA was extracted from the isolates using one of two methods. For immediate use, DNA was extracted by scraping a forcep across the edge of a mycelial culture growing on 3% MEA to collect a 1-2 mm^2 piece of mycelium. The tip of the forcep with the mycelium was then inserted in to a 1.5 ml microcentrifuge tube containing 100 µl 5X Tris-Borate (TBE) lysis buffer (54g Tris-base, 27.5g boric acid, 20ml 0.5M EDTA [pH 8.0]. The DNA sample was then boiled for 10 min at 100° C. DNA that was going to be saved for later analysis was isolated by taking 100 mg fresh weight mycelium from a culture growing on 3% MEA. The mycelium was placed in a 1.5 ml microcentrifuge tube with two sterilized 3 mm glass beads. The sample was placed in liquid nitrogen for two minutes, and then macerated using a FastPrep FP120 Homogenizer (Qbiogene Inc., Carlsbad, CA 92008) at a speed of 4.5 for 40 s. The last two steps were repeated two-three times or until samples looked completely macerated. The lysis buffer AP1 and the

RNase A stock solution from the Qiagen Dneasy® Plant DNA kit (Qiagen Sciences, Maryland 20874) were added to the macerated sample and homogenized using the FastPrep FP120 Homogenizer at a speed of 4.5 for 40 s. DNA was then extracted using the Qiagen Dneasy® Plant DNA kit following the manufactures instructions after the addition of the AP1 buffer and RNase A stock solution. A modification was made at the final step, where 50 µL of the elution buffer AE (10 mM Tris-Cl , 0.5 mM EDTA; pH 9.0) was added instead of 100 µL to increase the final DNA concentration. The elution buffer AE was also heated to 65° C before being added to help the long genomic DNA release from the silica membrane. The two final elutions were combined together in the same tube. DNA was stored at 4° C.

Established PCR and RFLP protocols for the analysis of the intergenic spacer 1 (IGS-1) region (Harrington and Wingfield 1995) were used for species determination. The primers used to amplify this region were LR12R (Veldman et al. 1981), and O-1 (Duchesne and Anderson 1990), as first recommended by Anderson and Stasovski (1992). The PCR reaction mixture was modified and included 2.5U of Taq polymerase, 1% 10X PCR buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs (Invitrogen, Carlsbad, California 92008), and 0.5 µM of each primer (Integrated DNA Technologies Inc., Coralville, Iowa 52241). DNA was diluted to a 1:10 ratio and 2 µl were added to the reaction mixture for a final reaction volume of 50 µl. The thermocycler used was a GeneAmp® PCR system 2720(Applied Biosystems, Foster City, CA 94404) and the conditions used were the same as described by Harrington and Wingfield (1995).

PCR-amplified DNA was first digested with the restriction enzyme AluI, and subsequently with BsmI and NdeI, as required (New England Biolabs, Ipswich, MA

01938-2723). The reaction mixture included 2.5 µl of the appropriate buffer supplied with the enzyme, 2.0 µl of deionized sterile water, and 0.75 µl of the enzyme. Amplified DNA (12 µl) was added to the reaction mixture for a final reaction volume of 17.25 µl and allowed to digest for 4-16 h at 37 C° for AluI and NdeI and 65 C° for BsmI.

Amplified DNA and the restriction enzyme fragments of these products were separated by electrophoreses in 1-2.5% agarose gels in 0.5X TBE buffer to determine the size of amplification and restriction products. For analysis of the amplified DNA, 2µl of loading dye (0.25% Bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia in water, for a total volume of 50ml) was added to the 12 µl PCR product and 12 µl of this mixture was loaded onto a 1% agarose gel run at 100 V for 1.5 h. For the restriction products, 2 µl of loading dye was added to the product and 15 µl of this mixture was loaded to a 2.5% agarose gel run at 60 V for 2.5 h. The banding patterns of the tested isolates were compared to those of eight different tester isolates of North American Biological Species (Table 2-2). Fragments longer than 100 bp were scored both visually and from sequenced isolates using the NEBcutter V2.0 (New England Biolabs, Ipswich, MA 01938-2723).

Some isolates could not be identified to species using the RFLP protocol of Harrington and Wingfield (1995). *Armillaria clavescens* and *A. gallica* can have the same AluI banding patterns making them indistinguishable from one another. *A. ostoyae* and *A. gemina* also have the same AluI banding patterns but can usually be distinguished from each other using different restriction enzymes such as NdeI or BsmI which have been shown to digest only the PCR product for *A. ostoyae* isolates. A mutation at these sites would make the species indistinguishable from one another. Some isolates were

identified to species using a modified version of the sexual mating tests described by Korhonen (1978). The sexual mating test involves pairing of haploid isolates with known haploid testers on culture media, which are then examined visually for the conversion of the haploid isolates to diploid form due to sexual compatibility. This test has also been found to work by pairing diploid isolates converting the haploid testers when sexually compatible. Using this modified technique, the species of diploid isolates, that could not be distinguished using the RFLP protocol, was determined. Plugs (5x5 mm) of the unidentified isolates were placed 10 mm apart from a plug (5x5 mm) of a tester isolate on a 60 mm by 15mm Petri dish containing 4% MEA amended with 20 g/l glucose and 5 g/l peptone. Four replications for each tester isolate and unidentified isolate were plated. Isolates were allowed to grow for four weeks in the dark at room temperature and then observed for the conversion of the haploid tester to the diploid form.

Table 2-2: Tester strains used as controls to compare and identify Michigan isolates of *Armillaria* to species

Name of Strain*	NABS**	Catalog Number	Depositor	Location Obtained From
Armillariella mellea 28-4 (A1B1)	NABS I	44396	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 28-6 (A1B2)	NABS I	44399	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 28-7 (A2B1)	NABS I	44401	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 28-9 (A2B2)	NABS I	44403	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 160-8	NABS II	52122	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 160-9	NABS II	52123	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 11-1	NABS III	52580	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 11-9	NABS III	52099	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 21-2	NABS III	52100	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 48-1	NABS V	52104	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 48-6	NABS V	52105	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 49-5	NABS VI	52106	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 49-8	NABS VI	52582	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 97-1	NABS VI	52111	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 90-4	NABS VII	52109	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 90-10	NABS VII	52110	JB Anderson	ATCC, Manassas, VA 20108

Continuation of Table 2-2: Tester strains used as controls to compare and identify Michigan isolates of *Armillaria* to species

Name of Strain	Species	Catalog Number	Depositor	Location Obtained From
Armillariella mellea 1-137	NABS VII	52114	JB Anderson	ATCC, Manassas, VA 20108
Armillariella mellea 121-2	NABS IX	52113	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 139-1	NABS IX	52116	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 140-6	NABS X	52119	AL Jones, TJ Proffer	Michigan State University
Armillariella mellea 140-9	NABS X	52121	AL Jones, TJ Proffer	Michigan State University
Scytinostroma galaectinum Smith T1	-	-	TJ Proffer	Michigan State University
Polyporus squamosis	-	-	TJ Proffer	Michigan State University
Schizophllum commune	-	-	TJ Proffer	Michigan State University

* *Armillariella* is a synonym for *Armillaria*. See the Taxonomy section in Chapter I for an explanation.
** NABS = North American Biological Species

DNA SEQUENCING OF THE IGS-1 REGIONS

A group of isolates were selected, representing all of the distinctive RFLP patterns, for sequencing. This is because the banding sizes from the restriction digests did not always add up to the proper fragment size for the IGS-1 region. This was also important for the isolates in the *A. ostoyae/A. gemina* group using the AluI restriction digest. DNA fragments representing all of the AluI, BsmI, and NdeI banding patterns were sequenced and compared with one another as well as with sequenced strains on Genbank. The PCR reaction mixture and the thermocycling conditions were the same as for species identification with the exceptions of the final reaction volume which was increased to 100 µl. Loading dye (3µl) was added to the entire PCR product. The entire PCR product, 20 µl per well (five wells), was loaded onto a 1% agarose gels which were run at 100 V for 1.5 h. The DNA was then extracted from the gel using a QIAquick® Gel Extraction Kit (Qiagen Sciences, Maryland 20874) following the instructions of the manufacturer. DNA sequencing was performed at the Research Technology Support Facility (RTSF) at Michigan State University. Sequences using direct PCR and those obtained from GenBank were analyzed both manually and using Lasergene® version 6 software (DNASTAR Inc., Madison, WI 53705).

SOMATIC INCOMPATIBILITY TESTS

The genet identification of isolates collected from the two orchard sites in the clonal study was determined using somatic incompatibility tests. Plugs (5x5 mm) of two different unidentified isolates were plated approximately 10 mm from each other on a 60 mm by 15mm Petri dish containing 4% MEA amended with 20 g/l glucose and 5 g/l

peptone. Isolates were then allowed to grow for six to eight weeks in the dark at 25 °C. After six to eight weeks the cultures were observed for the presence or absence of a barrage zone to determine the somatic incompatibility (SI) group of each isolate. For the first orchard, two rounds of pairings were done in order to determine the SI group of each isolate. Since the second orchard had more isolates, three rounds of pairings were needed to determine the SI group of each isolate.

Orchard A:

Isolates were paired in all possible combinations. In addition, self-crosses were included as controls. There were four replications (four plates) for each cross. After this first round of pairings, the SI group for each isolate could be determined; however, a second round of parings was used to confirm the results from the first round. In this second round, a representative isolate from an SI group identified in the first round was paired with another representative isolate from all other SI groups. Also, each isolate within an SI group was crossed with the other isolates within the same SI group to reconfirm compatibility. Self-crosses were again done as controls and three replications (three plates) were done for each cross in the second round.

Orchard B:

Because of the greater number of isolates collected from the second orchard, the isolates were divided into four groups of 20-21 isolates. In the first round of pairings, all possible crosses within a group were done, as well self-crosses as controls. There were four replications (four plates) for each cross. In the second round of pairings, representative isolates from each SI group found in one of the four groups were crossed with representative isolates from SI group in the three other groups. Self-crosses of each

representative isolate were also done as controls. In the second round of pairings, there were four replications (four plates) for each cross. After the two rounds of pairings were completed the SI group for each isolate could be determined. However, a third round of pairings was done to verify the results in the same way that the second round of pairings was done for the first orchard.

MICROSATTELITE ANALYSIS

Somatic incompatibility groups identified for the *A. ostoyae* isolates in both orchards, and *A. gemina* isolates in orchard B (site # 29) were confirmed to be genetically distinct using published microsatellite markers, specifically designed for *A. ostoyae* (Langrell et al. 2001; and Worrall et al. 2004) (Table 2-3). Two representative isolates from each SI group in orchard A (site # 14), one representative isolate for each SI group in orchard B (site # 29), and seven isolates from different orchards were used with the microsatellite markers to confirm that the *A. ostoyae* SI groups were genetically distinct from each other and to show that the two representative isolates in the same SI group had the same alleles. In addition, the seven other isolates of *A. ostoyae* from other sites were tested to see if the microsatellite markers would pick up global diversity between isolates. The DNA used for the microsatellites was extracted using the second method described utilizing the Qiagen Dneasy® Plant DNA kit. The DNA was diluted to a 1:10 concentration. The PCR reaction mixture was the same as previously described. The thermocycling conditions were the same as described by Langrell et al. (2001) with the annealing temperature ranging from 53-58° C depending on the fluorescence labeled primers (Applied Biosystems, Foster City, CA 94404). The primers were labeled with 6-

FAM or HEX at the 5'-end of the forward primer (Table 2-3). The PCR products for each primer set were purified using a QIAquick® PCR Purification Kit (Qiagen Sciences, Maryland 20874) following manufacture instructions, and then the products from each set of primers (5 μl) were combined for an isolate (20 μl final volume). Samples were submitted to the Research Technology Support Facility (RTSF) at Michigan State University for analyses via the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA 94404) and GeneScan® Analysis Software Version 3.7 (Applied Biosystems, Foster City, CA 94404) to identify the sizes of each of the four loci.

Table 2-3: Microsatellite markers used to genetically confirm SIG's from the two intensively sampled orchards

Locus	Primer Sequences (5'-3') and Label	Observed Range (bp)	EMBL No.	Source
CAG25	F: 6FAM-CATGACGCCACGGATACCA	356-359		Worrall et
	R: TCGCTGACATGTGCCGAGG			al. (2004)
CAG77	F: HEX-AGGCTGGCCGAATAGTGAAT	328-349		Worrall et
	R: CTGATCTGTGACCTCAAGCA			al. (2004)
AOSSR74	F: 6FAM-GCTCACCCTCAAACTTAACA	96-114	AJ307595	Langrell et
	R: GCAGGGCACAAATGAAACTA			al. (2001)
AOSSR84	F: HEX-ACACCACGAGTGCTTCTACTA	128-150	AJ307598	Langrell et
	R: GCT TGG TAA TGG GCA GAG			al. (2001)

RESULTS:
SPECIES IDENTIFICATION AND DISTRIBUTION

Amplification of the IGS-1 region and restriction fragments of this region were obtained for all isolates. The amplified product for the IGS-1 region was 875 bp for all *A. mellea* isolates and 920 bp for all other isolates. The amplified product from all isolates was first digested with AluI, and subsequently with NdeI and BsmI as required. The size of the resulting fragments was determined using electrophoresis and by comparison with results based on sequenced isolates using the NEBcutter V2.0 (New England Biolabs, Ipswich, MA 01938-2723). Using the restriction enzyme AluI, a total of three patterns were obtained from fragments longer than 100 bp, all of which corresponded with previously published patterns (Harrington and Wingfield 1995; White et al. 1998; Kim et al. 2000) (Fig. 2-1; Appendix A: Table A-1). From 18 isolates the PCR products yielded fragments of approximately 476 bp and 175 bp, corresponding to *A. mellea* RFLP pattern A, as designated by Harrington and Wingfield (1995). From these 18 isolates, the PCR products from five isolates belonging to the same SI group from orchard B (site # 29), yielded a strong fragment at approximately 240 bp. This band was often observed as a very faint fragment for the PCR products of other isolates identified as *A. mellea*. The fragment of 240 bp was due to partial digestion of the PCR product and was only observed when running the digested PCR product on agarose gels. Based on the sequenced data for these isolates, the restriction sites when cut should only produce fragments of 476 bp and 175 bp. The amount of restriction enzyme used in the reaction was unable to resolve the partial digestion of the PCR products for these isolates. There were 104 isolates whose PCR products yielded fragments of approximately 308 bp, 200 bp, and 135 bp. This pattern placed them into either *A. ostoyae* or *A. gemina*, as

designated by Harrington and Wingfield (1995). From 36 isolates, the PCR products yielded fragments at approximately 583 bp and 240 bp, identifying these isolates as either *A. calvescens* or *A. gallica*, as designated by Harrington and Wingfield (1995). Out of the 36 isolates, the PCR products of 15 isolates also yielded fragments at approximately 400 bp and 200 bp. The fragments of 476 bp and 175 bp were due to partial digestion of the PCR product observed when running the digested PCR product on agarose gels. Based on the sequenced data for these isolates the restriction sites only should produce fragments of 583 bp and 240 bp. The amount of restriction enzyme used in the reaction was unable to resolve the partial digestion of the PCR products for these isolates.

Isolates placed into the *A. ostoyae/A. gemina* group by AluI, are usually distinguished from one another by the restriction enzymes NdeI. The PCR product of most *A. ostoyae* isolates yield fragments of approximately 550 bp and 370 bp (Fig. 2-2; Appendix A: Table A-1). The digestion of the PCR products for *A. gemina* tester isolates and previous published reports indicate that no digestion with this enzyme occurred for this particular species accept in one rare case (Kim et al 2000). The PCR product of isolates of *A. ostoyae* rarely has not digested with this enzyme (Harrington and Wingfield 1995; Kim et al. 2000). The PCR products of 33 isolates from a total of eight SI groups from orchard B (site # 29) and two isolates from site # 35 in the *A. ostoyae/A. gemina* AluI group did not digest with NdeI. The PCR products of isolates with the AluI banding patterns for *A. mellea* pattern A and *A.calvescens/A. gallica* also did not digest with this enzyme.

Because the PCR product of some isolates identified as either *A. ostoyae* or *A. gemina* did not digest with NdeI, the restriction enzyme BsmI was used to try and

identify the isolates to species. The PCR products of isolates of *A. ostoyae* have been reported to digest with this enzyme, whereas *A. gemina* does not (Harrington and Wingfield 1995). The digestion of the PCR products with BsmI yielded products at approximately 620 bp and 300 bp, which distinguished the isolates as *A. ostoyae*. The PCR product of 20 of the isolates belonging to four SI groups from orchard B (site # 29) and the two isolates from site # 35 that did not digest with NdeI also did not digest with BsmI confirming their identification as *A. gemina*. The PCR products of two isolates from one SI group from orchard B (site # 29) that digested with NdeI, did not digest with BsmI. All PCR product for isolates with the AluI fragment pattern for *A. mellea* pattern A digested with BsmI yielding fragments at approximately 550 bp and 325 bp (Fig. 2-3; Appendix A: Table A-1). Isolates in the *A. calvescens/A. gallica* group based on AluI did not digest with BsmI.

While AluI can be used to distinguish isolates in the *A. calvescens/A. gallica* group, no restriction enzymes are reported to distinguish between these two species. Sexual compatibility tests still must be performed to identify these isolates to species. Sexual compatibility tests were performed for all isolates from the distribution survey in the *A. calvescens/A. gallica* group, except for orchard B (site # 29), where representatives isolate from each of the three SI groups in this group was tested. Based on the crosses all of these isolates were identified as *A. gallica*. The tester strain obtained from ATCC, *Armillariella mellea* 90-4 (NABS VII) was the source of confirmation and gave the best results (Fig. 2-4, 2-5; Appendix A: Table A-2).

Sexual compatibility tests were done for a representative isolate from each of the 15 SI groups whose PCR product yielded the AluI banding pattern for *A. ostoyae/ A.*

gemina from orchard B (site # 29), and both isolates from site #35 whose PCR product did not digest with NdeI and/or BsmI. The sexual mating tests confirmed that each representative isolate whose PCR product digested with NdeI and/or BsmI from orchard B (site # 29) to be *A. ostoyae*. The tester strains obtained from ATCC, *Armillariella mellea* 28-6 (NABS I) and *Armillariella mellea* 28-4 (NABS I) gave the best results. Some of the *A. ostoyae* isolates did not convert the tester *Armillariella mellea* 28-6, most likely due to incompatible mating types. However, although the tester strain *Armillariella mellea* 28-4 (NABS I) partially converted to the diploid form with out being mated the conversion to the diploid form was stronger with the isolates that did not convert with *Armillariella mellea* 28-6. The results for the other isolates whose PCR product did not digest with both NdeI and BsmI were identified as *A. gemina*. The tester strain, *Armillariella mellea* 160-9 (NABS II) was the source of confirmation and gave the best results. (Fig. 2-4, 2-5; Appendix A: Table A-3).

Sequencing of the IGS-1 region was also done for representative isolates with different AluI, NdeI, and BsmI banding patterns to help identify species and determine fragment sizes from the restriction digests. All sequenced isolates were compared with sequences on GenBank. Based on the comparison with strains of *Armillaria* submitted to GenBank, isolates that were identified as *A. mellea* were 99% homologous with other *A. mellea* isolates on GenBank compared to other species where some *A. tabescens* isolates were 95% homologous, and some *A. ostoyae* and *A. gallica* isolates were 94% homologous. Isolates identified as *A. ostoyae* that digested with both NdeI and BsmI were 99% homologous with other *A. ostoyae* isolates and 98% homologous with some *A. gemina* isolates on GenBank, whereas isolates that did not digest with NdeI or BsmI were

99% homologous with some *A. gemina* isolates and 98% homologous with some *A. ostoyae* isolates. Isolates of *A. ostoyae* that digested with either BsmI or NdeI were 99% homologous to isolates of *A. ostoyae* and *A. gemina.* Isolates identified as *A. gallica* were 99% homologous with other *A. gallica* isolates and 98% homologous with some *A. clavescens* isolates on GenBank.

This new PCR based survey of *Armillaria* species found in Michigan's cherry regions included new areas not surveyed by Proffer et al. (1987). The new orchard areas surveyed included: the orchard ridge area in Kent County, and Van Buren and Berrien Counties. Forested areas were surveyed in Clinton, Ionia, Ingham, and Tuscola Counties, and a sample from a Christmas tree plantation in Montcalm County was added to the survey. *A. ostoyae* was the most predominant species found infecting tart cherry and other stone fruit trees in this survey, especially in the Northwest region. *Armillaria mellea* was found infecting cherry trees in Berrien County and occasionally Oceana, and Leelanau Counties. *Armillaria gallica* was found on a tart cherry tree in Leelanau County and on two plum trees in Van Buren County. *A. gallica* is often noted to act as a saprophyte in the forest on dead or declining trees, stumps, fallen logs, or in the soil. However, based on field observations in this survey it appears capable of initiating disease in orchard grown *Prunus* spp. *Armillaria gemina*, never before reported in Michigan, appeared to be pathogenic on *Prunus* spp. in Leelanau County on tart cherry and also on sweet cherry in Oceana County (Table 2-4; Fig. 2-6).

Table 2-4: Number of isolates collected from each county, the host, and species distribution of *Armillaria* spp. in the cherry producing regions of Michigan

County	Host	Number of Isolates	Species
Antrim	Tart Cherry	7	*A. ostoyae*
Benzie	Tart Cherry	4	*A. ostoyae*
	Sweet Cherry	1	*A. ostoyae*
	Peach	1	*A. ostoyae*
Grand Traverse	Tart Cherry	22	*A. ostoyae*
Leelanau	Tart Cherry	38	*A. ostoyae*
		20	*A. gemina*
		14	*A. mellea*
		1	*A. gallica*
	Peach	1	*A. ostoyae*
	Beech	1	*A. ostoyae*
		2	*A. gallica*
	Maple	1	*A. gallica*
	Fallen Log	16	*A. gallica*
Manistee	Sweet Cherry	2	*A. ostoyae*
Oceana	Tart Cherry	4	*A. ostoyae*
		1	*A. mellea*
	Sweet Cherry	2	*A. gemina*
	Plum	1	*A. mellea*
Montcalm	Douglas Fir	1	*A. ostoyae*
Clinton	Fallen Log	2	*A. gallica*
Ingham	Fallen Log	6	*A. gallica*
Ionia	Fallen Log	5	*A. gallica*
Kent	Apple	0	
	Peach	0	
	Tart Cherry	0	

Continuation of Table 2-4: Number of isolates collected from each county, the host, and species distribution of *Armillaria* spp. in the cherry producing regions of Michigan

County	Host	Number of Isolates	Species
Berrien	Tart Cherry	2	*A. mellea*
	Apple	0	
Van Buren	Plum	1	*A. gallica*
Tuscola	Fallen Log	2	*A. gallica*

See Appendix 1 for complete data for all isolates including; site, tree type, banding size for the IGS-1 region, RFLP fragment sizes for AluI, BsmI, and NdeI, mating compatibility tests, and species.

Figure 2-1.

AluI banding patterns observed for Michigan Isolates of *Armillaria* spp.

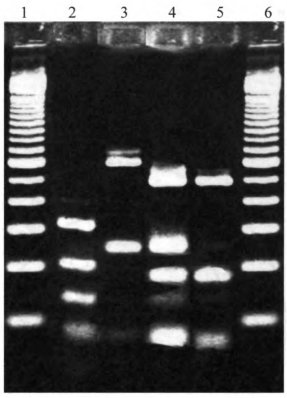


Fig. 2-1: RFLP analysis of the IGS-1 region amplified from *Armillaria* species. The DNA products were digested with AluI. Lanes 1 and 6, 100 bp ladder; lanes 2, *A. ostoyae* /*A. gemina*; lanes 3 *A. calvescens* /*A. gallica*; lanes 4-5 *A. mellea*.

Figure 2-2.

NdeI banding patterns observed for Michigan Isolates of *Armillaria* spp.

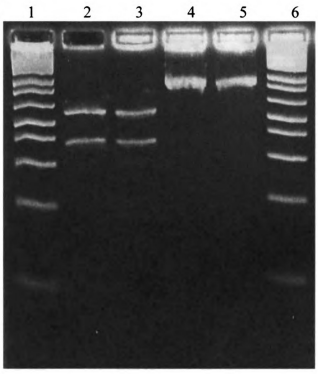


Fig. 2-2: RFLP analysis of the IGS-1 region amplified from *Armillaria* species. The DNA products were digested with NdeI. Lanes 1 and 6, 1KB plus DNA ladder; lanes 2 and 3, *A. ostoyae*; and lanes 4 and 5, *A. gemina*.

Figure 2-3.

BsmI banding patterns observed for Michigan Isolates of *Armillaria* spp.

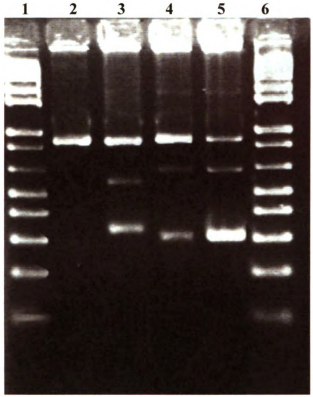


Fig. 2-3: RFLP analysis of the IGS-1 region amplified from *Armillaria* species. The DNA products were digested with BsmI. Lanes 1 and 6, 1KB plus DNA ladder; lane 2, *A. gemina*; lane 3, *A. mellea*; and lanes 4 and 5 *A. ostoyae*.

Figure 2-4

Sexual compatibility test results for species identification of *Armillaria* isolates, using haploid tester isolates

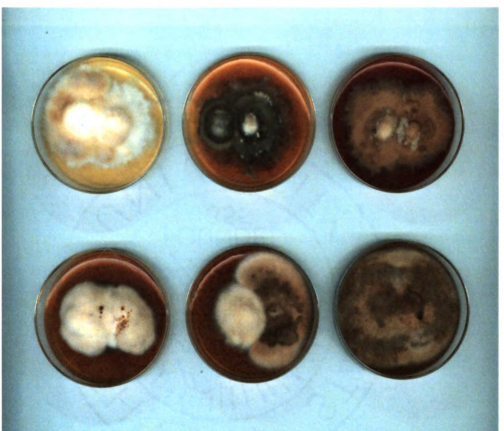


Fig. 2-4: Sexual compatibility tests: Starting from the top left: 90-4 x 90-4 (self cross); 90-4 x 137-1 (compatible); 137-1 x 137-1 (self cross); 28-6 x 28-6 (self cross); 28-6 x 160-9 (incompatible); 160-9 x 160-9 (self cross)

Figure 2-5

Sexual compatibility test results for species identification of *Armillaria* isolates, using haploid testers and unknown diploid isolates

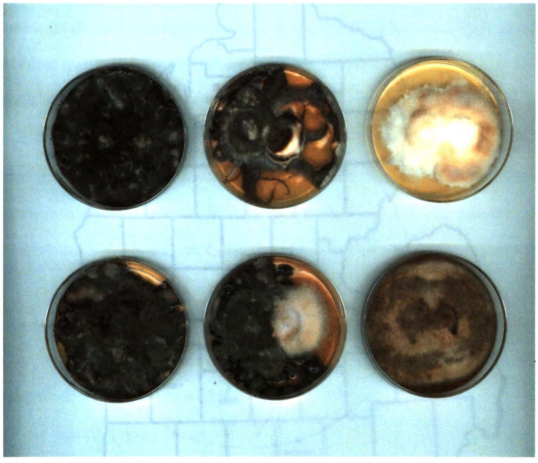
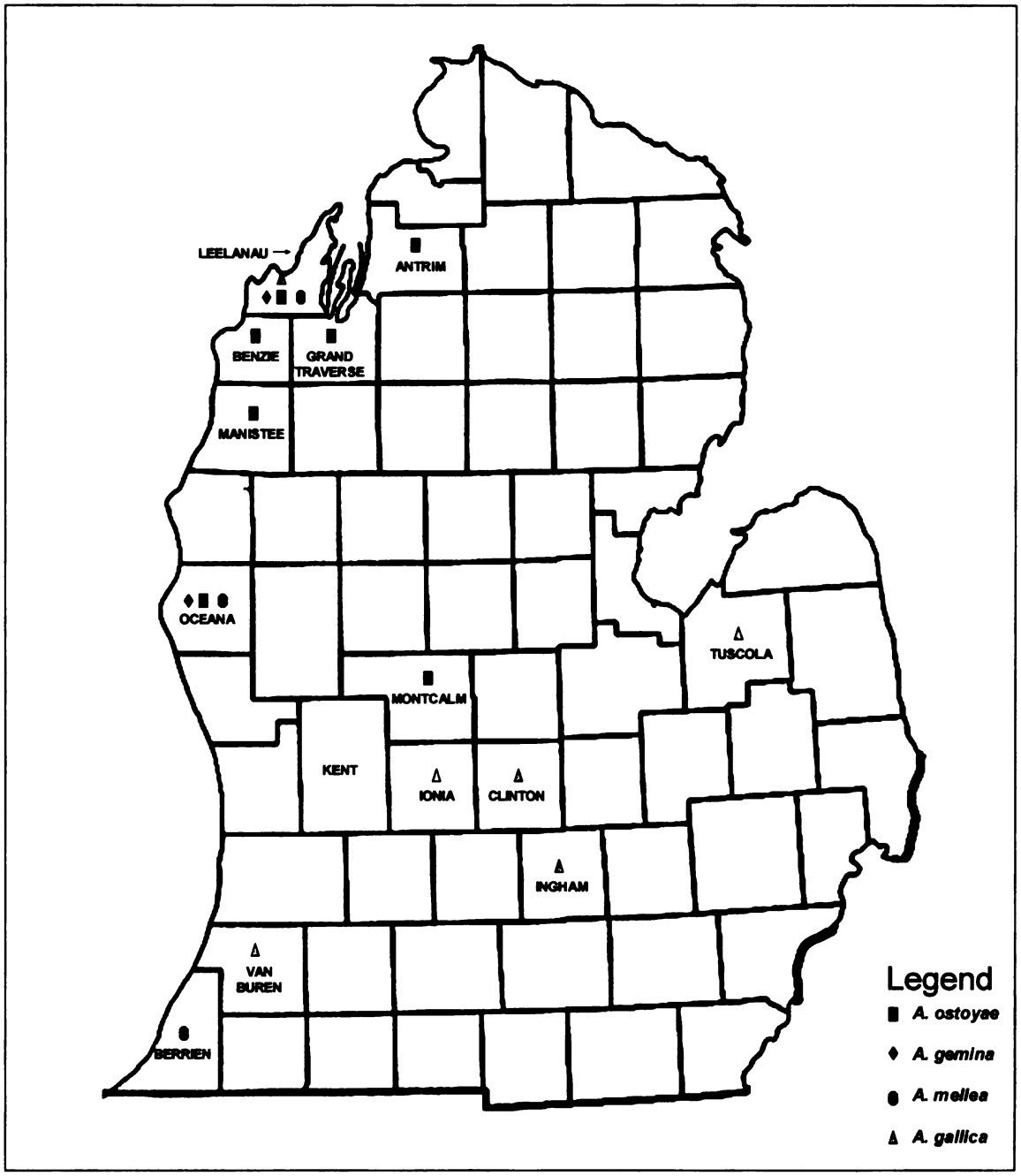


Fig. 2-5: Sexual compatibility tests: Starting from the top left: RR R4-T57 x RR R4T57 (self cross); RR **R4**T57 x 90-4 (compatible); 90-4 x 90-4 (self cross); LR R14T5 x LR R14T5 (self cross); LR R14T5 x **160**-9 (incompatible); 160-9 x 160-9 (self cross)

Figure 2-6.

Distribution map of species of *Armillaria* in Michigan's cherry producing regions



SOMATIC INCOMPATIBILITY AND MICROSATELLITE RESULTS:

The observed range of allele sizes for the four loci (CAG 25, CAG 77, AOSSR74, and AOSSR84) corresponded with previous reports by Langrell et al. (2001) and Worrall et al (2004). Each compatibility group of the *A. ostoyae* and *A. gemina* strains had a unique set of alleles for the four loci. However, similar alleles for each of the four loci were found at all the sites and the alleles of *A. gemina* were also the same sizes as many of the *A. ostoyae* strains. The similarity in allele size could be explained by the close relationship between the two species compared to the other species of *Armillaria*. PCR product for other species were preliminarily tested with each primer set and found that the primers for the loci CAG77 and AOSSR84 did not anneal to the other species, whereas strains of *A. ostoyae* and *A. gemina* always had PCR products for all four primer sets.

In orchard A (site # 14) a total of 22 isolates was collected and identified as *A. ostoyae*. From these 22 isolates, six genetically distinct genets were found using both somatic incompatibility tests and microsatellite analysis (Table 2-5; Fig. 2-7, 2-8). The alleles for orchard A (site # 14) were approximately 348, 353, 355, and 356 for locus CAG25; 322, 324, 327, 330, and 336 for locus CAG77; 126, 128, 130, 132, 135, and 137 for locus AOSSR84; and 82, 90, 92, 94, 96, 98, and 100 for locus AOSSR74. The isolates were homozygous or heterozygous for the loci CAG25 and CAG77, and they were all heterozygous for the locus AOSSR84 and AOSSR74.

Orchard B (site # 29) a total of 81 isolates were collected. From these 81 isolates 26 isolates representing 11 genets were identified as *A. ostoyae*, 20 isolates representing four genets were identified as *A. gemina*,15 isolates representing four genets were

identified as *A. mellea*, and 20 isolates representing three genets were identified as *A. gallica* (Table 2-5; Fig. 2-7, 2-9). Microsatellites were used to confirm genetic diversity between clones of *A. ostoyae* and *A. gemina* in orchard B (site # 29). The alleles for *A. ostoyae* isolates were approximately 348, 351, 353, and 356 for locus CAG25; 322, 327, 330 and 336 for locus CAG77; 122, 126, 128, 130, 132, 135, and 137 for locus AOSSR84; and 82, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, and 106 for locus AOSSR74. The isolates were homozygous or heterozygous for the four loci. The alleles for the *A. gemina* isolates were approximately 348 and 351 for locus CAG25; 330 and 346 for locus CAG77; 126, 128, 130, and 132 for locus AOSSR84; and 90, 92, 94, 96, 100, and 104 for locus AOSSR74. The isolates were homozygous or heterozygous for the loci CAG25 and CAG77, and they were all heterozygous for the locus AOSSR84 and AOSSR74.

Seven isolates from other orchards were analyzed to see if the markers could distinguish genetic diversity at a more global level. The alleles observed at these seven different orchards were approximately 342, 344, 346, 348, 353, and 356 for locus CAG25; 322, 327, 330, and 336 for locus CAG77; 120, 122, 124, 126, 128, 130, 132, and 134 for locus AOSSR84; and 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, and 102 for locus AOSSR84 (Table 2-5). The isolates were homozygous or heterozygous for the loci CAG77, and AOSSR74, and they were all heterozygous for the loci CAG25 and AOSSR84. The isolates were homozygous or heterozygous for the loci CAG25 and CAG77, and they were all heterozygous for the locus AOSSR84 and AOSSR74.

Table 2-5: Somatic incompatibility groups and microsatellite results for orchard A (site # 14), orchard B (site # 29), and seven miscellaneous strains of *A. ostoyae* collected from other sites

Site	SI group	# of Isolates in SI group	CAG25	CAG77	AOSRR84	AOSSR74
14	Ost1-1	3	356	327	126/137	92/94/96
14	Ost2-1	3	353	322/324	128/137	98/100
14	Ost3-1	3	353/356	322/324	135/137	96/100
14	Ost4-1	4	348/356	327/336	126/130/135/137	90/96
14	Ost5-1	4	353	327	128/130/132/135	82/92/94/96/98
14	Ost6-1	5	348/356	330	126/130/137	92/94/96/98
29	Ost1-2	1	351/353	327/330	122/137	82/86/92/104/106
29	Ost2-2	4	356	336	122/126/128/130	86/90/94
29	Ost3-2	1	356	327/336	122/128	94/96/98/100
29	Ost4-2	4	351	322/332	128/132	90/92/94/98/104
29	Ost5-2	2	348/356	327	126/128	86/92/94/98
29	Ost6-2	5	353/356	330/336	132/137	82/88/92
29	Ost7-2	2	351	332	128/130	88/90/92/94/98
29	Ost8-2	2	353/356	332	132/135/137	92/100/102/104/106
29	Ost9-2	3	351/353	330	132	92
29	Ost10-2	5	351/353	327	126/128/135/137	82/92/98/102
29	Ost11-2	2	351/356	327/336	128/137	90/94/100/102/104
29	Gem1-2	14	348/351	330	126/128	90/92/94/96
29	Gem2-2	1	348	330/346	128/130/132	90/92/94/96/100
29	Gem3-2	1	351	330/346	128/130/132	90/92/94/96/104
29	Gem4-2	1	348	330/346	128/130/132	90/92/94
29	Mel1-2	8	-	-	-	-
29	Mel2-2	5	-	-	-	-

Continuation of Table 2-5: Somatic incompatibility groups and microsatellite results for orchard A (site # 14), orchard B (site # 29), and seven miscellaneous strains of *A. ostoyae* collected from other sites

Site	SI group	# of Isolates in SI group	CAG25	CAG77	AOSRR84	AOSSR74
29	Mel3-2	1	-	-	-	-
29	Mel4-2	1	-	-	-	-
29	Cal1-2	1	-	-	-	-
29	Cal2-2	8	-	-	-	-
29	Cal3-2	11	-	-	-	-
3	PT1T2	-	342/356	330/336	124/130/132/134	94/98/100
17	CT12T1	-	344/356	330	126/128/130	90/94/100/102
26	CT19T2	-	356	322/327	124/128	80/84/86/90/92/102
30	SC2T1	-	356	322/327	130/132	82/92/94/96/98
32	CT23T1	-	346/ 356	330	126/132	84/88/90/102
33	XT1T1	-	353/356	327/330	132/134	84/92/98/100
35	SC3T1	-	344/348	327/330	120/122/124/126/128	80/90/92/94/100

See Appendix 2 for data for all isolates.

Figure 2-7.

Somatic incompatibility tests used to define genets of *Armillaria* in orchard A (site #14) and orchard B (site #29)

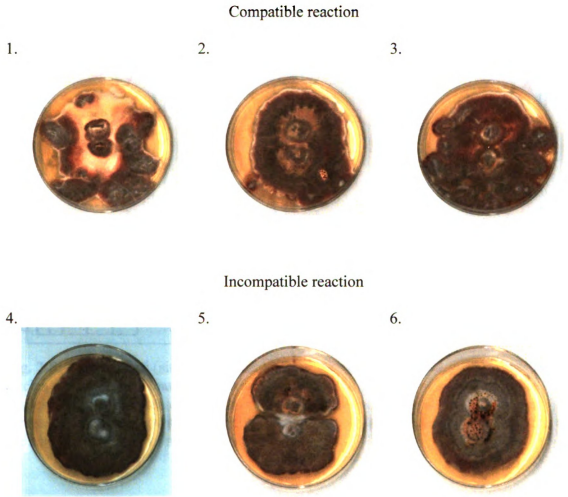


Fig. 2-7: Somatic Incompatibility Tests: Plugs (5x5 mm) of an unidentified isolate plated approximately 10 mm from each other on a 60 mm by 15mm Petri dish. Starting from the top left: plates 1, 3, 4, and 6, self-crosses or controls; plate 2, compatible reaction; and plate 5, incompatible reaction, seen by the presence of a barrage zone.

Figure 2-8.

Distribution of *A. ostoyae* genets in orchard A (site # 14) based on results from somatic incompatibility tests and microsatellite markers

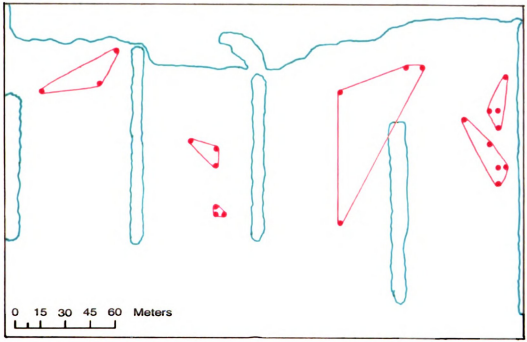


Fig. 2-8: First orchard (site # 14) distribution of six genets of *Armillaria ostoyae*: The red circles represent trees that isolates were collected from and the lines connecting the circles represent the area of possible spread of an individual genet. The green lines represent four single rows of poplar trees that separated the orchard into four sections and the surrounding stand of maple trees.

Figure 2-9.

Distribution results of *Armillaria* genets for orchard B (site #29) based on results from somatic incompatibility tests and microsatellite markers

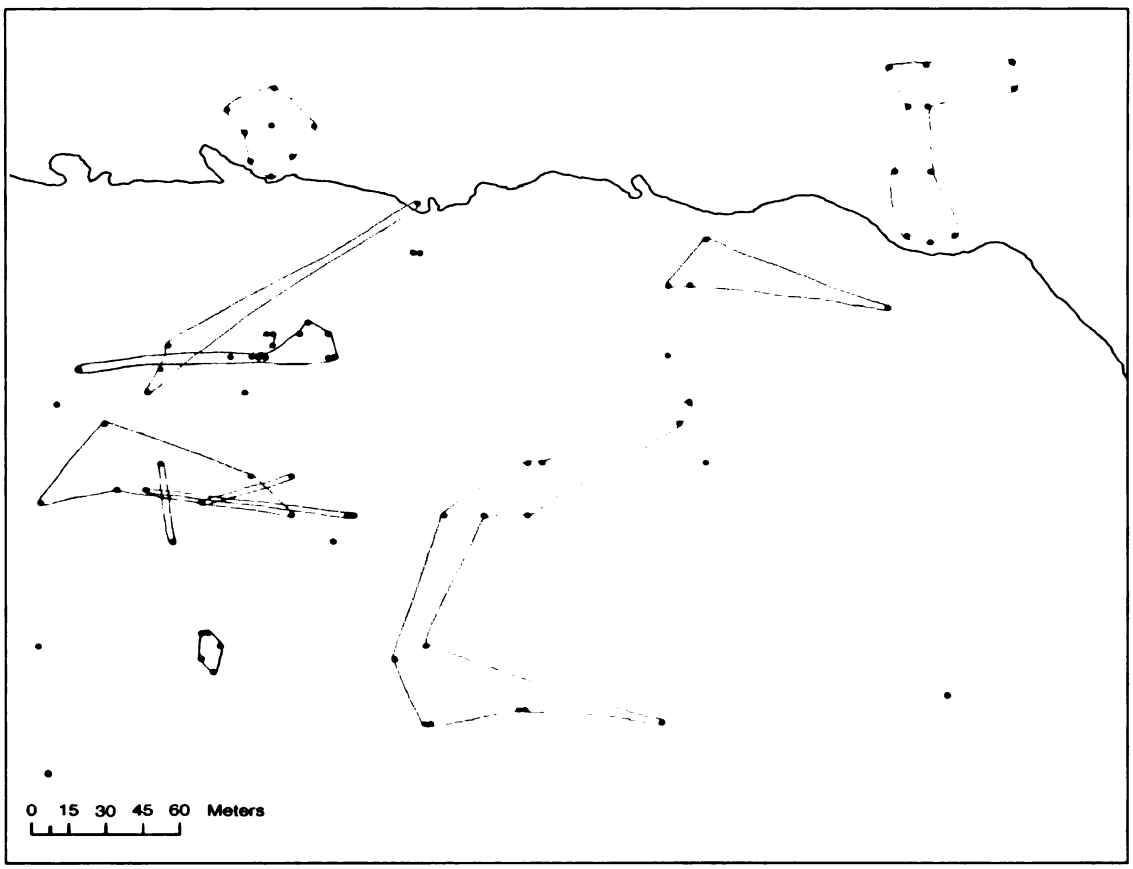


Fig. 2-9: Second orchard (site # 29) distribution of genets of *Armillaria* spp.: The circles represent trees that isolates were collected from and the lines represent the area of possible spread of an individual genet. The red represents *A. ostoyae*; the brown represents *A. gemina*; the blue represents *A. mellea*; and the green represent *A. gallica*. The black represent the edge of the surrounding forest.

DISCUSSION:

The amplification and RFLP analysis of the IGS-1 region using AluI, BsmI, and NdeI digests to identify isolates of *Armillaria* in this survey yielded RFLP patterns consistent with previous published reports (Harrington and Wingfield 1995; Kim et al. 2000; White et al. 1998). This method of identification was not reliant on basidiomes, like the 1987 survey (Proffer et al. 1987) and also allowed for faster identification than the sexual compatibility test. However this molecular method could not differentiate *A. calvescens* from *A. gallica*. Therefore, there is still a reliance on the sexual compatibility tests to confirm the species of *Armillaria* with the AluI banding patterns of *A. calvescens*/*A. gallica*.

Some isolates recovered in the study had RFLP patterns for *A. gemina*, which has never been reported in Michigan. Representative isolates placed in the *A. ostoyae*/*A. gemina* group with AluI were paired in sexual compatibility tests to confirm the species identification. The isolates with the RFLP patterns for *A. gemina*, whose PCR product did not digest with NdeI and BsmI, were confirmed to species using the sexual compatibility tests. The sexually compatibility tests were consistent with the observed RFLP patterns for the PCR products of *A. ostoyae* and *A. gemina* confirming that RFLP analysis is enough to identify between these two species.

Although sexual compatibility tests were able to distinguish the species of tested isolates, most of the established tester strains used for the sexual compatibility tests did not prove to be very efficient. Most of the tester strains were obtained from ATCC, and many were already in the diploid form or converted to the diploid form without being mated. Also some tester strains that maintained the haploid phenotype did not convert

even with the tester strains of the same species (Appendix A: Table A-2; Table A-3). In the future, the reliance on a few tester strains for each species using the ATCC as a source will make it difficult and perhaps impossible to identify some isolates of *Armillaria* to species. *Armillaria* has a bifactorial mating system and if the isolates being tested are incompatible with the mating type of the only reliable tester the species can not be determined. If this method is to be used in the future to identify unknown isolates to species, new testers for all of the biological species should be collected. These testers were first used by Anderson and Ullrich (1979), so the tester strains may have mutated, degenerated, or lost viability since they are almost 30 years old.

Further support for species identification can be seen from the sequencing results of select isolates, which corresponded with both the RFLP analyses and the sexual compatibility tests. Sequenced isolates were compared to other isolates submitted to Genbank and were found to be most closely related to isolates of the same species. However, isolates identified as *A. ostoyae* that digested with only one of the two enzymes, BsmI or NdeI, were 99% homologous to isolates of *A. ostoyae* and *A. gemina*. More isolates, should be tested with this method of identification, since many of the species are closely related, and should still be supported by other identification methods such as RFLP analysis and the sexual compatibility tests.

The results from this distribution survey correspond in most regards with the 1987 survey (Proffer et al. 1987). *A. ostoyae* was again found to be the predominant species infecting tart cherry in the Northwest cherry producing region. *Armillaria mellea* was still found to be more predominant as a pathogen on tart cherries in the southern regions of Michigan. In a new finding of the current survey, *A. mellea* was found further north

than previously reported. In the 1987 survey (Proffer et al. 1987), the farthest north that *A. mellea* was reported was in Manistee County on one oak tree. However, isolates of *A. mellea* were found farther north on tart cherry in orchard B (site # 29) in Leelanau County on the Old Mission Peninsula.

Armillaria gallica was found at orchard B (site #29) in Leelanau County on a tart cherry tree and in the forest as a saprophyte. This species was also found on a plum tree at site # 4 in Van Buren County. For the most part, *A. gallica* is considered a saprophyte and not pathogenic, however, based on field observation in both orchards it appeared to be acting as an opportunistic pathogen to orchard grown *Prunus* spp. To confirm the pathogenicity of *A. gallica* to *Prunus* spp. greenhouse inoculation trials should be done.

Isolates of *A. gemina* were found at orchard B (site #29) on tart cherry. *Armillaria gemina* was also identified from site # 35 in Oceana County on sweet cherry. *Armillaria gemina* is usually not known as a virulent pathogen. However, based on field observation at these two sites, *A. gemina* appeared to be the primary agent of disease and decline to many of the orchard trees. The pathogenicity of *A. gemina* to orchard grown *Prunus* spp. would not be surprising, since unlike *A. ostoyae* which tends to be found on conifers, *A. gemina* has only been reported on hardwood species (Blogdett and Worrall 1992; Harrington and Rizzo 1993; McLaughlin 2001; Rizzo and Harrington 1993; Volk and Burdsall). Based on the field observations and host specificity, pathogenicity tests should be done to confirm that *A. gemina* can be pathogenic to orchard grown *Prunus* spp.

The isolates of *A. gemina*, *A. mellea*, and *A. gallica* found at orchard B (site # 29) were not found when conducting the broad survey, where only a few isolates were

collected from each site. However, when this site was extensively surveyed isolates from all four species, as including *A. ostoyae*, were collected. The extensive survey from orchard B (site # 29) may indicate that other orchards are also diverse in the species of *Armillaria* infecting trees, however due to chance and because *A. ostoyae* is the most dominant species infecting cherry other species were not collected when broadly surveying an orchard site. This finding can also support the idea that some species may have not been identified in the 1987 survey by Proffer et al., which relied on the production of basidiomes. Species not producing basidiomes in an orchard at the time of the survey would have been missed.

Based on somatic incompatibility results and microsatellite results, two intensively surveyed orchards were occupied by multiple *Armillaria* genets. Identification of isolates using established microsatellite markers for *A. ostoyae* and *A. gemina* isolates prove to be a reliable and faster method to distinguish individual genets within Michigan populations. It appears that this method of identification can remove the reliance on somatic incompatibility identification tests for these Michigan populations. However, when initially looking at new populations, other available markers should be evaluated for the specific population to identify which markers show the best variation for the specific loci. Also, when investigating a new population other identification methods should still support the use of microsatellite markers.

The genets from both orchards occupied rather small and discrete areas. In orchard A (site # 14) there seemed to be no division or fragmentation between the genets. However, this may be due to the low number of sample found at the site. The low number of samples is due in part to the age and the lack of tree maintenance at the site,

resulting in fewer trees to sample. Many of the trees within infection pockets were no longer present and the existing stumps that looked as though they had been invaded by *Armillaria*, due to the presence of dried mycelial fans, wood decay, and/or the presence of zone lines, were too old to collect viable isolates. Many of the stumps were decorticated or secondary fungi had moved into the stumps. However, it is difficult to determine if the existing infestation occurring at this cherry site was subsequent to orchard establishment. Based on the radial growth rate of *A. ostoyae* of approximately 1 m per year for young conifer stands (Peet et al. 1996; and Shaw and Roth 1976) it appears that infestation occurred prior to orchards establishment. This rate of growth is supported by largest genet at the site, which can be estimated to be around 40 to 100 years in age. Many of the smaller genets may have been established after the orchards establishment. However based on sample size it is hard to determine if fungal establishment occurred subsequent to orchard establishment. Based on field observation and the pocket sizes of areas missing trees, most of the infetation probably occurred prior to orchard establishment.

At orchard B (site # 29) many of the genets appear to occupy the same space; suggesting that multiple ramets are at the site. A ramet is a term to describe a genet that has been fragmented into two or more pieces; usually due to invasion by other genets. Ramats could explain why many of the genets found at this site appear to overlie one another when mapped out. The mosaic pattern of genets at this orchard correspond with previous studies that looked at clonal spread in forests (Rizzo and Harrington 1993; Rizzo et al. 1995; Worrall 1994; and Worrall et al. 2004). This correspondence with forest populations indicates the probability that these genets are left over from the

existing forest and simply moved to the cherry when the orchard was planted. This can also be supported by the radial spread of the largest genet which at the rate of 1 m per year (Peet et al. 1996; Shaw and Roth 1976) can be estimated at approximately 70 to 140 years in age. However, the multiple genets infecting a single tree could suggest that these genets occurred after orchard establishment due to new infection from basidiospores. The population dynamics at orchard B (site # 29) could be a combination of old genets established prior to orchard establishment as well as new infection seen by a genet infecting a single tree.

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FUTURE WORK

This research has expanded the fundamental understanding of the population diversity of *Armillaria* spp. in Michigan, first initiated by Proffer et al. (1987). More orchards should be intensively surveyed to see if other orchards have the same diversity in species seen in orchard B (site # 29), or if a single species usually dominates an orchard site. Also a particular orchard could be studied over many years, particularly orchard B (site # 29), to examine the radial rate of spread in cherry, and to examine the dynamics between the different species of *Armillaria* found at the site. If this orchard was studied over more years, it could be possible to see if new infection is occurring at the site, as thought possible, based on genets infecting a single tree.

From the large collection of isolates from the survey, pathogenicity screening could be done to rate the virulence of individual isolates. Isolates of *A. gemina* and *A. gallica*, found on cherry, should be tested for pathogencity to cherry in order to prove Koch's postulate and that these species are indeed pathogenic to cherry. Since orchard site in Michigan are being converted to vineyards pathogenicity screening of the other species found on *Prunus* in Michigan, besides *A. ostoyae* and *A. mellea*, should be done including *A. gemina*, *A. calvescens* (reported by Proffer et al. 1987), and *A. gallica*. This also would be important since one of the species found on cherry, *A. gallica*, has been reported on grape by Aguín-Casal et al. (2004).

Another interesting study would be to look more closely at the life cycle of *A. ostoyae*. It has been shown that both *A. gallica* and *A. tabsecens* go through two diploidizations and two haploidization cycles prior to formation of the basidia and are also genetic mosaics. It would be interesting to see if this is also true for other species of

Armillaria, specifically *A. ostoyae*. Many of the strains collected from the survey of *A. ostoyae* converted back and forth between the diploid and haploid phase when cultured from the same source. From these isolates, basidiome development could be induced to study the nuclear state of single cells. Also the nuclear state of cells from basidiomes from nature could be examined.

APPENDIX A:

SPECIES IDENTIFICATION RESULTS

Table A-1: Location, tree type, banding size for the IGS-1 region, restriction enzyme digest results, and species determination, for established *Armillaria* isolates

Site #	Isolate	Tree Type	IGS-1 Region (bp)	AluI (bp)	NdeI (bp)	BsmI (bp)	Species
1	OcCo-5-19-05-1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
2	BW-6-2-05-1	Black Cherry	920	583, 400, 240, 200	-	-	*A. gallica*
2	BW-6-2-05-4	Black Cherry	920	583, 400, 240, 200	-	-	*A. gallica*
2	BW-6-8-05-2	Fallen Log	920	583, 400, 240, 200	-	-	*A. gallica*
2	BW-6-8-05-4	Fallen Log	920	583, 400, 240, 200	-	-	*A. gallica*
2	BW-6-16-05-3	Maple	920	583, 400, 240, 200	-	-	*A. gallica*
2	BW-6-23-05-1	Maple	920	583, 400, 240, 200	-	-	*A. gallica*
3	PT1T2	Peach	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*

4	PL1T1	Plum	920	583, 240	-	-	*A. gallica*
5	CT1T1	Sour Cherry	875	476, 175	-	-	*A. mellea*
5	CT1T2	Sour Cherry	875	476, 175	-	-	*A. mellea*
6	None	Apple	-	-	-	-	-
7	CT2T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
7	CT2T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
8	CT3T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
9	CT5T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
9	CT5T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
9	CT5T3	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
10	CT6T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
11	None	Sour Cherry	-	-	-	-	-
12	CT8T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
12	CT8T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
13	CT9T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
13	CT9T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	1-54	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	1-101	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*

14	1-217	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	2-27	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	2-43	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	2-44	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	2-48	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	2-49	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	2-91	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	3-64	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	3-79	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-35	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-36	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-55	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-57	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-58	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-59	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-82	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-86	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-148	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
14	4-280	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*

14	4-306	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
15	CT10T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
16	CT11T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
16	CT11T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
16	CT11T4	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
17	CT12T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
17	SC1T1	Sweet Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
17	PT2T1	Peach	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
18	CT13T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
19	None	-	-	-	-	-	-
20	None	-	-	-	-	-	-
21	None	-	-	-	-	-	-
22	SHSP1	Fallen Log	920	583, 400, 240, 200	-	-	*A. gallica*
22	SHSP4	Fallen Log	920	583, 400, 240, 200	-	-	*A. gallica*
23	ISRA1	Elm	920	583, 400, 240, 200	-	-	*A. gallica*
23	ISRA2	Elm	920	583, 400, 240, 200	-	-	*A. gallica*

23	ISRA3	Beech	920	583, 400, 240, 200	-	-	*A. gallica*
23	ISRA4	Balsam	920	583, 400, 240, 200	-	-	*A. gallica*
23	ISRA5	Beech	920	583, 400, 240, 200	-	-	*A. gallica*
24	None	Sour Cherry	-	-	-	-	-
25	CT18T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
26	CT19T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
26	CT19T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
27	CT20T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
27	CT20T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
28	CT21T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
28	CT21T3	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	CT22T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	CT22T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R1T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R1T73	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R2T9	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R2T19	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*

29	LR R4T6	Sour Cherry	875	476, 175	-	580 , 330	*A. mellea*
29	LR R4T7	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R5T6	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R5T9	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R5T10	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R5T11	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R5T15	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R5T16	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R5T56	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R6T0	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R6T7	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R7T6	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R7T7	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	LR R7T8	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R7T12	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R8T11	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	LR R9T80	Sour Cherry	920	308, 200, 135	-	620, 300	*A. ostoyae*
29	LR R11T38	Sour Cherry	920	308, 200, 135	-	620, 300	*A. ostoyae*
29	LR R11T40	Sour Cherry	920	308, 200, 135	-	620, 300	*A. ostoyae*

29	LR R14T4	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R14T5	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	LR R15T39	Sour Cherry	920	308, 200, 135	-	620, 300	*A. ostoyae*
29	RR R1T8	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R1T73	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R4T3	Sour Cherry	920	308, 200, 135	550, 370	-	*A. ostoyae*
29	RR R4T35	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R4T36	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R4T57	Sour Cherry	920	583, 240	-	-	*A. gallica*
29	RR R5T4	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R5T8	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	Stump	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R6T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R6T6	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R7T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R8T15	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R8T22	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R8T23	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*

29	RR R8T34	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R8T36	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R8T42	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R10T19	Sour Cherry	920	308, 200, 135	550, 370	-	*A. ostoyae*
29	RR R17T27	Sour Cherry	875	476, 240, 175	-	580, 330	*A. mellea*
29	RR R17T28	Sour Cherry	875	476, 240, 175	-	580, 330	*A. mellea*
29	RR R18T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	RR R18T29	Sour Cherry	875	476, 240, 175	-	580, 330	*A. mellea*
29	RR R18T50	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R19T18	Sour Cherry	875	476, 240, 175	-	580, 330	*A. mellea*
29	RR R19T45	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R20T25	Sour Cherry	875	476, 240, 175	-	580, 330	*A. mellea*
29	RR R22T141	Sour Cherry	875	476, 175	-	580, 330	*A. mellea*
29	RR R23T72	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R23T73	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R24T62	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R24T63	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R24T97	Sour Cherry	920	308, 200, 135	-	-	*A. gemina*
29	RR R28T3	Sour Cherry	875	490, 240	-	580, 330	*A. mellea*

29	F1	Beech	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
29	BT1	Beech	920	583, 240	-	-	*A. gallica*
29	BT3	Beech	920	583, 240	-	-	*A. gallica*
29	MT2	Maple	920	583, 240	-	-	*A. gallica*
29	F5	Fallen Log	920	583, 240	-	-	*A. gallica*
29	F6	Fallen Log	920	583, 240	-	-	*A. gallica*
29	F7	Fallen Log	920	583, 240	-	-	*A. gallica*
29	F8	Fallen Log	920	583, 240	-	-	*A. gallica*
29	F11	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-1	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-2	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-3	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-4	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-5	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-6	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-8	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-9	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-10	Fallen Log	920	583, 240	-	-	*A. gallica*

29	10-3-06-11	Fallen Log	920	583, 240	-	-	*A. gallica*
29	10-3-06-12	Fallen Log	920	583, 240	-	-	*A. gallica*
30	SC2T1	Sweet Cherry	920	310, 200, 135	550, 370	620, 300	*A. ostoyae*
30	SC2T2	Sweet Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
31	OcCo 6-22-06-C	Sour Cherry	875	476, 175	-	-	*A. mellea*
31	OcCo 6-22-06-P	Plum	875	476, 175	-	-	*A. mellea*
32	CT23T1	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
32	CT23T2	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
32	CT23T3	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
32	CT23T4	Sour Cherry	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
33	XT1T1	Frasier Fir	920	308, 200, 135	550, 370	620, 300	*A. ostoyae*
34	None	Sour Cherry	-	-	-	-	-
35	SC3T1	Sweet Cherry	920	308, 200, 135	-	620, 300	*A. gemina*
35	SC3T2	Sweet Cherry	920	308, 200, 135	-	620, 300	*A. gemina*
36	TG5	Fallen Log	920	583, 400, 240, 200	-	-	*A. gallica*
36	TG6	Fallen Log	920	583, 400, 240, 200	-	-	*A. gallica*

Table A-2: Sexual compatibility results for strains of *Armillaria* identified as *A. calvescens/ A. gallica* using PCR amplification of the IGS-1 region and RFLP patterns

Isolate	*Armillariella mellea* 11-1 (NABS III)	*Armillariella mellea* 11-9 (NABS III)	*Armillariella mellea* 21-2 (NABS III)*	*Armillariella mellea* 90-4 (NABS VII)**	*Armillariella mellea* 90-10 (NABS VII)	*Armillariella mellea* 137-1 (NABS VII)
BW-6-2-05-1	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
BW-6-2-05-4	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
BW-6-8-05-2	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
BW-6-8-05-4	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
BW-6-16-05-3	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
BW-6-23-05-1	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
PL1T1	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
SHSP1	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
SHSP4	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
ISRA1	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
ISRA2	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
ISRA3	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
ISRA4	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative

ISRA5	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
RR R4T57	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
F7	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
10-3-06-10	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
TG-5	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative
TG-6	Tester turning diploid by itself	Tester turning diploid by itself	Negative	Positive	Tester turning diploid by itself	Negative

* Testers never converted to the diploid form even when crossed with tester isolates of the same species.

** Tester slightly converted with testers 11-1 and 11-9. However the conversion with test isolates was much more dramatic.

Table A-3: Sexual compatibility results for strains of *Armillaria* identified as *A. ostoyae*/ *A. gemina* using PCR amplification of the IGS-1 region and RFLP patterns

Isolate	*Armillariella mellea* 28-4 (NABS I)	*Armillariella mellea* 28-6 (NABS I)	*Armillariella mellea* 28-7 (NABS I)	*Armillariella mellea* 28-9 (NABS I)	*Armillariella mellea* 160-8 (NABS II)	*Armillariella mellea* 160-9 (NABS II)
LR R1T1	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
LR R2T19	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
LR R4T35	Tester turning diploid by itself	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Positive
LR R5T6	Tester turning diploid by itself	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Positive
LR R5T56	Tester turning diploid by itself	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Positive
LR R6T0	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
LR R7T6	Tester turning diploid by itself	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Positive
LR R11T38	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
LR R14T5	Tester turning diploid by itself Positive	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
RR R5T4	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
RR R5T8	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
RR R8T23	Tester turning	Positive	Tester diploid	Tester diploid	Tester turning	Negative

	diploid by itself				diploid by itself	
RR R10T19	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
RR R10T40	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
RR R18T1	Tester turning diploid by itself	Positive	Tester diploid	Tester diploid	Tester turning diploid by itself	Negative
SC3T1	Tester turning diploid by itself	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Positive
SC3T2	Tester turning diploid by itself	Negative	Tester diploid	Tester diploid	Tester turning diploid by itself	Positive

APPENDIX B:

SOMATIC INCOMPATIBILITY TESTS AND MICROSATELLITE RESULTS

Table B-1: Results from somatic incompatibility test and microsatellites for strains of *A. ostoyae* collected from orchard A (site # 14) and orchard B (site # 29) and microsatellite results for six *A. ostoyae* isolates and one *A. gemina* from miscellaneous sites

Isolate	Site #	SIG	CAG25	CAG77	AOSSR74	AOSSR84
1-59	14	Ost1-1	356	327	126/137	92/94/96
1-101	14	Ost1-1	356	327	126/137	92/94/96
1-217	14	Ost1-1	-	-	-	-
2-27	14	Ost2-1	353	322/324	128/137	98/100
2-43	14	Ost2-1	353	322/324	128/137	98/100
2-44	14	Ost2-1	-	-	-	-
2-48	14	Ost3-1	353/356	322/324	135/137	96/100
2-49	14	Ost3-1	-	-	-	-
2-91	14	Ost3-1	353/356	322/324	135/137	96/100
3-64	14	Ost4-1	348/356	327/336	126/130/135/137	90/96
3-79	14	Ost4-1	-	-	-	-
4-280	14	Ost4-1	-	-	-	-
4-306	14	Ost4-1	348,/356	327/336	126/130/135/137	90/96
4-35	14	Ost5-1	353	327	128/130/132/135	82/92/94/96/98
4-55	14	Ost5-1	-	-	-	-
4-57	14	Ost5-1	353	327	128/130/132/135	82/92/94/96/98
4-82	14	Ost5-1	-	-	-	-
4-148	14	Ost5-1	-	-	-	-

4-36	14	Ost6-1	348/356	330	126/130/137	92/94/96/98
4-58	14	Ost6-1	-	-	-	-
4-59	14	Ost6-1	-	-	-	-
4-86	14	Ost6-1	348/356	330	126/130/137	92/94/96/98
LR R1T1	29	Ost1-2	351/353	327/330	122/137	82/86/92/104/106
LR R2T9	29	Ost2-2	-	-	-	-
LR R4T7	29	Ost2-2	356	336	122/126/128/130	86/90/94
LR R6T0	29	Ost2-2	-	-	-	-
F1	29	Ost2-2	-	-	-	-
LR R2T19	29	Ost3-2	356	327/336	122/128	94/96/98/100
LR R9T80	29	Ost4-2	-	-	-	-
LR R11T38	29	Ost4-2	-	-	-	-
LR R11T40	29	Ost4-2	-	-	-	-
LR R15T39	29	Ost4-2	351	322/332	128/132	90/92/94/98/104
LR R14T4	29	Ost5-2	-	-	-	-
LR R14T5	29	Ost5-2	348/356	327	126/128	86/92/94/98
RR R1T8	29	Ost6-2	-	-	-	-
RR R5T4	29	Ost6-2	-	-	-	-
RR R6T2	29	Ost6-2	353/356	330/336	132/137	82/88/92
RR R7T1	29	Ost6-2	-	-	-	-
RR R8T15	29	Ost6-2	-	-	-	-
RR R4T3	29	Ost7-2	351	332	128/130	88/90/92/94/98
RR R10T19	29	Ost7-2	-	-	-	-
RR R5T8	29	Ost8-2	-	-	-	-
Stump	29	Ost8-2	353/356	332	132/135/137	92/100/102/104/106

RR R6T6	29	Ost9-2	-	-	-	-
RR R8T22	29	Ost9-2	-	-	-	-
RR R8T23	29	Ost9-2	351/353	330	132	92
RR R10T40	29	Ost10-1	351/353	327	126/128/135/137	82/92/98/102
RR R18T1	29	Ost11-1	351/356	327/336	128/137	90/94/100/102/104
LR R1T73	29	Gem1-2	348/351	330	126/128	90/92/94/96
RR R1T73	29	Gem1-2	-	-	-	-
RR R4T35	29	Gem1-2	-	-	-	-
RR R4T36	29	Gem1-2	-	-	-	-
RR R8T34	29	Gem1-2	-	-	-	-
RR R8T36	29	Gem1-2	-	-	-	-
RR R8T42	29	Gem1-2	-	-	-	-
RR R18T50	29	Gem1-2	-	-	-	-
RR R19T45	29	Gem1-2	-	-	-	-
RR R23T62	29	Gem1-2	-	-	-	-
RR R23T63	29	Gem1-2	-	-	-	-
RR R24T72	29	Gem1-2	-	-	-	-
RR R24T73	29	Gem1-2	-	-	-	-
RR R24T97	29	Gem1-2	-	-	-	-
LR R5T6	29	Gem2-2	348	330/346	128/130/132	90/92/94/96/100
LR R5T9	29	Gem3-2	-	-	-	-
LR R6T7	29	Gem3-2	351	330/346	128/130/132	90/92/94/96/104
LR R7T6	29	Gem3-2	-	-	-	-
LR R7T7	29	Gem3-2	-	-	-	-
LR R5T56	29	Gem4-2	348	330/346	128/130/132	90/92/94
LR R4T6	29	Mel1-2	-	-	-	-

LR R5T10	29	Mel1-2	-	-	-	-
LR R5T11	29	Mel1-2	-	-	-	-
LR R5T15	29	Mel1-2	-	-	-	-
LR R5T16	29	Mel1-2	-	-	-	-
LR R7T8	29	Mel1-2	-	-	-	-
LR R7T12	29	Mel1-2	-	-	-	-
LR R8T11	29	Mel1-2	-	-	-	-
RR R17T27	29	Mel2-2	-	-	-	-
RR R17T28	29	Mel2-2	-	-	-	-
RR R18T29	29	Mel2-2	-	-	-	-
RR R19T18	29	Mel2-2	-	-	-	-
RR R20T25	29	Mel2-2	-	-	-	-
RR R22T141	29	Mel3-2	-	-	-	-
RR R28T3	29	Mel4-3	-	-	-	-
RR R4T57	29	Gal1-2	-	-	-	-
BT1	29	Gal2-2	-	-	-	-
BT3	29	Gal2-2	-	-	-	-
MT2	29	Gal2-2	-	-	-	-
F5	29	Gal2-2	-	-	-	-
F6	29	Gal2-2	-	-	-	-
F7	29	Gal2-2	-	-	-	-
F8	29	Gal2-2	-	-	-	-
F11	29	Gal2-2	-	-	-	-
10-3-06-1	29	Gal3-2	-	-	-	-
10-3-06-2	29	Gal3-2	-	-	-	-
10-3-06-3	29	Gal3-2	-	-	-	-

10-3-06-4	29	Gal3-2	-	-	-	-
10-3-06-5	29	Gal3-2	-	-	-	-
10-3-06-6	29	Gal3-2	-	-	-	-
10-3-06-8	29	Gal3-2	-	-	-	-
10-3-06-9	29	Gal3-2	-	-	-	-
10-3-06-10	29	Gal3-2	-	-	-	-
10-3-06-11	29	Gal3-2	-	-	-	-
10-3-06-12	29	Gal3-2	-	-	-	-
PT1T2	3	-	342/356	330/336	124/130/132/134	94/98/100
CT12T1	17	-	344/356	330	126/128/130	90/94/100/102
CT19T2	26	-	356	322/327	124/128	80/84/86/90/92/ 102
SC2T1	30	-	356	322/327	130/132	82/92/94/96/98
CT23T1	32	-	346/356	330	126/132	84/88/90/102
XT1T1	33	-	353/356	327/330	132/134	84/92/98/100
SC3T1	35	-	344/348	327/330	120/122/124/126/128	80/90/92/94/100

APPENDIX C:

GREENHOUSE ROOTSTOCK INOCULATION AND FIELD TRIAL

INTRODUCTION

Many orchard sites in Michigan are being converted into vineyards because grapes have more value per acre. However, growers are often unaware that *Armillaria* has been reported on grapes in Australia, Brazil, Central and Eastern Europe, and in California. Even if a grower is aware of the potential problem, new growers may not know the history of an orchard and whether or not *Armillaria* is present. Michigan's grape industry is much younger than those of both Europe and California and the effects or presence of infection with *Armillaria* have not yet been reported. Michigan does have one of the species of *Armillaria* reported on grapes, *A. mellea*, and another potentially pathogenic species, *A. ostoyae*. In the Northwestern cherry producing region of Michigan where many of the vineyards are currently being established, *A. ostoyae* tends to be the predominant species and it is known as a virulent pathogen with a wide host range. While most reports of *A. ostoyae* are on conifers, it is proven to be an aggressive pathogen of cherry in Northwest Michigan, as seen by Proffer et al. in the 1987 survey and rootstock inoculation trials (Proffer et al. 1998). Its continued impact on cherry was confirmed by the current survey.

In California, Baumgartner et al. (2006ab) have begun to test grape rootstock susceptibility to *A. mellea*. They tested eight different rootstocks finding rootstocks 3309C and Riparia Gloire, commonly used in Michigan, most susceptible to *A. mellea*, while Freedom, not commonly used in Michigan, was found to be most resistant. Other

rootstocks that commonly do well in Michigan soils were not tested by Baumgartner, including 5-BB, SO4, 101-14 Mgt (Howell et al. 1999). With the increase of viticulture in the state of Michigan, it is important to begin and assess *A. ostoyae* and *A. mellea* for their possible pathogenicity to grape rootstocks grown in Michigan. The hypothesis of this work was that the current *Armillaria* species known to infecting orchard grown *Prunus* in Michigan will move to grape when orchards are converted to vineyards. The hypothesis was tested by beginning greenhouse and field trial inoculations of grape rootstocks grown in Michigan for the future assessment of susceptibility to *A. ostoyae* and *A. mellea.*

MATERIALS AND METHODS: GRAPE ROOTSTOCK SELECTION

Four varieties of grape rootstocks, 50 rootstocks per variety, were ordered from California Grapevine Nursery Inc. (St. Helen, CA 94574-9790). The grape rootstocks included the following varieties: Freedom, Riparia Gloire, 3309C, and 101-14. Although not used in Michigan, Freedom was chosen as a negative control since it was previously shown to be the most resistant rootstock to *A. mellea* by Baumgartner et al. (2006ab). The positive controls included Riperia Glorie and 3309C. They were selected because they are commonly used in Michigan, and were known to be susceptible to *A. mellea* (Baumgartner et al 2006ab). The rootstock 101-14 was chosen because it is very commonly used in Michigan. Once received, the rootstocks were temporarily stored in a cold room until planting to prevent budding. The rootstocks were then brought to room temperature (20° C to 25° C) four days before planting.

INOCULUM PREPERATION

Inoculum was prepared three months prior to planting. To prepare the inoculum *Prunus mahaleb* twigs approximately 2.5 cm in diameter were cut from spot trees near an old orchard site at Michigan State University. The twigs were cut into approximately 8 cm pieces and were then autoclaved in a bag with 100mL water for 30 minutes. After being autoclaved 25 twigs per bag were placed into 16 autoclavable fungal spawn bags containing. These bags have an air vent and are sold commercially for culturing edible mushrooms. In addition to the twigs, 500mL of 4% MEA amended with 20 g/l glucose and 5 g/l peptone was added to each bag. The tops of the bags were folded and closed with autoclave tape, and autoclaved for 30 minutes. Once autoclaved the bags with the media and twigs were allowed to cool until the media was partially solid and 400 µL of 90% ethanol were added to the bag under aseptic conditions. The bags were inoculated with three 60 mm by 15 mm Pertri dishes of one isolate of *Armillaria* (Table C-1 for isolates of *Armillaria* used). For each strain there were a total of two bags. The *Armillaria* isolates used were primarily collected from Michigan orchards. One isolate of *A. mellea* was not a Michigan isolate, since at the time there were not enough strains of this species in the collection at the time of inoculation. The inoculated spawn bags were then sealed using a seal-a-meal machine and placed in the dark at 25° C for three months (Figure C-1 for prepared inoculum).

Table C-1: Strains of *Armillaria* used to inoculate grape rootstock

Strains of *Armillaria*	
A mellea isolates:	*A ostoyae* isolates:
1. B277 UNH Harrington	1. PT1T2
2. C2 23-2 x 10-1	2. CT9T1
3. 1-1 x 11-20 C1	3. CT20T1
4. CT1T2	4. Oc. Co. 5-19-05-1

GREENHOUSE SETUP

The 160 rootstocks, 40 of each variety, were planted in 3 gallon pots filled with one part sand and one part soil obtained from the greenhouse at Michigan State University. The rootstocks were then inoculated on May 19, 2006 with three different isolates of either *A. ostoyae* or *A. mellea* (Table C-2 for setup). There were four replicates for each inoculum combination. The inoculum pieces were placed next to the rootstock and were buried approximately 2.5 cm into the soil mix. A layer of mulch was then placed on top of the inoculated pot to keep the soil cool. The inoculated grape rootstocks were placed outside for the summer (Figure C-2). The pots were then moved into the greenhouse for the winter at the end of September and were again moved outside in May of the following spring.

The plants were checked on regular bases for signs of decline, such as defoliation. At nine months every rootstock was thoroughly checked for signs of mycelial fans under the bark of the roots, and also for signs of rhizomorph growth from the inoculum source. Each inoculum source was also checked to make sure it was still viable. All pots inoculated with *A. mellea* still had viable inoculum, however most of the *A. ostoyae* pots no longer had viable inoculum. All of the *A. ostoyae* pots were re-inoculated with the PT1T1 strain.

Table C-2: Pot setup for greenhouse inoculations for each rootstock variety

Rep	*A. ostoyae* isolates	Rep	*A. mellea* isolates	Rep	*A. ostoyae* isolates	Rep	*A. mellea* isolates
1	1,2,3	1	1,2,3	3	1,2,3	3	1,2,3
1	2,3,4	1	2,3,4	3	2,3,4	3	2,3,4
1	3,4,1	1	3,4,1	3	3,4,1	3	3,4,1
1	4,1,2	1	4,1,2	3	4,1,2	3	4,1,2
1	CK	1	CK	3	CK	3	CK
2	1,2,3	2	1,2,3	4	1,2,3	4	1,2,3
2	2,3,4	2	2,3,4	4	2,3,4	4	2,3,4
2	3,4,1	2	3,4,1	4	3,4,1	4	3,4,1
2	4,1,2	2	4,1,2	4	4,1,2	4	4,1,2
2	CK	2	CK	4	CK	4	CK

FIELD TRIAL SET UP

The four grape rootstocks were also used for a long term field trial to see if *Armillaria ostoyae* will move to grape in the field. The site chosen for the field trial was a peach orchard in Leelanau County where *A. ostoyae* was infecting peach trees. The growers were interested in converting the orchard to a vineyard, making this site ideal for a rootstock test. The results will give the grower, as well others, valuable information on the suitability of *Armillaria* sites for vineyard production. Ten rootstocks of each type were planted on May 16, 2006. The rootstocks were planted within an infected pocket with root debris and stumps with viable *Armillaria* infections present. Each of the rootstocks was planted 1 m apart in 2 rows (Figures C-3 and C-4).

FIGURE C-1

Prepared *Armillaria* inoculum for the greenhouse inoculation trial to test grape rootstocks to susceptibility to *A. ostoyae* and *A. mellea*

Fig C-1: Inoculum prepared using cherry twigs for greenhouse inoculation to test grape rootstocks for susceptibility to *Armillaria*.

FIGURE C-2:

Greenhouse inoculation trial to test four different grape rootstocks to their susceptibility to *A. ostoyae* and *A. mellea*

Fig C-2: Grape rootstocks inoculated in a greenhouse trial with *Armillaria ostoyae* and *Armillaria mellea* strains.

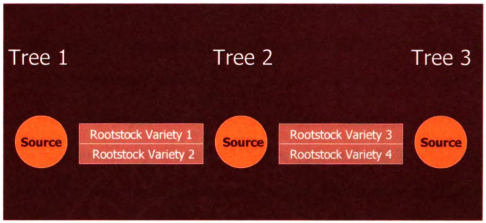
FIGURE C-3:

Grape rootstock field trial to test four rootstocks to susceptibility of *A. ostoyae*

Fig C-3: Grape rootstock planted in an infected peach orchard to test for susceptibility to *A ostoyae*. Grape rootstocks are surrounded by a layer of much and are planted in two row going out from a central source towards two other sources.

FIGURE C-4:

Field trial setup to test grape rootstock to susceptibility to *A. ostoyae*



CONCLUSIONS

To date there have been no signs of infection due to *Armillaria* from either the greenhouse or field trial. Symptoms caused by infection from Armillaria root rot should not be expected for a couple of more years in the field trial based on the rate at which symptoms are seen in tart cherry from field observations. The rootstocks from the greenhouse trial will be check at the end of August to see if any symptoms have developed. If *Armillaria* spp. found in Michigan are able to infect grape there will probably be more success with the field trial where syptoms should be seen if the rootstock are infected. In greenhouse inoculation trials, Baumgartner (2006a,b) reported that although infection occurred, no symptoms, such as defoliation, occurred even 12 months after inoculation.

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APPENDIX D:

GLOSSARY

GLOSSARY:

Acropetal: Proceeding from the base toward the apex or from below upward, used to describe the movement of fungicides in a plants, so in this case it would move into the stem or trunk of a tree.

Adnexed: Pertaining to the attachment of the fertile tissue (the gills, tubes, spines, etc.) to the stipe of the fungus in which the fertile tissue typically curves upwards towards the pileus of the fungus before attaching to the stipe.

Arachnoid: See Cortina

Basidiome: Mushroom, basidiocarp or fruiting body that bears basidiospores.

Basipetal: Proceeding from apex towards the base or from above downward, used to describe the movement of fungicides in a plants, so in this case it would move into the root system.

Binding hyphae: Thick-walled, typically aseptate, highly branched vegetative hyphae.

Caespitose: When basidiomes grow in dense clusters, with the stems fused together or packed right up against one another at the base.

Cortina (Cortinaceous): A special type of annulus that is filamentous, resembling a spider web, attached from the margin of the cap (pileus) to the stem (stipe) when young. In age only a few fibers may remain on the cap margin or the stipe.

Clamp connection: A bridge-like hyphal connection involved in maintaining the dikaryotic condition.

Decurrent: Pertaining to the attachment of the gills to the stipe, in which the gills curve partly down the stipe towards the base of the stipe.

Dichotomous: A type of hyphal branching into two equal forks.

Dikaryon: A hypha or portion of hyphae which contains two haploid nuclei in each cell.

Floccose: Having a cottony appearance, like a flocked Christmas tree. Seen in some species of *Amanita*.

Generative hyphae: Undifferentiated, thin-walled, usually have frequent septa, and may or may not have clamp connections.

Gregarious cluster: When basidiomes grow in a group that is a bit more scattered and irregular.

Karyogamy: Fusion of two nuclei.

Monokaryon: A hypha or portion of hyphae which contains one haploid nucleus in each cell.

Monopodial: A type of hyphal branching which maintains a single direction of growth and splits laterally.

Plasmogamy

Primary mycelium: Mycelium prior to dikaryotization, produced by basidiospores.

Pseudosclerotial plate: See zone line.

Secondary mycelium: Mycelium subsequent to dikaryotization, or the fusion of primary mycelium from two different mating types.

Skeletal hyphae: Thick-walled and very long and straight, with little cell content. They have few septa and lack clamp connections.

Sinuate: Referring to a type of gill attachment, specifically gills that are notched at their point of attachment to the stipe.

Subhymenium: Refers to the layer of cells between the hymenium, the layer of spore-bearing cells, and the main trama of the gills, or tubes, of a basidiome.

Zone line: black lines seen in decayed wood, which are curved planes in the wood, composed of thickend, dark fungal cells