ARMILLARIA SPECIES IN THE MISSOURI OZARK FORESTS

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Chapter 1

Introduction to the Experimental System

Armillaria biology

Armillaria (Fr:Fr) Staude is a genus of white rot wood decay fungi in the phylum Basidiomycota. Over 40 species of *Armillaria* occur throughout the world (Watling, Kile and Burdsall, 1991). Many of these species are facultative pathogens that cause serious root disease in forests and orchards (Lamour and Jeger, 2000). Other species, however, occur mainly as saprophytic species contributing to wood decay (Fox, 2000b).

In Missouri, each *Armillaria* species produces basidiomes for a relatively short period (up to approx. a month) in the fall; *A. tabescens* usually fruits first, followed by *A. mellea* with the onset of cool weather, and finally *A. gallica* following frost (J. N. Bruhn, personal communication). The fruiting bodies are gilled, honey-colored mushrooms found in clusters on stumps, at the base of trees, or fruiting around the forest floor from dead roots. Throughout the year, *Armillaria* can be diagnosed by other characteristic signs (i.e., mycelial fans and rhizomorphs). Mycelial fans develop under the bark of infected roots and represent the invasive growth of the fungus from the point of infection away from and toward the root crown (Fox, 2000a).

From colonized woody food bases, *Armillaria* forms rhizomorphs: root-like hyphal aggregates surrounded by a melanized rind. Rhizomorphs transport water, nutrients, and oxygen, permitting *Armillaria* to spread through the forest floor among woody food bases to form huge thalli over a long period of time (Fox, 2000a). One of these networks in Michigan was shown to cover 15 hectares and was estimated to be over 1500 years old (Smith, Bruhn and Anderson, 1992). For many species of *Armillaria*, rhizomorphs significantly contribute to infection by radiating out from a nutrient base to forage for other resources and new infection sites (Bruhn and Mihail, 2003). Once a new vulnerable food base is encountered, rhizomorphs dedifferentiate into mycelium in order to decay the wood (Fox, 2000a). This allows genets (*i.e.* genetically unique individuals) of *Armillaria* to simultaneously colonize multiple food bases thus forming a continuously expanding network with the soil (Smith *et al.*, 1992; Smith, Bruhn and Anderson, 1994).

Identification of *Armillaria* species

Prior to the development of new identification techniques for species delimitation in the 1970's, annulate *Armillaria* species were characteristically identified as *A. mellea sensu lato* (Gregory, Rishbeth and Shaw, 1991). Using this broad species concept, *A. mellea s.l.* was characterized by an extraordinarily wide host range, with highly variable virulence and morphology (Gregory *et al.*, 1991; Guillaumin, Anderson and Korhonen, 1991). Hintikka (1973) developed a

mating test technique based on culture morphology to discriminate species. This technique was utilized by Korhonen (1978) to distinguish five European biological species within *A. mellea s.l*. Simultaneously, 10 genetically isolated biological species were discovered in North America (Anderson and Ullrich, 1979; Anderson, 1986).

The mating test used to separate *A. mellea s.l*. into biological species involves pairing two single spore isolates on malt extract agar and observing morphological changes. Single spore isolates typically produce white and fluffy mycelium with aerial mycelia. If the two single spore isolates are of compatible mating types (conspecific), the mycelium will anastomose and form a crustose flattened morphology typical of diploid mycelium (Hintikka, 1973). If the single spore isolates either are incompatible or represent different species, the colonies will not anastomose and the morphology of each strain will remain white and fluffy. A similar paired mycelium test can be used to identify diploid field isolates obtained from mushrooms, mycelial fans or rhizomorphs. In this type of test, the field isolate is paired with single spore tester isolates of known species. If the paired mycelia are compatible, they anastomose freely and, the morphology of the haploid tester becomes flattened and crustose, indicating that the diploid field isolate is of the same species as the single spore tester isolate (Guillaumin *et al.*, 1991; Rizzo and Harrington, 1992).

Other useful techniques for distinguishing *Armillaria* spp. include isozyme analysis and mycelial growth characteristics (Bruhn, Johnson, Karr, Wetteroff *et al.*, 1998) and nuclear DNA content and RFLP analysis (Kim, Klopfenstein,

McDonald, Arumuganathan *et al.*, 2000). Isozyme analysis, using esterase and polyphenol oxidase enzymes, was used to separate species from a collection of isolates representing the Ozark forests of Missouri, USA (Bruhn *et al.*, 1998). Growth characteristics were also found to be sufficient to discriminate species in this collection (Bruhn *et al.*, 1998). Nuclear DNA content was characterized for nine North American *Armillaria* species (Kim *et al.*, 2000). The largest nuclear DNA content, that of *A. gallica*, was 40% larger than that of *A. ostoyae*, *A. gemina*, or *A. mellea*, 25% larger than *A. nabsnona*, and 10% larger than *A. calvescens*, *A. sinapina*, North American Biological Species (NABS) X, and NABS XI. DNA content was determined to be a useful diagnostic characteristic in distinguishing select species of *Armillaria* (Kim *et al.*, 2000).

Interpretation of mating tests and other morphological identification methods require substantial experience. Further, the time and labor required for morphological species determination can be prohibitive. Molecular methods for species identification of *Armillaria* are much more efficient, though the equipment and reagents are more expensive. Most commonly, restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) amplified rDNA region has been applied. The rDNA cluster contains the 18S, 5.8S, 26S, and 5S genes along with two internal transcribed spacers (ITS1 and ITS2) and two non-transcribed spacers (IGS-1 and IGS-2) (Perez-Sierra, Whitehead and Whitehead, 2000). While nuclear rDNA gene sequences are highly conserved, there is no evolutionary pressure for conservation of the non-coding ITS1, ITS2, IGS-1, and IGS-2 regions. Thus there is frequently high variability in the ITS and

IGS regions making them useful in discriminating species of *Armillaria* (Perez-Sierra *et al.*, 2000).

The foundation of PCR analysis in the identification of *Armillaria* spp. comes from a comprehensive study (Anderson and Stasovski, 1992). Primers CLR12 and O-1 (Duchesne and Anderson, 1990) were used to amplify and directly sequence the IGS-1 region between the 26S and 5S genes for eleven Northern Hemisphere species. Phylogenetic analysis of the sequence data revealed two distinct groups of species; one including *A. ostoyae*, *A. gemina*, *A. borealis* and a second including *A. lutea*, *A. calvescens*, *A. cepistipes*, *A. sinapina*, North American Biological Species (NABS) IX and NABS X. Divergence in the intergenic region supported the morphological divergence of *A. mellea* and *A. tabescens* from other *Armillaria* spp. (Anderson and Stasovski, 1992).

Sequence variation found within the intergenic region by Anderson and Stasovski (1992) suggested a possible method of RFLP analysis for rapid species diagnosis. Harrington and Wingfield (1995) applied this strategy using the primers LR12R and O-1 to amplify the IGS-1 region of 11 European and North American *Armillaria* spp. Amplifications were conducted from direct mycelial scrapes or extracted DNA, and PCR products were digested with the enzymes *Alu*I, *Nde*I, *Bsm*I, or *Hind*II for discrimination of species. Upon digestion with *Alu*I, most species produced distinct RFLP patterns with two exceptions. Identical RFLP patterns were observed among isolates of *A. ostoyae*, *A. gemina*, *A. borealis* and *A. cepistipes* and between isolates of North American *A. gallica*

and *A. calvescens*. *Armillaria ostoyae*, *A. gemina*, *A. borealis* and *A. cepistipes* could be distinguished by digestion of amplifications with restriction enzymes *Nde*I, *Bsm*I or *Hind*II. North American *A. gallica* and *A. calvescens* were found to differ at only a single base pair, as determined by IGS sequence analysis, resulting in a site differential for restriction enzyme *Tha*I. However, digestion of amplified products with *Tha*I was inconsistent, and therefore North American *A. gallica* and *A. calvescens* were unable to be distinguished (Harrington and Wingfield, 1995).

The combination of PCR amplification of the IGS-1 region using primers LR12R and O-1 followed by *Alu*I digestion has been applied to *Armillaria* isolates of many different species around the world. In the USA, this procedure was used to identify isolates collected from the Olympic Peninsula of Washington as *A. ostoyae*, *A. sinapina*, *A. nabsnona* (= NABS IX), *A. gallica*, and NABS XI (Banik, Volk and Burdsall, 1996) and from California as *A. mellea, A. gallica, A. nabsnona*, and NABS X (Baumgartner and Rizzo, 2001a; Baumgartner and Rizzo, 2001b). Also within North America, the same primer and restriction enzyme combination was used to identify isolates collected from Canada and the USA as *A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. mellea*, *A. gallica*, *A. nabsnona*, NABS X, and NABS XI (Kim *et al.*, 2000) and from British Columbia as *A. ostoyae*, *A. sinapina*, *A. nabsnona*, and *A. gallica* (White, Dubetz, Cruickshank and Morrison, 1998). However, in order to discriminate *A. gallica* and *A. sinapina* isolates from British Columbia, the IGS-2 region was amplified

using primers TWIL and 5S02R and the PCR product was digested with *Alu*I (White *et al.*, 1998).

The primer combination LR12R/O-1 was used with multiple restriction enzymes to identify 39 isolates of *Armillaria* collected from bigtooth aspen in Pennsylvania (Frontz, Davis, Bunyard and Royse, 1998). Digestions of PCR products were conducted with restriction enzymes *Alu*I, *Hha*I, *Hpa*II, *Hae*III, and *Hin*fI, although digestion with *Alu*I was again shown to provide the most discrimination among species. Isolates were identified as *A. calvescens* or *A. gallica*, *A. ostoyae*, *A. mellea*, and *A. sinapina*. Digests with the restriction enzymes *Alu*I, *Dpn*II, *Taq*I, *Rsa*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Hpa*I, and *Pst*I failed to discriminate isolates of *A. gallica* and *A. calvescens* (Frontz *et al.*, 1998)

Use of primers LR12R and O-1 to amplify the IGS-1 region followed by digestion with restriction enzyme *Alu*I has not been limited to North America. In Britain, 96 isolates of *Armillaria* were identified from the direct addition of mycelia or pieces of basidiomes to the reaction mixture (Perez-Sierra, Whitehead and Whitehead, 1999). Using *Alu*I for digestion, isolates of *A. mellea*, *A. tabescens*, and *A. gallica* were successfully distinguished. PCR products of *A. borealis* and *A. cepistipes* were distinguished following digestion with *Bsm*I (Perez-Sierra *et al.*, 1999).

Japanese *Armillaria* isolates were also successfully identified using PCR-RFLP of the IGS-1 region (Terashima, Cha, Yajima, Igarashi *et al.*, 1998a; Terashima, Kawashima, Cha and Miura, 1998b). Using primers LR12R and O-1 to amplify the IGS-1 followed by an *Alu*I digestion, *A. mellea* subsp. *nipponica*

was distinguished from the other species. Digestion with *Alu*I and *Dde*I was able to differentiate isolates of *A. ostoyae, A. gallica*, *A. jezoensis*, and *A. singula* (Terashima *et al.*, 1998b). African *Armillaria* isolates were identified as *A. heimii* and *A. fuscipes* using PCR-RFLP of the IGS-1 region as well, although a different forward primer, P-1, was used (Coetzee, Wingfield, Harrington, Steimel *et al.*, 2001b; Coetzee, Wingfield, Roux, Crous *et al.*, 2003; Mwenje, Wingfield, Coetzee and Wingfield, 2003; Perez-Sierra, Guillaumin, Spooner and Bridge, 2004).

PCR amplification of the IGS-1 has also been useful for phylogenetic studies in Europe (Chillali, Wipf, Guillaumin, Mohammed *et al.*, 1998), Australia and New Zealand (Coetzee, Wingfield, Bloomer, Ridley *et al.*, 2001a), and Japan (Terashima *et al.*, 1998a). Phylogenetic analysis of *A. mellea* s.s. based on IGS-1 (using primers P-1 and O-1) and ITS1 (using primers ITS1 and ITS4) sequences distinguished four geographic groups: Asia, western North America, eastern North American, and Europe (Coetzee, Wingfield, Harrington, Dalevi *et al.*, 2000).

In addition to the IGS region, amplification of ITS1 and ITS2 has been useful in distinguishing *Armillaria* spp. (Smith-White, Summerell, Gunn, Rinzin *et al.*, 2002; Sicoli and Stenlid, 2003; Lochman, Sery, Jankovsky and Mikes, 2004). Fungal primers ITS1 and ITS4 have been used to amplify the ITS1 region for direct sequencing analysis (Chillali *et al.*, 1998; Coetzee *et al.*, 2000; Coetzee *et al.*, 2001a). Species-specific primers were also developed for the ITS region of European *Armillaria mellea* and *A. tabescens* (Sicoli and Stenlid, 2003).

Oak decline and Armillaria root disease in the Missouri Ozark Forest Ecosystem Project (MOFEP)

Forest decline occurs as a result of a combination of abiotic and biotic factors. These factors are often grouped as predisposing, inciting, or contributing factors (Manion and Lachance, 1992). Predisposing factors are site and stand characteristics, such as soil conditions and genetic potential, which negatively affect the ability of susceptible tree species to endure stress. Inciting factors, including drought and defoliating insects, stress trees temporarily, allowing for recovery. Contributing factors exacerbate the disorder caused by the predisposing factors, most often to a point of no recovery (Manion and Lachance, 1992). In the context of forest decline, *Armillaria* spp. are often contributing factors to the fatal decline of trees stressed by defoliation or drought (Wargo and Harrington, 1991; Houston, 1992).

Decline has been observed for oak trees in the Missouri Ozark Mountains (Law and Gott, 1987; Johnson and Law, 1989a; Dwyer, Cutter and Wetteroff, 1995). Growth rates of a sub-population of oak trees did not recover following a severe drought and were further lowered by subsequent droughts (Dwyer *et al.*, 1995). Drought and other predisposing factors combined with Armillaria root disease have been linked to oak mortality and decline in the region (Johnson and Law, 1989b) Specifically, *A. mellea* and *A. tabescens* have been associated

with mortality of *Quercus coccinea*, *Q. marilandica*, and *Q. velutina* (Bruhn, Wetteroff, Mihail, Kabrick *et al.*, 2000).

In 1989, the Missouri Ozark Forest Ecosystem Project (MOFEP) was initiated to examine the impacts of forest management practices on the forest ecosystem. Nine sites, ranging from 266 ha to 527 ha in size, were chosen in upland forests of southeastern Missouri (Brookshire, Jensen and Dey, 1997). Each site was assigned a silviculture treatment of even-aged management, uneven-aged management, or no-harvest management resulting in three sites per treatment (Brookshire *et al.*, 1997). Across the study sites, overstory vegetation is upland forest with mature second-growth *Quercus*, *Carya*, and *Pinus echinata* Mill. (Kabrick, Larsen and Shifley, 1997). MOFEP sites occur in two land type associations (LTA): the 'Breaks' LTA is characterized by greater soil variety and more geological strata than the 'Hills' LTA, which has gentler relief (Meinert, Nigh and Kabrick, 1997).

Within MOFEP, *Armillaria mellea*, *A. tabescens*, and *A. gallica* have been identified (Bruhn *et al.*, 1998; Bruhn *et al.*, 2000). Initial investigations showed *A. mellea* to occur in 91% of 180 0.2-ha plots and to be associated with dead or dying oak trees. "Recently dead trees" were described as 'trees retaining fine twig structure and any dead leaves', whereas dying trees were described as 'living trees with abnormally few live leaves' (Bruhn, Wetteroff, Mihail and Burks, 1997; Bruhn *et al.*, 2000). *Armillaria gallica* was found in 64% of 180 plots. *Armillaria tabescens* prevalence was difficult to estimate accurately due to its lower abundance. *Armillaria gallica* and *A. tabescens* were less often associated

with mortality (Bruhn *et al.*, 1997) compared with *A. mellea*. Most mortality within the region was associated with two red oak species: *Quercus coccinea* and *Quercus velutina* (Bruhn *et al.*, 2000).

Distributions of *Armillaria mellea*, *A. tabescens*, and *A. gallica* were also evaluated in relation to site characteristics, overstory species composition, and oak decline (Bruhn *et al.*, 2000). *Armillaria mellea* and *A. gallica* occurred together under favorable conditions for *A. gallica* as well as on plots with greater proportions of dead *Quercus coccinea* and *Q. marilandica.* Oak decline was most severe where vulnerable host species were most abundant (in the Hills LTA) and on exposed and neutral west aspects (where *A. mellea* most often occurred in the absence of *A. gallica*) (Bruhn *et al.*, 2000).

In order to apply forest management techniques effectively it is important to understand the interactions of the organisms within the forest ecosystem. In the forests of the southern Missouri Ozark Mountains, *Armillaria* and oak species are important interacting components of the ecosystem. Elucidating the effects of *Armillaria* on oak decline requires a rapid, efficient and successful method for identification of *Armillaria* isolates to species. It was my objective for this thesis to develop and refine the technique for speciation of *Armillaria* so that identifications could be made in an efficient manner and to examine the association of the detected *Armillaria* species with their respective hosts.

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Chapter 2

Protocol Development

We first attempted to identify our *Armillaria* field isolates to species by following the method of Harrington and Wingfield (1995). Their protocol provided a rapid diagnosis of *Armillaria* spp. using a direct mycelial scrape for PCR. Although this method was successful for other groups (Terashima, Kawashima, Cha and Miura, 1998; Perez-Sierra, Whitehead and Whitehead, 1999; Kim, Klopfenstein, McDonald, Arumuganathan *et al.*, 2000), we did not obtain consistent results with *Armillaria* isolates from Missouri. Thus, I examined each step of the protocol along with methods for DNA extraction to obtain clear and consistent species identifications. Ultimately a protocol was developed with a high success rate which could justify the time and expense involved.

Following the method of Harrington and Wingfield (1995), samples for PCR were taken from a direct mycelial scrape. A pipette tip was used to scrape approximately 1 cm across the surface of an isolate grown on 1.5% malt agar. The tip was then immersed and agitated in the PCR mix. The PCR cycle settings and temperatures were taken from Frontz, Davis, Bunyard and Royse (1998). Because initial attempts gave inconsistent results, further modifications of the protocol were explored. Since the methods of Harrington and Wingfield (1995) and Frontz et al, (1998) differed in their annealing temperatures and elongation times, these were examined in more detail. Using 49° or 57.7° C, the annealing

temperature was tested, with no improvement in results. The differing elongation times (30 cycles at 72° C for 60 seconds or 5 cycles at 72° C for 3 minutes followed by 25 cycles at 72° C for 90 seconds) had no consistent effect on results. Later tests however showed stronger bands with the settings from Harrington and Wingfield (1995). These PCR conditions, initial denaturation at 95° C for 95 s, followed by 35 cycles at 60° C for 40 s, 72° C for 120 s, 90° C for 30 s, with a final elongation at 72° C for 10 min, were then used for all PCR reactions.

Next, I examined the sampling and culturing methods as sources of inconsistency. The brand of pipette tip (Eppendorf or Fisher), technique of sampling (scraping along or touching the surface of the culture), and agar media (water agar or malt extract agars) were all tested and found to be unrelated to success in obtaining a PCR product. However, differences in PCR success rates were found when the location of scrape and age of culture were considered. To test the location of the scrape, samples were taken from the outside growing edge, from the mid-point between edge and center, and from the center of actively growing cultures. Samples taken from the outside edge of the colony gave better PCR products, suggesting the need for fresh cultures. Therefore, the age of culture was examined by sampling from cultures 1, 2, and 3 weeks old. Samples from the two-week-old cultures were the most successful. Thus, young, fresh mycelium is best for PCR from direct mycelial scrapes.

PCR-RFLP from a direct mycelial scrape of two-week-old cultures was applied to 140 isolates collected in 2002. Once an isolate was identified to

species it was eliminated from investigation. Most isolates required multiple PCR attempts before amplifications were successful and identifications could be made. Even after multiple attempts, however, 45 of the 140 isolates did not yield detectable PCR products. These isolates were labelled 'difficult' and investigated using different culturing methods and DNA extractions.

The 'difficult' isolates were grown on a 2.5cm x 2.5cm overlay of dialysis membrane (23mm Spectra/Por® molecularporous membrane tubing, Spectrum Medical Industries, Inc., Los Angeles, California) on either 2% malt agar or carrot-orange juice agar media (Mihail, Obert, Bruhn and Taylor, 1995). Mycelial scrapes from the surface of the dialysis membrane were used for PCR. Using mycelium grown on dialysis membrane, four 'difficult' isolates were identified to species (Table 2.1). Eleven 'difficult' isolates were grown in liquid carrot-orange juice agar for production of rhizomorphs. Tips of the rhizomorphs were dissected under a microscope and placed in the PCR mixture. The rhizomorph tips yielded identifications for another four isolates (Table 2.1).

At this point, the focus of protocol development switched from use of actively growing mycelium to use of extracted DNA as a starting point for PCR. A DNA extraction method using CTAB (ICN Biomedicals, Inc., Aurora, Ohio) that successfully removes polysaccharide-like components (Murray and Thompson, 1980) was applied to all 45 'difficult' isolates. With the CTAB DNA extraction, 16 isolates were identified (Table 2.1). However, several extractions were often necessary for each isolate using CTAB. Because of the inefficiency of the CTAB extraction, other methods were explored.

The DNEasy Plant Mini kit (Qiagen, Germantown, Maryland) was used to extract DNA from the remaining 21 'difficult' field isolates collected in 2002. In a single attempt, DNA was successfully extracted and all isolates were identified using PCR-RFLP (Table 2.1). The Qiagen kit was the most successful method for identifying isolates that could not be amplified using PCR directly from mycelial scrapes.

If the 'difficult' isolates had remained unidentified, the percentage distribution of *Armillaria* spp. would have been skewed. The initial isolates identified using the mycelial scrape method (without a DNA extraction) represented a species distribution of 59% *A. mellea*, 6% *A. tabescens* and 35% *A. gallica*. However, the final distributions of *A. mellea* (48%) and *A. tabescens* (18%), after identification of the 'difficult' isolates, were different than the initial distributions as suggested by the mycelial scrape method.

A second group of *Armillaria* isolates, collected from stumps and snags in 2003 and 2004, was initially processed using the direct mycelial scrape PCR-RFLP protocol for identification. Using this method, 54 of the 196 isolates were identified to species (Table 2.2). Once an isolate was identified it was no longer processed using the other methods. Thus, for the remaining unidentified isolates, the PrepMan^{TM} Ultra reagent (Applied Biosystems, California, USA) was used to extract DNA. Unfortunately, using this reagent for DNA extraction resulted in amplifications for only 25 of the 142 isolates (Table 2.2). Since the Qiagen DNEasy Plant Mini extraction kit had worked on the difficult isolates from the 2002 pool, it was used for the remaining isolates from 2003 and 2004. Using

the Qiagen DNEasy kit for extraction prior to PCR-RFLP provided identifications for all but six isolates on the first attempt (Table 2.2). Once again, the Qiagen extraction kit was more successful than the mycelial scrape or other DNA extraction methods.

From the time and effort put into examining and developing an efficient protocol for speciation of *Armillaria* field isolates, a successful method was found. This method, fully described in Chapter 3, begins with culturing isolates on 2% malt agar media overlaid with dialysis membrane. After two weeks growth, mycelium is collected from the dialysis membrane. DNA is extracted from the mycelium using the Qiagen DNEasy Plant Mini kit following the manufacturer's recommended protocol. The DNA is then used for PCR-RFLP analysis, following the protocol of Harrington and Wingfield (1995). Using this procedure, *Armillaria* isolates can be identified to species in a successful and timely manner.

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Table 2.1. Identification of 21 'difficult' *Armillaria* isolates in 2002 collections using various sources of template DNA

	Armillaria species			
Method	*mellea*	*tabescens*	*gallica*	Total
Mycelial Scrape				
2M + d.m.[a]	0	0	3	3
COA + d.m.[b]	1	0	0	1
Rhizomorph	2	0	2	4
DNA Extraction				
CTAB[c]	2	6	8	16
Qiagen kit[d]	6	13	2	21

[a] Isolates were inoculated onto dialysis membrane overlaying 2% malt extract agar.

[b] Isolates were grown on dialysis membrane overlaying carrot-orange juice agar.

[c] DNA extraction was performed using the CTAB based protocol.

[d] DNA extraction was performed using the Qiagen DNEasy Plant Mini kit.

Table 2.2. The number of *Armillaria* isolates collected in 2003 and 2004 identified to species as a result of protocol refinement

	Armillaria species			
Method	*mellea*	*tabescens*	*gallica*	Total
Mycelial Scrape [a]	42	3	9	54
Prepman reagent [b]	24	1	0	25
Qiagen kit [c]	78	12	27	117
Total	144	16	36	196

[a] Mycelial scrape of 1-2 week old culture on 2% malt agar media used for PCR.

[b] DNA for PCR was extracted using the PrepMan™ Ultra reagent.

[c] DNA for PCR was extracted using the Qiagen DNEasy Plant Mini kit.

Chapter 3

Host relationships of *Armillaria* isolates collected within the Missouri Ozark Forest Ecosystem Project during 2002

Introduction

Armillaria is a genus of white rot fungi considered to contain some of the most significant tree pathogens in the world. Pathogenicity varies among *Armillaria* spp. Some species are highly pathogenic and others act primarily as saprophytes (Gregory, Rishbeth and Shaw, 1991). Propagation of *Armillaria* spp. occurs sexually by means of airborne haploid basidiospores produced by mushrooms, or vegetatively by diploid mycelial fans growing within the substrate and rhizomorphs growing through wood or soil. Relatively pathogenic species, such as *A. mellea,* appear able to colonize vulnerable living root systems through wounds as germinating basidiospores or as short rhizomorphs, giving rise to mycelial fan systems which may spread through connected root systems (Rishbeth, 1991). Relatively saprophytic species, however, proliferate especially efficiently by extensive rhizomorph systems spreading among woody nutrient bases, allowing genetically unique individuals (*i.e.* genets) to occupy large areas (Rishbeth, 1991; Bruhn and Mihail, 2003; Mihail and Bruhn, 2005)

Within the Missouri Ozark Mountains, both pathogenic species (*i.e*. *A. mellea* and *A. tabescens*) and primarily saprophytic species (*A. gallica*) coexist

and contribute to forest structure (Bruhn, Wetteroff, Mihail, Kabrick *et al.*, 2000). Explanation of the interactions among these coexisting species and the forest vegetation is important for elucidating effective forest management strategies. Since 1993, sites in southern Missouri have been examined for Armillaria root disease as well as for forest decline and mortality, in connection with the Missouri Ozark Forest Ecosystem Project (MOFEP). The Ozark Mountains are predominately upland forests with overstory vegetation of mature second-growth *Quercus*, *Carya,* and *Pinus* (Kabrick, Larsen and Shifley, 1997). In 1996, each of the nine MOFEP sites received a forest management treatment: either even-aged (sites 3, 5, and 9), uneven-aged (sites 2, 4, and 7), or no-harvest (sites 1, 6, and 8) (Brookshire, Jensen and Dey, 1997). *Armillaria* isolates have been collected on all nine MOFEP sites from mycelial fans, rhizomorphs, and fruiting bodies. Species identifications have been accomplished using a combination of isozyme analysis and diploid/haploid pairings (Bruhn *et al.*, 2000).

Identifying *Armillaria* isolates to species has been time and labor intensive. For example, isolates were identified to species using mycelial growth characteristics and isozyme production (Bruhn, Johnson, Karr, Wetteroff et al., 1998) or by diploid/ haploid mating tests with known haploid testers (Bruhn et al., 2000). These tests however require large amounts of labor and take at least six weeks to yield results from pure cultures. A more rapid molecular technique involving amplification of the Intergenic Spacer (IGS-1) of the ribosomal DNA region, followed by digestion using the restriction enzyme *Alu*I was developed for species identifcation of *Armillaria* isolates (Harrington and Wingfield, 1995). This

technique has found widespread application (Terashima, Kawashima, Cha and Miura, 1998; Perez-Sierra, Whitehead and Whitehead, 1999; Kim, Klopfenstein, McDonald, Arumuganathan *et al.*, 2000) This molecular identification method is efficient in terms of time required and provides consistent results.

In order to understand the host interactions of the *Armillaria* spp. and their contribution to oak decline within the MOFEP sites, field isolates have been collected from mushrooms, mycelial fans and rhizomorphs since 1993. During 2002, six years after the first MOFEP harvest, a new mycelial morphology pattern was detected, and the question arose whether this previously unknown morphology represented an anomaly or a new species. Therefore, isolates collected during 2002 were identified using the PCR-RFLP method and identifications were analyzed in the context of oak decline and mortality.

Materials and Methods

Origin of isolates

Armillaria field isolates were collected from seven of the nine MOFEP sites (266 ha to 527 ha in size) (Brookshire *et al.*, 1997). For each collection, host species and condition were recorded (Appendix 1). Recent host mortality was characterized as “currently dying” (trees with abnormally few or small live leaves) or “dead within 2 years” (trees retaining fine twig structure and dead leaves).

Samples were obtained and cultured from rhizomorphs, mycelial fans and/or fruiting bodies. Initial cultural isolations were made on 2% water agar supplemented with 200 $\mu g \cdot ml^{-1}$ streptomycin sulfate (Sigma Chemical Co., St. Louis, MO). Fungal tissue from fruiting bodies was taken from internal cap tissue just above the gills. For tissue from mycelial fans, the bark or root surface sample was sterilized with 1.05% NaOCl and carefully removed to expose the fan. Rhizomorphs were soaked in 1.05% NaOCl for 7 min, blotted dry using paper towels, and cut into 1 mm segments for culturing. When subculturing from the water agar plates, contaminations from bacterial colonies were evaded using van Tieghem cells (Tuite, 1969), and pure working cultures were maintained on 2% malt extract agar.

Polymerase chain reaction (PCR)

Amplification of the IGS-1 region was accomplished following a method adapted from Harrington and Wingfield (1995). PCR was conducted by directly adding a mycelial scrape or extracted DNA (as described below) to a 20 µl PCR mixture. To obtain the mycelial sample, a pipette tip was scraped along the growing margin of a 2-3 week-old culture actively growing on 2% malt agar. The tip was directly placed in the PCR mixture tube and stirred to suspend the mycelium. Each PCR mixture contained 2 units *Taq* polymerase (Promega, Madison, Wisconsin), 10X Mg free buffer supplied with the enzyme, 1 mM dNTPs (Promega, Madison, Wisconsin), 2.5 mM $MgCl_2$, and 1 µM of each primer. Primers used were O-1: 5' AGT CCT ATG GCC GRG GAT 3' (Duchesne and

Anderson, 1990) and LR12R: 5' CTG AAC GCC TCT AAG TCA GAA 3' (Veldman, Klootwijk, Regt and Rudi, 1981). PCR conditions were an initial denaturation at 95° C for 95 s, followed by 35 cycles of 60° C for 40 s, 72° C for 120 s, 90° C for 30 s, with a final elongation at 72° C for 10 min.

DNA Extraction

For isolates that could not be amplified directly from mycelial scrapes, DNA extraction was conducted using the DNEasy Plant Mini kit (Qiagen, Germantown, Maryland). Isolates were grown on 2% malt agar overlain with 2.5cm x 2.5cm dialysis membrane (23mm Spectra/Por® molecularporous membrane tubing, Spectrum Medical Industries, Inc., Los Angeles, California) for 2-3 weeks at room temperature in the dark. Mycelium was harvested using a sterile spatula and placed in a 1.5 ml microcentrifuge tube. A micropestle was then used to grind the mycelium in 100 µl of lysis buffer from the first step of the manufacturer's instructions for the Qiagen DNEasy Plant Mini kit. Once the mycelium was completely suspended, the remaining lysis buffer was added and the manufacturer's instructions were followed. For PCR, 1 µl of DNA extraction product was added to 19 µl of PCR mixture as above. PCR and RFLP analysis was run as described.

Restriction Fragment Length Polymorphism (RFLP)

Restriction enzyme digestion of PCR products was completed by directly adding 5 units of *Alu*I (Promega, Madison, Wisconsin) to 20µl of the PCR product

and incubating for 1-12h at 37° C. Restricted fragments were separated on a 2% agarose gel (UltraPure™ Agarose, Invitrogen, Carlsbad, California) in tris-acetate EDTA (TAE) buffer at 80 V for 1-2 h, stained with ethidium bromide, and visualized under UV illumination.

DNA Sequencing

The IGS region of representative isolates from each known RFLP pattern occurring in the MOFEP research area was sequenced using primers O-1 and LR12R. PCR products were purified using the Wizard System (Promega, Madison, Wisconsin) and sequenced at the DNA Core at the University of Missouri (Columbia, Missouri) using a 3730 DNA Analyzer with Applied Biosystems Big Dye Terminator cycle sequencing chemistry. Sequence analysis was performed using ChromasPro (Technelysium Pty Ltd) and Sequencher (Gene Codes Corporation).

Cloning of IGS-1

PCR products of *Armillaria* isolates that gave poor sequencing were cloned using a TOPO TA Cloning® Kit with pCR® 2.1-TOPO® (Invitrogen, Carlsbad, California). Cloned products were transformed with One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* (Invitrogen, Carlsbad, California) following the manufacturer's instructions. Positive inserts were selected from Luria-Bertani (LB) media with kanamycin after incubation

overnight at 37º C as recommended in the manufacturer's instructions. Positive clones were analyzed by PCR and *Alu*I digestion following the conditions described above. IGS-1 regions of clones representing each RFLP profile were sequenced as previously described by the DNA Core at the University of Missouri (Columbia, Missouri).

Statistical Analyses

Contingency table analysis was used to examine the association of tree condition and species with *Armillaria* spp. The χ^2 statistic was used to test hypotheses (Sokal and Rohlf, 1995).

Results

Armillaria spp. identification using PCR-RFLP

Although some isolates were identified using mycelial scrapes prior to PCR, most required DNA extraction. PCR product sizes for *Armillaria mellea, A. tabescens* and *A. gallica* were similar to those reported by Harrington and Wingfield (1995). Digestion of the amplified IGS region with *Alu*I produced sufficient restriction profiles for identification of isolates to species. Three of the RFLP profiles (mel 1, tab, and gal1) were consistent with the observations of Harrington and Wingfield (1995) as *A. mellea*, *A. tabescens*, and *A. gallica*,

respectively (Fig. 3.1). However, intraspecific variation was observed yielding a second RFLP profile for *A. mellea* (mel 2) and *A. gallica* (gal 2) (Fig. 3.1). None of the RFLP profiles found corresponded to the new mycelial morphology pattern detected in the isolates collected from 2002.

The mel 1 RFLP profile was similar to patterns reported for isolates from the USA, specifically Massachusetts, New Hampshire, and Wisconsin (Harrington and Wingfield, 1995; Kim *et al.*, 2000). However, the mel 2 pattern is previously unreported and is not similar to any other reported profiles for *A. mellea*. The tab RFLP profile has been reported for many isolates throughout the world including the USA (Ohio and Illinois), France, Italy, and Japan (Harrington and Wingfield, 1995; Perez-Sierra *et al.*, 1999; Matsushita and Suzuki, 2005). RFLP profiles similar to gal 1 have been found for isolates from New Hampshire, Vermont, Michigan and Wisconsin in the USA (Harrington and Wingfield, 1995; Kim *et al.*, 2000). The only reported profile similar to that of gal 2 was found in isolates from Michigan, USA (Kim *et al.*, 2000).

The RFLP profiles mel 1, mel 2, gal 1 and gal 2 were evaluated for patterns in association with host species, recent mortality and forest management treatments. Each RFLP profile was distributed over all host species, and no distinct pattern was detected (Table 3.1). There was also no specific pattern observed among the RFLP profiles and recent mortality hosts (Table 3.2). Although mel 1 RFLP profile isolates were distributed throughout the three forest management treatments, the majority of mel 2 isolates were collected from uneven aged MOFEP sites (Fig. 3.2). These sites also provided

the majority of isolates representing RFLP profile gal 2 (Fig 3.3). However, since representative isolates for both RFLP profiles mel 2 and gal 2 were collected from all three management treatments, and no patterns were observed for host preference, mel 1 and mel 2 isolates were considered together as *A. mellea,* and gal 1 and gal 2 isolates were considered *A. gallica* for host species analysis.

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Sequencing analyses

Representative isolates of each RFLP profile were sequenced using primers LR12R and O-1 directly from PCR amplifications of IGS-1. DNA sequences from mel 1 and mel 2 RFLP profile isolates were most similar to the sequence of an *A. mellea* from Massachusetts (GenBank accession number AF163615) as revealed by a BLAST (Altschul, Gish, Miller, Myers *et al.*, 1990) search (http://www.ncbi.nlm.nih.gov/) (Table 3.3). The IGS-1 sequence of the tab RFLP profile had highest identity with the sequence of an isolate from Africa representing a northern hemisphere *A. tabescens* (AF773966) (Table 3.3). A BLAST search using sequences of RFLP profiles gal 1 and gal 2 revealed that both had highest identity with a sequence from an *A. gallica* isolate from Serbia and Montenegro (DQ115580) (Table 3.3). It should be noted that there are no IGS-1 sequences of North American *A. gallica* in GenBank.

Isolates with RFLP patterns mel 2 and gal 2 could not be sequenced directly. Sequence heterogeneity was detected in IGS-1 amplifications from the same individual. Therefore, PCR products were cloned using a TOPO TA vector. Sequence analysis revealed that for mel 2, loss of an *Alu*I restriction site resulted

in fragments of 475 base pairs (bp) and *ca* 225 bp (Fig 3.4, pattern *a*) instead of fragments 475 bp and 175 bp (Fig 3.4, pattern *b*). Individuals exhibiting the mel 2 RFLP profile contained IGS-1 regions with and without the additional *Alu*I restriction site, resulting in the RFLP profile containing fragments of 475, 225 and 175 bp (Fig 3.1).

The gal 2 RFLP profile isolates also exhibited heterogeneity within the IGS-1 repeated regions. Some amplicons upon digestion with *Alu*I revealed the same RFLP profile as gal 1, i.e. fragments of 582 and 240 bp in length (Fig 3.5, pattern *a*). However, other amplicons contained a new *Alu*I restriction site within the 582 bp fragment resulting in two separate fragments of 401 and 181 bp in length (Fig 3.5, pattern *b*). Thus, the combination of these two IGS-1 region sequences resulted in the gal 2 RFLP profile containing fragment lengths of 582, 401, 240 and 181 bp (Fig 3.1).

Tree host associations

Of the 140 *Armillaria* isolates examined, 44%, 25%, 8%, and 3% were collected from dogwood (*Cornus florida*), red oak spp. (*Quercus coccinea, Q. velutina* and *Q. marilandica*), white oak species (*Q. alba* and *Q. stellata*), and short leaf pine (*Pinus echinata*), respectively (Fig 3.6). Other hosts, including hickory (*Carya* spp.), black walnut (*Juglans nigra*), black cherry (*Prunus serotina*), Carolina buckthorn (*Rhamnus caroliniana*) and maple (*Acer* spp.), as well as unidentified hosts or dead roots, accounted for the remaining 20% of collections (Fig 3.6).

Armillaria mellea, *A. tabescens* and *A. gallica* were recovered from dogwood, red oak and white oak spp. (Fig 3.7) However, because of the few isolates of *A. tabescens* recovered from white oak spp., both red oak and white oak spp. were grouped together for contingency table analysis. *Armillaria mellea* was recovered at similar frequencies from dogwood and all oaks (red and white oak spp.) (Fig 3.7). In contrast, *A. tabescens* was found significantly more often on oak species, whereas *A. gallica* was collected significantly more often from dogwood (χ^2=11.175, df=2, P≤0.01, Fig 3.7).

If the analysis includes only *A. mellea* and *A. gallica* isolates, the associations among red and white oak can be distinguished. These two *Armillaria* spp. were recovered at similar frequencies from dogwood and white oak spp. (*Q. alba* and *Q. stellata*) (Fig 3.7). In contrast, *A. mellea* was recovered significantly more frequently from red oak species (*Q. coccinea, Q. velutina* and *Q. marilandica*) than was *A. gallica* (χ^2=9.002, df=2, P≤0.05, Fig 3.7).

From the 140 isolates collected in year 2002, 40 were collected from "recent mortality" trees. The most common "recent mortality" host species were red oak and dogwood (Fig 3.8). The remaining "recent mortality" isolates were collected from white oak species and short leaf pine (Fig 3.8).

The 40 isolates collected from "recent mortality" were identified as *A. mellea* or *A. gallica*. Both of these species were equally distributed over dogwood (Fig 3.8). However, over all "recent mortality" oak species, including white and red oak species, *A. mellea* was found significantly more often than *A. gallica* (χ^2=10.94, df=1, P≤0.001, Fig 3.8).

Discussion

Assessing the complex interactions of *Armillaria* spp. with forest structure was successfully accomplished using an efficient method for identification of *Armillaria* collections to species. Analyzing *Armillaria* spp. associations with host species has shown that *Armillaria mellea* is a contributing factor to oak decline in the southern Missouri Ozark Mountains.

PCR-RFLP analysis and sequencing of the IGS-1 region from PCR product was successful in identifying isolates as *A. mellea, A. tabescens, or A. gallica.* However, direct sequencing was difficult in the isolates exhibiting heterogeneity (mel 2 and gal 2), thus cloning of their PCR products was necessary for full characterization. Digestion with *Alu*I and sequencing of the clones revealed that in both the mel 2 and gal 2 profiles, more than one sequence exists for the IGS-1 region and the mel 2 and gal 2 profiles are actually combinations of these separate sequences. Due to the nature of the repeated ribosomal region, different sequences are not surprising. Furthermore, heterogeneity for this region has been observed previously in unidentified *Armillaria* sp. isolates from Bhutan (Coetzee, Wingfield, Kirisits, Chhetr *et al.*, 2005).

The analyses of *Armillaria* spp. in association with tree host species contributes to our understanding of the interactions of *Armillaria* spp. in the Missouri Ozark Mountains. Dogwood and red oak species were both significant sources of *Armillaria* isolates. Red oak species (*Q. velutina* and *Q. coccinea*) and white oak species (*Q. stellata* and *Q. alba*) are important woody species

within the Missouri Ozark Forest Ecosystem Project in respect to basal area and density (Brookshire *et al.*, 1997). Dogwood (*Cornus florida*), although not as significant in respect to basal area and density (Brookshire *et al.*, 1997), is still important for both wildlife and aesthetic value.

Forest decline occurs as a result of a combination of abiotic and biotic factors. These factors are often grouped as predisposing, inciting, or contributing factors (Manion and Lachance, 1992). Predisposing factors are site and stand characteristics, such as soil conditions and genetic potential, which negatively affect the ability of susceptible tree species to endure stress. Inciting factors, including drought and defoliating insects, stress trees temporarily, allowing for recovery. Contributing factors exacerbate the disorder caused by the predisposing and inciting factors, often to a point of no recovery (Manion and Lachance, 1992). In the context of forest decline, *Armillaria* spp. are often contributing factors to the mortality of trees stressed by defoliation or drought (Wargo and Harrington, 1991; Houston, 1992).

The greatest number of *Armillaria* isolates from recent mortality trees were obtained from red oak species. Red oak species in the Missouri Ozark region have been predisposed to decline by a number of factors including severe drought, snowless winters and defoliations caused by insects and late spring frosts (Johnson and Law, 1989). Thus, contributing factors, including *Armillaria* as well as the two-lined chestnut borer, aggravate the declining condition sending the tree towards death rather than allowing for recovery (Johnson and Law, 1989). The majority of recent mortality isolates were collected from red oak

species but dogwood, white oak species, and short leaf pine were also affected. The small number of isolates collected from recent mortality short leaf pine and white oak species reflects the healthier condition of the populations of these species, so the effect of *Armillaria* on these species may be negligible.

Armillaria mellea, A. tabescens and *A. gallica* are all functioning as hardwood specialists (Bruhn *et al.*, 2000). *Armillaria mellea* was recovered with equal frequency from dogwoods and oak species. This is not surprising due to the high pathogenicity of *A. mellea.* However, when the oak species are considered separately as red oaks and white oaks, *A. mellea* occurs more often on the predisposed red oak species. Although relatively few isolates of *A. tabescens* were collected, this pathogenic species was found more often on red oaks than on any other host species. *Armillaria gallica* is a saprophytic species and was most often found on dogwood trees instead of oaks. This may be indicative of a higher susceptibility for dogwood to *Armillaria* infection. Though shortleaf pine was rarely associated with *Armillaria*, *A. gallica* was the only *Armillaria* species found associated with short leaf pine in 2002.

Oak decline has been documented for the Missouri Ozark forests (Law and Gott, 1987; Johnson and Law, 1989; Dwyer, Cutter and Wetteroff, 1995). Specifically, red oak species, including *Quercus coccinea* and *Q. velutina*, have been most affected. From this study, it is apparent that *Armillaria mellea* is an important contributing factor to this decline. Using PCR-RFLP of the IGS-1 region provided me with an efficient and successful method to speciate all collected isolates of *Armillaria* for effective analysis of the impact of *Armillaria* on

host species. This study has contributed to the knowledge of the relationship of *Armillaria* species with their hosts in the Missouri Ozark Mountains and to the role of *Armillaria* in determining forest structure.

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Table 3.1 Number of isolates representing RFLP profiles mel 1, mel 2, gal 1 and gal 2 collected from different host species

	RFLP profiles				
Host	mel 1	mel 2	gal 1	gal 2	Total
Cornus florida	18	13	23	5	59
Carya spp.	1	1	3	0	5
Juglans nigra	0	0	2	0	2
Pinus echinata	0	0	4	0	4
Prunus serotina	0	0	1	0	1
red oak spp.	0	1	0	0	1
Quercus coccinea	0	5	1	1	7
Quercus marilandica	1	1	0	0	2
Quercus velutina	11	5	2	0	18
Quercus alba	5	0	2	1	8
Quercus stellata	1	0	1	0	2
Unknown[a]	2	2	1	1	6
Total	39	28	40	8	115

[a] Isolates were collected from unidentifiable dead roots or hosts.

Table 3.2 Number of isolates representing RFLP profiles mel 1, mel 2, gal 1 and gal 2 collected from recent mortality host species

	RFLP profiles				
Host	mel 1	mel 2	gal 1	gal 2	Total
Cornus florida	5	1	6	1	13
Pinus echinata	0	0	3	0	3
Quercus coccinea	0	2	0	1	3
Quercus marilandica	1	1	0	0	2
Quercus velutina	8	5	2	0	15
Quercus alba	3	0	0	0	3
Quercus stellata	1	0	0	0	1
Total	18	9	11	2	40

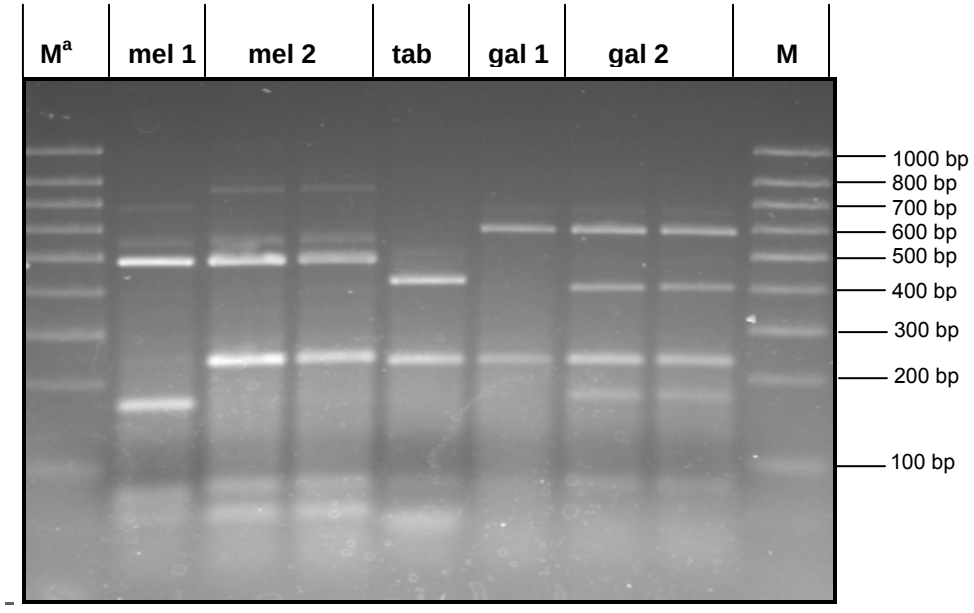
Table 3.3 Results of IGS sequence nucleotide BLAST search of GenBank using representative isolates for each RFLP profile

RFLP Profile	Most Similar Sequence Accession #[a]	Source	Identities[b]	*Armillaria* spp.	Publication
mel 1	AY509187	Wisconsin	704/707	*mellea*	Kim (unpublished)
mel 2 pattern *a*	AY509187	Wisconsin	704/707	*mellea*	Kim (unpublished)
mel 2 pattern *b*	AY509185	Virginia	737/740	*mellea*	Kim (unpublished)
tab	AY773966	Africa (introduced)	727/729	*tabescens*	Coetzee 2003
gal 1	AY509173	Wisconsin	675/681	*gallica*	Kim (unpublished)
gal 2 pattern *a*	DQ115580	Serbia and Montenegro	827/839	*gallica*	Keca (unpublished)
gal 2 pattern *b*	AY509172	Michigan	772/797	*gallica*	Kim (unpublished)

[a] Accession number in the GenBank database.

[b] Identities equal the number of matching bases out of the number of bases in the query sequence.

Figure 3.1 RFLP profiles of the IGS region for *Armillaria mellea* (mel 1 and mel 2), *A. tabescens* (tab), and *A. gallica* (gal 1 and gal 2) isolates collected from the Missouri Ozark Mountain forests in 2002



[a] Lanes labeled M contain the Bioline HyperLadder IV DNA marker.

Figure 3.2 The number of *Armillaria* isolates representing RFLP profiles mel 1 and mel 2 collected from no harvest, uneven-aged or even-aged forest treatments in 2002.

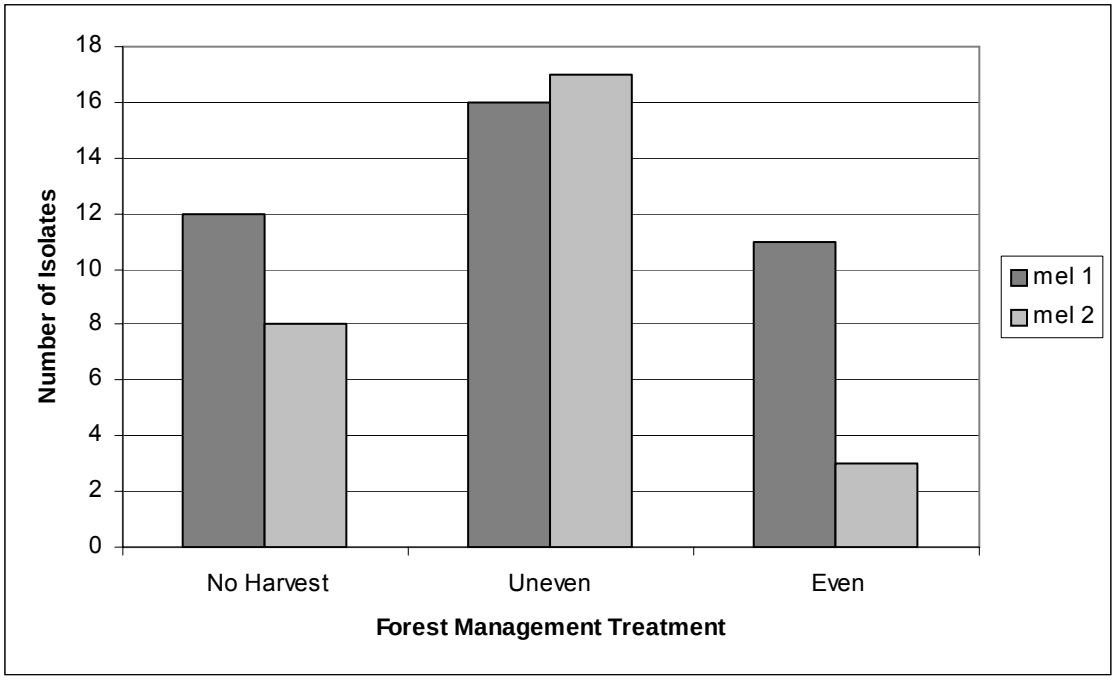


Figure 3.3 The number of *Armillaria* isolates representing RFLP profiles gal 1 and gal 2 collected from no harvest, uneven-aged or even-aged forest treatments in 2002.

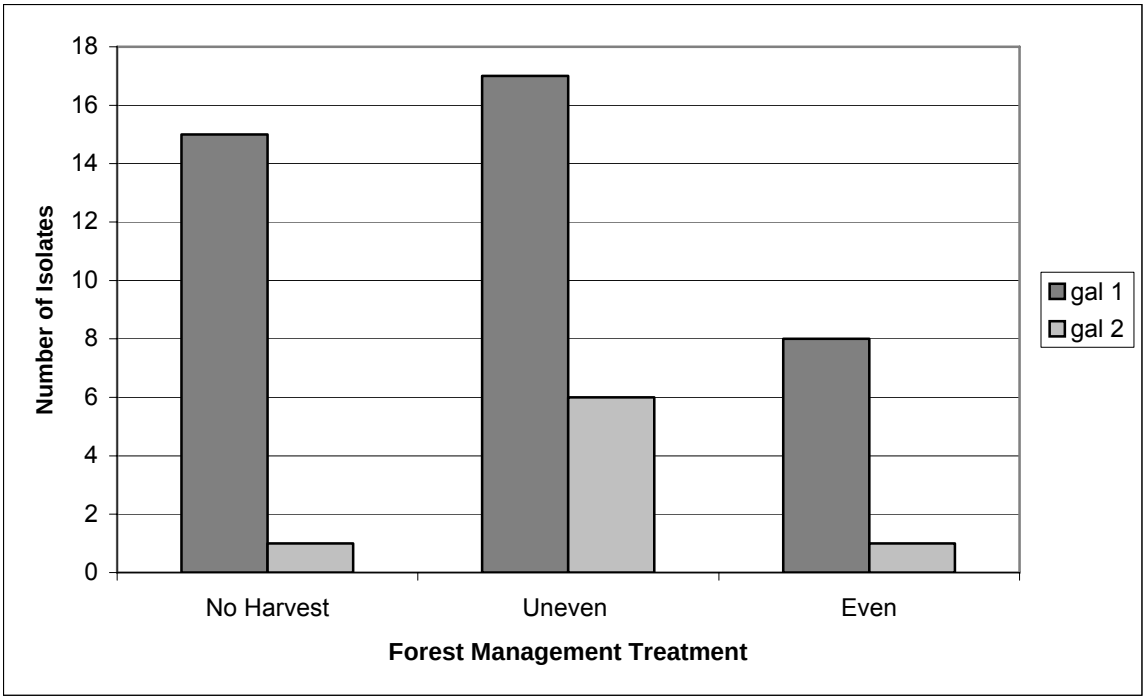
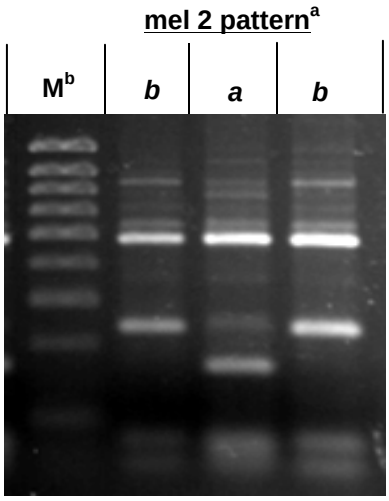


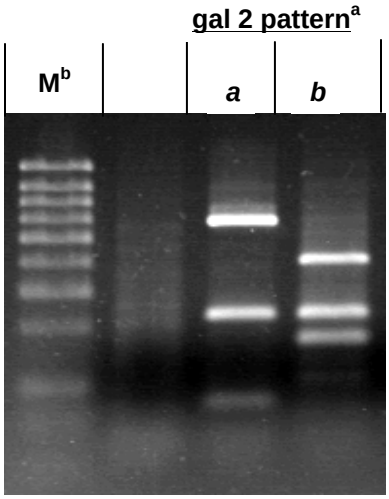
Figure 3.4 RFLP profiles of the IGS region for clones of mel 2 *Armillaria* isolates collected from the Missouri Ozark Mountain forests in 2002.



[a] PCR products of mel 2 isolates were cloned using a TOPO TA vector. Patterns *a* and *b* represent the two RFLP profiles observed among the clones. Pattern *a* contains fragments of 475 base pairs (bp) and 175 bp. Pattern *b* contains fragments of 475 bp and *ca* 225 bp.

[b] The lane labeled M contains the Bioline HyperLadder IV DNA marker.

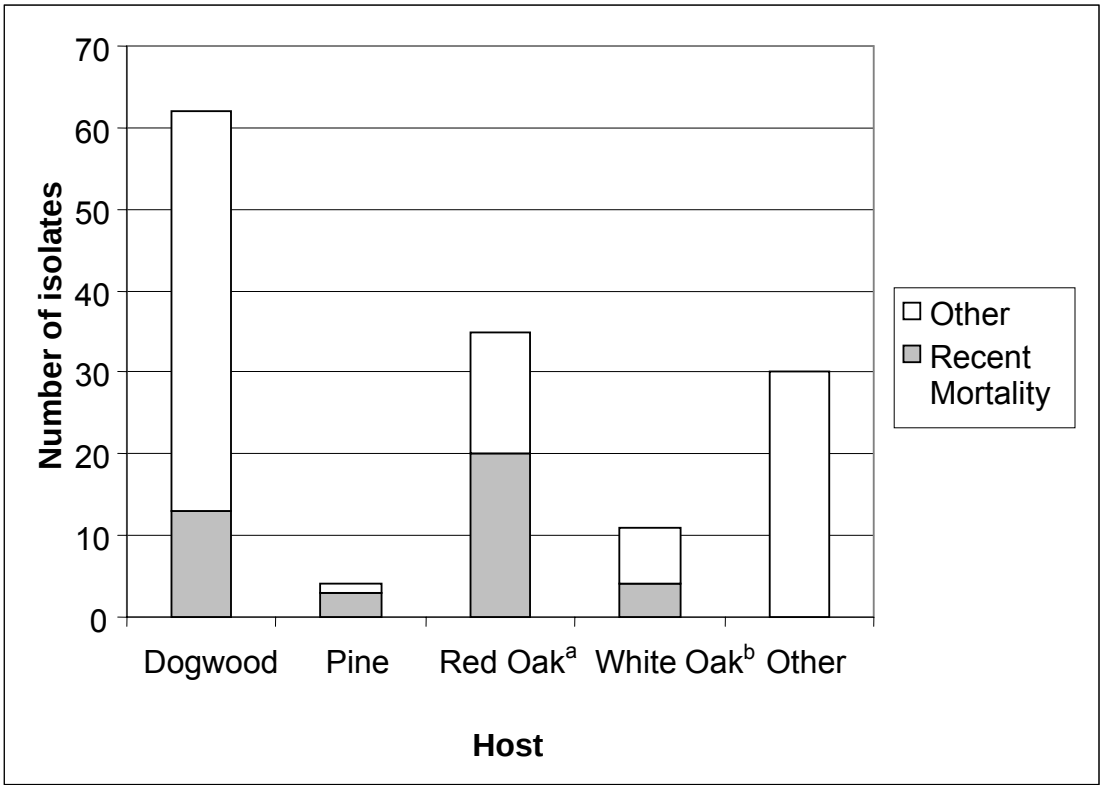
Figure 3.5 RFLP profiles of the IGS region for clones of gal 2 *Armillaria* isolates collected from the Missouri Ozark Mountain forests in 2002.



[a] PCR products of gal 2 isolates were cloned using a TOPO TA vector. Patterns *a* and *b* represent the two RFLP profiles observed among the clones. Pattern *a* contains fragments of 582 base pairs (bp) and 240 bp. Pattern *b* contains fragments of 401 bp, 240 bp, and 181 bp.

[b] The lane labeled M contains the Bioline HyperLadder IV DNA marker.

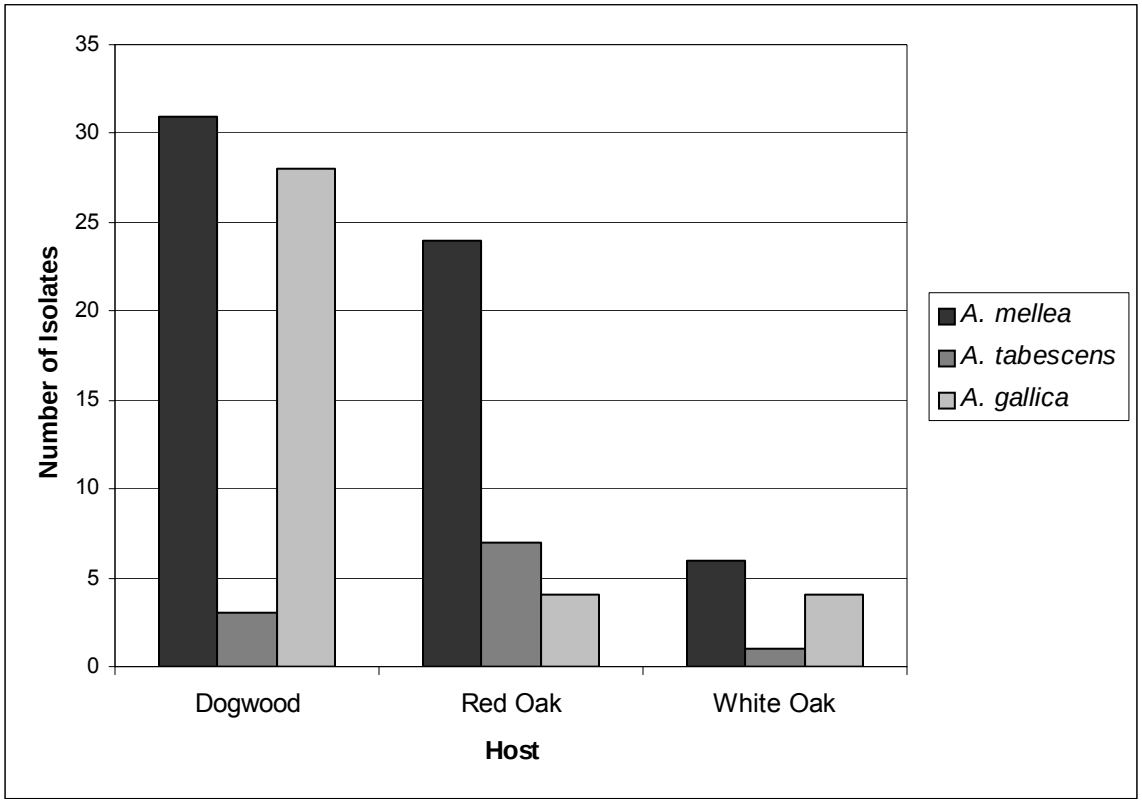
Figure 3.6 Distribution of *Armillaria* among all host species in the Missouri Ozark forests in 2002.



[a] Red oak species include *Quercus coccinea, Q. marilandica,* and *Q. velutina*

[b] White oak species include *Q. alba* and *Q. stellata*

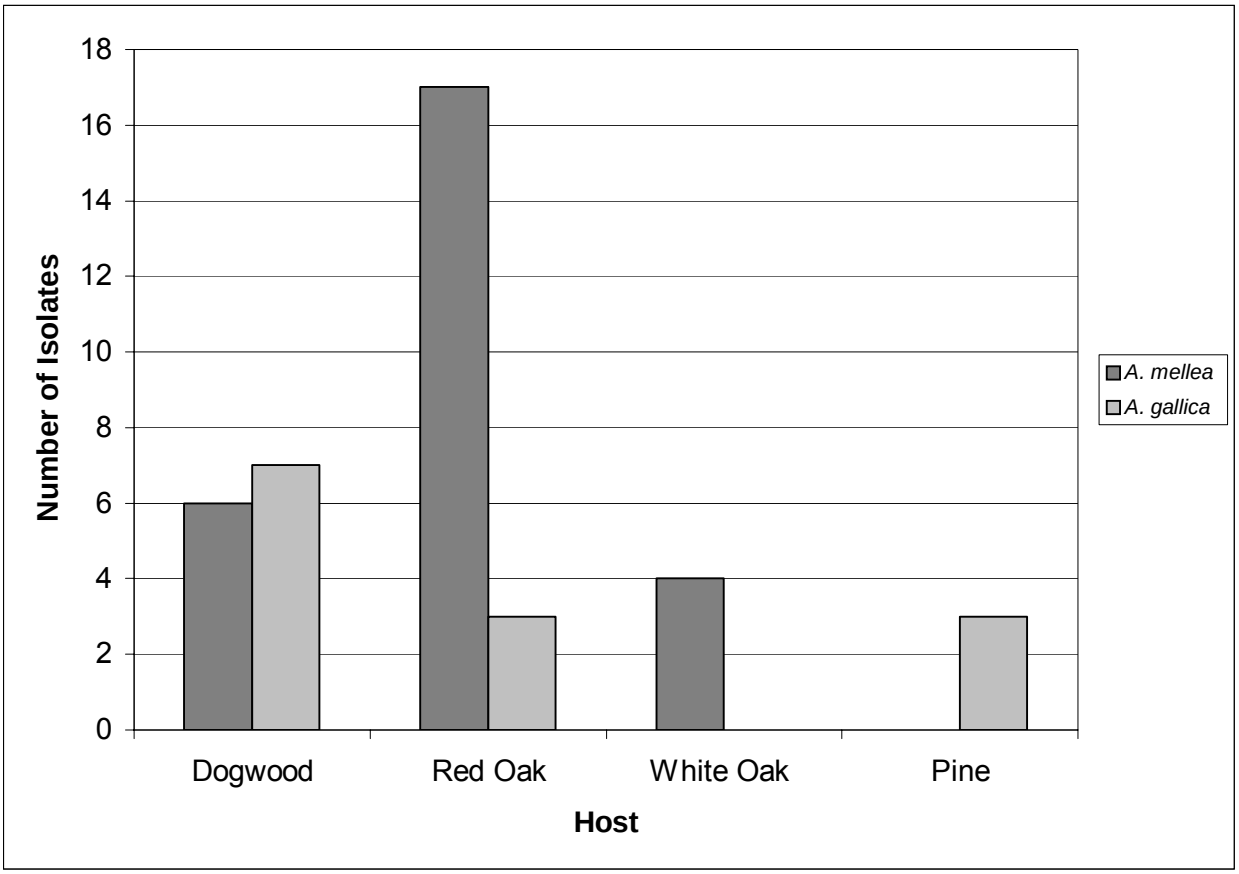
Figure 3.7 Distribution of *Armillaria mellea, A. tabescens,* and *A. gallica* among dogwood, red oak and white oak spp. in the Missouri Ozark forests in 2002.



[a] Red oak species include *Quercus coccinea, Q. marilandica,* and *Q. velutina*

[b] White oak species include *Quercua alba* and *Q. stellata*

Figure 3.8 *Armillaria mellea* and *A. gallica* isolates collected from recent mortality dogwood, pine, red and white oak spp. hosts in the Missouri Ozark forests in 2002.



[a] Red oak species include *Quercus coccinea, Q. marilandica,* and *Q. velutina*

[b] White oak species include *Quercua alba* and *Q. stellata*

Chapter 4

Association of *Armillaria* species with oak stumps in the Missouri Ozark Forest Ecosystem Project

Introduction

Armillaria (Fr:Fr) Staude is a genus of white rot fungi that plays multiple roles in forest ecosystems. All *Armillaria* spp. contribute to nutrient recycling through wood decay. In addition, *Armillaria* spp. vary greatly in pathogenicity toward their preferred host species, contributing to forest decline by blocking the recovery of stressed trees. Understanding the interspecific interactions of these *Armillaria spp.* and their influences on host vegetation is important to designing and implementing successful forest management techniques.

The Missouri Ozark Forest Ecosystem Project (MOFEP) is a comprehensive study examining multiple components of the forest ecosystem within the framework of three forest management systems: even-aged, uneven-aged and no-harvest management (Brookshire, Jensen and Dey, 1997). As a result of the 1996 MOFEP harvest treatments, oak stumps were created with the expectation that stump sprouting will contribute to stand regeneration. However, if *Armillaria* spp. are already associated with stump root systems, sprouting success may be influenced (Bruhn, Dey, Kromroy, Mihail *et al.*, 2004). Because

Armillaria spp. are associated with oak decline in the Missouri Ozark region (Johnson and Law, 1989; Bruhn, Wetteroff, Mihail, Kabrick *et al.*, 2000), it is important to understand their potential to alter the outcome of management treatments and specifically, the success of stumps to produce sprouts for stand regeneration.

Investigating the association of *Armillaria* spp. with stumps will contribute to a careful evaluation of the long term success of stump sprouting in forest regeneration. In order to elucidate the importance of *Armillaria* spp. in stump sprouting, it is necessary to identify isolates both to species and genet (i.e. genetically unique individual). Identification to species allows determination of whether pathogenic (*i.e*., *A. mellea* and *A. tabescens*) or saprophytic (*A. gallica*) *Armillaria spp.* are associated with stumps. These three species have been previously documented in the Missouri Ozark region (Bruhn *et al.*, 2000). Determining whether stumps are colonized by single or multiple genets provides insights into the competition among individuals during stump colonization.

In this study, 168 *Armillaria* field isolates were collected from stumps and identified to species. Where multiple isolates were collected from one stump, it was possible to examine the frequency of co-colonization by multiple *Armillaria* genets or species. My results contribute to a larger study of the feasibility of stump regeneration as a sound long-term reforestation technique.

Materials and Methods

Origin of Isolates

Oak stumps were selected from sites within MOFEP receiving even-aged or uneven-aged management treatments (Bruhn *et al.*, 2004). Two well-separated buttress roots, not associated with living sprouts and as nearly as possible on opposite sides of the stump, were evaluated for *Armillaria* infection (Bruhn *et al.*, 2004). Where *Armillaria* activity was observed samples were collected, isolated into pure culture and identified to species. Where *Armillaria* isolates were collected from both examined roots, isolates were further characterized to genet through vegetative incompatibility tests as described below.

Species Identification

Isolates were identified to species through PCR-RFLP of the intergenic spacer region (IGS-1) using a protocol modified from Harrington and Wingfield (1995). PCR was conducted by directly adding a mycelial scrape or extracted DNA (as described below) to a 20 µl PCR mixture. To obtain the mycelial sample, a pipette tip was scraped along the growing margin of 2-3 week-old culture actively growing on 2% malt agar. The tip was directly placed in the PCR mixture tube and stirred to suspend the mycelium. Each PCR mixture contained 2 units *Taq* polymerase (Promega, Madison, Wisconsin), 10X Mg free buffer supplied with the enzyme, 1 mM dNTPs (Promega, Madison, Wisconsin), 2.5 mM

$MgCl_2$, and 1 µM of each primer. Primers used were O-1: 5' AGT CCT ATG GCC GRG GAT 3' (Duchesne and Anderson, 1990) and LR12R: 5' CTG AAC GCC TCT AAG TCA GAA 3' (Veldman, Klootwijk, Regt and Rudi, 1981). PCR conditions were an initial denaturation at 95° C for 95 s, followed by 35 cycles at 60° C for 40 s, 72° C for 120 s, 90° C for 30 s, with a final elongation at 72° C for 10 min.

For isolates that could not be amplified directly from mycelial scrapes, DNA extraction was conducted using the DNEasy Plant Mini kit (Qiagen, Germantown, Maryland). Isolates were grown on 2% malt agar overlaid with 2.5cm x 2.5cm of dialysis membrane (23mm Spectra/Por® moleculardporous membrane tubing, Spectrum Medical Industries, Inc., Los Angeles, California) for 2-3 weeks at room temperature in the dark. Mycelium was harvested using a sterile spatula and placed in a 1.5 ml microcentrifuge tube. A micropestle was then used to grind the mycelium in 100 µl of lysis buffer from the first step of the manufacturer's instructions for the Qiagen DNEasy Plant Mini kit. Once the mycelium was completely suspended, the remaining lysis buffer was added and the manufacturer's instructions were followed. For PCR, 1 µl of DNA extract product was added to 19 µl of PCR mixture as above.

Restriction enzyme digestion of PCR products was completed by directly adding 5 units of *Alu*I (Promega, Madison, Wisconsin) to a 20 µl PCR mixture and incubating for 1-12 h at 37° C. Restricted fragments were separated on a 2% agarose gel (UltraPure™ Agarose, Invitrogen, Carlsbad, California) in tris-

acetate EDTA (TAE) buffer at 80 V for 1-2 h, stained with ethidium bromide, and visualized under UV illumination.

Genet identification

When two samples were collected from one stump, vegetative incompatibility tests were used to determine genet compatibility (Harrington, Worrall and Baker, 1992). Diploid field isolates were paired approximately 0.5 cm apart on Shaw-Roth medium (Shaw and Roth, 1976) and grown in the dark at room temperature for approximately six weeks. Culture pairs were then evaluated for visible incompatibility reaction along their interface to determine whether or not the paired isolates belong to the same genet.

Statistical Analyses

Contingency table analysis was used to examine the association of *Armillaria* species with oak stumps. The χ^2 statistic was used to test hypotheses (Sokal and Rohlf, 1995).

Results

From stumps of red and white oak species, 168 *Armillaria* field isolates were collected (Table 4.1). From white oak spp. stumps, 70%, 4% and 26% of

47 collected *Armillaria* field isolates were identified as *A. mellea*, *A. tabescens* and *A. gallica*, respectively. From red oak spp. stumps, 121 field isolates were collected and identified as 77% *A. mellea*, 6% *A. tabescens* and 17% *A. gallica*. Statistical analyses revealed that *A. mellea*, *A. tabescens* and *A. gallica* were recovered from red and white oak stumps in proportion to the frequency of each *Armillaria* spp. population with no significant difference (Table 4.1).

For 27 stumps, paired *Armillaria* isolates were collected from well-separated buttress roots. Species identifications revealed that 24 pairs of isolates represented the same species, while three represented different species (Table 4.2). Of the 24 pairs of isolates identified as the same species, fifteen pairs represented the same genet and nine pairs represented different genets (Table 4.2). Eight of these nine pairs were identified as *A. mellea* and one as *A. gallica* (Table 4.2). Only three pairs of isolates represented two species collected from the same stump. One stump yielded *A. mellea* with *A. tabescens* and two stumps yielded *A. mellea* with *A. gallica* (Table 4.2).

Discussion

The influence of *Armillaria* on stump regeneration should be considered when developing and predicting forest management models. Evaluating the interactions of *Armillaria* was facilitated by identification of *Armillaria* isolates to species and genet levels. In this study, isolates were identified as *A. mellea*, *A. tabescens* and *A. gallica*. Analysis of *Armillaria* spp. collected from both white

and red oak spp. stumps did not reveal any evidence of host preference by the *Armillaria* spp. Because *A. mellea* has been shown to influence red oak decline in the Missouri Ozark region (Johnson and Law, 1989; Bruhn *et al.*, 2000) it was surprising that it was not found significantly more often on red oak species than on white oak species. Further, the frequency of *A. mellea* recovery from white oak should be of considerable interest in forecasting long-term effects of forest management practices. The longevity of white oak stump sprouts may be influenced by preexisting *Armillaria* infections.

Examination of paired isolates from stumps revealed that most stumps are colonized by a single species, predominately *Armillaria mellea.* On almost half of the stumps colonized by *A. mellea*, the pair of isolates collected represented different genets. This evidence supports the view that *A. mellea* effectively establishes new genets through basidiospore production, with limited genet spread by mycelial fans and rhizomorphs (Rishbeth, 1985). In the three cases where two species were found on one stump, *A. mellea* was present (once with *A. tabescens* and twice with *A. gallica*). The fact that *A. mellea* was found infecting 21 of the 27 stumps is consistent with its description as one of the more pathogenic *Armillaria* species (Gregory, Rishbeth and Shaw, 1991).

In order to apply effective forest management techniques it is important to investigate the possible effects of *Armillaria* spp. on stand management treatments. This study has contributed to this evaluation by demonstrating the potential for *Armillaria* stump infections to reduce the stand regenerating capacity of both red and white oak stumps. Forest management models relying solely on

sprout survival and growth for stand regeneration should consider the effects of *Armillaria* when evaluating the efficacy of the model. As the results of this study are incorporated into the broader evaluation of sprout regeneration and survival, it will be possible to refine forest management objectives and model predictions.

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Table 4.1. The percentage of *Armillaria* field isolates recovered from red and white oak stumps in 2003 and 2004 identified to species

	Armillaria species			
Stump oak group	*mellea*	*tabescens*	*gallica*	n[a]
white oak[b]	70	4	26	47
red oak[c]	77	6	17	121

[a] n equals the total number of isolates collected from the stump species.

[b] Includes *Quercus alba* and *Q. stellata*

[c] Includes *Quercus coccinea, Q. velutina* and *Q. marilandica*

Table 4.2. Number of *Armillaria* isolate pairs collected from stumps in 2003 and 2004

	Armillaria					
	mellea	*gallica*	*tabescens*	*mellea/tabescens*	*mellea/gallica*	Total
Same species						
same genet	10	4	1	-	-	15
different genet	8	1	0	-	-	9
Different species	-	-	-	1	2	3

Appendix 1. *Armillaria* isolates, host species, condition and location within MOFEP

Armillaria species	RFLP profile	Host	Host condition	Collection[a]	MOFEP Site	MOFEP Plot	Isolate number
gallica	gal 1	*Carya* spp.	dead	rhiz	6	19	1150
	gal 1	*Carya* spp.	dead	rhiz	6	19	1188
	gal 1	*Carya* spp.	stump	rhiz	4	60	1174
	gal 1	*Cornus florida*	dead	fan	2	68	1134
	gal 1	*Cornus florida*	dead	fan	2	16	1186
	gal 1	*Cornus florida*	dead	fan	3	51	1159
	gal 1	*Cornus florida*	dead	fan	3	56	1163
	gal 1	*Cornus florida*	dead	fan	3	51	1171
	gal 1	*Cornus florida*	dead	fan	3	51	1179
	gal 1	*Cornus florida*	dead	fan	5	1	1139
	gal 1	*Cornus florida*	dead	fan	5	3	1195
	gal 1	*Cornus florida*	dead	fan	6	19	1141
	gal 1	*Cornus florida*	dead	fan	6	19	1142
	gal 1	*Cornus florida*	dead	fan	6	63	1157
	gal 1	*Cornus florida*	dead	fan	6	53	1167
	gal 1	*Cornus florida*	dead	fan	6	20	1192
	gal 1	*Cornus florida*	dead	fan	6	19	1197
	gal 1	*Cornus florida*	dead 2001	fan	7	55	1248
	gal 1	*Cornus florida*	dead 2002	rhiz	7	55	1256
	gal 1	*Cornus florida*	dead 2002	rhiz	8	17	1244
	gal 1	*Cornus florida*	dead bent	rhiz	4	60	1137
	gal 1	*Cornus florida*	dead, broken	rhiz	4	38	1138
	gal 1	*Cornus florida*	down dead	fan	6	53	1190
	gal 1	*Cornus florida*	near death	fan	2	72	1196
	gal 1	*Cornus florida*	recently dead	fan	2	25	1205
	gal 1	*Cornus florida*	recently dead	fan	5	30	1175
	gal 1	*Juglans nigra*	broken but alive	rhiz	4	70	1172
	gal 1	*Juglans nigra*	fan on adjacent root	fan	4	70	1164
	gal 1	*Pinus echinata*	dead 3-5 yrs	rhiz	7	55	1247
	gal 1	*Pinus echinata*	recently killed	fan	6	63	1155

Appendix 1. Continued

Armillaria species	RFLP profile	Host	Host condition	Collection[a]	MOFEP Site	MOFEP Plot	Isolate number
gallica	gal 1	*Pinus echinata*	recently killed	fan	6	63	1156
	gal 1	*Pinus echinata*	recently killed	fan	6	2	1176
	gal 1	*Prunus serotina*	dead	fan	4	60	1187
	gal 1	*Quercus alba*	dead	rhiz	4	35	1212
	gal 1	*Quercus alba*	dead partner	fan	4	35	1211
	gal 1	*Quercus coccinea*	girdled	fan	2	72	1198
	gal 1	*Quercus stellata*	dead	fan	5	12	1180
	gal 1	*Quercus velutina*	dead 2001	rhiz	8	17	1231
	gal 1	*Quercus velutina*	dead 2002	fan	6	63	1177
	gal 1		stump	rhiz	7	55	1226
gallica	gal 2	*Cornus florida*	dead	fan	4	70	1136
	gal 2	*Cornus florida*	dead	fan	4	49	1199
	gal 2	*Cornus florida*	dead	rhiz	5	1	1129
	gal 2	*Cornus florida*	down dead	fan	2	67	1158
	gal 2	*Cornus florida*	recently killed	fan	2	67	1178
	gal 2	*Quercus alba*	dead	fan	2	67	1170
	gal 2	*Quercus coccinea*	dead 2002	fan	2	32	1182
	gal 2		root under old exanulate	rhiz	8	13	1203
mellea	mel 1	*Carya* spp.	dead	fan	5	48	1130
	mel 1	*Cornus florida*	all dead	fan	4	49	1200
	mel 1	*Cornus florida*	dead	fan	2	67	1146
	mel 1	*Cornus florida*	dead	fan	2	16	1166
	mel 1	*Cornus florida*	dead	fan	2	30	1206
	mel 1	*Cornus florida*	dead	fan	2	33	1208
	mel 1	*Cornus florida*	dead	fan	3	61	1140
	mel 1	*Cornus florida*	dead	fan	3	51	1160
	mel 1	*Cornus florida*	dead	fan	3	51	1161
	mel 1	*Cornus florida*	dead	fan	5	2	1154

Appendix 1. Continued

Armillaria species	RFLP profile	Host	Host condition	Collection[a]	MOFEP Site	MOFEP Plot	Isolate number
mellea	mel 1	*Cornus florida*	dead	fan	5	2	1168
	mel 1	*Cornus florida*	dead	fan	6	3	1169
	mel 1	*Cornus florida*	dead	fan	6	19	1184
	mel 1	*Cornus florida*	dead 0-1yrs	fan	7	53	1241
	mel 1	*Cornus florida*	dead 2002	fan	3	61	1149
	mel 1	*Cornus florida*	dead 2002	fan	7	45	1237
	mel 1	*Cornus florida*	dead 5+yrs stub	fan	7	55	1249
	mel 1	*Cornus florida*	recently dead	fan	2	34	1209
	mel 1	*Cornus florida*	recently killed	fan	2	18	1145
	mel 1	*Quercus alba*	dead	fan	3	56	1162
	mel 1	*Quercus alba*	dead	fan	5	48	1144
	mel 1	*Quercus alba*	dead ~2yrs	fan	7	45	1236
	mel 1	*Quercus alba*	dead 2002	fan	8	17	1233
	mel 1	*Quercus alba*	dead 2002	fan	8	17	1250
	mel 1	*Quercus marilandica*	dead 2002	fan	8	1	1266
	mel 1	*Quercus stellata*	recently killed	fan	6	19	1189
	mel 1	*Quercus velutina*	dead	fan	5	2	1153
	mel 1	*Quercus velutina*	dead ~2yrs	fan	7	53	1259
	mel 1	*Quercus velutina*	dead 1-5yrs	fan	3	51	1147
	mel 1	*Quercus velutina*	dead 2001	fan	8	17	1232
	mel 1	*Quercus velutina*	dead 2001	fan	8	7	1242
	mel 1	*Quercus velutina*	dead 2002	fan	7	58	1229
	mel 1	*Quercus velutina*	dead 2002	fan	7	53	1255
	mel 1	*Quercus velutina*	dead 2002	fan	7	58	1257
	mel 1	*Quercus velutina*	dead 2002	fan	8	2	1221
	mel 1	*Quercus velutina*	dead 5-10yrs	fan	8	7	1230
	mel 1	*Quercus velutina*	recently killed	fan	2	33	1207
	mel 1		fine root	bas	8	4	1202
	mel 1		sprouting	fan	8	7	1243

Appendix 1. Continued

Armillaria species	RFLP profile	Host	Host condition	Collection[a]	MOFEP Site	MOFEP Plot	Isolate number
mellea	mel 2	*Carya* spp.	dead	fan	4	24	1152
	mel 2	*Cornus florida*	---	fan	6	3	1131
	mel 2	*Cornus florida*	---	fan	6	3	1133
	mel 2	*Cornus florida*	dead	fan	2	69	1210
	mel 2	*Cornus florida*	dead	fan	4	15	1126
	mel 2	*Cornus florida*	dead	fan	4	70	1135
	mel 2	*Cornus florida*	dead	fan	4	38	1143
	mel 2	*Cornus florida*	dead	fan	4	49	1216
	mel 2	*Cornus florida*	dead	fan	5	1	1127
	mel 2	*Cornus florida*	dead	fan	5	1	1128
	mel 2	*Cornus florida*	dead	fan	6	3	1132
	mel 2	*Cornus florida*	dead	fan	6	53	1181
	mel 2	*Cornus florida*	dead 2002	fan	8	17	1245
	mel 2	*Cornus florida*	dead to dying clump	fan	4	49	1213
	mel 2	dead root	dead root	fan	4	15	1125
	mel 2	*Quercus coccinea*	died 2001	fan	7	53	1239
	mel 2	*Quercus coccinea*	long dead stub	fan	7	53	1240
	mel 2	*Quercus coccinea*	recenly killed	fan	3	52	1148
	mel 2	*Quercus coccinea*		fan	7	53	1238
	mel 2	*Quercus marilandica*	dead 2002	fan	8	7	1227
	mel 2	*Quercus velutina*	dead 1-2yrs	fan	7	25	1217
	mel 2	*Quercus velutina*	dead 2002	fan	2	16	1173
	mel 2	*Quercus velutina*	dead 2002	fan	2	69	1193
	mel 2	*Quercus velutina*	dead 2002	fan	4	24	1151
	mel 2	*Quercus velutina*	dead 2002	fan	8	2	1220
	mel 2	Red Oak spp.	dead	fan	4	36	1194
	mel 2	Red Oak spp.	dead several yrs	bas	8	4	1228
	mel 2		old stump adjacent to Qc	fan	7	65	1201

Appendix 1. Continued

Armillaria species	RFLP profile	Host	Host condition	Collection[a]	MOFEP Site	MOFEP Plot	Isolate number
tabescens	tab	*Acer* spp.	dead	fan	3	30	1185
	tab	*Cornus florida*	dead	fan	2	25	1215
	tab	*Cornus florida*	dead	fan	4	60	1204
	tab	*Cornus florida*	dead	fan	6	63	1191
	tab	Ct	stump	bas	7	63	1254
	tab	*Quercus alba*	dead	fan	2	19	1183
	tab	*Quercus coccinea*	dead	fan	7	65	1264
	tab	*Quercus coccinea*	stump	bas	7	63	1261
	tab	*Quercus velutina*	dead	fan	2	25	1214
	tab	*Quercus velutina*	dead 1-3yrs	bas	8	24	1234
	tab	*Quercus velutina*	stump	fan	7	63	1253
	tab	*Quercus velutina*	stump	bas	8	13	1222
	tab	Red Oak spp.	dead (under old exanulate)	fan	8	13	1260
	tab	*Rhamnus caroliniana*	dead	fan	5	12	1165
	tab		---	bas	8	13	1246
	tab		old buried stump	fan	7	65	1258
	tab		old buried stump	fan	7	65	1263
	tab		on half buried branch	bas	8	13	1265
	tab		outside plot	bas	8	13	1218
	tab		root	fan	7	65	1262
	tab		root under old exanulate	fan	8	13	1251
	tab		small dead root	bas	8	13	1219
	tab		small dead root	bas	8	13	1235
	tab		small dead root	bas	8	13	1252
	tab		substrate unknown	bas	8	13	1223

[a] Form in which sample was collected; fan = mycelial fan, bas = basidiomes, and rhiz = rhizomorph