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Armillaria in Massachusetts Forests: Ecology, Species Distribution, and Population Structure, with an Emphasis on Mixed Oak Forests

Nicholas Justin Brazee
University of Massachusetts Amherst, nbrazee@psis.umass.edu

ARMILLARIA IN MASSACHUSETTS FORESTS: ECOLOGY, SPECIES DISTRIBUTION, AND POPULATION STRUCTURE, WITH AN EMPHASIS ON MIXED OAK FORESTS

A Dissertation Presented

by

NICHOLAS JUSTIN BRAZEE

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Approved as to style and content by:

_____
Robert L. Wick, Chair

_____
Robert E. Marra, Member

_____
Geunhwa Jung, Member

_____
Matthew J. Kelty, Member

_____
Stephen M. Rich, Department Head
Department of Plant, Soil, and Insect Sciences

DEDICATION

This work is dedicated to the countless trees that have been killed by *Armillaria*, for surely the number of deceased is too large to fathom.

ACKNOWLEDGEMENTS

This work would not have been possible without the continued support of Dr. Robert L. Wick. His expertise, interest in the field plant pathology, and sincere desire to educate is an inspiration to all his students. I would like to sincerely thank Dr. Robert E. Marra for all his support during the course of this project. I would also like to thank Dr. Geunhwa Jung and Dr. Matthew J. Kelty for their support.

ABSTRACT

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MAY 2011

NICHOLAS J. BRAZEE, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M.S., UNIVERSITY OF WASHINGTON

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Robert L. Wick

The ecology, species distribution, and population structure of *Armillaria* was investigated in the forests of Massachusetts. From 64 plots at 16 sites, 640 isolates of *Armillaria* were collected from six forest types (northern hardwoods, mixed oak, pitch pine, white pine, white pine/mixed oak, and eastern hemlock). *Armillaria gallica* proved to be the most abundant species, making up 316/640 (52%) of all isolations. This was followed by *A. solidipes* (219/640; 34%), *A. mellea* (46/640; 7%), *A. calvescens* (36/640; 6%), *A. gemina* (16/640; 3%), and *A. sinapina* (7/640; 1%). *Armillaria gallica* was routinely encountered causing significant decay of the lower bole on living hardwood hosts, especially oaks. The population structure of 153 isolates of *A. gallica* collected from mixed oak forests was investigated using amplified fragment length polymorphisms (AFLPs). From a total sampling area of 4.51 ha, 38 AFLP genotypes were discovered, yielding a figure of eight genets per hectare with the average *A. gallica* genet occupying 0.13 ha. When the effects of hydrolyzable tannins on *in vitro* growth were compared between *A. calvescens* and *A. gallica*, it was *A. gallica* that appeared better at oxidizing and metabolizing commercial tannins (tannic acid and gallic acid) along with black oak root bark extracts. This was determined through measurements of colony area and dry biomass, and suggests that *A. gallica* may be a better adapted pathogen of oak. In order to more accurately distinguish between isolates of *A. calvescens* and *A. gallica*, a three-gene phylogeny was reconstructed, using partial sequences of the elongation factor 1-alpha (*tef1*), RNA polymerase II (*rpb2*) and nuclear large subunit (nLSU) genes. After comparing 12 isolates each of *A. calvescens* and *A. gallica* that originated from across northeastern North America, only the *tef1* gene could accurately distinguish these two species. Five single nucleotide polymorphisms were present between the two species and maximum likelihood and maximum parsimony methods grouped *A. calvescens* and *A. gallica* into monophyletic clades.

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CHAPTER 1

ARMILLARIA SPECIES DISTRIBUTION ON SYMPTOMATIC HOSTS IN NORTHERN HARDWOOD AND MIXED OAK FORESTS IN WESTERN MASSACHUSETTS

Introduction

Armillaria species are some of the most important forest pathogens to be found in the two forest types – northern hardwood, and mixed oak – that dominate much of New England (Wargo and Houston 1974, Wargo 1977, Twery et al. 1990, Bauce and Allen 1992, Blodgett and Worrall 1992a, Burrill et al. 1999, McLaughlin 2001, Horsley et al. 2002). The contrasting disturbance regimes (Westveld et al. 1956) of these two forest types are worth noting here. Whereas northern hardwood forests have long intervals between stand reinitiation disturbance events and consist of tree species that are very susceptible to decay (Lorimer and White 2003, Sinclair and Lyon 2005), mixed oak forests were typically subjected to low to mid-severity forest fires, but in recent decades have experienced over-crowding and severe defoliation events (Abrams 1992, Davidson et al. 1999). Both of these conditions have allowed for considerable decay and mortality due to *Armillaria* (Wargo and Houston 1974, Wargo 1977, Bauce and Allen 1992).

Northern hardwood forests of New England are principally composed of sugar maple (*Acer saccharum* Marsh.), yellow birch (*Betula alleghaniensis* Britton) and American beech (*Fagus grandifolia* Ehrh.) along with various other associated hardwoods and conifers (Eyre 1980, Hornbeck and Leak 1992). In New England, this forest type covers over four million hectares from western Connecticut and Massachusetts, throughout Vermont and New Hampshire, and in scattered areas of Maine (Hornbeck and Leak 1992). Mixed oak forests in New England are mostly confined to Massachusetts, Connecticut, and Rhode Island (Westveld et al. 1956). These stands are dominated by mixtures of red oak (*Quercus rubra* L.), black oak (*Quercus velutina* Lam.) and locally abundant white oak (*Quercus alba* L.), scarlet oak (*Quercus coccinea*

Muenchh.) and chestnut oak (*Quercus prinus* L.) (Westveld et al. 1956, Eyre 1980, Abrams 1992).

The species distribution of *Armillaria* has been investigated previously in greatest detail in New York and southern Ontario, where large collections were carried out at broad geographic scales (Blodgett and Worrall 1992a, McLaughlin 2001). Conversely, numerous smaller-scale studies across varied forest types have occurred in northern Ontario, Newfoundland, New Hampshire, and Pennsylvania, respectively (Bérubé 2000, Dumas 1988, Harrington and Rizzo 1993, Marçais and Wargo 2000). However, a more detailed examination of species distribution in southern New England has been lacking, given the importance of *Armillaria* in these forests. Such a study would require a large number of isolates collected from a relatively small geographic area comprising several distinct forest types, making Massachusetts a prime study area (Westveld et al. 1956).

Although North American *Armillaria* species differ in their pathogenicity and virulence, every species has been shown to cause infection on live hosts (Gregory et al. 1991). This is a key point when considering *Armillaria* as a mortality agent, as there is considerable debate about each species designation (i.e. primary or secondary pathogen). Laboratory and potted seedling experiments that have separated and compared *Armillaria* species by their pathogenicity and virulence are indeed relevant (Morrison 2004), but cannot be directly comparable in the forest setting, since trees are often exposed to multiple, interacting biotic and abiotic stresses (Sinclair and Lyon 2005). For example, a species of *Armillaria* described as 'weakly virulent', such as *A. gallica*, working in concert with an insect defoliator (e.g. gypsy moth) can be just as successful in causing mortality as a 'highly virulent' species, such as *A. solidipes*, working without another stress agent.

Individual *Armillaria* species have such a broad host range (Raabe 1962) that strong associations with particular hosts or landscape characteristics can be difficult to identify. Yet, associations by forest type and major landscape characteristics (e.g. soil type, parent material, and

elevation) can be significant (Blodgett and Worrall 1992b, McLaughlin 2001, Wargo et al. 1987). Because armillaria root rot is caused by a complex of different species, it is critical to understand which species inhabit distinct forest types, so that pathogenicity and virulence of individual species can be better understood at the forest level. The goal of this study was to determine the species distribution of *Armillaria* in western Massachusetts in eight separate stands of northern hardwood and mixed oak forests. An additional goal was to collect a large number of isolates from living but infected hosts to compare the *Armillaria* species' pathogenicity and distribution among trees from the two most common forest types in this region.

Materials and Methods

Site Selection

Four northern hardwood and four mixed oak sites on state-owned lands were chosen in western Massachusetts using forest type maps created by the Massachusetts Department of Conservation and Recreation (DCR) (Figure 1.1). Northern hardwood stands were chosen in northwestern Massachusetts. Parent material across this region is homogeneous, so sites that occupy the two dominant mountain ranges in the region (Taconic and Hoosac) were randomly chosen. Southwestern Massachusetts, while also dominated primarily by northern hardwoods, was excluded because *Quercus rubra* is a major component of the forest (Westveld et al. 1956). The mixed oak forests were randomly chosen in central Massachusetts east of the Connecticut River. Sites were stratified by parent material and lie within the central hardwood zone that dominates most of Southern New England (Westveld et al. 1956). Site characteristics are listed below (Table 1.1).

The largest contiguous block of forest that contained the desired forest type was selected for use at a given site. Using ArcMap v. 9.3 (ESRI, Redlands, CA), a polygon was constructed around the target study area. Four study plots were established at each site. To remove any bias in plot selection, plot centers were generated using a random integer generator (http://random.org).

The range of possible random numbers fell within the range of latitude and longitude values for the polygon of the selected study area. Coordinate combinations were used in the order they were generated. Random points that were within 100 m of an already generated point or an established trail or road were discarded. Extra random points were generated to account for rare occasions when points fell upon non-forest or recently harvested areas. Therefore, 2 forest types × 4 sites within each forest type × 4 plots at each site = 32 total study plots.

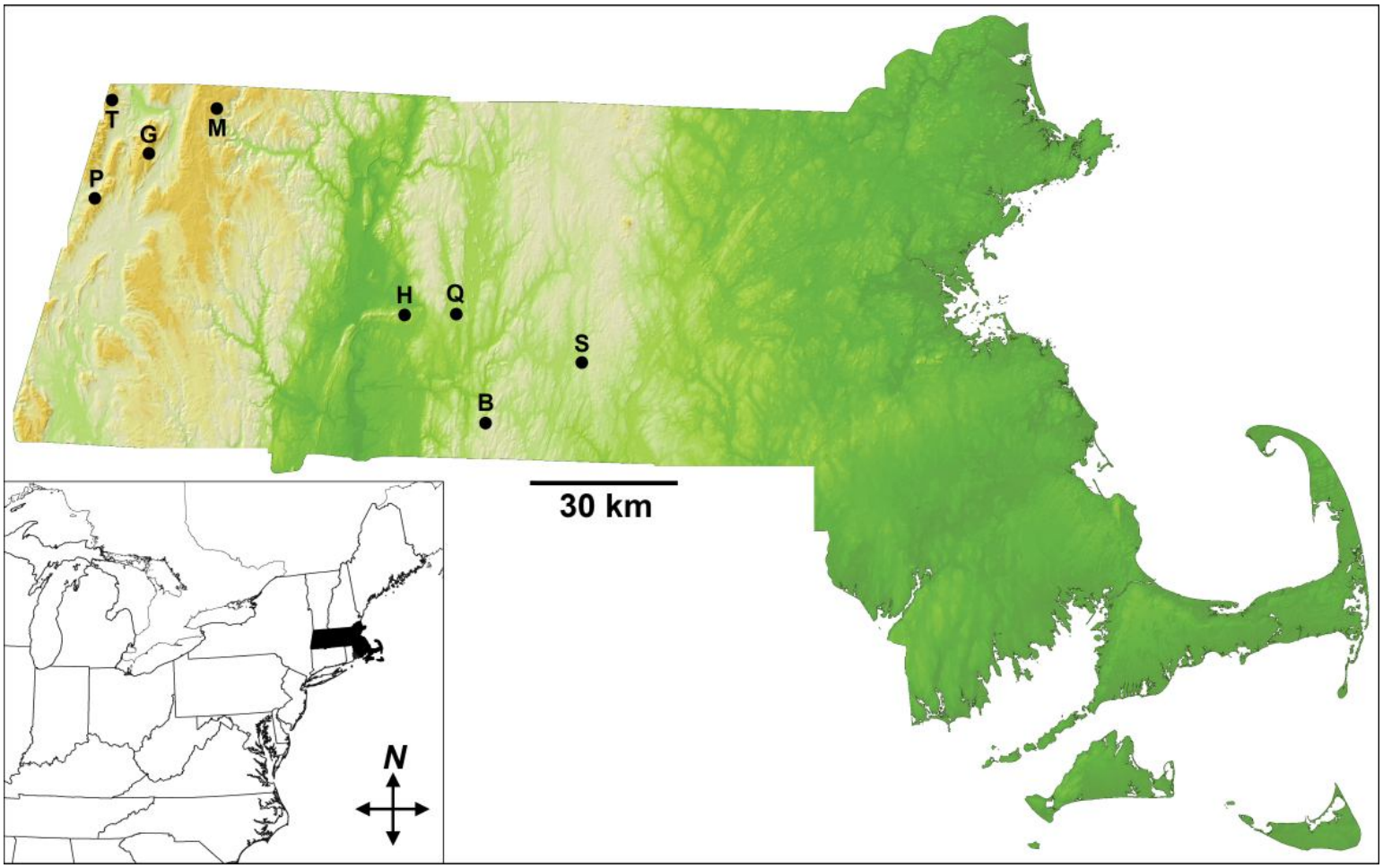

Figure 1.1: Location of study sites in northern hardwood and mixed oak sites in Massachusetts. Northern hardwood sites are denoted as: G=Mount Greylock State Reservation; M=Monroe State Forest; P=Pittsfield State Forest; and T=Taconic Trail State Forest. Mixed oak sites are denoted as: B=Brimfield State Forest; H=Holyoke Range State Reservation; Q=Quabbin Park; and S=Spencer State Forest.

Table 1.1. Characteristics of Study Sites Where *Armillaria* Was Sampled.

Site Name	Plot Size (ha)[Z]	Elevation (m)[Y]	Dominant Forest Cover[X]	Basal Area (m^2/ha)[W]	Parent Material	Soil Drainage Classes[V]
N. Hardwoods						
Monroe	0.36	698 – 767	*Acer rubrum – Fagus grandifolia – A. saccharum*	28	Pelitic Rocks	2.5[a]
Greylock	1.73	624 – 818	*Fagus grandifolia – Acer rubrum – Betula alleghaniensis*	22	Calcgranofels and Pelitic Rocks	2.6[a]
Pittsfield	0.48	500 – 630	*Fraxinus americana – Acer rubrum – A. saccharum*	30	Pelitic Rocks	2.8[a]
Taconic	0.27	433 – 633	*Fagus grandifolia – Acer saccharum – A. rubrum*	25	Pelitic and Carbonate Rocks	2.5[a]
Mixed Oak						
Brimfield	0.43	282 – 332	*Quercus alba – Betula lenta – Q. velutina*	28	Sulfidic Schists	3.4[b]
Quabbin	0.47	176 – 224	*Quercus velutina – Acer rubrum – Q. rubra*	28	Mafic Rocks, Sulfidic Schists, and Granite	3.1[b]
Holyoke	0.10	173 – 248	*Quercus prinus – Q. rubra – Carya glabra*	22	Basalt	2.1[c]
Spencer	0.11	249 – 285	*Quercus rubra – Q. velutina – Acer rubrum*	22	Calcgranofels	2.8[a]

[Z] Mean area of four variable size plots used to sample *Armillaria* per site.
[Y] Range of elevations across four plots per site.
[X] Based upon importance values listed from highest to lowest for the three most common tree species, summed from four 0.01 ha vegetation plots per site.
[W] Mean basal area across the four plots per site.
[V] Soil drainage classes rank from 1 to 4: Soils from group 1 are excessively drained while soils from group 4 are moderately well-drained. Values with different letters are significantly different at $p = 0.05$ using the Tukey test.

Vegetation Sampling and Site Characteristics

Plot centers were established at the canopy dominant/codominant nearest to the Euclidean center. Basal area was determined from the plot center using a basal area factor of 10. Forest composition was sampled using 0.01 ha (100 m^2) rectangular plots (7.07 m × 14.14 m) (Brower et al. 1990). All trees > 5 cm in dbh were sampled for species, dbh, height, and crown class. Coordinates of point centers were recorded with a hand held GPS unit. Tree species importance values (IV) [(relative density + relative dominance)/2] were generated for each vegetation plot. Relative density is calculated by dividing the number of individuals of a species by the total number of all individuals within a plot. Relative dominance is calculated by dividing the basal area of a particular species by the total basal area of all trees in a plot.

Elevation for each plot was determined using a 1:5,000 digital elevation model (MassGIS 2010) (Table 1.1). Soil types and parent material for the sites were determined using digitized datalayers of published soil and bedrock surveys conducted by the USDA Natural Resources Conservation Service (NRCS), and USGS, respectively (MassGIS 2010). Soil drainage classes were determined using published soil surveys by the USDA Natural Resources Conservation Service (Table 1.1).

Sampling of *Armillaria* and Pathogenicity

From the plot center, starting from a north azimuth and moving counter-clockwise (360º to 0º), all trees > 5 cm in dbh were assessed for symptoms and signs of *Armillaria* until 10 were found. Therefore, plots were variable in size, being as large as was necessary to find 10 isolates. No preference was given to any particular tree species. Symptoms included crown dieback, basal cavities, chlorotic or undersized foliage, basal flaring, basal resinosus, and basal seams. An emphasis was placed upon finding live trees infected with *Armillaria* to determine which species were capable of infecting a live host. Additionally, we were eager to find out if *Armillaria* species

that are considered low virulence or non-pathogenic would be encountered infecting living trees. Dead trees were chosen when obvious signs of the pathogen (mycelial fans, rhizomorphs, pseudosclerotia) were visible. Species, dbh, crown class, and the type of infection/association (butt rot, root rot, superficial attachment) were recorded. When live trees showed symptoms of root and butt rot, the lower bole and main lateral roots were excavated for signs of *Armillaria*. When *Armillaria* was present, rhizomorphs, mycelial fans, pseudosclerotia, and/or infected woody tissue were collected.

The intent of this sampling design was to locate live trees showing outward expression of infection by *Armillaria* at the time of sampling. It was not designed to determine species incidence within a defined area of the forest. Trees harboring infections without any symptoms or signs of infection were invariably missed. While this sampling structure may not provide an accurate depiction of overall *Armillaria* species incidence at the sites chosen, it does produce an accurate incidence of the *Armillaria* species capable of causing disease severe enough so that hosts exhibit symptoms of infection.

Penetration and/or death of the outer bark was required as proof of pathogenicity. Root decay, butt rot, and cambial infection of the lower bole were regarded as successful colonization of the host. Basal cavities were determined to have been caused from *Armillaria* if white rot was present and rhizomorphs or mycelial fans were present within the heartwood, or within rotted wood tissue at ground level within the lower bole. Rhizomorph attachment to the roots or lower bole with no evidence of invasion or bark necrosis was deemed superficial attachment. Infections were listed as root rot and butt rot.

Isolation, PCR, and Sequencing

Armillaria rhizomorphs, mycelial fans, and pseudosclerotia were washed in warm water and then under aseptic conditions soaked in a 2% solution of sodium hypochlorite for four to six minutes, rinsed in sterile distilled water, air dried, cut into small segments, and placed on 2% malt

extract agar amended with 200 mg/L each of neomycin sulfate, streptomycin sulfate, and chlortetracycline, and 5 mL/L of benomyl solution (20 mg of 95% benomyl in 50 mL of warm 95% ethanol diluted to 100 mL with sterile distilled water) (McLaughlin 2001, Worrall 1991). Cultures were incubated in the dark at 25º C and subsequently subcultured to unamended 1% glucose, 2% malt extract agar. In preparation for DNA extraction, isolates were transferred to 1% glucose, 0.5% yeast extract, 2% malt extract broth with 300 ppm of 95% ethanol.

Harvested mycelium was lyophilized in 2 mL centrifuge tubes using a Labconco FreeZone 12 (Labconco, Kansas City, MO) lyophilizer for 48 hours. Genomic DNA was then extracted using the Qiagen DNeasy plant tissue kit (Qiagen, Valencia, CA) following the manufacturers protocol. Isolates were identified using a slightly modified version of the PCR-RFLP protocol developed by McLaughlin and Hsiang (2010). This protocol has proven very reliable in distinguishing *A. gemina* from *A. solidipes* and also most isolates of *A. calvescens*, *A. gallica*, and *A. sinapina* from one another. A total of 49 tester isolates spanning six species (*A. calvescens*, *A. gallica*, *A. gemina*, *A. mellea*, *A. sinapina*, and *A. solidipes*) were obtained for reference from the Moscow forestry sciences laboratory, Moscow, ID, and from Dr. John McLaughlin, Ontario Forest Research Institute, Sault Ste. Marie, Ontario. These were used as positive controls within the PCR-RFLP protocol.

PCR was performed with 10X Tsg reaction buffer and Tsg DNA polymerase (Bio Basic, Ontario, Canada). Reaction conditions using primers Ao-f700 and 18S-rev (McLaughlin and Hsiang 2010) were as follows: 1X Tsg concentration, 1 unit of Tsg DNA polymerase, 0.2 µM primer concentrations and 200 µM concentrations of each dNTP (Fermentas, Glen Burnie, MD). Thermocycler conditions were: initial denature 94º C for 3 min; 35 cycles of denaturing at 94º C for 30 s, annealing at 58º C for 45 s, and extension at 72º C for 90 s; and a final extension of 72º C for 10 min. Reaction conditions using primers 5S-1f and 5S-1r (McLaughlin and Hsiang 2010) were as follows: 1X Tsg concentration, 1 unit of Tsg DNA polymerase, 0.2 µM primer concentrations and 200 µM concentrations of each dNTP. Thermocycler conditions were: initial

denature 94º C for 3 min; 35 cycles of denaturing at 94º C for 30 s, annealing at 61º C for 40 s, and extension at 72º C for 50 s; and a final extension of 72º C for 10 min. All PCR reactions were performed using an Eppendorf Mastercycler gradient thermocycler (Eppendorf, Westbury, NY).

Representative isolates from each species were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA) after amplification with the 5S primers at the Genomics Resource Laboratory, University of Massachusetts, Amherst. Sequences were manually edited and aligned using ClustalW in MEGA version 4 (Tamura et al. 2007). Restriction sites were then located to verify fragment sizes listed by McLaughlin and Hsiang (2010).

Statistical Analysis

Chi-square goodness-of-fit was used to determine if *Armillaria* species incidence differed significantly by: i) forest type; ii) host tree species; and iii) mode of infection (root or butt rot). Chi-square was also used to determine if the crown classes of each tree found infected with *Armillaria* differed by forest types. Columns consisted of individual *Armillaria* species incidence while rows consisted of the desired category being tested. Expected values used were (S X C)/N, where S is the number of observations for an *Armillaria* species, C is the total number of observations within a row of the category under consideration, and N is the total number of observations for all *Armillaria* species. Exact *p*-values were generated during Chi-square analysis when low cell counts (< 5 observations) would have compromised the Chi-square distribution (Zar 2007). A general linear model (GLM) was used to determine if i) the median number of *Armillaria* species present in individual plots differed by site and forest type, and ii) soil drainage classes were different by forest type. Linear regression was used to compare the percentage of occurrence of *Quercus* species from vegetation plots and *Armillaria* sampling plots. All post-hoc analysis was done using the Tukey's HSD test. All statistical analysis was done using SPSS 16.0 (SPSS Inc., Chicago, IL).

Results

Vegetation Sampling

Within northern hardwood forests, *Acer rubrum, A. saccharum, Betula alleghaniensis*, and *Fagus grandifolia* were the only species to occur at all four sites. *Fagus grandifolia* was the most abundant species at all four sites, making up 74/207 total trees with a total IV of 0.272 (Table 1.2). However, with the exception of Taconic, which was dominated by *F. grandifolia*, all of the northern hardwood sites were dominated by *Acer* species. Across all four sites, *Acer* species (*A. pennsylvanicum*, *A. rubrum*, and *A. saccharum*) had a total combined IV of 0.429 (Table 1.2).

Within mixed oak forests, *A. rubrum, Quercus rubra*, and *Q. velutina* were the only species found at all four sites. *Acer rubrum* was the most abundant species by density (36/144 total trees), but was third in IV behind *Q. velutina* (0.224), and *Q. rubra* (0.197) (Table 1.2). A different *Quercus* species was dominant at each site. *Quercus alba* had the highest IV at Brimfield, yet had only a minor role at the other three sites, while *Q. prinus* was only present on the rocky, exposed southern slopes of the Holyoke site (Table 1.2).

Armillaria Species Incidence, Distribution, and Infection Type

Overall, five *Armillaria* species were located from 23 host tree species, with *Armillaria* species incidence showing significant differences within both northern hardwood ($p < 0.001$) and mixed oak forests ($p = 0.004$) (Table 1.3). Northern hardwoods had greater species diversity, as five *Armillaria* species were encountered, while mixed oak forests had only two species present. *Armillaria gallica* was the dominant species encountered in both forest types, yet was less abundant in northern hardwood forests compared to mixed oak (Table 1.3).

Table 1.2. Incidence of *Armillaria* by Forest Type and Individual Host with Vegetation Plot Characteristics.

Host Tree Species	Total Trees (Vegetation)[Z]	IV Values (Vegetation)[Y]	No. Infected Trees[X]	*A. calvescens*	*A. gallica*	*A. gemina*	*A. sinapina*	*A. solidipes*
N. Hardwoods								
Acer pennsylvanicum L.	29	0.080	1	1	0	0	0	0
Acer rubrum L.	39	0.212	36	3	22	2	2	7
Acer saccharum Marsh.	20	0.137	62	15	27	2	3	15
Betula alleghaniensis Britton	16	0.092	6	1	2	2	0	1
Betula lenta L.	5	0.016	1	0	1	0	0	0
Betula papyrifera Marsh.	2	0.016	6	1	4	0	0	1
Fagus grandifolia Ehrh.	74	0.272	27	4	17	5	1	0
Fraxinus americana L.	4	0.065	12	4	7	0	1	0
Prunus serotina Ehrh.	2	0.028	1	0	0	1	0	0
Quercus rubra L.	4	0.040	8	0	8	0	0	0
N. Hardwood Total	207[W]		160	29	88	12	7	24
Mixed Oak								
Acer rubrum L.	36	0.160	15	---	15	---	---	0
Acer saccharum Marsh.	8	0.033	2	---	2	---	---	0
Betula alleghaniensis Britton	0	0	1	---	1	---	---	0
Betula lenta L.	15	0.084	3	---	3	---	---	0
Carya glabra (Mill.)	12	0.084	13	---	12	---	---	1
Carya ovata (Mill.) K. Koch	3	0.015	2	---	2	---	---	0
Pinus strobus L.	3	0.015	1	---	1	---	---	0
Populus tremuloides Michx.	1	0	1	---	1	---	---	0
Quercus alba L.	12	0.125	19	---	19	---	---	0
Quercus prinus L.	6	0.039	8	---	8	---	---	0
Quercus rubra L.	19	0.197	39	---	36	---	---	3

Quercus velutina Lam.	24	0.224	55	---	52	---	---	3
Tilia americana L.	1	0	1	---	1	---	---	0
Mixed Oak Total	144		160	---	153	---	---	7
Overall Total			320	29	241	12	7	31

[Z] Total number of trees sampled from 16 0.01 ha vegetation plots in each forest type.
[Y] Importance values (IV) [(relative density + relative dominance)/2] generated for each species were pooled from 16 0.01 ha vegetation plots per forest type.
[X] Total number of infected trees encountered within each forest type.
[W] Tree species occurring in vegetation plots that were not found infected with *Armillaria* are not listed: (northern hardwoods = *Carpinus caroliniana, Ostrya virginiana, Picea rubens*, and *Tilia americana*; mixed oak = *O. virginiana*).

Table 1.3. Incidence of *Armillaria* from Northern Hardwood and Mixed Oak Forests.

Forest Type[Z]	Total[Y]	*A. calvescens*	*A. gallica*	*A. gemina*	*A. sinapina*	*A. solidipes*	*p*-value[X]
NH	160	29↑[W]	88↓	12↑	7	24↑	<0.001
MO	160	0	153↑	0	0	7↓	0.004
Overall	320	29	241	12	7	31	

[Z] Forest types are listed as: NH = northern hardwoods; and MO = mixed oak.
[Y] Total number of isolates collected from each forest type.
[X] Probability that there are no significant differences among frequencies of incidence by forest type based on Chi-square analysis (using expected values) at $p = 0.05$.
[W] Arrows denote whether actual values were higher (+5) or lower (-5) than expected values. No arrow indicates actual values were within ±5 occurrences from expected values.

At the four northern hardwood sites, five *Armillaria* species were found on ten hardwood host species (Table 1.2). One hundred forty six of the 160 isolates came from live hosts (91%). *Armillaria gallica* was the most common species encountered, comprising 88 of 160 isolates (55%) (Table 1.2). This was followed by *A. calvescens* (29/160; 18%), *A. gemina* (12/160; 8%), *A. sinapina* (7/160; 4%), and *A. solidipes* (24/160; 15%) (Table 1.2). Three or more *Armillaria* species occurred in a single plot on eight occasions, yet when the median number of species per plot was compared across the 4 sites there were no significant differences ($p = 0.098$). When *Armillaria* species diversity by plot was compared between forest types (northern hardwoods and mixed oak), significant differences became apparent ($p < 0.01$) (Table 1.4).

Armillaria gallica was the most common species found at three of the four northern hardwood sites. This species was observed most often causing butt rot, making up 69/88 of all butt-rot isolations (Table 1.5). Across all species, butt rot was the dominant mode of infection, making up 117/160 of all isolations (Table 1.5). Severe root and butt rot caused by *A. gallica* was observed on live trees, especially *A. rubrum* and *A. saccharum*. Large basal cavities were encountered routinely, with decay in the heartwood tissue extending out into the sapwood. *Armillaria calvescens*, while less abundant, also produced a very similar type of decay to live trees.

Armillaria solidipes was uncommon at three of the four northern hardwood sites (Table 1.4). Yet at Mount Greylock, *A. solidipes* was the most abundant species encountered, making up 19/40 (48%) total isolates collected at this site. Based on Chi-square analysis, the distribution of *A. solidipes* at Mount Greylock was significantly different than the other three northern hardwood sites ($p < 0.010$).

From the mixed oak sites, two *Armillaria* species were found on one coniferous and twelve hardwood host species (Table 1.2). One-hundred-forty of 160 isolates (88%) came from live hosts. Again, butt rot was the dominant mode of infection, making up 118/160 isolations (74%) (Table 1.5). *Armillaria gallica* was the overwhelmingly dominant species, as it accounted for 153 of 160 isolates (96 %) (Table 1.2). It was found infecting all 13 host tree species, and caused a range of disease symptoms from minor butt rot with basal resinosus, to extensive decay of the heartwood. *Armillaria solidipes* was the only other species encountered. It was found rarely, making up 7/160 (4%) of isolates (Table 1.2). Six of these seven isolates were found only at the Spencer site. There were no significant differences in species diversity by plot across the four sites ($p = 0.248$) (Table 1.4).

Table 1.4. *Armillaria* Species Incidence by Individual Site in Northern Hardwood and Mixed Oak Forests.

		Northern Harwood Sites				Mixed Oak Sites			
Armillaria spp.	Total[Z]	Monroe[Y]	Greylock	Pittsfield	Taconic	Brimfield	Quabbin	Holyoke	Spencer
A. calvescens	29	14	5	9	1	0	0	0	0
A. gallica	241	17	9	26	36	40	39	40	34
A. gemina	12	2	7	1	2	0	0	0	0
A. sinapina	7	3	0	4	0	0	0	0	0
A. solidipes	31	4	19	0	1	0	1	0	6
Total	320	40	40	40	40	40	40	40	40
Median no. of spp. per plot[X]		2.5^{ab}	3.5^{a}	2.5^{abc}	1.5^{bc}	1.0^{c}	1.3^{bc}	1.0^{c}	1.5^{bc}

[Z] Total number of isolates collected from both forest types.
[Y] Number of isolates for each species from four plots at each site.
[X] Values with different letters are significantly different at $p = 0.05$ using the Tukey test.

Table 1.5. Number of Infected Trees by *Armillaria* Species and Mode of Infection for Northern Hardwood and Mixed Oak Forests.

Armillaria spp.	Total[Z]	Mode of Infection	
		Butt Rot	Root Rot
N. Hardwoods			
A. calvescens	29	18	10
A. gallica	88	69	18
A. gemina	12	8	3
A. sinapina	7	5	2
A. solidipes	24	17	6
Total	160	117	39
p-value[Y]		0.914	0.788
Mixed Oak			
A. gallica	153	113	38
A. solidipes	7	5	2
Total	160	118	40
p-value		0.925	1.000

[Z] Total number of isolates collected from each forest type.
[Y] Probability that there are no significant differences among frequencies of *Armillaria* species by infection type based on Chi-square analysis at $p = 0.05$ (using expected values).

Host Preference

Acer saccharum was the most common host across all four northern hardwood stands, accounting for 39% of all infected trees encountered (62/160) (Table 1.2). It was the most common host, or tied for the most, for four of the five *Armillaria* species collected in this forest type (Table 1.2). However, *A. saccharum* was never the most dominant tree species at any stand, making up only 20/207 total trees from the 16 vegetation plots with a total IV of 0.137 (Table 1.2). In contrast, *F. grandifolia* made up only 17% of infected hosts (27/160), while dominating stands (74/207 total trees, 0.272 IV) that are classified as in the aftermath stage of beech bark disease (Houston 1975) (Table 1.2). Hepting (1971) has also noted that *F. grandifolia* is relatively

resistant to infection by *Armillaria* species despite significant stresses brought on by beech bark disease.

Acer saccharum and *A. rubrum* were the only hosts that supported all five *Armillaria* species, thus providing enough data to perform a meaningful Chi-square analysis. In comparison to expected values, there were no significant differences in *Armillaria* species incidence on these two host species ($p = 0.129$ and $p = 0.610$, respectively). While not statistically significant, *A. saccharum* seemed to be the preferred host for *A. calvescens* (15/29; $p = 0.346$) and *A. solidipes* (15/24; $p = 0.060$) in northern hardwood forests (Table 1.6). The difference in host preference between *Acer rubrum* and *A. saccharum* was interesting. *Acer rubrum* was almost twice as common as *A. saccharum* in the vegetation plots (39 vs. 20 total trees), with an IV more than 1.5 times greater (0.212 vs. 0.137). Relative dominance and relative density values were not significantly different between the two species ($p = 0.794$ and $p = 0.669$, respectively). However, *Armillaria* was isolated from *A. saccharum* 62 times while it was found on *A. rubrum* only 36 times (Table 1.2). *Armillaria gallica* was the most abundant species isolated from *A. saccharum*, but was also found on 9/10 hosts sampled in this forest type, illustrating the wide host range for this species (Table 1.2). Both *A. gemina* and *A. sinapina* were sampled too infrequently to show any strong trends towards a particular host (Table 1.2).

Table 1.6. Incidence of *A. calvescens, A. gallica,* and *A. solidipes* by Sugar Maple Compared to All Other Hosts in Northern Hardwood Forests Only.

		Host		
Armillaria spp.	Total[Z]	Sugar Maple	All Others	*p*-value[Y]
A. calvescens	29	15	14	0.346
A. gallica	88	27	61	0.065
A. solidipes	24	15	9	0.060
Total	141	57	84	

[Z] Total number of isolates from northern hardwood forests only.
[Y] Probability that there is no difference among frequencies of *Armillaria* isolates based on Chi-square analysis (using expected values).

Armillaria solidipes was the most common species found within northern hardwoods at Mount Greylock. The only major difference between Mount Greylock and the three other northern hardwood sites was a higher proportion of *B. alleghaniensis* along with the presence of the spruce/fir forest type at elevations above roughly 900 m (2,900 ft.). However, there were no significant differences in the abundance of *A. solidipes* by relative dominance of *B. alleghaniensis* in comparison to the other *Armillaria* species ($p = 0.152$).

Quercus velutina was the most common host across all four mixed oak stands, making up 55/160 (34%) of infected trees (Table 1.3). *Quercus rubra* (39/160; 24%) and *Q. alba* (19/160; 12%) were also abundant hosts for *Armillaria* (Table 1.3). Using a linear regression to test the association between the proportion of occurrence for the four *Quercus* species (*Quercus alba*, *Q. prinus*, *Q. rubra*, and *Q. velutina*) encountered in the 0.01 ha vegetation plots and those sampled with *Armillaria* infections was highly significant ($p < 0.001$, $r^2 = 0.98$) (Figure 1.2). This illustrates that *A. gallica* does not preferentially select specific *Quercus* species within a given mixed oak stand, further evidence of this species wide host range.

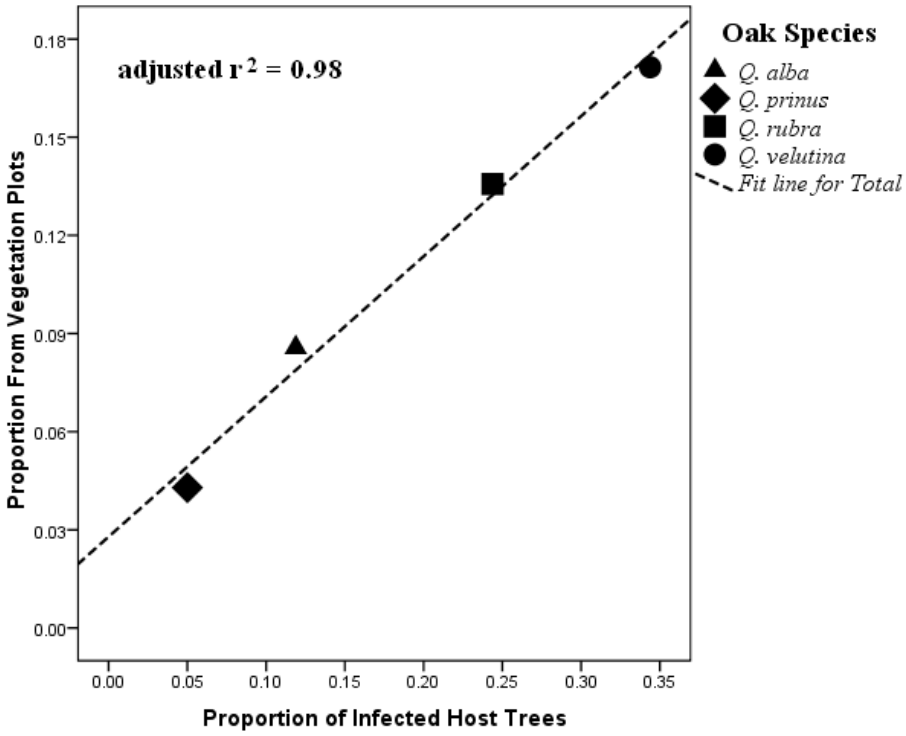

Figure 1.2. Association between *Quercus* Species from Vegetation Plots and *Armillaria* Sampling Plots. X-axis Represents Proportion of all *Quercus* Species Sampled from 16 0.01 ha Vegetation Plots While Y-axis Represents Proportion of *Quercus* Species Sampled with *Armillaria* Infections.

Incidence by Crown Class and Soil Drainage Classes

Host incidence was significantly different by crown class (Table 1.7). For both forest types, trees from the codominant crown class were most often found infected with *Armillaria*, making up 177/320 (55%) total isolations. Meanwhile, trees from the suppressed crown class were only rarely encountered, making up only 13/320 (4%) isolations. This is most likely explained by the fact that the predominant *Armillaria* species found in this study primarily cause butt rot, an infection process that takes years or decades to develop before disease symptoms are visible (Table 1.7).

Soil drainage classes were significantly different by forest type ($p = 0.010$) and by site ($p < 0.001$) (Table 1.1). The northern hardwood forests had more excessively drained soils than

the mixed oak; not because of soil texture but due to depth to impermeable bedrock. *Armillaria gallica* was the only species to occur on all soil drainage classes, where incidence was not significantly different ($p = 0.582$).

Table 1.7. Number of Infected Hosts by Crown Class in Northern Hardwood and Mixed Oak Forests.

Forest Type	Total[Z]	Dom[Y]	Cod	Int	Sup	*p*-value[X]
N. Hardwoods	160	46	94	17	3	<0.001
Mixed Oak	160	28	83	39	10	<0.001
Total	320	74	177	56	13	<0.001

[Z] Total number of isolates collected from each forest type.
[Y] Crown classes are listed as: Dom = dominant; Cod = codominant; Int = intermediate; and Sup = suppressed.
[X] Probability that there are no significant differences among frequencies of *Armillaria* species by crown class based on Chi-square analysis at $p = 0.05$ (all values equal).

Discussion

The sampling design used to find infected trees in this study was biased to those trees with visible symptoms and signs of infection. Therefore, minor to moderate infections of roots and decay of the heartwood on asymptomatic trees were surely missed. Morrison et al. (2000) found that climatic region was a significant factor in whether trees with root infections displayed aboveground symptoms, with wetter forests showing fewer aboveground symptoms. Mixed hardwood forests in Massachusetts are relatively wet, with an average annual precipitation rate (weighted statewide) from 1992 through 2010 of 125 cm (NCDC 2011). Additional studies have confirmed that relying on aboveground symptoms underestimates the incidence of root disease (Filip 1986, Whitney et al. 1989). Yet, several studies have proven just the opposite, that aboveground symptoms are reliable indicators of root disease in both wet and dry forests (Shaw and Toes 1977, Wallis and Bloomberg 1981, Omdal et al. 2004). All of these studies were performed in conifer forests where the most abundant *Armillaria* species (*A. solidipes*) is

predominantly root infecting. In mixed hardwood forests of northeastern North America, butt rot infecting species (*A. calvescens* and *A. gallica*) dominate, so these comparisons have only limited value.

In contrast to Blodgett and Worrall (1992b), Marçais and Wargo (2000), and McLaughlin (2001), *A. gallica* was more abundant in *Acer*-dominated stands than *A. calvescens* in western Massachusetts. I believe this dominance of *A. gallica* over *A. calvescens* in northern hardwood forests of western Massachusetts is due to my study sites being located in the southernmost extent of northern hardwoods in New England, and their proximity to mixed oak forests that are dominated by *A. gallica* (Figure 1.1; Table 1.3). Despite their overlapping ranges, it would appear that *A. gallica* can outcompete *A. calvescens* in *Acer*-dominated forests that are in close proximity to *Quercus*-dominated forests.

Within both northern hardwood and mixed oak forests in Massachusetts, *A. gallica* was observed as an aggressive pathogen, causing severe root and butt rot on a wide range of hosts. It was collected on 22 of the 23 tree species encountered in this study, and caused butt rot on 182/241 hosts it had infected. *Armillaria gallica* has been shown to be associated with oak in the northeast previously (Blodgett and Worrall 1992b, McLaughlin 2001), but never has it been so definitively linked with this forest type as it has in this study. Within mixed oak forests, *A. gallica* was found dominating on a range of different sites, from a mesic *Q. alba* forest in deep coarse loam to a dry *Q. prinus*/*Q. rubra*-dominated forest on exposed basalt. Based on observations, infections of oaks were not nearly as severe as they were on *A. rubrum* and *A. saccharum* in northern hardwood stands. Infections were severe, however, when oaks regenerated from stump sprouts. Stump sprout regeneration leads to multiple stems, which can lead to one stem expressing dominance over the remainders. These out-competed stems become suppressed and die, thus creating an infection site for *A. gallica*. This was the most common mode of entry when aggressive butt rot on oak was encountered. This mode of infection has also been documented in upland oak sites in Missouri (Bruhn et al. 2005). Crown dieback, basal resinosus, and basal

flaring were also routinely encountered disease symptoms, regardless of site and tree species composition.

While trees with butt rot may survive for many years even with a substantial infection, much of the structural core is destroyed, which can lead to stem breakage from strong winds and colonization by other decay fungi. Kile et al. (1991) considered butt rot caused by *Armillaria* as primary parasitism, since live tissues may first be colonized prior to establishment within the heartwood. Furthermore, the decay may expand from the heartwood into live sapwood tissue, which was encountered in this study. While the heartwood is dead tissue, it does contain extractives, a chemical defense against microorganisms that *Armillaria* must overcome in order to colonize a host (Garraway et al. 1991). From an economic perspective, butt rot is very damaging as much of the monetary value of a tree, especially *A. saccharum*, is lost by the destruction of the butt log (Nordin 1950, 1954, Ohman 1968, Silverborg 1954, Zillgitt and Gevorkiantz 1948).

The findings in this study have important implications towards how we view *A. gallica* in mixed oak forests. Over the past several decades, northeastern oak forests have experienced widespread mortality due to the interacting effects of *Armillaria*, overcrowding, and extensive defoliation by insects, most notably from gypsy moth (Wargo 1977, Twery et al. 1990, Burrill et al. 1999, Davidson et al. 1999, Abrams 2003). Some researchers have characterized *A. gallica* as a saprophyte or weak pathogen only capable of colonizing hosts at or near death (Burdsall and Volk 1993, Legrand et al. 1996, Bruhn et al. 2000). Yet, temperate hardwoods are almost always affected by multiple biotic and/or abiotic stresses in the forest, which range from drought, defoliating and wood-boring insects, decay fungi, freeze damage, ice/windstorms, among others (Houston 1987, Wargo and Harrington 1991, Sinclair and Lyon 2005). My ability to locate *A. gallica* causing disease to live oak trees indicates that regardless of how severe an initial infection is, ultimately a combination of stresses will interact allowing *A. gallica* to play an important role in tree mortality. This assertion is supported by other studies of *A. gallica* in mixed oak forests (Wargo 1977, Marçais and Breda 2006).

Armillaria calvescens was most frequently collected from *A. saccharum*, which is consistent with other reports of this species (Table 1.3) (Bauce and Allen 1992, Blodgett and Worrall 1992a, McLaughlin 2001). It was most often observed causing severe butt rot to live trees. This species was present at all four sites but was most abundant at Monroe, the most northern site sampled, where it comprised 14/40 (35%) of isolates collected (Table 1.2). This species is very similar to *A. gallica* in host range, behavior, and genetic profile (Kim et al. 2006). Yet, based on this survey, it is of secondary importance to *A. gallica* in western Massachusetts northern hardwood forests.

Armillaria gemina was collected a total of 12 times (Table 1.2), with all of those isolations coming from living hosts showing visible symptoms of decay. Eight of the 12 collections came from trees with butt rot. Furthermore, 10 of 12 isolations came from trees occupying the dominant and codominant crown class. This is in agreement with McLaughlin (2001) who found *A. gemina* causing butt rot infections on *A. rubrum* and *A. saccharum*, and with Bérubé and Dessureault (1989), who described *A. gemina* as having a similar pathogenicity to *A. solidipes*. Morrison (2004) found that *A. gemina* was more virulent than *A. gallica* and *A. calvescens* in a study of pathogenicity with potted *Pseudotsuga menziesii* seedlings. However, *A. gemina* has also been reported to be a very weak pathogen, only able to colonize trees at or very near death (Blodgett and Worrall 1992a, Rizzo and Harrington 1993). Blodgett and Worrall (1992b) found *A. gemina* in higher abundance in stands with a greater proportion of *F. grandifolia*. This trend was not encountered in this study but could be masked by the proliferation of beech across northern hardwoods in western Massachusetts following the introduction of beech bark disease. *Armillaria gemina* is considered rare throughout most of its range, but it was found routinely at Mount Greylock, accounting for 18% of all isolates collected at this site (Table 1.4). This species may indeed be rare compared to other *Armillaria* species throughout the range of northern hardwoods in eastern North America, but it can be locally abundant as indicated here.

Armillaria solidipes was found to be the most abundant species within northern hardwood forests at Mount Greylock, the only site in close proximity to a high elevation spruce-fir forest. This species comprised 7/10 isolates from the highest elevation plot at Mount Greylock (818 m), and has been shown to be most abundant in high elevation spruce-fir forests of New Hampshire (Rizzo and Harrington 1988, Worrall et al. 2005). The predominance of *A. solidipes* in northern hardwoods at this site also illustrates how species abundance in one forest type can influence its abundance in neighboring forest types. The spruce-fir at Mount Greylock is the southernmost tract of this forest type in New England. While *A. solidipes* is known to prefer conifer-dominated forests, its ability to thrive in hardwood forests that reside near conifer forests suggests this species is very abundant in northern hardwoods across northern New England where the spruce-fir forest type is common.

The presence of *A. solidipes* in northern hardwoods has important implications for forest management activities considering this species can spread very effectively via root to root contacts. In addition, the predominant silvicultural prescription for northern hardwoods in this region is a shelterwood cut (Hornbeck and Leak 1992), which requires multiple stand entries to complete and in doing so constantly creates new sources of inoculum, in the form of infected stumps. This inoculum can be utilized by *A. solidipes* to build up energy reserves so that it may colonize not only residual trees in the stand, but newly regenerating trees as well.

Armillaria mellea, a species common in oak forests of Missouri (Bruhn et al. 2000) and known to occur in Pennsylvania (Marçais and Wargo 2000), was not encountered in this study and to date is considered rare in northeastern North America. Including the results from this study, only 14 isolates of *A. mellea* have been identified from a total of 1,508 collected from Massachusetts, New Hampshire, New York, and Ontario (Dumas 1988, Blodgett and Worrall 1992a, Harrington and Rizzo 1993, Rizzo and Harrington 1993, McLaughlin 2001).

To conclude, *Armillaria* species are important components of northern hardwood and mixed oak disturbance regimes in western Massachusetts. The information presented here is

critical to the argument that *Armillaria* is a dominant agent of mortality in hardwood forests of Southern New England. Five species were located in varying levels of incidence, yet all were able to successfully colonize and cause disease to a live host. This study has supported the notion that certain *Armillaria* species show a strong host preference for particular forest types. Mixed oak forests in western Massachusetts are dominated almost exclusively by *A. gallica*, with rare occurrences of *A. solidipes*. This is the first study to clearly make this association in northeastern mixed oak forests with a robust sample size. Northern hardwood forests, in contrast, are much more diverse in their assemblage of species. Seven of the eight sites sampled in this study were dominated by *A. gallica*. The remaining site, Mount Greylock, was dominated by *A. solidipes* and was the only northern hardwood site in close proximity to a high elevation spruce-fir forest. It was hypothesized that the abundance of one *Armillaria* species within a particular forest type can influence the species incidence in surrounding forest types. This trend appears to be most apparent with *A. gallica*, a species abundant in mixed oak forests, as the most encountered species in northern hardwood stands in northwest Massachusetts.

CHAPTER 2

ARMILLARIA SPECIES DISTRIBUTION AND SITE RELATIONSHIPS IN *PINUS*- AND *TSUGA*-DOMINATED FORESTS IN MASSACHUSETTS

Introduction

Armillaria root disease, caused by *Armillaria* spp. ((Fr.:Fr.) Staude), is widely regarded as one of the most important diseases of coniferous trees throughout the world (Hansen and Lewis 1997), yet little is known about the distribution of *Armillaria* species in *Pinus* and *Tsuga* forests of northeastern North America. Armillaria root disease is especially important in northeastern conifer forests because many destructive root decay fungi common in other areas of North America are either uncommon (e.g. *Heterobasidion irregulare* Garbelotto & Otrosina and *Onnia tomentosa* (Fr.: Fr.) P. Karst.) or are not known to occur (e.g. *Phellinidium sulphurascens* Pilát and *Leptographium wageneri* (Kendr.) M.J. Wingf.) (Sinclair and Lyon 2005). Additionally, climate change, pollution, native and non-native insect outbreaks (i.e. hemlock woolly adelgid and elongate hemlock scale), threaten to make *Pinus* and *Tsuga* forests susceptible to attack from *Armillaria* (McLaughlin et al. 1982, Orwig et al. 2002, Dukes et al. 2009). Determining the distribution and behavior of *Armillaria* species is necessary to predict how interacting disturbance agents will ultimately affect these forests.

Conifer forests from southern New England to the mid-Atlantic are generally dominated by pitch pine (*Pinus rigida* Mill.), eastern white pine (*P. strobus* L.), and eastern hemlock (*Tsuga canadensis* (L.) Carr.) (Eyre 1980). Pitch pine is a shade intolerant species that relies on fire to successfully regenerate (Motzkin et al. 1999). The wood of this tree is very resinous, making it resistant to decay from fungi (Hepting 1971). Forests dominated by pitch pine are scattered throughout the eastern U.S., and this forest type covers over 85,000 ha in Massachusetts (Burns and Honkala 1990), typically occupying dry, sandy soils on glacial outwash along the Atlantic coast (Westveld et al. 1956). Prior to European settlement, pitch pine forests were also common

in the Connecticut River valley in central Massachusetts, but due to housing and agricultural development this type has been vastly reduced (Motzkin et al. 1999). There are very few studies involving pitch pine and its associated decay pathogens (Hepting 1971, Roth 1952).

Eastern white pine is a long-lived, mid-tolerant tree species that occupies a range of sites in New England, but grows best on sandy, well-drained soils (Eyre 1980). In Massachusetts, eastern white pine is the most abundant tree, making up 28% of total live tree volume and 58% of all sawtimber with a DBH greater than 50 cm (USDA 2006). White pine is susceptible to several important root-rot fungi (Sinclair and Lyon 2005). However, studies documenting the extent and distribution of root disease on eastern white pine are lacking. *Armillaria* has been reported to cause significant mortality of eastern white pine seedlings and saplings within 9 m of colonized hardwood stumps (Hepting 1971). However, Gerlach et al. (1997) reported negligible levels of white pine mortality from Armillaria root disease in a study of seedling composition and density in Minnesota, where *A. solidipes* was assumed to be the species present. Results from earlier studies suggest that white pine is resistant to fungal decay compared to other conifers. Mwangi et al. (1990) found that extracts from fresh sapwood were inhibitory to *in vitro* growth of *A. solidipes*, and in an assessment of heartwood resistance to three different decay fungi for 14 North American conifer species, including nine species of *Pinus*, eastern white pine ranked as the second most resistant, behind longleaf pine (Clark 1957).

Eastern hemlock is the most shade tolerant of all North American tree species (Burns and Honkala 1990), and is the fourth most abundant tree species in the state, making up over 10% of total tree volume (USDA 2006). Although the wood is not considered very durable due to ring-shake (Scheffer and Morrell 1998), eastern hemlock is resistant to stem-decay fungi (Spaulding 1914, Hepting 1971). *Armillaria* has been associated frequently with eastern hemlock, but it has never been known to cause significant decay or mortality (Hepting 1971, Wargo and Fagan 2000). Pure stands of hemlock are generally small in area because hemlock is often mixed with white pine along with northern and central hardwoods (Eyre 1980). Eastern hemlock has received

considerable attention the past two decades due to the spread of the non-native hemlock woolly adelgid (Orwig et al. 2002). A reduction in extreme winter temperatures has allowed the adelgid to continue its northward spread from heavily infested areas in Connecticut and parts of Massachusetts into the southern regions of Vermont, New Hampshire, and Maine (Dukes et al. 2009, Onken and Souto 2009).

Overall, data are lacking regarding the incidence of decay fungi on northeastern conifer forests. In order to better explain the role of *Armillaria* in northeastern *Pinus* and *Tsuga* forests, a comprehensive survey of the *Armillaria* species present was conducted. The specific goals of this study were to, i) determine the distribution of *Armillaria* species in forests dominated by *Pinus* and *Tsuga* in Massachusetts; ii) determine if soil type and soil drainage classes are associated with particular *Armillaria* species; and iii) observe the behavior of *Armillaria* in each conifer-dominated forest type to better understand its role as a disturbance agent.

Materials and Methods

Site Selection

Four distinct forest types were examined in this study, and are listed with the forest cover type numbers assigned by the Society of American Foresters (Eyre 1980). Chosen forest types include: i) pitch pine (45), ii) eastern white pine (21), iii) eastern white pine/mixed oak (20 and 52), and iv) eastern hemlock (23). A pure stand is defined as having at least 80% of the basal area occupied by a single tree species (Eyre 1980). Plantation forestry is uncommon in Massachusetts, and as a result none of the sites sampled were entirely composed of the desired tree species. The three conifer species chosen for study (pitch pine, white pine, and hemlock) provide a gradient of shade tolerance levels and site preferences: (i) intolerant and dry (pitch pine); (ii) mid-tolerant and moist (white pine); and (iii) tolerant and wet (hemlock). Sites were chosen to fit the following criteria:

1) Pure pitch pine: the largest contiguous stands occupying glacial outwash in the central region (Montague) and the coastal lowlands (Myles Standish) (Figure 2.1). Both pitch pine sites have been disturbed by fire suppression activities for over 50 years, with large fires last occurring at Montague in 1957 and Myles Standish in 1957 and 1964. In addition, pitch pines at Myles Standish have experienced infestations of the pine needle miner, with moderate to severe damage occurring in 1978-80 and 2004-2006.

2) Pure eastern white pine: the largest contiguous stands occupying glacial outwash in north-central Massachusetts, an area that contains extensive stands of pure white pine (Westveld et al. 1956) (Figure 2.1). The central region site (Quabbin Gate 29) experienced damage from gypsy moth (1980-81) while the coastal region site (Townsend) experienced widespread defoliation by gypsy moth (1980-1982 and 1990-91) along with timber harvesting activities (shelterwood cut) in the late 1990s.

3) Eastern white pine/mixed oak: the largest contiguous stands with one site occurring in the central region (Erving/Warwick), and one site occurring in the coastal lowlands in southeastern Massachusetts (Freetown/Fall River) (Figure 2.1). The central region site has experienced damage from oak leaftier (1976-1979) followed by widespread gypsy moth defoliation (1980-82). The coastal region site experienced major defoliation from gypsy moth (1981-82, 1985, and 1995) and recently has been heavily defoliated, initially by forest tent caterpillar and gypsy moth, and currently from winter moth. The defoliation events have occurred in scattered areas of southeastern Massachusetts from 2003 to 2010, and as a result, severe oak mortality has occurred at this site over the past three to five years.

4) Pure eastern hemlock: the largest contiguous stands in areas where the hemlock woolly adelgid has either not been found, or has only recently infested (Figure 2.1). Additionally, the sites were stratified by elevation and surrounding forest type. One site occurred at a higher elevation (>350 m) in the western region (Tolland/Sandisfield) and was surrounded by a northern hardwood (beech-birch-maple) forest. This site was lightly defoliated by hemlock looper in 1994-

95 and has suffered from widespread canopy damage due to a severe ice storm in late 2008. The second site (Quabbin Gate 15) occurred at a lower elevation (<350 m) in the central region and was surrounded by a white pine/mixed oak forest. This site has experienced defoliation from gypsy moth (1978-1981) and oak leaf skeletonizer (1985-86). Stand disturbance information was obtained from USDA forest health monitoring reports (http://fhm.fs.fed.us/fhh/neregion.shtml), DCR aerial survey detection maps (MassGIS 2010), and the DCR forest health office.

In addition to the site selection criteria above, one site for each forest type (four total) were grouped within a relatively small geographic area (~200 km^2) to determine how strongly forest type and site variables influence species incidence (Figure 2.1). Details on how individual plots were selected are presented in Chapter 1. Briefly, the largest contiguous block of forest that contained the desired forest type was selected for use at a given site. Using ArcMap v. 10.0 (ESRI, Redlands, CA) and a random integer generator (random.org), the coordinates for four study plots were randomly chosen for each site. Therefore, 4 forest types × 2 sites within each forest type × 4 plots at each site = 32 total study plots.

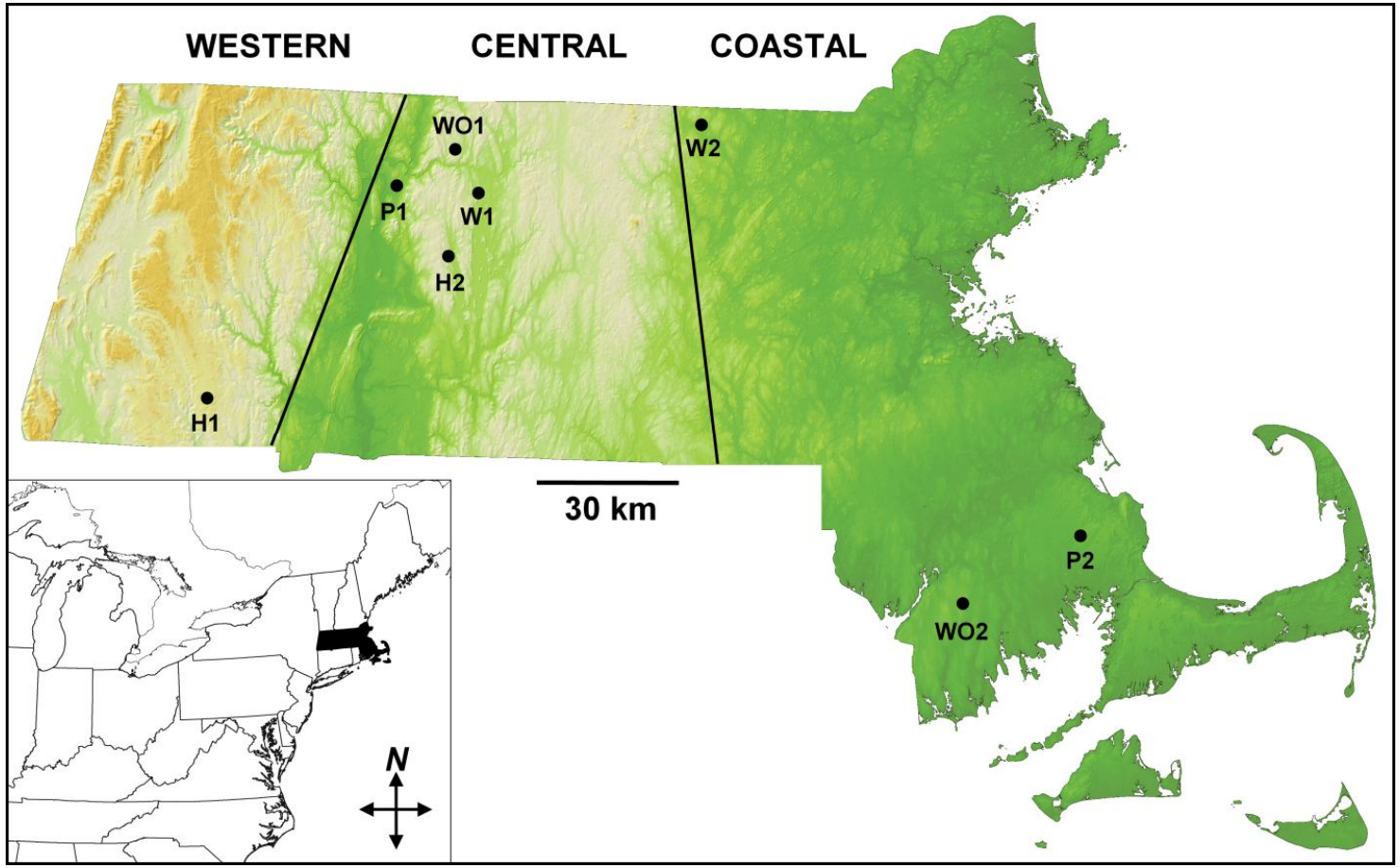

Figure 2.1. Location of Study Sites in *Pinus*- and *Tsuga*-Dominated Forests in Massachusetts. Eastern hemlock Sites are Denoted as: H1 = Tolland/Sandisfield State Forest and H2 = Quabbin Gate 15; White Pine Sites are Denoted as: W1 = Quabbin Gate 29 and W2 = Townsend State Forest; White Pine/Mixed Oak Sites are Denoted as: WO1 = Erving/Warwick State Forest and WO2 = Freetown-Fall River State Forest; Pitch Pine Forests are Denoted as PP1 = Montague Plains Wildlife Management Area and PP2 = Myles Standish State Forest.

Table 2.1. Characteristics of Study Sites Where *Armillaria* Was Sampled.

Forest Types & Site Names	Elevation (m)[Z]	Dominant Forest Cover[Y]	IV[X]	Basal Area (m^2/ha)[W]	Parent Material	Soil Type	Soil Drainage Class[V]
Pitch Pine							
Montague	97 – 100	*Pinus rigida – Quercus coccinea – P. strobus*	0.82	32	Mesozoic basin sediments	loamy sand	excessively drained
Myles Standish	42 – 51	*Pinus rigida – Acer rubrum – P. strobus*	0.92	24	granite	loamy sand	excessively drained
White Pine							
Quabbin G29	175 – 195	*Pinus strobus – Quercus velutina – Tsuga canadensis*	0.81	38	granite	sandy loam	well drained
Townsend	95 – 108	*Pinus strobus – Quercus velutina – Betula lenta*	0.80	33	granite	sandy loam	excessively drained
White Pine – Oak							
Erving-Warwick	234 – 314	*Pinus strobus – Quercus rubra – Acer rubrum*	0.54	33	granite, peltic, and mafic rocks	sandy loam	moderately well drained
Freetown-Fall River	54 – 80	*Pinus strobus – Quercus coccinea – Q. velutina*	0.51	26	granite	sandy loam	moderately well drained
Hemlock							
Tolland-Sandisfield	391 – 503	*Tsuga canadensis – Acer rubrum – Betula lenta*	0.89	45	granite, and metamorphic rocks	medium loam	moderately well drained
Quabbin G15	176 – 242	*Tsuga canadensis – Acer rubrum – Betula lenta*	0.96	45	sulfidic schists, peltic, and mafic rocks	medium loam	well drained

[Z]Range of elevations across four plots per site.
[X]Based on importance values listed from highest to lowest for three most common tree species, summed from four 0.01 ha vegetation plots per site.
[W]Mean basal area across the four plots per site.
[V]Soil drainage classes rank from 1 to 4: Soils from group 1 are excessively drained while soils from group 4 are moderately well-drained.

Vegetation Sampling, Elevation, Parent Material, and Soils

Plot centers were established at the nearest canopy dominant/codominant tree. Basal area was determined from the plot center using a prism with basal area factor of 10. Forest composition was sampled using 0.01 ha (100 m^2) rectangular plots. All trees > 5 cm in DBH were sampled for species, DBH, height, and crown class. Coordinates of point centers were recorded with a hand held GPS unit. Tree species importance values (IV) [(relative density + relative dominance)/2] were generated for each vegetation plot. Elevation for each plot was determined using a 1:5,000 digital elevation model (MassGIS 2010), while parent materials for the sites were determined using digitized data layers of published bedrock surveys conducted by the USDA Natural Resources Conservation Service (NRCS) and the U.S. Geological Survey (USGS) (MassGIS 2010; Table 2.1). Soil types and drainage classes were determined using published soil surveys by the NRCS (2011).

Sites supporting pitch pine were designated as dry. Soils at these sites are collectively described as very deep, excessively drained, loamy sand on glacial outwash. Soils at the central region site are Windsor series (mixed, mesic Typic Udipsamments), while soils at the coastal site are Carver series (mesic, coated Typic Quartzipsamments) (NRCS 2011). Sites supporting white pine are designated as moist. Soils at these sites are collectively described as deep, well drained, sandy loam on glacial outwash. Soils at the central region site are Merrimac/Canton series (coarse-loamy over sandy, sandy-skeletal, mixed, mesic Typic Dystrudepts) while soils at the coastal sites are Hinckley/Paxton series (sandy-skeletal, mixed, mesic Typic Udorthents and coarse-loamy, mixed, active, mesic Oxyaquic Dystrudepts) (NRCS 2011).

Despite the similarities between the soils at the pitch pine and white pine sites, the soils differ in texture and water holding capacities. In addition, the white pine-dominated sites occurred in areas where water table was much closer to the surface than pitch pine-dominated sites, evident by the presence of nearby wetlands. While these two tree species can occupy the

same soils, pitch pine cannot compete with white pine on richer sites, restricting pure pitch pine forests to soils that are both very deep and excessively drained. These are sites where white pine does not grow well (Westveld et al. 1956) and fires were historically common (Motzkin et al. 1999).

Soils supporting hemlock are designated as wet. Soils at these sites are collectively described as shallow to moderately deep, moderately well drained, medium loam over glacial till. Soils at the western region site are Lyman/Tunbridge series (loamy/coarse-loamy, isotic, frigid Lithic Haplorthods) while soils at the central region site are Chatfield series (coarse-loamy, mixed, superactive, mesic Typic Dystrudepts) (NRCS 2011).

Sampling and Isolation of *Armillaria*

A hierarchical approach was used to sample *Armillaria*-infected trees. From the plot center, starting from a north azimuth and moving counter-clockwise (360º to 0º), trees > 5 cm in DBH were assessed for symptoms and signs of *Armillaria* until 10 were found. Live, symptomatic trees were preferentially selected for sample. When live, symptomatic trees were not present, dead trees were chosen. The primary tree species in each forest type was preferentially selected, yet several minor tree species in the stands were also sampled. Average plot size was 0.3 ha, ranging between 0.1 and 0.8 ha. Symptoms used to locate trees with active infections included crown dieback, basal cavities, chlorotic or undersized foliage, basal flaring, basal resinosus, and basal seams. While an emphasis was placed upon finding live trees infected with *Armillaria*, conifers growing in moist/wet regions generally do not show symptoms of dieback the way hardwoods do (Morrison et al. 2000). Therefore, instead of sampling asymptomatic trees, dead trees were selected for sampling according to their decay class rank, as listed below (Table 2.2). Species, DBH, crown class, type of infection/association (butt rot, root rot, superficial attachment), condition of tree if dead (standing or wind thrown), and decay class were recorded. Dead trees exhibiting decay classes >3 (i.e. bark missing, sapwood and heartwood spongy, bole

has loss of structural integrity) were not sampled. When live trees showed symptoms of root and butt rot, the lower bole and main lateral roots were excavated for signs of *Armillaria*. When *Armillaria* was present, rhizomorphs, mycelial fans, and/or infected bark/wood tissue were collected.

Table 2.2. Decay Classes Used to Hierarchically Sample *Armillaria*.

Decay Class[z]	Description
0	Dead needles/leaves are present; fine twigs are present on branches; medium branches are present; bark is tight.
1	Needles/leaves absent, fine twigs are present on branches; medium branches are present; bark is present and sapwood is hard.
2	Fine twigs are absent; medium branches are present; bark is present and sapwood is hard.
3	Fine twigs are absent; medium branches are absent; bark is present or sapwood is hard.

[z]Decay classes slightly modified from Fast et al. (2008).

Tissues of *Armillaria* were washed in warm water and then under aseptic conditions soaked in a 2% solution of sodium hypochlorite for two to five minutes, rinsed in sterile distilled water, air dried, cut into small segments, and placed on 1.5% malt extract agar amended with 200 mg/L each of neomycin sulphate, streptomycin sulphate, and chlortetracycline, and 5 mL/L of benomyl solution (20 mg of 95% benomyl in 50 mL of warm 95% ethanol diluted to 100 mL with sterile distilled water) (McLaughlin 2001, Worrall 1991). Cultures were incubated in the dark at 25º C and subsequently sub-cultured to unamended 2% malt extract agar. In preparation for DNA extraction, isolates were transferred to malt-yeast extract broth (2% malt and 0.5% yeast extract) amended with 300 ppm of 95% ethanol, which stimulates mycelial growth and rhizomorph production (Weinhold 1963).

Identification of Unknown Isolates

A slightly modified version of the PCR-RFLP protocol designed by McLaughlin and Hsiang (2010) was used to identify unknown isolates. This protocol targets variation within the intergenic spacer (IGS) regions of the rDNA cluster. The IGS-1 cannot distinguish between *A. solidipes* and *A. gemina*, nor can it distinguish *A. calvescens*, *A. gallica*, or *A. sinapina* from one another (Kim et al. 2006). However, the IGS-1 is very effective when used to initially screen northeastern isolates for designation into three subgroups: (*A. solidipes* / *A. gemina*); (*A. mellea*); and (*A. calvescens* / *A. gallica* / *A. sinapina*), at which point individual species can then be separated by targeting variation in the IGS-2 region (McLaughlin and Hsiang 2010).

Harvested mycelium was lyophilized in 2 mL centrifuge tubes using a Labconco FreeZone 12 (Labconco, Kansas City, MO) lyophilizer for 48 hours. Genomic DNA was then obtained using a phenol-chloroform extraction, modified from Marra and Corwin (2009). Tester isolates spanning six species (*A. calvescens*, *A. gallica*, *A. gemina*, *A. mellea*, *A. sinapina*, and *A. solidipes*) were used as positive controls within the protocol. They were obtained from the Moscow forestry sciences laboratory, Moscow, ID, and from the Ontario Forest Research Institute, Sault Ste. Marie, Ontario.

PCR was performed with 10X Platinum Taq reaction buffer and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Reaction conditions using primers Ao-f700/18S-rev (McLaughlin and Hsiang 2010) were as follows: 1X buffer concentration, 0.25 units of Platinum Taq DNA polymerase, 0.2 μM primer concentrations and 200 μM concentrations of each dNTP (Fermentas, Glen Burnie, MD). Thermocycler conditions were: initial denature 94 C for 3 min; 35 cycles of denaturing at 94 C for 30 s, annealing at 58 C for 45 s, and extension at 72 C for 90 s; and a final extension of 72 C for 10 min. Reaction conditions using primers 5S-1f/5S-1r (McLaughlin and Hsiang 2010) were as follows: 1X buffer concentration, 0.5 units of Platinum Taq DNA polymerase, 0.2 μM primer concentrations and 200 μM concentrations of each dNTP.

Thermocycler conditions were: initial denature 94 C for 3 min; 35 cycles of denaturing at 94 C for 30 s, annealing at 61 C for 40 s, and extension at 72 C for 50 s; and a final extension of 72 C for 10 min. All PCR reactions were performed using an Eppendorf mastercycler gradient thermocycler (Eppendorf, Westbury, NY). An isoschizomer (NsiI) of the restriction enzyme MpH1103I and a neoschizomer (HpyCH4IV) of the restriction enzyme TaiI were used (New England Biolabs, Ipswich, MA).

Because *A. mellea* is one of the most genetically divergent *Armillaria* species in North America, the nuclear large subunit (nLSU) gene can accurately distinguish this species from the other nine North American *Armillaria* species, as shown by Kim et al. (2006). For further verification of this species identification, an approximately 950 bp amplicon from the 5' end of the nLSU was sequenced using the primers LROR and LR5 (Moncalvo et al. 2000). Prior to sequencing, PCR products were purified using ExoSAP-IT (USB, Cleveland, OH). Isolates were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA) at the Genomics Resource Laboratory, University of Massachusetts, Amherst. Sequences were manually edited and aligned using ClustalW in MEGA v. 4 (Tamura et al. 2007) and compared with nLSU sequences from GenBank using the BLAST program (Altschul et al. 1997) and from tester strains of the other five northeastern North American *Armillaria* species.

Statistical Analysis

Chi-square goodness-of-fit was used to determine if *Armillaria* species incidence differed significantly by:

i) forest type (pitch pine, white pine, white pine - oak, and hemlock)

ii) host conifer species (pitch pine, white pine, and hemlock)

iii) soil type (loamy sand, sandy loam, and medium loam)

iv) soil drainage class (excessively drained, well-drained, and moderately well-drained)

v) crown class (dominant, codominant, intermediate, and suppressed)

vi) host oak species (white oak, scarlet oak, red oak, and black oak)

Columns consisted of individual *Armillaria* species incidence while rows consisted of the desired category being tested. When necessary, expected values were used; (S X C)/N, where S is the number of observations for an *Armillaria* species, C is the total number of observations within a row of the category under consideration, and N is the total number of observations for all *Armillaria* species (Blodgett and Worrall 1992*a*). Exact *p*-values were generated during Chi-square analysis when low cell counts (< 5 observations) would have comprised the Chi-square distribution (Zar 2007). A general linear model (GLM) was used to determine if there were significant differences between the diameters of the primary conifers sampled in 0.01 ha vegetation plots and those found infected with *Armillaria*. All statistical analysis was done using SPSS 16.0 (SPSS Inc., Chicago, IL).

Results

All Forest Types

Six *Armillaria* species representing 320 isolates were collected from 19 host tree species across four forest types in this study (Table 2.3). However, three species (*A. gallica, A. mellea,* and *A. solidipes*) made up 97% (309/320) of all isolates, while *A. calvescens, A. gemina,* and *A. sinapina* made up the remaining 3%. Overall, *A. solidipes* was the most abundant species, making up 188/320 (59%) isolates (Table 2.3). It was the most abundant species collected from all three conifers in this study, and was also frequently encountered infecting various hardwood hosts, which made up 39/188 (21%) of all isolations (Table 2.4). *Armillaria gallica* was the second most abundant species, occurring at six of eight sites. This species was predominately found on hardwoods, but was sampled 26 times from all three conifers, including 19 isolations from eastern hemlock (Table 2.4). *Armillaria mellea* was isolated 46 times at five of eight sites, of which, 21 came from white pine and six from pitch pine (Table 2.4).

Table 2.3. Incidence of *Armillaria* Species by Forest Type and Individual Host.

Tree Species	Total[Z]	*A. calvescens*	*A. gallica*	*A. gemina*	*A. mellea*	*A. sinapina*	*A. solidipes*	*p*-value[Y]
Pitch Pine								
Betula populifolia Marsh.	2	--	0	--	0	--	2	
Castanea dentata (Marsh.) Borkh.	1	--	0	--	0	--	1	
Pinus rigida Mill.	76	--	3	--	6	--	67	
Quercus coccinea Muenchh.	1	--	0	--	0	--	1	
Pitch Pine Total	80	0	3	0	6	0	71	<0.001
White Pine								
Acer rubrum L.	2	--	2	--	0	--	0	
Betula lenta L.	2	--	0	--	1	--	1	
Betula papyrifera Marsh.	2	--	1	--	1	--	0	
Castanea dentata (Marsh.) Borkh.	1	--	0	--	1	--	0	
Fraxinus americana L.	3	--	3	--	0	--	0	
Pinus strobus L.	46	--	2	--	8	--	36	
Populus grandidentata Michx.	3	--	3	--	0	--	0	
Prunus serotina Ehrh.	1	--	0	--	1	--	0	
Quercus alba L.	8	--	4	--	4	--	0	
Quercus rubra L.	1	--	0	--	0	--	1	
Quercus velutina Lam.	11	--	3	--	8	--	0	
White Pine Total	80	0	18	0	24	0	38	0.001
White Pine/Mixed Oak								
Acer rubrum L.	2	--	1	--	0	--	1	
Betula papyrifera Marsh.	1	--	0	--	1	--	0	
Pinus strobus L.	28	--	0	--	13	--	15	
Prunus serotina Ehrh.	1	--	1	--	0	--	0	
Quercus alba L.	8	--	1	--	0	--	7	
Quercus coccinea Muenchh.	14	--	0	--	2	--	12	
Quercus rubra L.	12	--	12	--	0	--	0	
Quercus velutina Lam.	13	--	7	--	0	--	6	
Sassafras albidum (Nutt.) Nees	1	--	0	--	0	--	1	

White Pine/Mixed Oak Total	80	0	22	0	16	0	42	0.328
Eastern Hemlock								
Acer rubrum L.	6	0	5	0	0	0	1	
Betula alleghaniensis Britton	3	1	2	0	0	0	0	
Betula lenta L.	5	1	0	0	0	0	4	
Betula papyrifera Marsh.	1	0	0	0	0	0	1	
Fagus grandifolia Ehrh.	5	3	2	0	0	0	0	
Fraxinus americana L.	1	1	0	0	0	0	0	
Pinus strobus L.	2	0	2	0	0	0	0	
Quercus alba L.	1	0	1	0	0	0	0	
Quercus bicolor Willd.	1	0	1	0	0	0	0	
Tsuga canadensis (L.) Carr.	55	1	19	3	0	1	31	
Eastern Hemlock Total	80	7	32	3	0	1	37	0.002
Overall Total	320	7	75	3	46	1	188	

[Z] Total number of isolates sampled in each forest type.

[Y] Probability that there are no significant differences among *Armillaria* species frequency by forest type based on Chi-square analysis (using expected values) at $p = 0.05$.

Table 2.4. Incidence of *Armillaria* Species Isolated and Host List From All 32 Plots at Eight Sites.

Tree Species	Total[z]	*A. calvescens*	*A. gallica*	*A. gemina*	*A. mellea*	*A. sinapina*	*A. solidipes*
Acer rubrum L.	10	0	8	0	0	0	2
Betula alleghaniensis Britton	3	1	2	0	0	0	0
Betula lenta L.	7	1	0	0	1	0	5
Betula papyrifera Marsh.	4	0	1	0	2	0	1
Betula populifolia Marsh.	2	0	0	0	0	0	2
Castanea dentata (Marsh.) Borkh.	2	0	0	0	1	0	1
Fagus grandifolia Ehrh.	5	3	2	0	0	0	0
Fraxinus americana L.	4	1	3	0	0	0	0
Pinus rigida Mill.	76	0	3	0	6	0	67
Pinus strobus L.	76	0	4	0	21	0	51
Populus grandidentata Michx.	3	0	3	0	0	0	0
Prunus serotina Ehrh.	2	0	1	0	1	0	0
Quercus alba L.	17	0	6	0	4	0	7
Quercus bicolor Willd.	1	0	1	0	0	0	0
Quercus coccinea Muenchh.	15	0	0	0	2	0	13
Quercus rubra L.	13	0	12	0	0	0	1
Quercus velutina Lam.	24	0	10	0	8	0	6
Sassafras albidum (Nutt.) Nees	1	0	0	0	0	0	1
Tsuga canadensis (L.) Carr.	55	1	19	3	0	1	31
Hardwood Total	113	6	49	0	19	0	39
Conifer Total	207	1	26	3	27	1	149
Overall Total	320	7	75	3	46	1	188

[z] Total isolates collected for each host.

Table 2.5. Incidence of *Armillaria* Species by Site and Region in Massachusetts *Pinus*- and *Tsuga*-Dominated Forests.

Site Name	Region[Z]	Total[Y]	*A. calvescens*	*A. gallica*	*A. gemina*	*A. mellea*	*A. sinapina*	*A. solidipes*	*p*-value[X]
Pitch Pine									
Montague	Central	80	0	3↓[W]	0	0	0	37↑	0.002
Myles Standish	Coastal	80	0	0	0	6	0	34↑	0.003
White Pine									
Quabbin Gate 29	Central	80	0	15	0	13↑	0	12↓	0.001
Townsend	Coastal	80	0	3↓	0	11	0	26	0.011
White Pine – Oak									
Erving-Warwick	Central	80	0	22↑	0	10	0	8↓	<0.001
Freetown-Fall River	Coastal	80	0	0	0	6	0	34	0.441
Hemlock									
Tolland-Sandisfield	Western	80	7	14	3	0	1	15↓	<0.001
Quabbin Gate 15	Central	80	0	18	0	0	0	22	0.057
Total		320	7	75	3	46	1	188	

[Z] Region in Massachusetts where site occurred
[Y] Total isolates collected within each region and total.
[X] Probability that there are no significant differences among *Armillaria* species frequency by site based on Chi-square analysis (using expected values) at $p = 0.05$.
[W] For rows with significant *p* values, arrows denote whether actual values were higher (+5) or lower (-5) than expected values. No arrow indicates actual values were within ±5 occurrences from expected values.

Pitch Pine Forest Type

Of the 80 infected trees sampled in eight plots at the two pitch pine forest type sites, 76 (95%) were pitch pine. *Armillaria solidipes* was the overwhelmingly dominant species encountered, making up 71/80 (89%) isolates collected, while *A. mellea* (6/80; 8%) and *A. gallica* (3/80; 4%) were the only other species isolated (Table 2.3). *Armillaria* species incidence from pitch pine was significantly different ($p < 0.001$), with a higher incidence of *A. solidipes* compared to expected values (Table 2.3). There were no major differences in forest composition, elevation, soil type, and soil drainage classes between the two sites (Table 2.1). The only difference in *Armillaria* species incidence between the two sites was the occurrence of *A. mellea* and *A. sinapina* in the coastal lowlands of southeastern Massachusetts (Table 2.5).

All 76 isolations of *Armillaria* from pitch pine came from dead trees (Table 2.6). Overall, trees in decay class 1 (47/76; 62%) were most abundant, and 89% (68/76) of all sampled trees were determined to be recently killed by *Armillaria* (Table 2.6). There were significant differences in the isolation of *Armillaria* from pitch pine by crown class ($p = 0.001$), with 40/76 isolations coming from trees in the suppressed crown class (Table 2.7). In addition, non-symptomatic pitch pines in 0.01 ha vegetation plots had a significantly larger DBH than those infected with *Armillaria* (19.4 vs. 13.1 cm; $p < 0.001$) (Table 2.8). *Armillaria* species typically associated with hardwoods (*A. gallica* and *A. mellea*) were found on pitch pine, although the frequencies were low (Table 2.4).

White Pine & White Pine/Mixed Oak Forest Types

White pine constituted 76/160 (48%) of the trees sampled in 16 plots at four sites. The relatively low number of times this host was sampled, considering the site type, is reflective of the lack of symptoms on living trees in comparison to other trees in the stands, most notably *Quercus* species. *Armillaria solidipes* was again the dominant species in both forest types,

making up 42/80 (53%) isolates from the white pine/mixed oak forest type, and 38/80 (48%) isolates from pure white pine forests (Table 2.3). *Armillaria* species incidence from white pine/mixed oak was not significantly different ($p = 0.328$), yet incidence was significantly different from pure white pine ($p = 0.001$) (Table 2.3). Overall, incidence of individual *Armillaria* species from white pine was significantly different, with a higher incidence of *A. mellea* compared to expected values ($p < 0.001$; Table 2.4).

Interesting differences in *Armillaria* species incidence occurred by site between the pure white pine and white pine/mixed oak forests. *Armillaria gallica* was the most commonly isolated species in the central region, making up 37/80 total isolates from two sites (Erving-Warwick and Quabbin Gate 29) (Table 2.5; Figure 2.1). At the white pine/mixed oak site in this region, 20/22 *A. gallica* isolations came from oak, with *Q. rubra* as the dominant oak species (Table 2.1). *Armillaria mellea* was also present in the central region, occurring 10/40 times (25%) in white pine/mixed oak, and 13/40 times (33%) from pure white pine (Table 2.5). From both sites, 17/23 *A. mellea* isolates (74%) came from white pine. *Armillaria solidipes* was the least commonly occurring species, making up 20/80 isolates from both central region sites (Table 2.5).

In contrast, the coastal sites (Freetown-Fall River and Townsend) occupying sandy, glacial outwash in eastern Massachusetts were dominated by *A. solidipes*, which made up 59/80 (74%) total isolations (Table 2.5; Figure 2.1). At the white pine/mixed oak site in southeastern Massachusetts, 21/34 (62%) of all *A. solidipes* isolates came from *Quercus* spp. (Table 2.3), with *Q. coccinea* the dominant oak species (Table 2.1). At both coastal sites, *A. gallica* was rare, making up only 3/80 isolates (Table 2.5), but *A. mellea* was again observed, making up 11/40 isolates from the pure white pine, and 6/40 isolates from white pine/mixed oak. In contrast to the central region, *A. mellea* did not show as strong a host preference for white pine in the eastern region, occurring 5/17 times (29%) on this host.

Nearly every isolate of *Armillaria* from white pine came from dead trees (70/76; 92%) (Table 2.6). Overall, trees in decay class 1 (56/76; 74%) were most abundant, with 88% (67/76)

being deemed recently killed by the fungus (Table 2.6). Once again, there were significant differences in the isolation of *Armillaria* from white pine by crown class ($p < 0.001$), with 65/76 (86%) isolations coming from trees in the suppressed crown class (Table 2.7). Non-symptomatic white pine sampled in 0.01 ha vegetation plots also had a significantly larger DBH than those infected with *Armillaria* (26.6 vs. 10.1 cm; $p < 0.001$) (Table 2.8).

Eastern Hemlock Forest Type

Eastern hemlock constituted 55/80 (69%) of the trees sampled in eight plots at two sites (Table 2.3). *Armillaria solidipes* was the most commonly isolated species, making up 31/55 isolations on eastern hemlock (56%) and 37/80 overall isolations (46%) (Table 2.3). Overall, *Armillaria* species incidence from hemlock was significantly different ($p = 0.002$) (Table 2.3). There was a clear difference in species distribution between the two sites sampled. The higher elevation hemlock site surrounded by northern hardwoods contained five species (*A. calvescens*, *A. gallica*, *A. gemina*, *A. sinapina*, and *A. solidipes*), while the lower elevation site surrounded by white pine/mixed oak contained only two (*A. gallica* and *A. solidipes*) (Table 2.5). However, the proportion of the two most abundant species (*A. gallica* and *A. solidipes*) was the same between the two sites (Table 2.5).

Of the 55 sampled hemlocks, 11 isolations came from living trees showing symptoms of root disease, while 44 came from dead trees (Table 2.6). Dead trees were sampled from all four decay classes, with decay class 2 (22/44; 50%) being the most abundant, and only 36% (20/55) being recently killed by *Armillaria* (Table 2.6). There were no significant differences in the isolation of *Armillaria* from hemlock by crown class ($p = 0.327$; Table 2.7). Also, there were no significant differences in DBH between non-symptomatic hemlocks in 0.01 ha vegetation plots and those infected with *Armillaria* ($p = 0.277$; Table 2.8). *Armillaria* species typically associated with hardwoods were also found on eastern hemlock (Table 2.4). Most notably, *A. gallica* made up 19/55 (35%) total isolations for hemlock. Also of note was the occurrence of *A. gemina* on

eastern hemlock, which has been reported only rarely. One of the three isolations of *A. gemina* on hemlock came from mycelial fans at the base of a recently dead, suppressed hemlock. *Armillaria mellea* was the only species that was not isolated from eastern hemlock.

Table 2.6. Frequency of Dead Coniferous Hosts Sampled by Decay Class.

				Decay Class				
Tree species	Total[Z]	Living	Dead	0	1	2	3	Recently Killed[Y]
Pinus rigida	76	0	76	21	47	7	1	68 (89%)
Pinus strobus	76	6	70	11	56	3	0	67 (88%)
Tsuga canadensis	55	11	44	2	18	22	2	20 (36%)
Total	207	17	190	34	121	32	3	155 (75%)

[Z] Total number of trees sampled in eight plots at two sites for *T. canadensis* and *P. rigida*, and 16 plots at four sites for *P. strobus*.
[Y] Values for recently killed are a summation of decay classes 0 and 1. The percentage of recently killed trees compared to the total number sampled is in parenthesis.

Table 2.7. Frequency of Coniferous Hosts Infected With *Armillaria* by Crown Class.

Tree species	Total[Z]	Dom[Y]	Cod	Int	Sup	*p*-value[X]
Pinus rigida	76	0	13	23	40	0.001
Pinus strobus	76	0	0	11	65	<0.001
Tsuga canadensis	55	9	16	12	18	0.327
Total	207	9	29	46	123	<0.001

[Z] Total number of trees sampled in eight plots at two sites for *T. canadensis* and *P. rigida*, and 16 plots at four sites for *P. strobus*.
[Y] Crown classes are listed as: Dom = Dominant; Cod = codominant; Int = intermediate; and Sup = suppressed.
[X] Probability that there are no significant differences among frequencies of isolation by crown class based on Chi-square analysis (all values equal) at $p = 0.05$.

Table 2.8. Mean Diameter at Breast Height (dbh) in cm Between Trees in 0.01 ha Vegetation Plots and Trees Sampled With *Armillaria*.

	Vegetation Plots		Infected by *Armillaria*		
Tree Species	Total[Z]	DBH	Total	DBH	*p*-value[Y]
Pinus rigida	73	19.4 (10)[X]	76	13.1 (7)	<0.001
Pinus strobus	91	26.6 (17)	76	10.1 (5)	<0.001
Tsuga canadensis	74	27.5 (14)	55	30.4 (16)	0.277

[Z] Total number of trees sampled in eight plots at two sites for *T. canadensis* and *P. rigida*, and 16 plots at four sites for *P. strobus*.
[X] Values are significantly different at $p = 0.05$.
[Y] Standard deviations in parenthesis.

Site Variables

Because three species (*A. calvescens*, *A. gemina*, and *A. sinapina*) were encountered in such low numbers, differences in site variables were compared to the three most common species, *A. gallica*, *A. mellea*, and *A. solidipes* (Table 2.9). The frequency of these species was significantly different by soil type and soil drainage classes, and the dominant conifer at each site. The biggest disparity among soil types was the highly significant difference in incidence on loamy sand (p <0.001), which was dominated almost exclusively by *A. solidipes* (Table 2.9). Meanwhile, both *A. mellea* (sandy loam) and *A. gallica* (medium loam) occurred in significantly higher frequencies compared to expected values (Table 2.9).

In addition to dominating sites with soils composed of loamy sand, *A. solidipes* was also dominant on soils that were excessively drained (p <0.001) compared to *A. gallica* and *A. mellea* (Table 2.9). Soils that were well-drained had a higher incidence of *A. gallica* and a lower incidence of *A. solidipes* than expected. *Armillaria mellea*, meanwhile, showed no significant differences in incidence by soil drainage, being evenly distributed across the three classes (Table 2.9). On moderately well-drained soils with better water-holding capacities, there were no significant differences in incidence by species ($p = 0.164$) (Table 2.9).

Sites where pitch pine was the dominant conifer had a higher incidence of *A. solidipes* and lower incidence of *A. gallica* and *A. mellea* compared to expected values (Table 2.9). Sites dominated by white pine had a higher incidence of *A. mellea* and a lower incidence of *A. solidipes* compared to expected values (Table 2.9). For sites dominated by hemlock, *A. gallica* was more abundant and *A. solidipes* less abundant than was expected (Table 2.9).

Table 2.9. Frequency of *Armillaria gallica*, *A. mellea*, and *A. solidipes* by Site Characteristics (Soil Type, Soil Drainage Class, and Dominant Conifer).

		Armillaria species			
Site Variable	Total[Z]	*A. gallica*	*A. mellea*	*A. solidipes*	*p*-value[Y]
Soil Type					
loamy sand	80	3↓[X]	6↓	71↑	<0.001
sandy loam	160	40	40↑	80↓	0.001
medium loam	69	32↑	0	37	0.001
Total	309	75	46	188	
Soil Drainage Class					
excessively drained	130	10↓	17	103↑	<0.001
well-drained	76	32↑	13	31↓	0.001
moderately well-drained	103	33	16	54	0.164
Total	309	75	46	188	
Dominant Conifer					
pitch pine	76	3↓	6	67↑	0.030
white pine	76	4↓	21↑	51	<0.001
hemlock	50	19↑	0	31↓	<0.001
Total	202	26	27	149	

[Z] Total number of *Armillaria* species sampled for each site variable.
[Y] Probability that there are no significant differences among *Armillaria* species frequency by site variables based on Chi-square analysis (using expected values) at $p = 0.05$.
[X] For rows with significant p values, arrows denote whether actual values were higher (+5) or lower (-5) than expected values. No arrow indicates actual values were within ±5 occurrences from expected values.

Table 2.10. Frequency of *Armillaria gallica, A. mellea,* and *A. solidipes* by *Quercus* Species.

Quercus species[Z]	Total[Y]	*A. gallica*	*A. mellea*	*A. solidipes*	*p*-value[X]
Quercus alba	17	6	4	7	0.753
Quercus coccinea	15	0	2	13	0.005
Quercus rubra	13	12	0	1	0.002
Quercus velutina	24	10	8	6	0.607
Total	69	28	14	27	0.070

[Z] *Quercus bicolor* was excluded due to being sampled only once.
[Y] Total isolates of *Armillaria* collected from each *Quercus* species.
[X] Probability that there are no significant differences among *Armillaria* species frequency by each *Quercus* species based on Chi-square analysis (all values equal) at $p = 0.05$.

Discussion

All Forest Types

Armillaria was located in every *Pinus*- and *Tsuga*-dominated forest sampled across Massachusetts. Overall, the majority of infected conifers (155/207; 75%) were described as recently killed by *Armillaria*, with over half of those trees (123/207) in the suppressed crown class. Infected *Pinus* species had significantly smaller diameters compared to a random sample of *Pinus* in vegetation plots. Based on these results, it appears that *Armillaria* is preferentially attacking young, light-stressed *Pinus* species while canopy dominant/co-dominant trees are relatively resistant to infection. However, it cannot be disproved that a non-lethal infection of *Armillaria* occurred in the sapling stage of development, leading to reduced growth and suppression by neighboring trees, which has been shown to occur in British Columbia (Cruickshank et al. 2010). Infected hemlocks showed no pattern of *Armillaria* incidence by crown class or diameter, and a lower percentage of hemlocks were recently killed (36%) by the fungus compared to white and pitch pine (88% and 89%, respectively).

To my knowledge, there are no published studies documenting the co-occurrence of *A. solidipes* and *A. mellea* in natural forests of North America, as was the case at five of eights sites sampled in this study. Both are widely regarded as the two most virulent species of

Armillaria in the northern hemisphere (Gregory et al. 1991). In general, *A. mellea* occupies the southern and central hardwood region, while *A. solidipes* tends to occupy the northern conifer region of North America (Burdsall and Volk 1993). In Massachusetts, not only are these species co-occurring but at times they are exhibiting atypical behavior; *A. mellea* attacking coniferous hosts in *Pinus*-dominated forests and *A. solidipes* attacking hardwood hosts, *Quercus* species in particular, in pure conifer and mixed conifer-hardwood forests.

Pitch Pine Forest Type

The sampled pitch pine forests in Massachusetts were dominated by *A. solidipes*. These dry, fire-adapted forests occupying sites with excessively drained, sandy soils are drought-prone despite average annual precipitation rates in Massachusetts near 125 cm (NCDC 2010). Pitch pine forests in the northeast share many similarities to dry pine forests of the interior west and upper Midwest where *A. solidipes* also dominates (Rizzo et al. 1995, Mallet and Maynard 1998, Ferguson et al. 2003). Similar conditions in Western Europe also favor *A. solidipes*, like the dry, coastal pine forests in southwestern France and northwestern Spain (Zas et al. 2007, Prospero et al. 2008). In Massachusetts, the dominance of *A. solidipes* in pitch pine forests was made even more evident by the four sites grouped within a small geographic area in central Massachusetts (Figure 1). The 611 ha Connecticut River valley pitch pine forest, which occupies a former glacial lake outwash delta, represents an island population of this forest type (Motzkin et al. 1999). Despite *A. gallica* and *A. mellea* occurring at nearby sites, they were unable to establish themselves as successfully as *A. solidipes* on the excessively drained, sandy soils that support pitch pine. *Armillaria mellea* was not present at the Connecticut River valley site, but its minor presence in the coastal pitch pine forest is not altogether surprising. In an earlier study, Wargo et al. (1993) isolated *A. mellea* (*sensu stricto*) from dying *Vaccinium* and from an adjacent pitch pine/scrub oak forest in southeastern Massachusetts.

When the results of this study are considered along with studies from western North America and Western Europe, further evidence is provided that *A. solidipes* has a distinct competitive advantage over all other northern hemispheric *Armillaria* species on sites with excessively drained, sandy soils that support *Pinus* spp., regardless of stand size. What remains to be determined is how far south *A. solidipes* can be found in Atlantic coast forests. Based on this study, *A. solidipes* has shown a strong preference for pitch pine forests, but was also quite abundant in coastal white pine and white pine/mixed oak forests. The New Jersey Pine Barrens comprise the largest expanse of pitch pine in North America at roughly 550,000 ha, and extends along the Atlantic coast to 39° latitude (McCormick and Forman 1998). *Armillaria solidipes* has been located as far south as latitude 38° in southern Italy (Guillaumin et al. 1993), and latitude 32° in southeastern Arizona (Gilbertson and Bigelow 1998), but only at high elevations. Additionally, Blodgett and Worrall (1992*a*) were not able to locate *A. solidipes* in southern New York or on Long Island. Yet overall, little data exists about *Armillaria* from the middle-Atlantic region, aside from a small study of *Armillaria* species in Virginia and Maryland, which found only *A. gallica* and *A. mellea* (Motta and Korhonen 1986).

White Pine and White Pine/Mixed Oak Forest Types

Both pure white pine and white pine/mixed oak forests were dominated by *A. solidipes*, but this species was not nearly as abundant as it was in pitch pine forests. There were significant differences in the incidence of *A. solidipes* based on site, with coastal white pine-dominated forests having a much higher frequency than sites in the upland hills of central Massachusetts. When white pine is considered alone, *A. solidipes* was the most abundant species, as expected, making up 67% (51/76) of all isolations. The significantly larger number of infected trees occupying the intermediate and suppressed crown classes coupled with the significantly smaller diameter of infected trees (compared to non-symptomatic trees in the vegetation plots), illustrates that *Armillaria* is attacking younger, light-stressed white pines. In British Columbia, Douglas-fir

has been reported to be most susceptible to *A. solidipes* during the first 20 years of growth in both managed and unmanaged forests (Morrison and Mallett 1996). In New England, height growth of white pine can be slow during the first ten years of development and white pine does not compete well with hardwoods until age 25 on better sites (Lancaster and Leak 1978). While eastern white pine is classified as mid-tolerant of shade, young white pines growing in low light conditions can be susceptible to infection by *Armillaria*. However, a non-lethal infection that occurred early in the development of white pine could also explain the results found in this study.

The occurrence of *A. mellea* on *Pinus* was unexpected because reports of this species on coniferous hosts are rare in eastern North America. Harrington and Rizzo (1993) reported finding a single isolate of *A. mellea* from white pine in New Hampshire. The authors speculated that *A. mellea* is restricted to mild, coastal regions in New England. While the presence of *A. mellea* at my sites in central Massachusetts disproves this theory, these were lower elevation sites east of the Appalachian Mountains. In California, Baumgartner and Rizzo (2001) isolated *A. mellea* 40/61 (66%) times from Douglas-fir in natural forests. In addition, the occurrence of *A. mellea* on conifers is well documented across Eastern Europe. Keča et al. (2009) collected *A. mellea* 18 times from three *Pinus* species (including *P. strobus*) in Serbian plantations. Also, *A. mellea* has been isolated from *Abies*, *Picea*, and *Pinus* from natural forests in Albania, Greece, and Serbia (Tsopelas 1999, Keča et al. 2009, Lushaj et al. 2010). Furthermore, in a controlled study of root bark penetration of Sitka spruce, *A. mellea* colonized superficial wounds faster than *A. solidipes*, and caused greater overall necrosis in deep wounds compared to *A. solidipes* and *H. annosum* (Solla et al. 2002).

Bruhn et al. (2000) found *A. mellea* in greater abundance on warmer and drier sites with more susceptible *Quercus* species (*Q. coccinea* and *Q. velutina*) in the Missouri Ozarks. In this study, dry sites where *Q. coccinea* occurred supported higher populations of *A. solidipes*. When *Armillaria* species incidence is evaluated on the four most abundant *Quercus* species, *A. solidipes* shows a strong host preference for *Q. coccinea*, in comparison to *A. gallica* and *A. mellea* (Table

10). An additional six isolates of *A. solidipes* were isolated from *Q. coccinea* only in a mixed oak forest at the tri-state border between Massachusetts, Connecticut, and Rhode Island. Meanwhile, richer sites that support *Q. rubra* were more heavily dominated by *A. gallica* (Table 2.10).

Eastern Hemlock Forest Type

Both hemlock sites were dominated by *A. gallica* and *A. solidipes*, which occurred in roughly equal proportions on the two sites. However, the higher elevation hemlock site that developed from a northern hardwood forest also contained *A. calvescens*, *A. gemina*, and *A. sinapina* while the lower elevation site that developed from white pine/mixed oak did not. This suggests that because eastern hemlock is a climax forest type (Eyre 1980), the occurrence of particular *Armillaria* species may be less dependent upon the presence of hemlock, and more dependent upon site variables (soil type, elevation, and parent material) and potentially the forest type that dominated during the earlier stages of stand development. This would explain why *A. calvescens* and *A. gemina* were absent at the lower elevation site, as these species appear to be restricted to forests composed primarily of northern hardwoods and hemlock (Blodgett and Worrall 1992*b*, Brazee and Wick 2009). Aside from elevation and surrounding forest type, there were no major differences in parent material, soil type, soil drainage, and forest composition between the two sites that could explain differences in incidence of those three species (Table 2.1). *Armillaria calvescens*, *A. gallica*, and *A. sinapina* have been previously isolated from eastern hemlock in Ontario (McLaughlin 2001) and New York (Blodgett and Worrall 1992*a*). In south-central Pennsylvania, *Armillaria* was collected from both an old-growth hemlock forest and adjacent hardwood forest (Fromm and Davis 2007). While the isolates were not identified to individual species, 60/71 fell into the *A. calvescens* / *A. gallica* / *A. sinapina* group according to IGS-1 sequences.

The occurrence of *A. gemina* on eastern hemlock in this study is particularly interesting. It was located only three times, with only one of those occurrences coming from mycelial fans at

the base of a recently dead hemlock. This species has been collected almost exclusively from hardwoods, but these results show that like all other northeastern *Armillaria* species, *A. gemina* can infect both hardwoods and conifers. Blodgett and Worrall (1992*b*) found that *A. gemina* was more prevalent in beech-dominated forests in New York. Because beech is very shade tolerant, it is frequently present in pure hemlock stands in New England (Eyre 1980).

None of the *Armillaria* species encountered on hemlock were observed as an aggressive pathogen, even *A. solidipes*. Yet, from an ecological perspective, the presence of *A. gallica* on hemlock provides further evidence of this species' broad host range. Mortality for which *Armillaria* was a contributing factor mostly occurred to scattered, individual trees. In addition, there were no significant differences in the incidence of *Armillaria* by crown class or host diameter. This conclusion is in agreement with previous studies, which found that *Armillaria* is not an aggressive pathogen of hemlock (Spaulding 1914, Secrest et al. 1941, Wargo and Fagan 2000, Fromm and Davis 2007). *Armillaria* still plays an important role in hemlock forests by contributing to the mortality of suppressed and weakened trees. While healthy stands of hemlock appear to be relatively resistant to attack from *Armillaria* species, the interacting effects of hemlock woolly adelgid, hemlock scale, and drought may allow the fungus to behave more aggressively in the future (Wargo 1996, Desprez-Loustau et al. 2006).

To conclude, this study provides evidence of site, forest type, and host preference of certain *Armillaria* species in northeastern *Pinus* and *Tsuga* forests. It appears that *Armillaria* is attacking young, small diameter conifers that are occupying the intermediate and suppressed crown classes, and that older, large diameter conifers are more resistant to infection. In the absence of low to mid-severity fires in pitch pine and white pine forests, *Armillaria* is aiding in the stand development process by killing weak trees in the understory. The occurrence of *A. solidipes* on oaks, *A. mellea* on white and pitch pine, and *A. gallica* on hemlock illustrates the inaccuracy of broadly characterizing certain *Armillaria* species by preference on hardwoods or conifers alone. While researchers will continue to focus on *A. mellea* as an aggressive pathogen

of hardwoods, its role as an important pathogen white pine, especially in declining stands, should be more closely studied. In addition, this study shows for the first time that *A. solidipes* should be considered an important pathogen of oak in coastal forests of New England, especially those suffering from insect defoliation.

CHAPTER 3

GENOTYPIC DIVERSITY OF *ARMILLARIA GALLICA* FROM MIXED OAK FORESTS USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

Introduction

The soilborne fungal genus *Armillaria* (Fr.:Fr.) Staude, in the phylum Basidiomycota, includes species that are among the largest and oldest organisms on Earth (Smith et al. 1992). Additionally, *Armillaria* species are some of the most ecologically significant plant pathogens in temperate forests worldwide (Kile et al. 1991). *Armillaria gallica* Marxmuller & Romagnesi is widely distributed across a range of different forest types throughout North America, Europe, and Asia (Burdsall and Volk 1993, Guillaumin et al. 1993, Ota et al. 1998), but in northeastern North America this species is most abundant in *Quercus*-dominated forests (Blodgett and Worrall 1992b, Brazee and Wick 2009). While sometimes broadly described as only a weak pathogen, *A. gallica* can cause extensive root and butt rot to hardwoods and is one of the primary disturbance agents in temperate, mixed oak forests (Luisi et al. 1996, Marçais and Bréda 2006, Brazee and Wick 2009). Perhaps more than any other *Armillaria* species, descriptions of pathogenicity for *A. gallica* vary substantially, suggesting a great deal of plasticity in this species' ability to cause disease. However, in order to fully understand the source of variability in pathogenicity, genetic diversity must be better understood at the landscape level.

The population structure of *A. gallica* has been studied previously at varying levels of detail. This species is probably best known for developing large, asexual clones that establish by means of a vast, melanized rhizomorph network. In northern Michigan, Smith et al. (1992) identified a single genet occupying at least 15 hectares (ha), with an estimated age of over 1,500 years. Additional studies have also documented a low level of genotypic diversity of *A. gallica* at small geographic scales within intensively sampled populations in North America and Europe (Legrand et al. 1996, Hodnett and Anderson 2000). When the population structure was examined

at a very broad geographic scale, Saville et al. (1996) found a high level of genotypic diversity between isolates of *A. gallica* from Michigan, Ontario, Rhode Island, and North Carolina. While there have been several studies that have intensively sampled arbitrarily defined populations of *Armillaria* (i.e. 1 to 3 ha) (Legrand et al. 1996, Hodnett and Anderson 2000, Prospero et al. 2003, Bendel et al. 2006), there are few studies describing the population structure at larger scales (i.e. 25,000 to 100,000 ha). It is also surprising that few researchers have used amplified fragment length polymorphisms (AFLPs) to better understand genotypic variation in populations of *Armillaria*. AFLPs can be a powerful tool to examine population subdivision in diploid species with inherently low levels of genetic variability (Meudt and Clarke 2007). Despite the interpretive problems associated with dominant marker systems, AFLPs create a large, reproducible multilocus dataset that can be informative for an intraspecific study such as this one.

One of the main drivers of population subdivision for soilborne fungi is precipitation rate, since soil moisture strongly influences whether or not fungi like *Armillaria* produce fruiting bodies. Low levels of genotypic variation in populations of *A. solidipes* have been found in dry conifer forests of the interior west in North America (Shaw and Roth 1976, Anderson et al. 1979, Ferguson et al. 2003), and in cold, high elevation conifer forests in Switzerland (Bendel et al 2006). Low precipitation rates and the subsequent lack of fruiting bodies may inhibit the formation of new genets, allowing existing genets to expand free from competition (Worrall 1994, Ferguson et al. 2003, Bendel et al. 2006). Precipitation rates in northeastern North America are relatively high, and more importantly do not significantly vary over the course of the year. The annual precipitation rate in Massachusetts over the past 19 years averaged 125 cm (1992 through 2010; weighted state-wide), with 26% of that total (32 cm) falling from August through October, the period when annual fungi are most likely to fruit in southern New England (NCDC 2011).

In Vermont, New Hampshire, and New York, fruiting body production and subsequent spore dissemination have helped create a mosaic of small genets in northern hardwood forests (Ullrich and Anderson 1978, Rizzo and Harrington 1993, Worrall 1994). However, northern

hardwood forests have higher rates of *Armillaria* species diversity than other forest types (Blodgett and Worrall 1992b, Brazee and Wick 2009), and competition among species can also restrict large genet development (Legrand et al. 1996). In contrast to northern hardwoods, mixed oak forests primarily occur at lower elevations, have drier soils, warmer average temperatures, and have reduced *Armillaria* species diversity (Westveld et al. 1956, Twery et al. 1990, Brazee and Wick 2009). Because of the reduced *Armillaria* species diversity in mixed oak forests, and the prevalence of *A. gallic*a, we could expect a smaller number of larger *A. gallica* genets on the landscape. Conversely, relatively high precipitation rates may inhibit large genet formation, because new genets are continuously established by spore dispersal.

It was hypothesized that *A. gallica* is not composed of a few large genets dominating the landscape in central Massachusetts, but rather numerous, smaller genets driven, in part, by basidiospore dispersal. This hypothesis was tested by analyzing 153 isolates previously collected from four sites where the fungus had been isolated primarily from living trees that were showing symptoms of dieback and decline. Our goal was to determine the genotypic diversity among an arbitrarily defined population of *A. gallica* from four mixed oak forests in central Massachusetts.

Materials and Methods

Study Sites, Isolation, and Identification of Isolates

Study sites and procedures used to sample *Armillaria* have been described in detail previously (Brazee and Wick 2009). Briefly, four sites dominated by different *Quercus* species were sampled from July to September, 2008. At each site, four plots were randomly established within the largest contiguous block of the mixed oak forest type on state-owned lands. From each plot center, a variable radius plots was established to locate ten isolates of *Armillaria* primarily from living trees exhibiting symptoms and signs of infection. Therefore, a total of 16 plots were used to sample *Armillaria* from four arbitrarily defined sites. The four sites encompass a total area of approximately 510 km^2 in south-central Massachusetts, a region dominated by several

Quercus species (Figure 3.1). Mean plot size was 0.28 ha with a range of 0.04 to 0.87 ha (Table 3.1). The total area sampled, summed from all 16 plots, was 4.51 ha (Table 3.1). The sampling scheme used did not allow for an intensive investigation of the population structure of *A. gallica* at any individual site. However, it does provide the basis for an estimation of the number of genets per hectare at multiple sites, which is an important figure in understanding the overall makeup of the population across the landscape.

An isolate is described as an individual sample of *Armillaria* collected from a single tree. All 153 isolates were collected from rhizomorphs, and are therefore diploid. Isolates were previously identified to species using a PCR-RFLP protocol designed by McLaughlin and Hsiang (2010) as described in Brazee and Wick (2009).

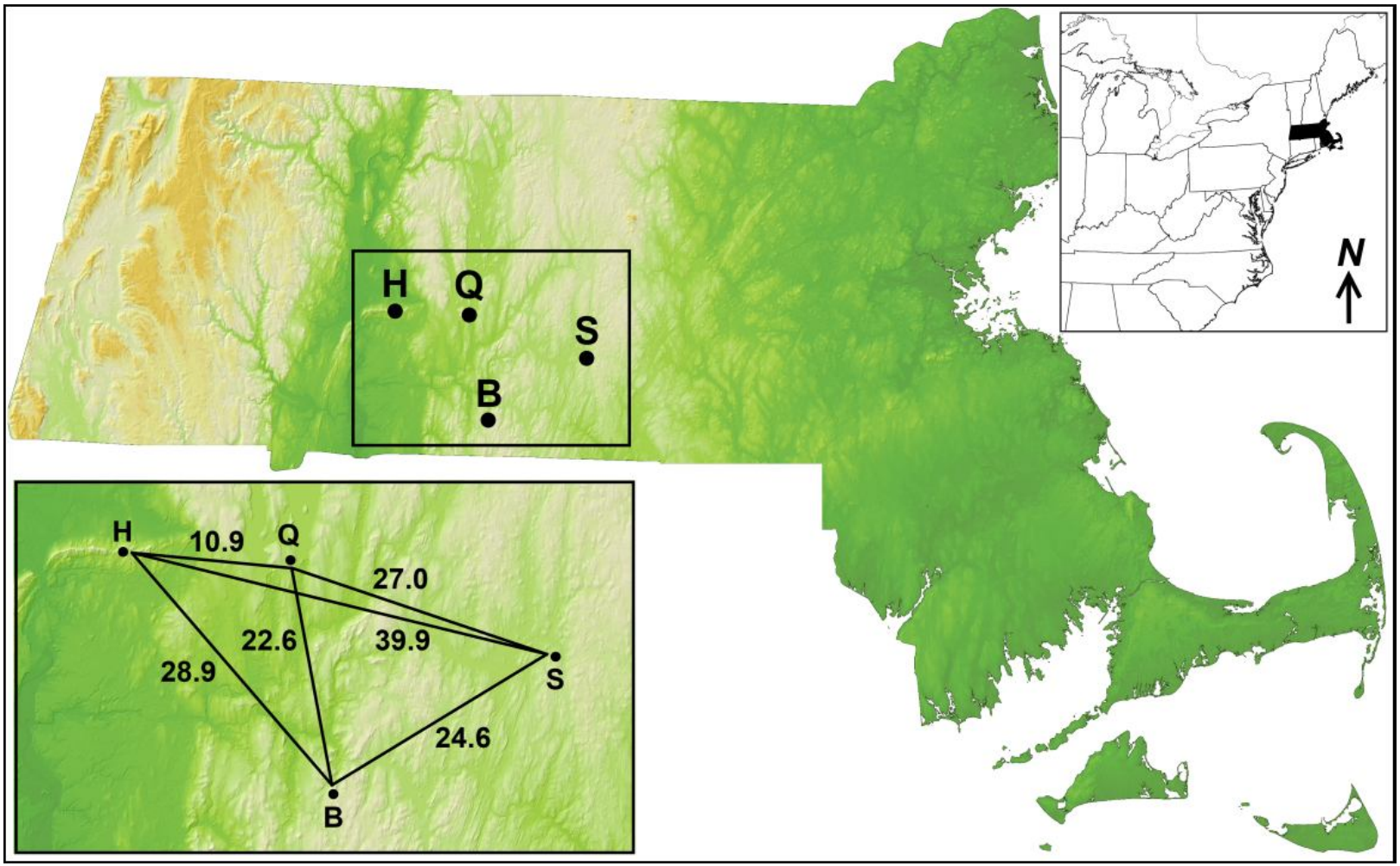

Figure 3.1. Location of Study Sites Used to Sample *A. gallica* in Central Massachusetts Oak Forests. Letters Denote Individual Sites, and are Described as: B = Brimfield State Forest; H = Holyoke Range State Park; Q = Quabbin Park; and S = Spencer State Forest. Within the Enlarged Figure (Bottom Left), Distances between Each Study Site are Shown in Kilometers.

Table 3.1. Characteristics of Study Sites Used to Sample *A. gallica* in Central Massachusetts Mixed Oak Forests.

Site	No. of Plots	Total Plot Area (ha)[Z]	Mean Plot Size (ha)	Range of Plot Sizes (ha)	Total Site Area (ha)[Y]	Maximum Distance (km)[X]	Dominant Oak Species
Brimfield	4	1.72	0.43	0.25 to 0.87	23	1.0	*Quercus alba*
Holyoke	4	0.40	0.10	0.06 to 0.17	29	3.3	*Quercus prinus*
Quabbin	4	1.89	0.47	0.22 to 0.79	110	2.4	*Quercus velutina*
Spencer	4	0.50	0.13	0.04 to 0.20	101	1.5	*Quercus rubra*
Total	16	4.51	0.28				

[Z] Total plot area is a summation of all four plot areas per site.
[Y] Total site area was determined by constructing a polygon linking all four plots at each site.
[X] Maximum distance between plots at each site.

DNA Extraction and AFLP Analysis

Isolates were grown in malt-yeast extract broth (2% malt extract and 0.5% yeast extract) amended with 300 ppm of 95% ethanol. Mycelium was lyophilized and then ground with glass beads. High quality genomic DNA was then obtained using an extraction protocol modified from Marra and Corwin (2009).

AFLP analysis was carried out using the procedure described by Vos et al. (1995) with slight modifications. Restriction digests were carried out at 37º C for 3 hours in 25 µl reactions consisting of 19 µl of extracted DNA (totaling roughly 250 ng), 1X *Eco*RI buffer, 1X bovine serum albumin, and 5 U of *Eco*RI and *Mse*I (New England Biolabs, Beverley, MA). Random samples were visualized on a 1.5% agarose gel to verify that DNA was completely digested. Ligation of oligonucleotide adaptors was carried out at 37º C for 3 hours in 20 µl reactions consisting of 4.5 µl of molecular grade H_2O, 1X T4 DNA ligase buffer, 1.5 U of T4 DNA ligase (Promega, Madison, WI), 5 µM of *Eco*RI adaptor, 25 µM of *Mse*I adaptor, and 10 µl of the *Eco*RI/*Mse*I digestion product.

Both pre-selective and selective PCR used primers that were complementary to each of the two adaptors (*Eco*RI and *Mse*I). No nucleotide extensions were used on the pre-selective *Eco*RI and *Mse*I primers. The *Eco*RI selective primers, Eaa-FAM and Eag-FAM, included a two-nucleotide extension (AA or AG, respectively) on the 3' end of the primer while the 5' end was labeled with 6-carboxyfluorescein (6-FAM). The *Mse*I selective primers, Mctg and Mctt, included a three-nucleotide extension (CTG or CTT, respectively) on the 3' end. Adaptors and primers were supplied by Integrated DNA Technologies (Coralville, IA).

Pre-selective PCR was carried out in 15 µl reactions consisting of 8.6 µl of molecular grade H_2O, 1X Platinum Taq buffer, 2.5mM $MgCl_2$, 0.25 U of Platinum Taq (Invitrogen, Carlsbad, CA), 250 µM of each dNTP (Fermentas, Glen Burnie, MD), 10 µM pre-*Eco*RI, 10 µM pre-*Mse*I, and 1 µl of the undiluted ligation product. Pre-selective PCR conditions were 2 min at

72°C, 5 min at 94° C; 20 cycles at 94° C for 30 s, 56° C for 30 s, and 72° C for 1 min; and 72° C for 5 min. Four selective primer combinations were used: (i) Eaa-Mctg; (ii) Eaa-Mctt; (iii) Eag-Mctg; and (iv) Eag-Mctt. The selective PCR conditions were 5 min at 95° C; 9 cycles at 94° C for 30 s, 65° C for 30 s, with a decrease of 1° C every cycle, and 72° C for 1 min; and 40 cycles of 94° C for 30 s, 56° C for 30 s, and 72° C for 1 min.

The selective PCR products were electrophoresed using an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA) at the Genomics Resource Laboratory (University of Massachusetts, Amherst) under the default run module for GeneScan version 4.0 according to the manufacturer's instructions. Data were collected and fragment sizes were determined by using GeneScan version 3.X analysis software and a 500 LIZ internal size standard (Applied Biosystems, Foster City, CA). Data were analyzed using GeneMarker v. 1.8 (Softgenetics, State College, PA) and scored manually. Polymorphic loci were defined as bright bands of the same size that were present in some but not all isolates. The reproducibility of the AFLP analysis was determined by scoring fragments from triplicate runs (same extraction) for 12 isolates from three sites, and quadruplet runs (same extraction) for eight isolates from one site. In addition, duplicate runs (different extraction) were performed for 20% of all isolates.

Statistical Analysis

Based on the fragment scoring in GeneMarker, a binary data matrix of presence (1) or absence (0) of peaks for each isolate was constructed. Within GenAlEx v. 6.4 (Peakall and Smouse 2006), Nei's unbiased genetic distance and identity, along with the average, unbiased expected heterozygosity (H_E) for the population were generated assuming HWE for a known diploid population. We used AMOVA (Excoffier et al. 1992) to measure genotypic diversity (Φ_{PT}), where Φ_{PT} is a measure of population subdivision (analogous to F_{ST}), calculated as the proportion of the variability among populations relative to the total variability. Specifically, $\Phi_{PT} = V_{AP} / (V_{AP} + V_{WP})$, where V_{AP} is the genotypic variability among populations and V_{WP} is the

variability within populations. The test was calculated using 999 permutations at a significance level of $p < 0.05$.

Population genetic structure and genetic diversity were also analyzed with AFLP-SURV v. 1.0 (Lynch and Milligan 1994, Vekemans et al. 2002). AFLP-SURV estimates allele frequencies at each marker locus within each population, under the assumption that each locus comprises only two alleles, presence (dominant) and absence (recessive). Model number 4 with 1,000 bootstrap replicates was chosen, which uses a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999), and assumes HWE for a diploid population.

In addition, two dissimilarity matrices, geographic and genetic distances, were generated to test an isolation-by-distance hypothesis (IBD) (Wright 1943) using the Mantel test (Mantel 1967). Within GenAlEx, the null hypothesis of no association between the elements in one matrix (X) and those in the other (Y) ($R_{XY} = 0$) was determined with 999 permutations at $p < 0.05$. The closer the R_{XY} value is to -1 or 1, the stronger the correlation between genetic and geographic distances. The IBD hypothesis was also tested using the online platform Isolation by Distance Web Service (IBDWS) v. 3.16 (Jensen et al. 2005). The correlation between the two matrices is determined by the *r* statistic, again ranging from -1 to 1 based on the relationship, with significance of *r* determined with 1,000 randomizations at $p < 0.05$. Significance using the Mantel test in both GenAlEx and IBDWS is assessed by comparing actual values (R_{XY} and *r*) to a distribution of scores obtained by randomizing the values from one axis of the matrix. Geographic distances between individual plots and sites were measured in meters using ArcMap v. 10.0 (ESRI, Redlands, CA) and then log-transformed prior to use in the matrix.

The genetic distance matrix was then imported into TreeFit (Kalinowsi 2009), which compares distances between populations in neighbor-joining (NJ) (Saitou and Nei 1987) and UPGMA (Sokal and Michener 1958) trees with the observed genetic distances. The relationship between these distances is expressed using the r^2 statistic. Based on this analysis, the NJ method ($r^2 = 0.95$) was found to better describe the distance data compared to UPGMA ($r^2 = 0.65$). The

distance matrix was then imported into MEGA v. 5 (Tamura et al. 2011) where the NJ method was used to produce a dendrogram of genotype clustering.

Results

Genetic Structure and Diversity

In total, 204 polymorphic peaks were scored from fragments ranging in size from 77 to 497 bp. Thirty-eight AFLP genotypes, composed of one to 10 total isolates each, were present in the population, with 12 genotypes (32%) represented by a single isolate (Table 3.2). There was a mean of eight genotypes per hectare (GPH), but that figure ranged from five to 33 depending on site (Table 3.2). For example, five genets were discovered within a 0.07 ha plot at the Holyoke site, which when extrapolated yields a figure of 71 genets per ha (GPH). Also, two additional plots at this site yielded figures of 40 GPH (four total genets), and 18 GPH (three total genets). In contrast, four plots ranging in size from 0.04 to 0.30 ha (one each at Brimfield and Holyoke, and two at Spencer), were composed of only one genet. By site, or subpopulation, the number of polymorphic loci ranged from 64 to 103, while the number of private alleles ranged from 17 to 39 (Table 3.2).

Table 3.2. Genotype Information for the Population of *A. gallica* From Mixed Oak Forests.

Site Name	No. of Isolates	Total Genotypes	Single Isolate Genotypes	Genets per ha[Z]	Polymorphic Loci[Y]	Private Alleles[X]
Brimfield	40	8	1	5	83 (41%)	33
Holyoke	40	13	5	33	76 (37%)	29
Quabbin	39	9	3	5	103 (51%)	39
Spencer	34	8	3	16	64 (31%)	17
All	153	38	12	8	204	118

[Z] Genets per hectare was determined by dividing the total number of genets by the total plot area.
[Y] polymorphic loci within each subpopulation with percent total in parenthesis.
[X] private alleles within each subpopulation.

Allele frequencies of the 204 loci produced an unbiased expected heterozygosity (H_E) of 0.112 (SE = 0.006) for the entire population, with subpopulation values ranging from 0.083 to 0.119 (Table 3.3). Nei's gene diversity (H_J), a unit analogous to H_E, was 0.190 (S.E. = 0.009) for the entire population, with values ranging from 0.123 to 0.183 within each subpopulation (Table 3.3). Total gene diversity, H_T, was 0.205, mean gene diversity within populations, H_W, was 0.143 (S.E. = 0.014), and the average gene diversity within populations, H_B, was 0.062 (S.E. <0.001) (Table 3.4). For all 153 isolates, Nei's unbiased genetic distance (0.040), and genetic identity (0.961) illustrate a close genetic relationship among isolates within this population of *A. gallica* (Table 3.3).

The AMOVA statistic, Φ_{PT} was 0.301 ($p < 0.001$), indicating a high level of genetic differentiation within the sampled *A. gallica* population (Wright 1978), with 70% of molecular variance explained within the subpopulations (site) and 30% among subpopulations within the entire population. Variance within each site, using plot as the subpopulation, produced Φ_{PT} values that were significant ($p < 0.001$) for all four sites (Table 3.4). But at this scale, the majority of the variance was not explained at the plot level, with most of the variance explained from within the entire population (site) (Table 3.4).

Table 3.3. Population Genetic Structure of *A. gallica*.

Total Isolates	Total Gene Diversity (H_T)	Mean Gene Diversity (H_W)	Average Gene Diversity (H_B)	Nei's Genetic Diversity	Genetic Identity
153	0.2051	0.1432 (0.0136)[Z]	0.0619 (<0.001)	0.040	0.961

[Z] Standard error in parenthesis.

Table 3.4. Heterozygosity, AMOVA Population Variance, and Mantel Test Results for the *A. gallica* Population.

	Heterozygosity								Mantel Test			
	AFLP-SURV		GenAlEx		AMOVA Population Variance				IBDWS		GenAlEx	
Site	H_J[Z]	S.E.	H_E[Y]	S.E.	Φ_{PT}	*p*-value	Within[X]	Among[W]	*r*	*p*-value[V]	R_{XY}	*p*-value[U]
Brimfield	0.137	0.013	0.090	0.009	0.656	<0.001	34%	66%	-0.047	0.602	0.665	<0.001
Holyoke	0.123	0.013	0.084	0.009	0.569	<0.001	43%	57%	0.338	<0.001	0.628	<0.001
Quabbin	0.183	0.014	0.121	0.010	0.710	<0.001	29%	71%	-0.033	0.615	0.708	<0.001
Spencer	0.129	0.014	0.090	0.010	0.778	<0.001	22%	78%	-0.038	0.437	0.666	<0.001
All Sites	0.190	0.009	0.112	0.006	0.301	<0.001	70%	30%	0.294	<0.001	0.577	<0.001

[Z] expected heterozygosity under Hardy-Weinberg genotypic proportions, also called Nei's gene diversity.
[Y] unbiased expected heterozygosity = $(2N / (2N-1)) \times H_E$.
[X] proportion of genetic variability within each subpopulation relative to the total variability.
[W] proportion of genetic variability among each subpopulation relative to the total variability.
[V] probability that there is a positive correlation between genetic and geographic distances at $p = 0.05$ after 1,000 randomizations.
[U] probability that there is a significant correlation between genetic and geographic distances at $p = 0.05$ after 999 permutations.

Isolation by Distance

The Mantel test in GenAlEx produced an R_{XY} value of 0.577 ($p < 0.001$) for the entire population, illustrating a positive correlation between genetic and geographic distances for genotypes of *A. gallica*. When the Mantel test was used to characterize each subpopulation, results were once again positive and significant ($p < 0.001$) for all four sites, with R_{XY} values ranging from 0.628 to 0.708 (Table 3.4). When the Mantel test was run in IBDWS for the entire population, the test produced a positive *r* value of 0.294 ($p < 0.001$). Yet, when the test was run using each subpopulation alone, three of the four subpopulations showed no significant correlation between genetic and geographic distances (Table 3.4).

Results of the NJ analysis based on genetic distances illustrated a clustering of genotypes by subpopulation (site), and a clustering of genotypes originating from the same plot at the Holyoke site (Figure 3.2). However, genotypes originating from the same plot at Brimfield, Quabbin, and Spencer did not consistently cluster together. In addition, the two sites (Holyoke and Quabbin) that were closest by geographic distance (10.9 km; Figure 3.1) were the most distant from one another on the NJ tree (Figure 3.2), a result that contradicts the positive correlation between genetic and geographic distances. When the Mantel test was performed again, this time comparing mean genetic distances among the subpopulations only (excluding mean genetic distances within each subpopulation), the two matrices were found to be inversely related, yet values were not significant at $p = 0.05$ ($R_{XY} = -0.628$; $p = 0.128$) and ($r = -0.632$; $p = 0.085$).

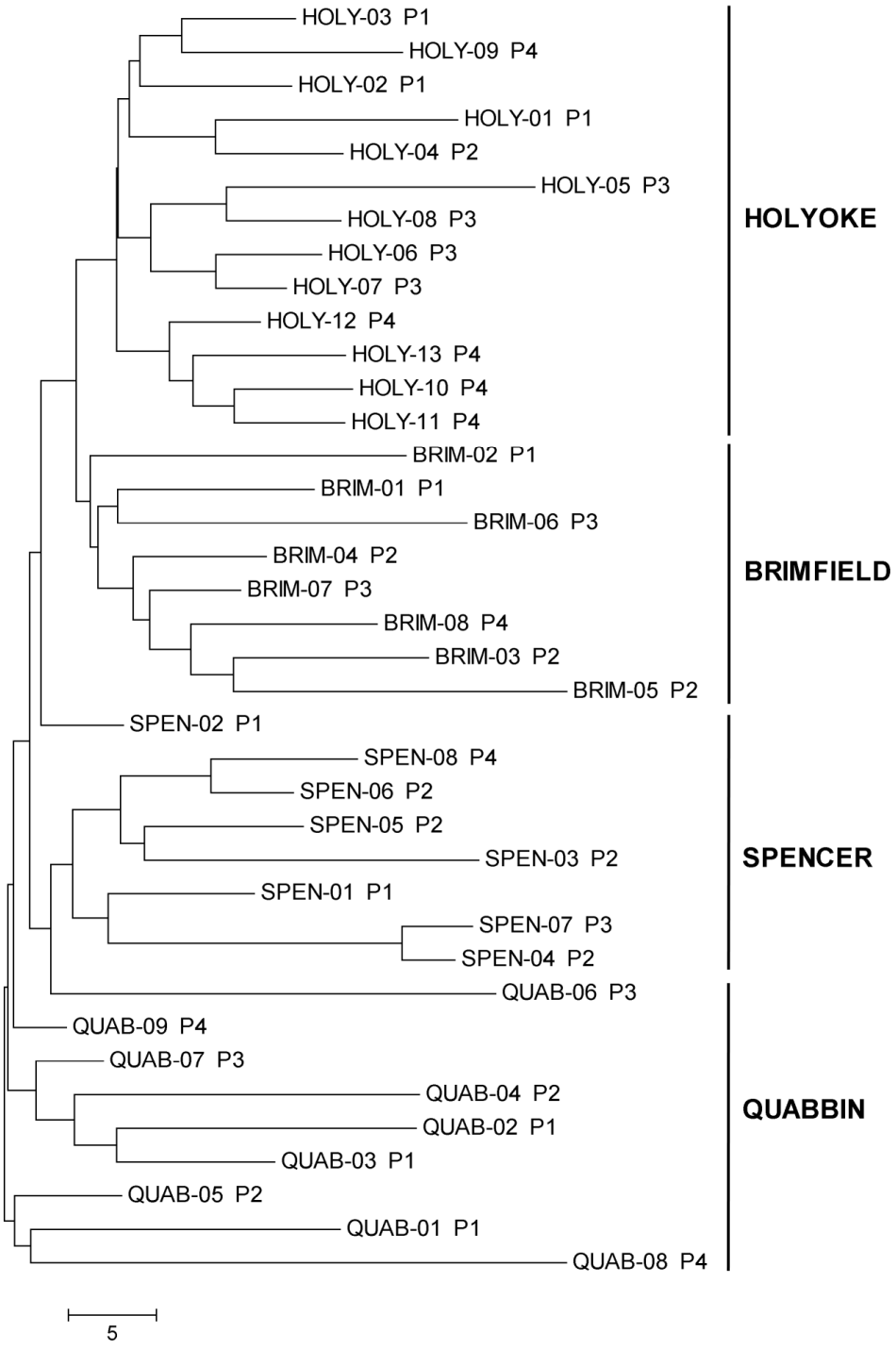

Figure 3.2. Neighbor-joining dendrogram using genetic distances to determine individual genotype grouping (n = 38). Genotypes are listed by site (BRIM = Brimfield; HOLY = Holyoke; QUAB = Quabbin; and SPEN = Spencer), followed by genotype number (01 to 13) and individual plot (P1 to P4).

Discussion

Previous studies of population subdivision within populations of *A. gallica* in North America have revealed varying levels of genotypic variation when differing geographic sampling scales were employed. After intensively sampling a single site occupying 100 ha in northern Michigan, low levels of genotypic diversity were found within an *A. gallica* population (Smith et al. 1992, Hodnett and Anderson 2000). In central New York, at geographic scales of 0.11 to 1.06 ha, populations of four species of *Armillaria* (including *A. gallica*) exhibited high levels of genotypic variation (Worrall 1994). When populations of *A. gallica* were compared at a large regional level in the eastern U.S. and Canada (Michigan, North Carolina, Rhode Island, and Ontario), significant genetic variation was also observed (Saville et al. 1996). Therefore, although genetic data exist at both small (0.1 to 100 ha) and large (millions of ha) geographic scales, an intermediate geographic scale has, until this study, been overlooked.

At the plot level, where sampling occurred at a small geographic scale (0.04 to 0.87 ha), both high and low levels of genotypic diversity were revealed at each of the four sites. Based on the sampling scheme used in this study, I was unable to determine the overall size of these genets, and cannot determine whether or not individual genets approach the large size found by Smith et al. (1992). My intent was to better understand what sampling scale is necessary to detect differentiation within the population at the landscape level.

Overall, the sampled population of *A. gallica* proved to have relatively low in genetic variability, with expected heterozygosities less than 0.2. Despite the low overall genetic variability, this assessment of the molecular variance within the sampled *A. gallica* population shows that significant genetic differentiation exists within subpopulations at the site level, areas that ranged in size from 23 to 110 ha and captured 57 to 78% of the overall genetic variability. But at the landscape level, 510 km^2 (510,000 ha), molecular variance was substantially less, with only 30% of the variability explained. The lack of diversity at the landscape level is in agreement

with Wright's theory of hierarchical population structure (Wright 1943), even though none of the study sites were isolated from one another by geographic barriers that would limit gene flow within the *A. gallica* population. My results show that sampling at a geographic scale of up to 100 ha can be sufficient to characterize the majority of the molecular variance within the overall population if more plots were sampled, and thus more genotypes discovered. The results also show that intensive sampling at one or a few locations, rather than an expanded scale of sampling that includes more nearby plots, has the potential to falsely characterize the overall population as having low genotypic diversity. While this could be anticipated, as basidiospores produced by *Armillaria* and other forest-inhabiting basidiomycetes are not known to travel long distances (Stenlid and Gustafsson 2001, Power et al. 2008), this study provides evidence of the geographic scale at which genotypic diversity can be expected.

The results of the Mantel test and genotype clustering in the NJ tree are seemingly at odds with one another. There was a positive correlation found between genetic and geographic distances, yet the two sites that are closest geographically to one another grouped distal to one another in the NJ tree. While the correlation in GenAlEx was over 0.5 ($R_{XY} = 0.577$), the correlation in IBDWS was less convincing ($r = 0.294$), and neither value comes close to approaching a value of 1 (complete isolation by distance). Because of the small sample size within each subpopulation (34 to 40) and the low number of genotypes within each subpopulation (eight to 13), the dataset is probably insufficient to properly test the IBD hypothesis. In addition, because *Armillaria* has two primary modes of dispersal, clonal spread via rhizomorphs and airborne basidiospores, a larger number of genotypes and individual isolates may do little to resolve the IBD question.

However, because the association between actual genetic distances and the observed distances in the NJ tree were so strong ($r^2 = 0.95$), the random clustering of genotypes within three of the four sites (Brimfield, Quabbin, and Spencer) is in agreement with Worrall's (1994) assertion that dispersal and germination is an important means of new genet formation for

A. gallica. But, new genet formation by basidiospores is primarily a local event, occurring at geographic scales of 20 to 100 ha in this study, while long distance (i.e. > 10 km) dispersal events were not observed.

While large genets, such as the one described by Smith et al. (1992), may exist on the landscape in central Massachusetts, the population we sampled is composed of numerous genets that are likely small in area. Thus, the effect of precipitation on fruiting body formation and spore dispersal appears to have created a mosaic of small genets in central Massachusetts mixed oak forests, similar to what has been reported in northern hardwood forests in New Hampshire and New York (Rizzo and Harrington 1993; Worrall 1994). In support of this assertion, the mean number of GPH in this study was eight (38 AFLP genotypes over 4.51 hectares), with a range of five to 33 GPH by site. Therefore, the average *A. gallica* genet occupies an area of 0.13 ha, with a range of 0.03 to 0.2 ha per genet. This figure is similar to Worrall's estimate of 11 GPH for *A. solidipes* in New York (1994), but is lower than what was determined for *A. calvescens* (24 GPH) from the same region. A mean of seven to nine GPH was found for *A. cepistipes* and *A. solidipes* in Norway spruce stands in the Alps (Prospero et al. 2003). For three species of *Armillaria* (*A. borealis*, *A. cepistipes*, and *A. solidipes*) occupying cold, high elevation forests in the Swiss Alps, genets occupied a mean area of 0.2 to 6.8 ha (Bendel et al. 2006).

To conclude, the *A. gallica* population used in this study exhibited high genetic variability at the site level (23 to 110 ha), but at the landscape-level (510 000 ha) the population was clearly divided into subpopulations. Our data show that the mixed oak forest landscape in Massachusetts is composed of numerous *A. gallica* genets that are relatively small in area. This is in agreement with results from northern hardwood forests in New England, where high precipitation rates appear to be influencing population subdivision. The genotypic variation we have described for this population of *A. gallica* suggests that pathogenicity studies should include several isolates representing a variety of unique genotypes before categorizing the species from a particular region. Only then can a more accurate representation of this species be obtained, since

currently there seems to a dichotomy between those who believe *A. gallica* is only weakly pathogenic and unable to successfully colonize healthy trees compared to those who believe that *A. gallica* can behave as an aggressive pathogen under certain circumstances.

CHAPTER 4

EFFECTS OF HYDROLYZABLE TANNINS ON *IN VITRO* GROWTH OF *ARMILLARIA CALVESCENS* AND *A. GALLICA*

Introduction

Armillaria calvescens Bérubé and Dessureault and *A. gallica* Marxmüller and Romagnesi are two of the most closely related species of *Armillaria* in North America. Both species create large, monopodial rhizomorph networks, primarily cause butt rot of hardwoods, produce nearly identical fruiting bodies, and have highly similar genetic profiles (Bérubé and Dessureault 1989, Burdsall and Volk 1993, Kim et al. 2006). Surveys of *Armillaria* species distribution in northeastern North America have shown that *A. calvescens* occurs most frequently in *Acer*-dominated forests, while *A. gallica* is more abundant in *Quercus*-dominated forests (Blodgett and Worrall 1992b, Brazee and Wick 2009, Marçais and Wargo 2000, McLaughlin 2001). However, while *A. gallica* can be found regularly in many forest types, *A. calvescens* appears to be restricted to northern hardwood - hemlock forests (Bérubé and Dessureault 1989, Blodgett and Worrall 1992a, Brazee and Wick 2009, McLaughlin 2001), with very few exceptions (Mallet 1990). Because of this disparity in incidence by forest type, the question arises as to whether *A. gallica* is better adapted to overcome the chemical host defenses of *Quercus* spp. than *A. calvescens*. If true, this would suggest that *A. calvescens* is a less virulent pathogen, since the primary northern hardwood tree species (*Acer* spp., *Betula* spp., and *Fagus grandifolia*) present a weaker physical defense (thinner bark) and chemical defense (reduced quantity and diversity of polyphenols in bark and wood) compared to *Quercus* spp. (Rowe 1979). In addition, *A. calvescens* exhibits a strong host preference for *Acer saccharum* (Blodgett and Worrall 1992a, McLaughlin 2001), a tree that can average up to 5% sugar in its sap (Taylor 1956). This suggests that *A. calvescens* has difficulty parasitizing trees with more typical, lower cambial sugar

concentrations. *Armillaria gallica* has never been shown to exhibit a significant association to any particular host species (Blodgett and Worrall 1992a, Brazee and Wick 2009).

Plant polyphenols, the primary chemical defense of higher plants, are secondary metabolites that are known to inhibit the growth of parasitic fungi by restricting the production of cell-wall degrading enzymes, and also by disrupting and killing fungal cells through toxicity (Goldstein and Swain 1965). Polyphenols are subdivided into two groups: one having a condensed flavinoid core (condensed tannins), and the other with a D-glucose core (hydrolyzable tannins) (Haslam 1998). Hydrolyzable tannins are the most abundant type in the leaves, bark, and wood of *Quercus* spp. and consist of various esters of gallic acid and ellagic acid (a dimer of gallic acid) (Haslam and Scalbert 1987, Mämmelä et al. 2000, Parker 1977). Hydrolyzable tannins can be broken down into monomeric units by certain microorganisms, including *Armillaria*, which allows the glucose core to be metabolized as a food source (Bhat et al. 1998, Shaw 1985, Wargo 1981, 1983).

When available carbon concentrations are low, polyphenols are effective at inhibiting the growth of *Armillaria* (Entry et al. 1992, Garraway et al. 1991, Shaw 1985, Wargo 1980, 1981, 1983). However, if there are sufficient carbon and nitrogen sources for *Armillaria*, the fungus can overcome the inhibitory effects of polyphenols and oxidize and metabolize these compounds, thus stimulating growth (Garraway et al. 1991, Wargo 1983, Shaw 1985). This is visible at the leading edge of mycelial fans produced by *Armillaria*. As the fans actively secrete polyphenol oxidases, the infected wood tissue becomes discolored as host polyphenols are oxidized (Marsh and Wargo 1989). Wargo (1984) found that in *Quercus alba* and *Q. velutina* bark colonized by *Armillaria*, total phenols were reduced by 78% and 54% compared to concentrations in uncolonized bark tissues, showing that *Armillaria* had oxidized host polyphenols. The oxidized polyphenol levels in colonized bark were up to 3.5 times higher when compared to healthy, uncolonized bark. When *Armillaria* isolates were grown in extracts of *Quercus alba* and *Q. velutina* root bark, the addition of glucose and ethanol stimulated growth over carbon-unamended

extracts (Wargo 1984). Yet, an increase in available carbon does not always correlate to enhanced degradation of polyphenolic compounds. Entry et al. (1992) showed that while higher concentrations of simple sugars (glucose, fructose, and sucrose) increased *in vitro* growth of *A. solidipes,* the source and concentration of carbon had no effect on the degradation of catechol and *para*-hydroxybenzoic acid, two important plant defense compounds.

Along with reducing sugars, ethanol is present within the vascular cambium and xylem sap of trees, and increases considerably when trees are exposed to hypoxic or anoxic conditions (MacDonald and Kimmerer 1991). Root disease can also cause ethanol concentrations to increase in root phloem and sapwood. Kelsey and Joseph (1998) found that *Pseudotsuga menziesii* infected with *Leptographium wageneri* had significantly elevated levels of ethanol in roots when compared to healthy trees. *In vitro* studies of *Armillaria* have proven that both rhizomorph and mycelium production is highly stimulated when ethanol is added to the growth medium (Weinhold 1963, Weinhold and Garraway 1966). When trees suffer defoliation from insects or wind/ice storms, root starch is converted back to reducing sugars for mobilization to the crown to refoliate (Wargo et al. 1972). There is some evidence that increases of simple sugars in the cambial tissue can stimulate growth of *Armillaria* (Wargo et al. 1972). However, it may be the increase of ethanol in conjunction with the increase in sugars that is stimulating the growth of *Armillaria* on host cambial tissues. For tree species that do not have naturally high levels of sugars in their cambium (i.e. *Quercus* spp.), stress-induced increases of available carbon are usually required for successful colonization by *Armillaria* (Wargo 1981, 1983, Twery et al. 1990, Marçais and Breda 2006).

All of the tree species present in northern hardwood and mixed oak forests are known to produce hydrolyzable and condensed tannins (Bates-Smith and Metcalfe 1957, Haslam 1989), yet there is considerable variation in the quantity and type produced. For example, there are at least 750 metabolites of gallic acid, and over 500 metabolites of ellagic acid produced within higher plants (Haslam 1998, Quideau and Feldman 1996). *Quercus* species produce many of the

important gallotannins and ellagitannins found in nature (Haslam 1989). Scalbert et al. (1989) found that ellagic acid was detectable in some central hardwood trees (*Quercus* and *Juglans*), but was absent in certain northern hardwood trees (*Fagus*, *Fraxinus*, *Populus*, *Prunus*, and *Ulmus*). In addition, Rowe (1979) reported that hydrolyzable tannins are either not present or found only in low concentrations within the bark and wood of *Acer* spp., *Betula* spp., and *Fagus grandifolia*. Direct, accurate comparisons of polyphenol concentrations in bark and wood tissue among the important temperate tree species are very few, due mostly to different extraction methods. However, a study of gallic acid concentrations in the wood tissue of three *Quercus* species (*Q. alba*, *Q. garryana*, and *Q. prinus*) produced values that ranged from 0.15% to 0.31%, while ellagic acid concentrations ranged from 0.63 to 1.04% (Lei et al. 2001). *Quercus* species have been shown to produce polyphenols in the foliage and within the vascular cambium, where these compounds are mobilized and stored within the heartwood, sapwood, and the bark tissues for defense (Bates-Smith and Metcalfe 1957, Hathaway 1959, Seikel et al. 1971, Parker 1977). While variation occurs in the type and concentration of polyphenols in leaf tissue as the growing season progresses, the concentrations of bark polyphenols are stable (Parker 1977).

Previous work on this subject was performed using various isolates of *Armillaria mellea* (*sensu lato*), since the complex of species that comprise the genus *Armillaria* had not been fully elucidated (Wargo 1980, 1983, 1984). Therefore, any variation encountered among *A. mellea* s.l. isolates could be potentially explained by differences among the species used. Shaw (1985) tested various concentrations of gallic acid, tannic acid, and ethanol on six species of *Armillaria* in an attempt to use growth rates to discriminate between different biological species, but found that significant differences within species rendered that technique unsuccessful.

The primary goals of this study were to: (i) determine whether there are significant differences in growth rates between *A. gallica* and *A. calvescens* on polyphenol media, and (ii) determine the effect that various glucose and ethanol amendments have on growth of each species on polyphenol media. It was hypothesized that *A. gallica* would exhibit higher growth rates over

A. calvescens within each polyphenol treatment, regardless of glucose and ethanol amendments. In addition, it was hypothesized that growth of both species would be inhibited by increasing concentrations of purified polyphenols, but that root bark extracts will enhance growth. If these hypotheses are correct, this could help to explain why *A. gallica* dominates mixed oak forests in northeastern North America, while *A. calvescens* is rarely found in this forest type.

Materials and Methods

Preparation of Polyphenol Media

Two commercially available hydrolyzable tannins were used; tannic acid (TA) (95% pure, Acros Organics, Morris Plains, N.J.) and gallic acid (GA) (98% pure, Acros Organics). In addition, black oak (*Q. velutina*) root bark extracts (RBE) were used in this study. Root bark was collected just below the soil surface at the base of healthy trees by separating the bark from live sapwood using a chisel and hammer. The root bark was immediately placed on ice and returned to the laboratory within one hour of harvest. It was then rinsed under tap water to remove soil, wrapped in cheese cloth, and then freeze-dried in a lyophilizer for 72 h. The outer suberized cork layer (phellem) was scraped off with a razor blade and the remaining bark tissue (phloem) was ground to a fine powder in a Wiley mill using a 20-mesh screen.

The polyphenol media was prepared as follows: a 5% solution of each commercial polphenol, TA or GA, was made by adding 40 g to 500 ml of Weinhold's basal medium (BM) (Weinhold 1963). The pH was then adjusted to 5.7 using 1M NaOH and the volume increased to 800 ml with BM. The solution was then filter-sterilized under aseptic conditions. To create the RBE medium, a 1% solution of *Q. velutina* root bark extracts was produced by dissolving the ground root bark in warm BM at a concentration of 10% (w/v) for 15 min. The contents were then centrifuged for 15 min at 3,500 rpm. The supernatant was removed and pre-filtered through a 1 µm filter, then filter-sterilized with a 0.45 µm bottle-top filter under aseptic conditions. It was determined that approximately 10% of the RBE dissolves in solution, which was in agreement

with previous work (Wargo 1983, 1984). Each of the TA/GA/RBE treatments was made up of the proper volume of BM (adjusted to a pH of 5.7 with 1M NaOH), with and without D-glucose when necessary. Once the BM was autoclaved and cooled to 55ºC, 95% EtOH and the 5% TA/GA or 1% RBE solutions were added under aseptic conditions, as necessary, to produce the desired concentrations.

For the first phase of the study, seven concentrations of TA (0, 0.12, 0.25, 0.50, 0.75, 1.0, and 2.0%) and GA (0, 0.12, 0.25, 0.38, 0.50, 1.0, and 2.0%) were tested. Four concentrations of RBE were used (0, 0.12, 0.25, and 0.50%). To assess the influence of simple carbon sources on polyphenol metabolism, six glucose and ethanol combinations were incorporated. These included; (i) control (BM only); (ii) glucose = 0.5%; (iii) glucose = 1%; (iv) ethanol = 0.1% (v/v); (v) ethanol = 0.5% (v/v); and (vi) glucose = 0.5% + ethanol = 0.1% (v/v). For the first phase of the study, three isolates of each species were used (six total), that had been collected previously from western Massachusetts (Table 4.1). Therefore, for TA and GA, seven TA/GA concentrations × six glucose/ethanol concentrations × six isolates of *Armillaria* = 252 cultures per species (504 total). For RBE, four concentrations × six glucose/ethanol concentrations × six isolates of *Armillaria* = 144 cultures.

Table 4.1. *Armillaria* Isolates Tested on Polyphenol Media.

Species	No.	Isolate Code	Host	Origin
A. calvescens	1	M2-6[Z]	*Acer saccharum*	Massachusetts
	2	G2-4[Z]	*Acer rubrum*	Massachusetts
	3	P3-8[Z]	*Acer saccharum*	Massachusetts
	4	G4-4[Y]	*Acer saccharum*	Massachusetts
	5	TO2-12[Y]	*Fagus grandifolia*	Massachusetts
	6	TO3-9[Y]	*Fraxinus americana*	Massachusetts
	7	ST17 (PR-3)[Y]	*Acer saccharum*	Michigan
	8	Ac98[Y]	*Acer saccharum*	Ontario
	9	Ac154[Y]	*Acer saccharum*	Ontario
A. gallica	1	B2-6[Z]	*Quercus velutina*	Massachusetts
	2	Q3-9[Z]	*Quercus velutina*	Massachusetts
	3	H1-6[Z]	*Quercus velutina*	Massachusetts
	4	W2-5[Y]	*Acer rubrum*	Massachusetts
	5	S2-9[Y]	*Quercus velutina*	Massachusetts
	6	D2-1[Y]	*Quercus alba*	Massachusetts
	7	MT5-1[Y]	*Pinus rigida*	Massachusetts
	8	ST22 (EL-1)[Y]	*Betula papyrifera*	Michigan
	9	ST23 (MA-1)[Y]	*Acer saccharum*	Wisconsin

[Z] isolates used in the first phase of the study (three isolates per species);
[Y] additional isolates used in the second phase of the study (nine isolates per species).
Alternate isolate codes published previously listed in parenthesis.

After phase one was completed, it was determined that more isolates per species were necessary and that the high polyphenol concentrations were not informative because they were very inhibitory to growth. For the second phase of this study, nine isolates of each species (18 total isolates, including the six isolates used in phase one) were used to test three GA/RBE concentrations (0, 0.12, and 0.25%). Once again, six glucose and ethanol combinations were incorporated into each polyphenol treatment. Therefore, three GA/RBE concentrations × six glucose/ethanol concentrations × 18 isolates of *Armillaria* = 324 cultures per species (648 total). In total, 548 cultures were produced in phase one, while 648 cultures were produced in phase two for a combined total of 1,296 cultures.

To ensure that growth on the polyphenol media was not influenced by any carryover of nutrients from the colonized plugs, all isolates were grown on water agar for roughly three weeks prior to transfer. Cultures older than four weeks were not used. A 5 mm plug was cut from the margins of each isolate colony for transfer to the polyphenol media. Within each 60 × 15 mm petri dish, 12 ml of medium was added to maintain consistency across treatments. TA and GA isolates were incubated in the dark at 25ºC for 25 days, while RBE isolates were grown for 18 days due to substantially faster growth.

Colony area (mm^2) was measured by capturing digital images in ImageJ (Rasband 2009). Petri plates were scanned top-down alongside a metric ruler. Within ImageJ, a straight line was then drawn over a known length of the ruler (e.g. 50 mm) to set the scale. A polygon was then manually constructed around the mycelial colony and area was measured. This method is very accurate and better suited for a fungus like *Armillaria* which can produce diffuse, irregular colonies. The borders of each colony were set at the limits of thallus growth, while expanding rhizomorph networks were excluded from the total area. To determine dry biomass, cultures were removed from the plastic petri dishes and placed in open, glass petri dishes filled with water. The cultures were then placed in a steam autoclave for one minute at operating temperature and pressure. Mycelial colonies were then removed from the dish with forceps, rinsed twice in water, and placed into 2 ml microcentrifuge tubes. The mycelium was then dried at 80ºC for 48 h. Dry weights (in mg) were obtained by weighing the tubes with mycelium and subtracting the original tube weight. Despite establishing several treatments of *Armillaria* in polyphenol broth using vented-cap, 25 cm^2 tissue culture flasks and thick plugs that were elevated above the broth surface, several isolates simply did not grow, regardless of polyphenol concentration or glucose/ethanol amendments.

Identification of Isolates Used

Proper species identification was a critical component of this study. All isolates were initially identified using a PCR-RFLP protocol developed by McLaughlin and Hsiang (2010) that targets variation in both the IGS-1 and IGS-2 regions of the rDNA cluster (Brazee and Wick 2009). Further confirmation of species identification was done by analyzing partial sequences from the elongation factor 1-alpha (*tef1*) gene for 24 isolates (12 each) of *A. calvescens* and *A. gallica*, a set that includes all of the isolates used in this study. Regions of *tef1* have been shown to accurately distinguish an array of *Armillaria* species from both the northern and southern hemisphere (Hasegawa et al. 2010, Maphosa et al. 2006).

Statistical Analysis

In addition to residual plots, the raw data was analyzed using the Box-Cox procedure to determine if transformation of the data was necessary (Zar 2007). Based on these analyses, colony area and dry biomass were square root transformed. A general linear model (GLM) was used to determine if variation in colony area and biomass values could be explained by; (i) polyphenol (tannic acid, gallic acid, and root bark extracts) concentration; (ii) glucose/ethanol concentration (control, glucose = 0.5%, glucose = 1.0%, ethanol = 0.1%, ethanol = 0.5%, and glucose = 0.5% + ethanol = 0.1%) or presence of ethanol alone; and (iii) species (*A. calvescens* and *A. gallica*) or individual isolate. In addition, isolates of *A. calvescens* were separated to test whether colony area and biomass were dependent upon their origin. Specifically, *A. calvescens* isolates that originated from forests with significant components of red oak (*Quercus rubra*) and/or eastern hemlock (*Tsuga canadensis*) were tested against isolates that originated from northern hardwood forests without red oak or hemlock. Both red oak and hemlock are minor components of the northern hardwood forest type (Eyre 1980) and have higher concentrations and broader arrays of polyphenols present in their root bark. Forest composition data, collected previously with individual *Armillaria* isolates, were used to determine origin criteria (Brazee and

Wick 2009). Post-hoc analyses were done using the Tukey's HSD test at $P = 0.05$. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL).

Results

Three Isolates per Species - Seven TA/GA Concentrations and Four RBE Concentrations

Colony Area

For TA, the majority of the variation was explained by the GLM using TA concentration, isolate, and ethanol (adjusted $r^2 = 0.96$). However, when the model was run with just TA concentration, adjusted r^2 was 0.87, illustrating how strongly colony area was dependent upon TA concentration alone. *Armillaria gallica* produced significantly larger colony areas than *A. calvescens* at the three highest TA concentrations (0.75, 1, and 2%), while at all others, differences were not significant (Table 4.2). At the highest TA concentration (2%), *A. gallica* had a mean colony area more than 2.5 times that of *A. calvescens* (Table 4.2). Both species produced their largest colony areas at the lowest TA concentration (0.12%) (Table 4.2), illustrating the stimulative effect of low TA concentrations. When variation in colony areas was tested against the six amendments of glucose and ethanol, there were no significant differences (data not shown).

For GA, the GLM using GA concentration, isolate, and ethanol explained a high level of variation (adjusted $r^2 = 0.96$). Once again, when the model was run with GA concentration only, adjusted r^2 was 0.84, which illustrated the effect of the polyphenol alone on overall growth of both species. While *A. gallica* produced its highest mean colony area at the lowest GA concentration (0.12%), mean colony areas for both species were not significantly different between the control and 0.12% treatments, showing that low levels of GA were neither stimulative or inhibitory (Table 4.2). Once again, there were no significant differences in colony area by glucose/ethanol concentrations on GA media (data not shown). *Armillaria gallica* produced significantly larger colony area than *A. calvescens* at GA concentrations of 0.12% and 0.25% (Table 4.2). At GA concentration 0.25%, *A. gallica* had a mean colony area almost twice

that of *A. calvescens* (660 to 350 mm^2; $p = 0.007$; Table 4.2), while at GA concentrations 0.38 and 0.50% there were no significant differences by species (Table 4.2). Growth of both species was entirely inhibited at 1 and 2%, although the media was oxidized at the margins of the plug for both species, indicating that both *Armillaria* species were actively producing polyphenol oxidases. The addition of ethanol led to significant increases in colony area and biomass for both species, especially at higher concentrations of GA (Table 4.3).

For RBE colony area, the GLM using RBE concentration, isolate, and ethanol explained the majority of the variation (adjusted $r^2 = 0.89$). In contrast to the TA and GA treatments, *A. gallica* mean colony area remained constant as RBE concentration increased (Table 4.2). All three RBE concentrations produced colony areas that were significantly larger than the control treatment, while mean colony areas for *A. calvescens* weren't significantly different across the four concentrations (Table 4.2). *Armillaria gallica* produced significantly greater colony area values than *A. calvescens* at 0.12, 0.25, and 0.50% (Table 4.2). At 0.50%, *A. gallica* had a mean colony area more than twice that of *A. calvescens* (1163 to 519 mm^2; $p < 0.001$) (Table 4.2). In addition, *A. gallica* produced a significantly higher colony area than *A. calvescens* within each one of the six glucose/ethanol treatments (data not shown). Once again, colony areas were not significantly different across the gradient of glucose/ethanol concentrations tested (data not shown).

Biomass

For TA, the highest level of variation was explained in the GLM using TA concentration, isolate, and ethanol (adjusted $r^2 = 0.84$). Significant differences in mean biomass between the two species by TA concentration were present only in the control treatment and at the highest (2%) TA concentration (Table 4.2). *Armillaria calvescens* produced significantly larger biomass values than *A. gallica* in the control treatment, yet overall values for *A. calvescens* were not significantly different across TA concentration, with the exception of the highest concentration (2%) (Table 4.2). For *A. gallica*, the largest mean biomass occurred at the lowest concentration (0.12%) and

was more than double the control mean (Table 4.2). Even with TA at 0.25%, biomass was still significantly larger than the control treatment (Table 4.2). The presence of ethanol in the TA treatments didn't produce significant differences in biomass for *A. gallica* but did produce significantly larger biomass for *A. calvescens* ($p = 0.027$) (data not shown). The presence of TA did not stimulate the production of rhizomorphs over control treatments and no significant differences in rhizomorph production were present between species (data not shown).

For GA, the GLM with the least amount of error (adjusted $r^2 = 0.93$) was achieved using GA concentration, isolate, and ethanol. Mean biomass values were significantly different between species only within the control treatment, where *A. calvescens* produced a mean biomass more than double that of *A. gallica* (31.6 to 15.2 mg; $p < 0.001$) (Table 4.2). For *A. calvescens*, mean biomass values decreased as GA concentration increased, and while *A. gallica* produced its largest biomass levels at 0.12% GA (Table 4.2), statistically, biomass values for *A. gallica* at 012% GA were not significantly different from the control when all treatments were compared together ($p = 0.077$). The addition of ethanol produced significantly larger biomass values for both *A. calvescens* and *A. gallica* within the GA treatment ($p = 0.001$ and $p < 0.001$, respectively), and in the highest concentrations (0.38 and 0.50%) the presence of ethanol increased biomass more than 10-fold (Table 4.3). Just as in the TA treatments, the addition of GA did not stimulate the production of rhizomorphs over control treatments and there were no differences in rhizomorph production between species (data not shown).

For RBE, the least amount of variation was achieved with the GLM using RBE concentration, isolate, and ethanol (adjusted $r^2 = 0.92$). Despite the strong effect of isolate, mean biomass values were significantly different between species within every treatment (Table 4.2). *Armillaria calvescens* produced a significantly higher mean biomass compared to *A. gallica* within the control treatment, but *A. gallica* produced a significantly higher biomass in each of the RBE treatments (Table 4.2). Mean biomass values for *A. calvescens* were not significantly different across the four treatments ($p = 0.055$), while *A. gallica* produced a significantly larger

biomass in each treatment ($p < 0.001$; Table 4.2). Mean biomass produced by *A. gallica* at 0.5% RBE was over 10 times higher than that of the control treatment (104.7 to 10.6 mg; $p < 0.001$) (Table 4.2).

None of the glucose/ethanol treatments were significantly different between the two species (Table 4.2). Also, none of the isolates for either species produced rhizomorphs in the control treatment (BM only), but when RBE was added to the BM, the presence of rhizomorphs was significantly higher for replicates of *A. gallica* compared to *A. calvescens* at concentrations of 0.12% ($p < 0.034$), 0.25% ($p < 0.005$), and 0.5% ($p < 0.006$). Across all three RBE concentrations (0.12%, 0.25%, and 0.5%; n = 108), *A. gallica* produced rhizomorphs in 27/54 replicates (50%), while *A. calvescens* produced rhizomorphs in 7/54 replicates (13%) ($p < 0.001$).

Table 4.2. Mean Colony Area (mm^2) and Mean Biomass (mg) Produced by *A. calvescens* and *A. gallica* by Concentration of Tannic Acid (TA), Gallic Acid (GA), and Root Bark Extracts (RBE) in Basal Medium.

% Polyphenol	Colony Area			Biomass		
TA	*A. calvescens*[z]	*A. gallica*	*p*-value	*A. calvescens*	*A. gallica*	*p*-value
0	822 (152)[a]	764 (250)[a]	0.303	34.5 (19)[a]	20.7 (13)[ab]	0.014
0.12	1051 (213)[b]	1074 (103)[b]	0.584	37.5 (23)[a]	42.3 (13)[d]	0.201
0.25	886 (135)[a]	810 (133)[a]	0.089	28.3 (17)[a]	30.4 (9)[c]	0.309
0.50	597 (88)[c]	618 (134)[c]	0.648	26.7 (17)[a]	22.8 (5)[bc]	0.790
0.75	468 (81)[d]	541 (97)[cd]	0.021	26.7 (12)[a]	22.4 (5)[bc]	0.318
1.00	336 (65)[e]	456 (68)[d]	<0.001	26.5 (16)[a]	22.1 (4)[bc]	0.417
2.00	98 (27)[f]	251 (41)[e]	<0.001	11.0 (6)[b]	14.4 (3)[a]	0.017
p-value	<0.001	<0.001		0.001	<0.001	
GA						
0	788 (126)[a]	689 (249)[ab]	0.080	31.6 (16)[a]	15.2 (7)[ab]	<0.001
0.12	733 (298)[a]	998 (257)[a]	0.005	21.2 (14)[ab]	25.4 (13)[a]	0.277
0.25	350 (247)[b]	660 (356)[b]	0.007	14.3 (14)[bc]	18.0 (12)[a]	0.267
0.38	127 (132)[c]	242 (219)[c]	0.114	8.8 (11)[c]	9.3 (9)[bc]	0.707
0.50	82 (66)[c]	118 (125)[c]	0.378	6.4 (7)[c]	8.1 (10)[c]	0.708
1.00	*no growth*	*no growth*	--	*no growth*	*no growth*	--
2.00	*no growth*	*no growth*	--	*no growth*	*no growth*	--
p-value	<0.001	<0.001		<0.001	<0.001	
RBE						
0	432 (103)	458 (129)[a]	0.569	17.7 (10)	10.6 (7)[a]	0.012
0.12	670 (151)	967 (207)[b]	<0.001	40.3 (28)	60.8 (18)[b]	0.008
0.25	580 (301)	1098 (324)[b]	<0.001	48.3 (40)	90.2 (36)[c]	0.001
0.50	519 (461)	1163 (452)[b]	<0.001	46.0 (46)	104.7 (49)[c]	<0.001
p-value	0.044	<0.001		0.055	<0.001	

[z] n = 18 replicates per treatment. Standard deviations in parenthesis. Values are significantly different between species (row) and within species (column) at $p = 0.05$. Values with different letters are significantly different within species at $p = 0.05$ using the Tukey's HSD test.

Table 4.3. Colony Area (mm^2) and Biomass (mg) Produced by *A. calvescens* and *A. gallica* on Gallic Acid Medium by Presence of Ethanol (Three Isolates per Species).

		Colony Area			Biomass		
%GA[Z]	Ethanol	*A. calvescens*[Y]	*A. gallica*	*p*-value	*A. calvescens*	*A. gallica*	*p*-value
0	No	714 (83)^{ab}	548 (79)^{cd}	< 0.001	25.2 (12)^{abc}	10.1 (6)^{d}	0.003
0	Yes	862 (121)^{a}	829 (284)^{bc}	0.627	38.1 (17)^{a}	20.3 (3)^{abc}	0.007
0.12	No	512 (97)^{b}	770 (125)^{c}	< 0.001	14.1 (7)^{bcd}	17.7 (9)^{bcd}	0.338
0.12	Yes	955 (263)^{a}	1225 (93)^{ac}	0.010	28.3 (16)^{ab}	33.1 (12)^{a}	0.381
0.25	No	177 (115)^{c}	376 (247)^{d}	0.033	5.3 (4)^{de}	9.2 (7)^{d}	0.138
0.25	Yes	524 (221)^{b}	944 (165)^{ab}	0.001	23.3 (14)^{abc}	26.7 (9)^{ab}	0.409
0.38	No	52 (23)^{d}	43 (14)^{f}	0.371	1.9 (1)^{e}	1.6 (1)^{e}	0.856
0.38	Yes	202 (154)^{c}	440 (113)^{d}	0.002	15.7 (12)^{bcd}	17.0 (5)^{bcd}	0.451
0.50	No	41 (11)^{d}	41 (8)^{f}	0.930	1.3 (0)^{e}	1 (0)^{e}	0.178
0.50	Yes	123 (73)^{cd}	195 (141)^{e}	0.234	11.4 (6)^{cd}	15.2 (9)^{cd}	0.317
p-value		<0.001	<0.001		<0.001	<0.001	

[Z] Concentrations of gallic acid above 0.5% were completely inhibitory to growth.
[Y] n = 9 replicates per treatment. Standard deviations in parenthesis. Values are significantly different between species (row) and within species (column) at $p = 0.05$. Values with different letters are significantly different within species at $p = 0.05$ using the Tukey's HSD test.

Nine Isolates per Species - Three GA/RBE Concentrations

Colony Area

For GA, the GLM using GA concentration, ethanol, and isolate, produced the least amount of variability (adjusted $r^2 = 0.93$). Significant differences in colony areas existed for *A. calvescens* and *A. gallica*, especially by isolate ($p < 0.001$ and $p = 0.001$, respectively). Overall, *A. gallica* produced larger colony areas than *A. calvescens* only at 0.12% GA ($p = 0.008$) (Table 4.4). While *A. calvescens* colony area decreased significantly and progressively from the control to 0.12% to 0.25% GA (937 to 653 to 328 mm^2; $p < 0.001$), *A. gallica* remained unchanged at 0.12% GA and decreased significantly only after the increase from 0.12 to 0.25% GA (798 to 795 to 394 mm^2; $p = 0.001$) (Table 4.4). The addition of ethanol played an important role in the oxidation and metabolism of GA. Significant differences in colony areas were present

within both species when ethanol was added to the medium across all three GA concentrations (Table 4.5).

For RBE colony area, concentration, isolate, and ethanol accounted for most of the variability (adjusted r^2 of 0.96). Colony areas for *A. gallica* increased as RBE concentration increased, and growth at both concentrations (0.12 and 0.25%) was significantly greater than the control treatment (Table 4.4). *Armillaria gallica* once again produced significantly greater colony area values than *A. calvescens* at both RBE concentrations (Table 4.4). At 0.25%, *A. gallica* had a mean colony area more than 1.5 times that of *A. calvescens* (1128 to 629 mm^2; $p < 0.001$) (Table 4.4). In contrast to the earlier RBE treatment (three isolates per species), *A. gallica* produced a significantly higher colony area in only one of the six glucose/ethanol treatments at $p = 0.05$ (data not shown).

Biomass

For GA, the GLM using GA concentration, ethanol, and isolate once again yielded the lowest level of variation (adjusted $r^2 = 0.83$). Interestingly, when GA concentration is used alone, adjusted r^2 is extremely low (0.062) illustrating the effect of individual isolate and ethanol on oxidation of GA. The effect of isolate in the GLM was again very strong for both *A. calvescens* and *A. gallica* ($p < 0.001$ and $p < 0.001$, respectively). Across the three GA concentrations, pair-wise comparisons showed that *A. calvescens* biomass decreased significantly from the control to 0.25%, while *A. gallica* biomass remained statistically unchanged (Table 4.4).

When biomass values on GA medium were analyzed by the presence or absence of ethanol, *A. gallica* biomass values were all statistically larger when ethanol was added. Also, at the 0.25% GA treatment amended with ethanol, *A. gallica* biomass more than tripled compared to the unamended treatment (Table 4.5). The addition of ethanol was significant for *A. calvescens* biomass only at 0.25% GA (Table 4.5). Despite the positive ethanol effect, the only significant differences between species occurred in the control treatment (Table 4.5). However, from the control to 0.12% GA, *A. calvescens* biomass decreased with and without ethanol, while *A. gallica*

biomass increased (Table 4.5). Again, the addition of GA to the medium did not stimulate production of rhizomorphs, as there were no significant differences the number of replicates that produced rhizomorphs between species (data not shown).

For RBE biomass, the GLM using RBE concentration, isolate, and ethanol yielded an adjusted r^2 of 0.95. Despite the strong isolate effect (Figure 4.1), species was a significant predictor variable when used with RBE concentration alone in the GLM ($p = 0.025$). Mean biomass was significantly different between species at the control and 0.25% RBE. *Armillaria calvescens* produced a significantly higher mean biomass in the absence of RBE, while *A. gallica* produced significantly larger mean biomass at 0.25% RBE (Table 4.4). While the two species were not statistically different at 0.12% RBE, they were at 0.25%, where the mean biomass produced by *A. gallica* was over seven times higher than the control treatment (9.2 to 73.1 mg; $p < 0.001$) and almost two times higher than growth by *A. calvescens* (73.1 to 41.2 mg; $p < 0.001$) (Table 4.4). *Armillaria calvescens* biomass, meanwhile, only doubled from the control to 0.25% (19.1 to 41.2 mg; $p < 0.001$) (Table 4.4).

None of the glucose/ethanol treatments were significantly different between the two species (data not shown). None of the isolates for either species produced rhizomorphs in the control treatment (BM only). However, when RBE was added to the BM, rhizomorph development occurred for both species but was significantly higher in isolates of *A. gallica* compared to *A. calvescens*, both in the 0.12% and 0.25% treatments (Table 4.6).

Table 4.4. Mean Colony Area (mm^2) and Biomass (mg) by Gallic Acid (GA) and Root Bark Extract (RBE) Concentration by Species.

% Polyphenol	Colony Area			Biomass		
GA	*A. calvescens*[Z]	*A. gallica*	*p*-value	*A. calvescens*	*A. gallica*	*p*-value
0	937 (396)[a]	789 (214)[a]	0.023	27.7 (25)[a]	15.0 (11)[ab]	0.001
0.12	653 (468)[b]	795 (290)[a]	0.008	22.5 (23)[a]	19.0 (11)[a]	0.277
0.25	348 (438)[c]	374 (324)[b]	0.329	15.0 (19)[b]	12.1 (10)[b]	0.779
p-value	<0.001	<0.001		0.001	0.001	
RBE						
0	489 (227)[a]	414 (147)[a]	0.047	19.1 (18)[a]	9.2 (7)[ab]	<0.001
0.12	734 (241)[b]	973 (326)[b]	<0.001	35.6 (21)[b]	42.8 (23)[b]	0.113
0.25	629 (368)[a]	1128 (538)[b]	<0.001	41.2 (31)[b]	73.1 (44)[c]	<0.001
p-value	0.170	<0.001		<0.001	<0.001	

[Z] n = 54 replicates per treatment. Standard deviations in parenthesis. Values are significantly different between species (row) and within species (column) at $p = 0.05$. Values with different letters are significantly different within species at $p = 0.05$ using the Tukey's HSD test.

Table 4.5. Colony Area (mm^2) and Biomass (mg) Produced by *A. calvescens* and *A. gallica* on Gallic Acid (GA) Medium by Presence of Ethanol.

		Colony Area			Biomass		
% GA	Ethanol	*A. calvescens*[Z]	*A. gallica*	*p*-value	*A. calvescens*	*A. gallica*	*p*-value
0	No	917 (419)[a]	714 (134)[ab]	0.024	22.0 (25)[ab]	9.2 (6)[cd]	0.009
0	Yes	957 (379)[a]	864 (253)[a]	0.320	33.4 (23)[a]	20.7 (11)[a]	0.017
0.12	No	502 (448)[b]	638 (185)[b]	0.022	16.4 (21)[bc]	13.1 (8)[bc]	0.968
0.12	Yes	803 (446)[a]	952 (293)[a]	0.084	28.6 (23)[ab]	24.9 (11)[a]	0.800
0.25	No	230 (442)[c]	172 (202)[c]	0.998	9.6 (19)[c]	5.4 (7)[d]	0.492
0.25	Yes	466 (419)[b]	575 (299)[b]	0.118	20.4 (17)[ab]	18.8 (9)[ab]	0.774
p-value		<0.001	<0.001		<0.001	<0.001	

[Z] n = 27 replicates per treatment. Standard deviations in parenthesis. Values are significantly different between species (row) and within species (column) at $p = 0.05$. Values with different letters are significantly different within species at $p = 0.05$ using the Tukey's HSD test.

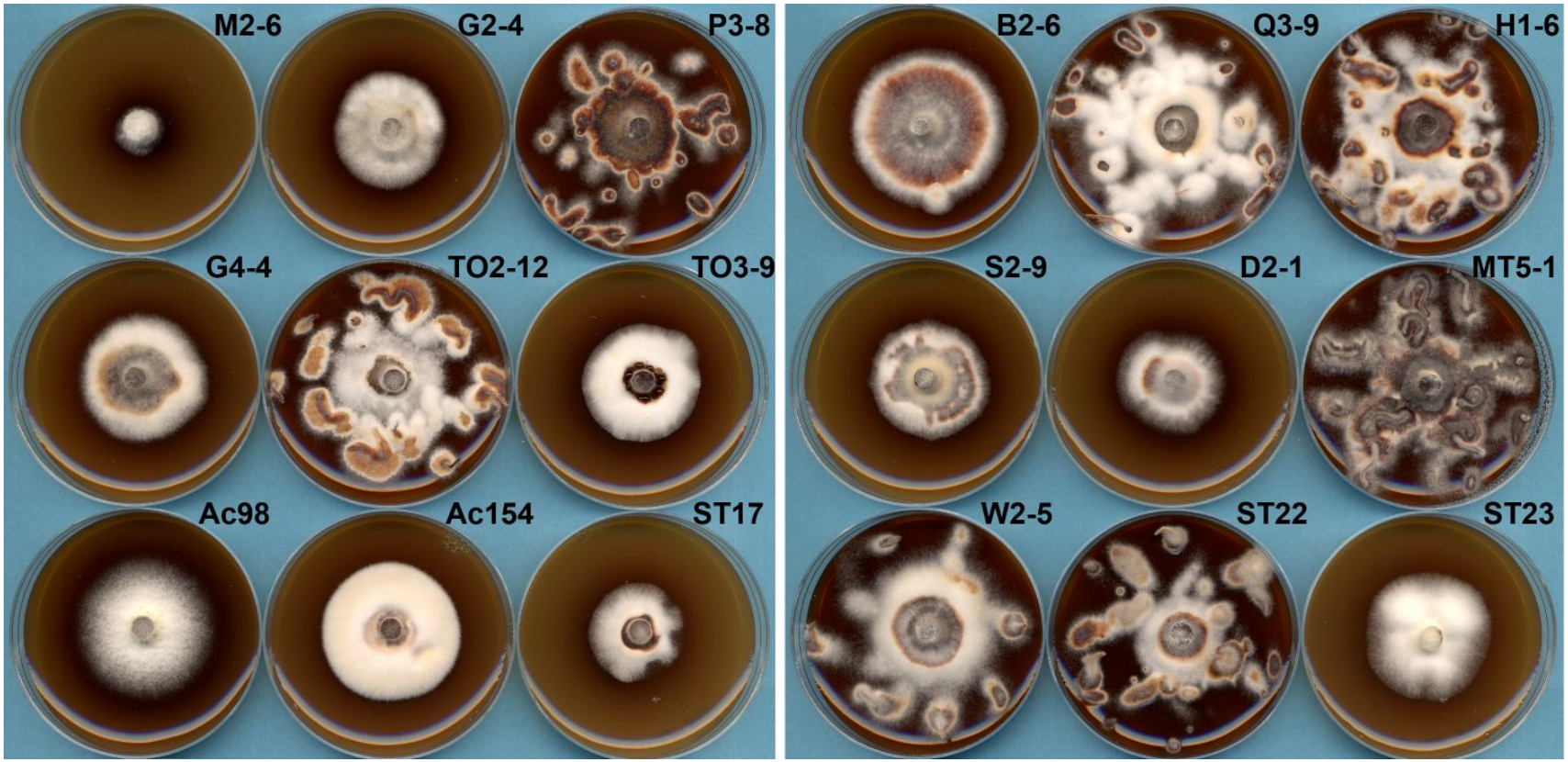

Figure 4.1. Growth of Nine *A. calvescens* (Left), and Nine *A. gallica* Isolates (Right) after 18 Days on *Q. velutina* Root Bark Extracts (0.25% with 0.5% (v/v) of 95% EtOH). Identification Codes for Each Isolate Appear in the Upper Right Corner.

Table 4.6. Rhizomorph Production (Presence or Absence) Between *A. calvescens* and *A. gallica* on Root Bark Extract (RBE) Medium.

% RBE	Rhizomorphs	*A. calvescens*	*A. gallica*	*p*-value
0	absent	54	54	--
0	present	0	0	--
0.12	absent	52	34	<0.001
0.12	present	2	20	<0.001
0.25	absent	45	15	<0.001
0.25	present	9	39	<0.001

Origin of *A. calvescens* Isolates

Five of nine *A. calvescens* isolates originated from northern hardwood forests that had significant components of red oak and/or hemlock, whereas four came from pure northern hardwood stands. When tested by their origin, significant differences between origins occurred in both colony area and biomass (Table 4.7). On both GA and RBE media, mean biomass of isolates originating from forests with oak/hemlock was more than twice as large as the mean biomass of isolates from pure northern hardwoods (Table 4.7). One isolate of *A. calvescens* (Ac98), originating from a forest with red oak, had large biomass values on GA medium that significantly skewed the overall mean of isolates originating from forests with oak/hemlock. When this isolate was removed from the analysis as an outlier, mean biomass decreased from 25.3 to 15.8 mg, but this mean was still significantly larger than the mean biomass of isolates originating from pure northern hardwoods (Table 4.7).

Table 4.7. Colony Area (mm^2) and Biomass (mg) of *A. calvescens* Isolates that Originated from Forests with and Without Significant Components of Oak and/or Hemlock on Gallic Acid and Root Bark Extract Media.

	Gallic Acid				Root Bark Extracts		
Colony Area	0.12	0.25	0.12 & 0.25	0.12 & 0.25[Y]	0.12	0.25	0.12 & 0.25
P-NH[Z]	444 (316)	146 (176)	295 (295)	295 (295)	577 (138)	347 (184)	462 (198)
OH-NH	820 (507)	510 (514)	500 (476)	459 (366)	791 (206)	828 (352)	857 (277)
p-value	0.001	0.001	<0.001	0.009	<0.001	<0.001	<0.001
Biomass							
P-NH	13.7 (12)	7.4 (9)	10.5 (11)	10.5 (11)	23.1 (12)	23.2 (16)	23.1 (14)
OH-NH	29.4 (27)	21.1 (22)	25.3 (25)	15.8 (17)	47.1 (22)	59.3 (35)	50.7 (27)
p-value	0.006	0.005	<0.001	0.031	<0.001	<0.001	<0.001

[Z] (P-NH) isolates that originated from pure northern hardwood forests (beech-birch-maple) and; (OH-NH) isolates that originated from northern hardwood forests with red oak and/or hemlock. Standard deviations in parenthesis.
[Y] excluding Ac98 from analysis as an outlier.

Control vs. 0.12% Treatments

The control treatment (no polyphenols added, six concentrations of glucose/ethanol) was carried out three times in the first phase of the study (three isolates per species, three polyphenol treatments) and twice in the second phase of the study (nine isolates per species, two polyphenol treatments). In each of these five control treatments, *A. calvescens* produced a significantly larger biomass than *A. gallica* (Table 4.8). In addition, within the two control treatments using nine isolates per species, *A. calvescens* produced significantly larger colony areas than *A. gallica* (Table 4.8). These results illustrate that *A. calvescens* exhibited higher growth rates in the absence of polyphenols.

When TA was added to the BM, *A. gallica* biomass values more than doubled from the control to 0.12% TA (20.7 to 42.3 mg; $p < 0.001$), while biomass values for *A. calvescens* were unchanged ($P = 0.965$) (Table 4.8). When GA was added to the BM (three isolates per species), *A. gallica* biomass again increased significantly from the control to 0.12% GA (15.2 to 25.4 mg; $p = 0.006$), while *A. calvescens* biomass decreased significantly from the control to 0.12% GA

(31.6 to 21.2 mg; $p = 0.033$) (Table 4.8). When GA was tested with nine isolates per species, the results were similar but with less significant *P* values. Biomass values for *A. gallica* increased significantly from the control to 0.12% GA ($p = 0.039$), while *A. calvescens* biomass remained statistically unchanged from the control to 0.12% GA ($p = 0.160$) (Table 4.8).

The differences in growth on the *Q. velutina* RBE were the most pronounced. When using only three isolates per species, biomass values for *A. calvescens* more than doubled from the control to 0.12% RBE (17.7 to 40.3 mg; $p = 0.01$), while *A. gallica* biomass increased more than six times from the control to 0.12% RBE (10.6 to 60.8 mg; $p < 0.001$) (Table 4.8). When the number of isolates per species was expanded to nine, *A. calvescens* biomass again increased significantly from the control to 0.12% RBE, but growth for *A. gallica* was again more substantial, producing a mean biomass over four times larger than the control (9.2 to 42.8 mg; $p < 0.001$) (Table 4.8).

Table 4.8. Mean Dry Weights (mg) by Tannic Acid (TA), Gallic Acid (GA), and Root Bark Extracts (RBE) for Control and 0.12% Treatments by Species.

% Polyphenol	*A. calvescens*	*A. gallica*	*p*-value
TA (n = 18)[z]			
0	34.5 (19)	20.7 (13)	0.014
0.12	37.5 (23)	42.3 (13)	0.201
p-value	0.965	<0.001	
GA (n = 18)			
0	31.6 (16)	15.2 (7)	< 0.001
0.12	21.2 (14)	25.4 (13)	0.277
p-value	0.033	0.006	
GA (n = 54)			
0	27.7 (25)	15.0 (11)	0.001
0.12	22.5 (23)	19.0 (11)	0.277
p-value	0.160	0.039	
RBE (n = 18)			
0	17.7 (10)	10.6 (7)	0.012
0.12	40.3 (28)	60.8 (18)	0.008
p-value	0.010	< 0.001	
RBE (n = 54)			
0	19.1 (18)	9.2 (7)	<0.001
0.12	35.6 (21)	42.8 (23)	0.113
p-value	<0.001	<0.001	

[z] n = number of replicates per species within each polyphenol treatment. Treatments with n = 18 were composed of three total isolates per species, while treatments with n = 54 were composed of nine total isolates per species. Standard deviations are in parenthesis.

Discussion

The primary goal of this study was to determine if differences in *in vitro* growth rates of *A. calvescens* and *A. gallica* occurred when the two species were challenged with varying concentrations of polyphenols. Despite the strong isolate effect and large standard deviations within treatments, the results of this study show that *A. gallica* is better at oxidizing and metabolizing polyphenols than *A. calvescens* across the gradient of polyphenol concentrations tested. This was most apparent when comparing results from the controls to those that were

grown on media supplemented with 0.12% polyphenol. Despite the fact that *A. calvescens* produced a significantly larger colony area and biomass in the control treatments, *A. gallica* yielded a larger colony area and biomass when low levels of polyphenolic compounds were added to the medium. In addition, *A. gallica* produced a significantly larger number of rhizomorphs on *Q. velutina* RBE medium compared to *A. calvescens*.

Therefore, it appears that *A. gallica* is better able to oxidize and metabolize polyphenolic compounds present in the root bark of *Quercus* spp. in comparison to *A. calvescens*. This helps to explain why in *Quercus*-dominated forests, where broad arrays of polyphenols are produced in higher concentrations compared to northern hardwood forests, *A. gallica* has a competitive advantage over *A. calvescens*. Exceptions do exist, as some *A. calvescens* isolates were effective at oxidizing and metabolizing polyphenols (Figure 4.1). There were significant differences in both colony area and biomass among *A. calvescens* isolates depending on whether or not they originated from forests with red oak and hemlock, trees that produce polyphenols in higher concentrations than the core northern hardwoods (beech, birch, and maple). This would explain why at times *A. calvescens* can be isolated from forests where oak is present (Blodgett and Worrall 1992b, Brazee and Wick 2009, McLaughlin 2001). The isolates of *A. calvescens* that performed best on *Q. velutina* RBE in this study were isolated from forests with significant components of *Quercus rubra* (P3-8) and *Tsuga canadensis* (TO2-12) in western Massachusetts (Figure 4.1).

The addition of available carbon to the polyphenol media showed that ethanol was the preferred carbon source over glucose. Glucose concentrations were doubled and tripled from the initial concentration (0.5%) in the basal medium with little effect on growth. No significant differences in colony area and biomass were found between the control treatment and the two glucose treatments (G = 0.5% and G = 1.0%) across all three polyphenols tested in this study, regardless of the number of isolates per species. This was surprising because previous work by Wargo (1980, 1983) showed that glucose had a significant effect on *in vitro* growth rates of

Armillaria in the presence of polyphenolic compounds. The addition of ethanol at 0.1 and 0.5% produced highly significant differences in both colony area and biomass, especially within the GA treatments. Wargo (1975, 1981) found root starch levels to be a good overall indicator of tree health, and that oaks with higher levels of root starch were more vigorous when faced with defoliation stress compared to oaks with lower levels of root starch. In contrast, elevated levels of ethanol indicate stress, as ethanol production in the stems of hardwoods increases dramatically when trees are exposed to hypoxic or anoxic conditions (MacDonald and Kimmerer 1991). Low oxygen conditions in roots, and subsequent ethanol production, can be induced in drier soils during seasonal wet periods, especially when trees are defoliated, which leads to reduced water uptake and reduced transpiration (Crawford and Baines 1977, Stephens et al. 1972). Therefore, *Armillaria* may have evolved to respond to increases in ethanol because of the correlation with elevated stress levels, which was clearly shown with wood-boring insects and oak (Montgomery and Wargo 1983). In comparison, elevated levels of simple sugars may not always stimulate growth of *Armillaria* as effectively because high starch levels have been shown to correspond with greater tree vigor against attack from insects and pathogens (Dunn et al. 1987).

In contrast to other published reports of *Armillaria* growth on tannic acid- and gallic acid-supplemented media (Cheo 1982, Shaw 1985), neither of these compounds stimulated rhizomorph development in this study over control treatments. This could be an isolate effect, or could be related to the purity or composition of commercial tannins available today. In addition, concentrations of gallic acid higher than 0.25% were very inhibitory to growth of *A. calvescens* and *A. gallica*, as growth was completely inhibited at concentrations above 0.5%. Shaw (1985) used GA at a 0.5% concentration exclusively, added to the same basal medium that was used in this study. The only difference was that Shaw grew his isolates on 3% MEA, while my isolates were grown on water agar. The carryover of nutrients or the initiation of polyphenol oxidase production on MEA could have allowed *Armillaria* to oxidize GA with greater success than seen here. The *Q. velutina* root bark extracts were highly stimulatory to rhizomorph development

(Table 4.6; Figure 4.1), which is consistent with field observations of *A. gallica* in mixed oak forests (Wargo and Montgomery 1983, Twery et al. 1990).

I have shown that certain *A. calvescens* isolates are effective at oxidizing and metabolizing polyphenols, but this still does not explain why *A. calvescens* is not present in mixed oak forests, (also known as the "oak/hickory" or "central hardwood forest type" (Eyre 1980)). While the focus has been on the polyphenols present in the host tissue, it should not be overlooked that host polyphenols ultimately become incorporated into the soil as trees die and decay. Despite the high similarity to *A. gallica*, *A. calvescens* appears to exhibit reduced plasticity in regards to the range of sites it can occupy. Soil temperature and moisture could play a role in restricting *A. calvescens* to cooler, wetter forest types like northern hardwoods (Blodgett and Worrall 1992b). Likewise, *A. gallica* may grow best in warmer, drier soil types, thereby restricting it from occupying northern hardwood forests above certain latitudes or elevations. Elevation has proven to have a significant role in the distribution of *Armillaria* species across a range of forest types (Keča et al. 2009, Wargo et al. 1987). While *A. gallica* is widely distributed throughout temperate regions in the northern hemisphere (Guillaumin et al. 1993), *A. calvescens* only occurs in the upper Midwest and Appalachian Mountains in eastern North America (Burdsall and Volk 1993). This study sheds some light onto why *A. calvescens* does not occur in mixed oak forests, but there are still several questions that remain unanswered, such as why *A. gallica* is not as abundant in maple-dominated forests as *A. calvescens*, and what site factors are influencing the distribution of these two species.

CHAPTER 5

SEQUENCE-BASED IDENTIFICATION OF *ARMILLARIA CALVESCENS* AND *A. GALLICA* FROM NORTHEASTERN NORTH AMERICA

Introduction

Armillaria calvescens and *A. gallica* are two very closely related biological species of *Armillaria* in North America, according to DNA sequence data (Kim et al. 2006). While *A. gallica* is cosmopolitan across temperate forests in the northern hemisphere (Burdsall and Volk 1993, Guillaumin et al. 1993, Ota et al. 1998), *A. calvescens* is mostly restricted to northern hardwood forests in eastern North America (Bérubé and Dessureault 1989). In addition to being important disturbance agents (Bauce and Allen 1992, Marçais and Bréda 2006), both species are major decay pathogens of northern hardwoods, with significant volume losses of merchantable timber reported from Ontario and Michigan (Nordin 1954, Nordin and Cafley 1950, Ohman 1968).

Based on the morphological and genetic relatedness of these two species, the relatively small geographic range of *A. calvescens*, and results from phylogenetic analyses, researchers have hypothesized that *A. calvescens* is derived from *A. gallica* (Anderson and Stasovski 1992, Piercey-Normore et al. 1998). Separating these two species from one another using both morphological and molecular techniques has proven to be difficult, yet important to fully understand the ecology of *Armillaria* in northeastern North America, where these two species dominate. To illustrate this point, 79% (712/906) of all *Armillaria* isolates collected from hardwood hosts in Ontario, New York and Massachusetts were either *A. calvescens* or *A. gallica* (Blodgett and Worrall 1992a, McLaughlin 2001, Brazee and Wick 2009).

Traditionally, *A. calvescens* and *A. gallica* have been distinguished by pairing unknown haploid or diploid isolates with known haploid tester strains (Anderson and Ullrich 1979). Incompatibility between known biological species would lead to the development of a visually

distinguishable barrage zone between paired isolates. Recently, it was determined by Maphosa et al. (2006) that the elongation factor-1 alpha (*tef1*) gene could be used to differentiate more than 15 species of *Armillaria* from the northern and southern hemisphere. While *A. gallica* was included in this study, *A. calvescens* was not. More recently, *tef1* sequences were used by Hasegawa et al. (2010) to successfully distinguish eight Japanese species of *Armillaria*, and by Antonin et al. (2009) to discriminate isolates in the *A. gallica* / *A. cepistipes* complex in Eastern Europe. In addition to the *tef1* gene, the RNA polymerase II (*rpb2*) gene has proven to be useful in discriminating closely related species of basidiomycete fungi (Matheny et al. 2007). To date, *rpb2* has not been used to examine closely related species of *Armillaria*. The large subunit (nLSU) gene in the rDNA repeat has been used successfully to distinguish species in several genera of basidiomycete fungi (Moncalvo et al. 2000), but provided poor resolution in a phylogenetic study of all 10 North American biological species of *Armillaria* (Kim et al. 2006).

Recently, McLaughlin and Hsiang (2010) developed a PCR-RFLP protocol that distinguishes among all six northeastern North American *Armillaria* isolates by targeting variation in both the IGS-1 and IGS-2 regions of the rDNA repeat. While this protocol affords a level of resolution previously unavailable, it cannot resolve *A. calvescens* from *A. gallica* with 100% accuracy. To address this issue, we generated partial sequences from the *tef1*, *rpb2*, and nLSU genes for 32 total isolates, 12 for each species of *A. calvescens* and *A. gallica*, and additional isolates for each of the remaining four northeastern North American *Armillaria* species. The goals of this study were to: (i) determine if two single-copy genes (*tef1* and *rpb2*) could accurately discriminate between *A. calvescens* and *A. gallica* individually or when concatenated with the nLSU gene, and (ii) determine if these single-copy genes could distinguish all six northeastern North American *Armillaria* species individually or when concatenated as a single data set.

Materials and Methods

Isolates Used and DNA Extraction

Isolates used in this study originated from Massachusetts, along with additional areas from northeastern North America (Table 5.1). For *A. calvescens* and *A. gallica*, 12 isolates per species were used that represent a geographically disparate population within both Massachusetts and northeastern North America. By using 12 isolates for each of these two species, I aimed to reduce the risk of characterizing an isolate that is not representative of the species as a whole. In addition to *A. calvescens* and *A. gallica*, two isolates of each of the remaining four northeastern North American *Armillaria* species (*A. gemina*, *A. mellea*, *A. sinapina*, and *A. solidipes*) were analyzed, for a total of 32 isolates used in this study (Table 5.1). Isolates from Massachusetts were obtained from rhizomorphs and mycelial fans on various hardwood and coniferous hosts as described in chapters 1 and 2 (Table 5.1). These isolates were initially identified using a PCR-RFLP protocol (McLaughlin and Hsiang 2010). All other isolates were previously identified through pairings with haploid tester strains, but were screened with the PCR-RFLP protocol as well. Isolates originating from areas outside of Massachusetts were obtained from the Moscow Forestry Sciences Laboratory, Moscow, ID, and from the Ontario Forest Research Institute, Sault Ste. Marie, Ontario.

Table 5.1. *Armillaria* isolates used to generate partial *tef1, rpb2,* and nLSU sequences.

Armillaria spp.	Overall Number	Species Number	Isolate Code[Z]	Host	Origin	Original Identification
A. calvescens	1	1	M2-6	*Acer saccharum*	Massachusetts	PCR-RFLP
	2	2	G2-4	*Acer rubrum*	Massachusetts	PCR-RFLP
	3	3	G4-4	*Acer saccharum*	Massachusetts	PCR-RFLP
	4	4	P2-1	*Acer saccharum*	Massachusetts	PCR-RFLP
	5	5	P3-8	*Acer saccharum*	Massachusetts	PCR-RFLP
	6	6	TO2-12	*Fagus grandifolia*	Massachusetts	PCR-RFLP
	7	7	TO3-9	*Fraxinus americana*	Massachusetts	PCR-RFLP
	8	8	ST3 (JB56A)	*Acer saccharum*	Quebec	Haploid pairing
	9	9	ST17 (PR-3)	*Acer saccharum*	Michigan	Haploid pairing
	10	10	ST18 (FFC-7)	Hardwood	Michigan	Haploid pairing
	11	11	Ac98	*Acer saccharum*	Ontario	Haploid pairing
	12	12	Ac154	*Acer saccharum*	Ontario	Haploid pairing
A. gallica	13	1	B2-6	*Quercus velutina*	Massachusetts	PCR-RFLP
	14	2	Q3-9	*Quercus velutina*	Massachusetts	PCR-RFLP
	15	3	H1-6	*Quercus velutina*	Massachusetts	PCR-RFLP
	16	4	W2-5	*Acer rubrum*	Massachusetts	PCR-RFLP
	17	5	S2-9	*Quercus velutina*	Massachusetts	PCR-RFLP
	18	6	D2-1	*Quercus alba*	Massachusetts	PCR-RFLP
	19	7	MT5-1	*Pinus rigida*	Massachusetts	PCR-RFLP
	20	8	ST22 (EL-1)	*Betula papyrifera*	Michigan	Haploid pairing
	21	9	ST23 (MA-1)	*Acer saccharum*	Wisconsin	Haploid pairing
	22	10	Aga33	*Prunus nigra*	Ontario	Haploid pairing
	23	11	Aga81	*Acer rubrum*	Ontario	Haploid pairing
	24	12	Aga235	*Acer saccharum*	Ontario	Haploid pairing

A. gemina	25	1	M3-3	*Fagus grandifolia*	Massachusetts	PCR-RFLP
	26	2	TO4-15	*Tsuga canadensis*	Massachusetts	PCR-RFLP
A. sinapina	27	1	P2-7	*Acer saccharum*	Massachusetts	PCR-RFLP
	28	2	TO3-7	*Tsuga canadensis*	Massachusetts	PCR-RFLP
A. solidipes	29	1	MS2-11	*Pinus rigida*	Massachusetts	PCR-RFLP
	30	2	W1-9	*Tsuga canadensis*	Massachusetts	PCR-RFLP
A. mellea	31	1	Am115	Hardwood	Ontario	Haploid pairing
	32	2	E2-14	*Pinus strobus*	Massachusetts	PCR-RFLP

[z] Additional isolate codes used previously are listed in parenthesis.

PCR and DNA Sequencing

Primer names and sequences, sequenced product sizes, and sources are listed below (Table 5.2). PCR was performed with 10X Platinum Taq reaction buffer and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). All PCR reactions were performed using an Eppendorf Mastercycler gradient thermalcycler (Eppendorf, Westbury, NY). Prior to sequencing, PCR products were purified using ExoSAP-IT (USB, Cleveland, OH). Isolates were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA) at the Genomics Resource Laboratory, University of Massachusetts, Amherst.

Table 5.2. Primers Used to Amplify and Sequence *tef1*, *rpb2*, and nLSU Genes.

Gene	Primer Names	Primer Sequence 5' to 3'	Source
tef1	EF595F	CGTGACTTCATCAAGAACATG	Kauserud and Schumacher (2001)
	EF1160R	CCGATCTTGTAGACGTCCTG	
rpb2	RPB2-6F	TGGGGKWTGGTYTGYCCTGC	Liu et al. (1999)
	RPB2-7R	CCCATWGCYTGCTTMCCCAT	
nLSU	LR0R	ACCCGCTGAACTTAAGC	Rehner and Samuels (1994)
	LR5	TCCTGAGGGAAACTTCG	Hopple and Vilgalys (1999)

Phylogenetic Analysis

Because *A. calvescens* is known only to occur in eastern North America and is believed to have descended from *A. gallica* (Anderson and Stasovski 1992, Piercey-Normore et al. 1998) I used sequences of *A. gallica* that originated from northeastern North America only. *Armillaria gallica* isolates that have originated from western North America, Europe, and Asia, were excluded from the analysis to perform a stricter phylogenetic analysis between only these two biological species.

Sequences were manually edited using BioEdit v. 7 (Hall 1999) and aligned using the online platform MAFFT v. 6 using the G-INS-i option (Katoh 2008). Phylogenetic analyses of partial *tef1*, *rpb2*, nLSU, and concatenated (*tef1* + *rpb2* + nLSU) sequences were performed using MEGA v. 5 (Tamura et al. 2011) with the following steps taken. Phylogenetic reconstructions were carried out separately for each gene region and with concatenated sequences using both the maximum-likelihood (ML), and the maximum parsimony (MP) methods. The best-fit ML nucleotide substitution model was chosen based on log-likelihood values generated from the data within MEGA; the model with the highest log-likelihood (-*lnL*) score was chosen to describe the substitution pattern. For all four data sets, a general time reversible (GTR) (Felsenstein 2004) substitution model was chosen. For the *tef1* data set, a GTR substitution model assuming an estimated proportion of invariant sites (+I) and 5 gamma-distributed rate categories (+G) was used to account for rate heterogeneity across sites. For the *rpb2* and concatenated data sets, a GTR+G substitution model was used, while for the nLSU data set, a GTR substitution model with uniform distribution rates was chosen. The gamma shape parameter was estimated directly from the data within MEGA.

For the MP analysis, the phylogeny was reconstructed using the Close-Neighbor-Interchange (CNI) heuristic search method (level 3) to find the most parsimonious trees. The initial trees were obtained with the random addition of sequences (10 replicates). Confidence for internal branches using both the ML and MP methods was obtained through bootstrap analysis (1,000 replicates) (Felsenstein 1985), retaining clades compatible with the 50% majority rule in the bootstrap consensus tree. Bootstrap support (BS) values greater than 70% were considered significant in this study. Nucleotide site information and ML model parameters are listed below in Table 5.3.

For the analysis of *tef1* sequences, the following *Armillaria* isolates described previously in Maphosa et al. (2006) were included (with GenBank accession numbers in parenthesis): *A. cepistipes* CMW 6909 (DQ435630); *A. gallica* CMW 3717 (DQ435629) and CMW 6901

(DQ435628); *A. gemina* CMW 6888 (DQ435626); *A. mellea* CMW 3956 (DQ435632); *A. nabsnona* CMW 6905 (DQ435631); and *A. solidipes* CMW 3162 (DQ435625). *Schizophyllum commune* (X94913) was used as an outgroup isolate.

Table 5.3. Summary of Datasets Used to Distinguish Six *Armillaria* Species Using *tef1, rpb2,* and nLSU Sequences.

	Dataset			
Characteristics	*tef1*	*rpb2*	nLSU	concatenated
Specimens analyzed	40	32	32	32
Nucleotide sites				
Total	511	700	907	2118
Conserved	433	627	877	1937
Variable	78	73	30	181
Parsimony-informative	71	65	29	165
Final dataset	475	650	871	1998
ML Phylogenetic Reconstruction				
Model Used	GTR+G+I	GTR+G	GTR	GTR+G
ML score	-1421.809	-1133.370	-1336.805	-3596.633
Gamma shape	1.372	1.344	--	0.501
Proportion of invariable sites	0.29	--	--	--

Results

tef1 Sequences

The phylogenetic analyses using ML and MP methods of partial *tef1* sequences both produced six monophyletic clades that distinguished all six northeastern *Armillaria* species from one another (Figure 5.1). However, only two species (*A. calvescens* and *A. sinapina*) were supported by BS greater than 95% using both methods. The *tef1* amplicon contained five single nucleotide polymorphisms (SNPs) that were able to distinguish all 24 *A. calvescens* and *A. gallica* isolates used for comparison. Two SNPs occurred in the first intron at bp 234 and 237, while the remaining three SNPs occurred in the second intron at bp 297, 361, and 370 (Table 5.4). None of the original species identifications made by haploid tester pairings or PCR-RFLP were refuted by

tef1 sequences. Isolates of *A. gallica* grouped within a monophyletic clade, and while BS values from the ML analysis were robust (76%), BS values from the MP analysis were not significant (59%) (Figure 5.1). In addition, there was no grouping of *A. gallica* isolates by region of origin (Figure 5.1). Meanwhile, the *A. calvescens* clade was strongly supported by the *tef1* data set (98/98% BS; ML/MP) (Figure 5.1). Ten of the 12 *A. calvescens* isolates formed a subclade with 98/98% BS values, while two remaining *A. calvescens* isolates, which originated from Ontario (Ac98) and Massachusetts (G4-4), formed a separate subclade with 86/99% BS.

When pairwise genetic distances were generated between Ac98 & G4-4 and (i) the 10 isolate subclade of *A. calvescens*, (ii) all 12 isolates of *A. gallica*, and (iii) two *A. sinapina* isolates, results were similar for *A. calvescens* and *A. gallica* (Table 5.5). Among the remaining four species, the *A. sinapina* (100/99%), *A. gemina* (78/81%), *A. solidipes* (75/80%) and *A. mellea* (90/99%) clades were all supported by significant BS values (1,000 replicates) (Figure 5.1).

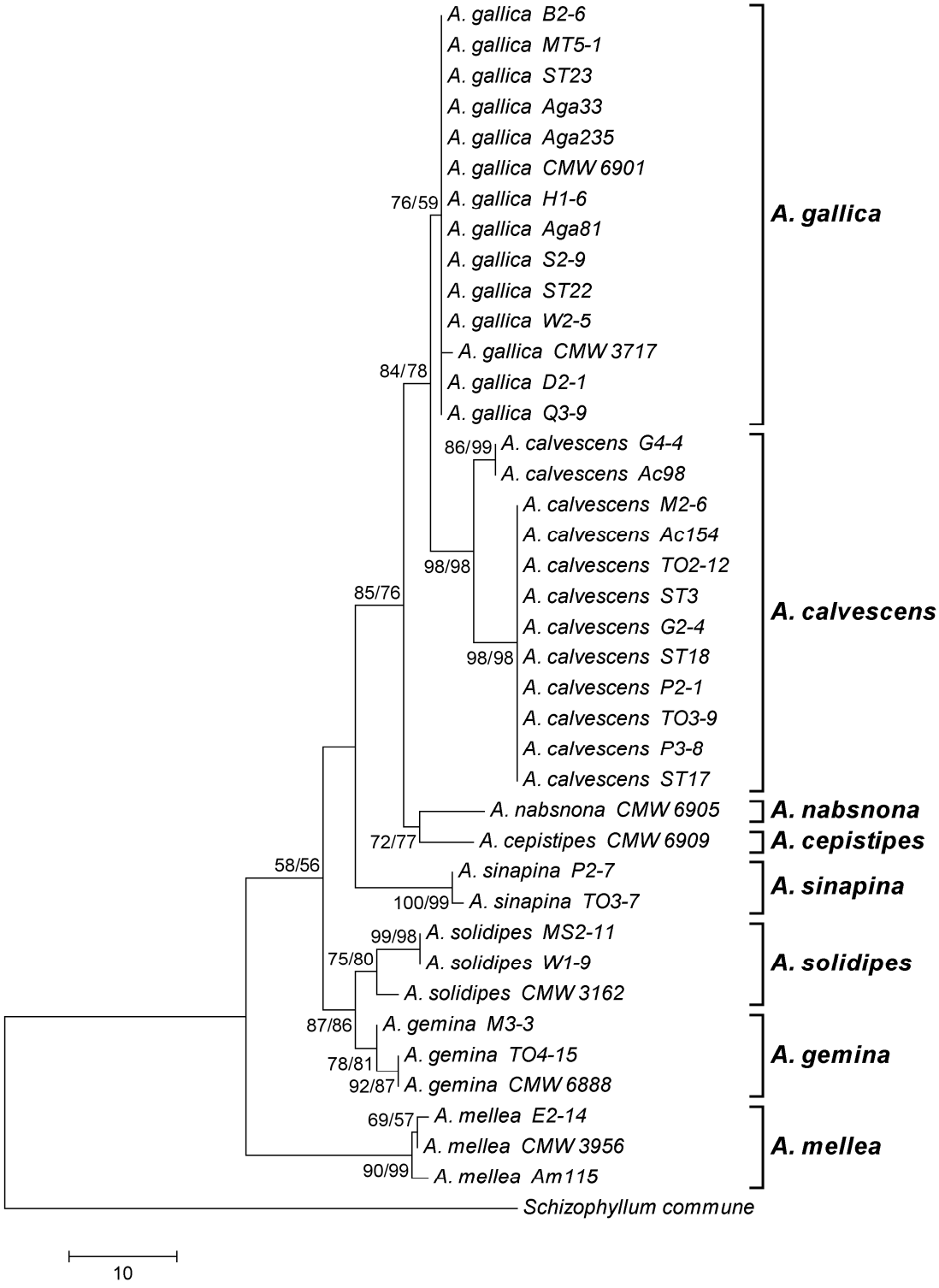

Figure 5.1. Maximum Parsimony (MP) Bootstrap Consensus Tree of *tef1* Sequences (n = 40) with Gaps and Missing Data Excluded from the Analysis. Bootstrap Support Percentages for Maximum Likelihood and MP (1,000 replicates) with Values Greater than 50% are Listed Next to the Branches.

Table 5.4. Location and Description of Single Nucleotide Polymorphisms Present in *tef1* Sequences between *A. calvescens*, *A. gallica*, and *A. sinapina*.

Armillaria Species	No. of Isolates[z]	*tef1* position number				
		234	237	297	361	370
A. calvescens	12	C	T	T	A	A
A. gallica	12	T	C	C	C	G
A. sinapina	2	C	C	C	C	G

[z] Number of individual isolates examined in this study.

Table 5.5. Pairwise Genetic Distances Within *tef1* and Concatenated Data Sets between *A. calvescens* Isolates Ac98 and G4-4 and *A. calvescens*, *A. gallica*, and *A. sinapina*.

Data Set	Genetic Distances[z]	
	Within species	Ac98 & G4-4
tef1		
A. calvescens (n = 10)	0	0.013
A. gallica (n = 12)	0-0.002	0.015-0.017
A. sinapina (n = 2)	0.002	0.048-0.050
Concatenated		
A. calvescens (n = 10)	0	0.003
A. gallica (n = 12)	0	0.003
A. sinapina (n = 2)	0.001	0.012

[z] Pairwise genetic distances were computed by using the maximum composite likelihood method (Tamura et al. 2004) with bootstrap support (1,000 replicates) and 5-gamma rate substitution rates.

rpb2 and nLSU Sequences

The partial *rpb2* sequences between domains six and seven failed to resolve all six northeastern species of *Armillaria*. *Armillaria calvescens*, *A. gallica*, and *A. sinapina* grouped together with 99/95% BS values (Figure 5.2). *Armillaria calvescens* and *A. gallica* could not be distinguished from one another, yet *A. sinapina* did form a monophyletic clade with low BS (63/63%). There were no SNPs present among *rpb2* sequences between *A. calvescens* and *A. gallica*. However, *rpb2* was able to distinguish the *A. gemina* and *A. solidipes* isolates used in this study, with both species grouped into monophyletic clades with strong BS (99/100% and

99/99%, respectively). *Armillaria mellea* isolates also formed a strongly-supported clade (99/100% BS), basal to the other five species.

The nLSU, as expected, also failed to resolve the six tested *Armillaria* species from one another. Five of the six species tested (*A. calvescens, A. gallica, A. gemina, A. sinapina,* and *A. solidipes*) grouped within a single clade with 99/99% BS, with *A. mellea* being the only species distinguished by nLSU sequences (Figure 5.3). There were only three variable sites between all 24 isolates of *A. calvescens* and *A. gallica*, illustrating the highly conserved nature of the nLSU gene.

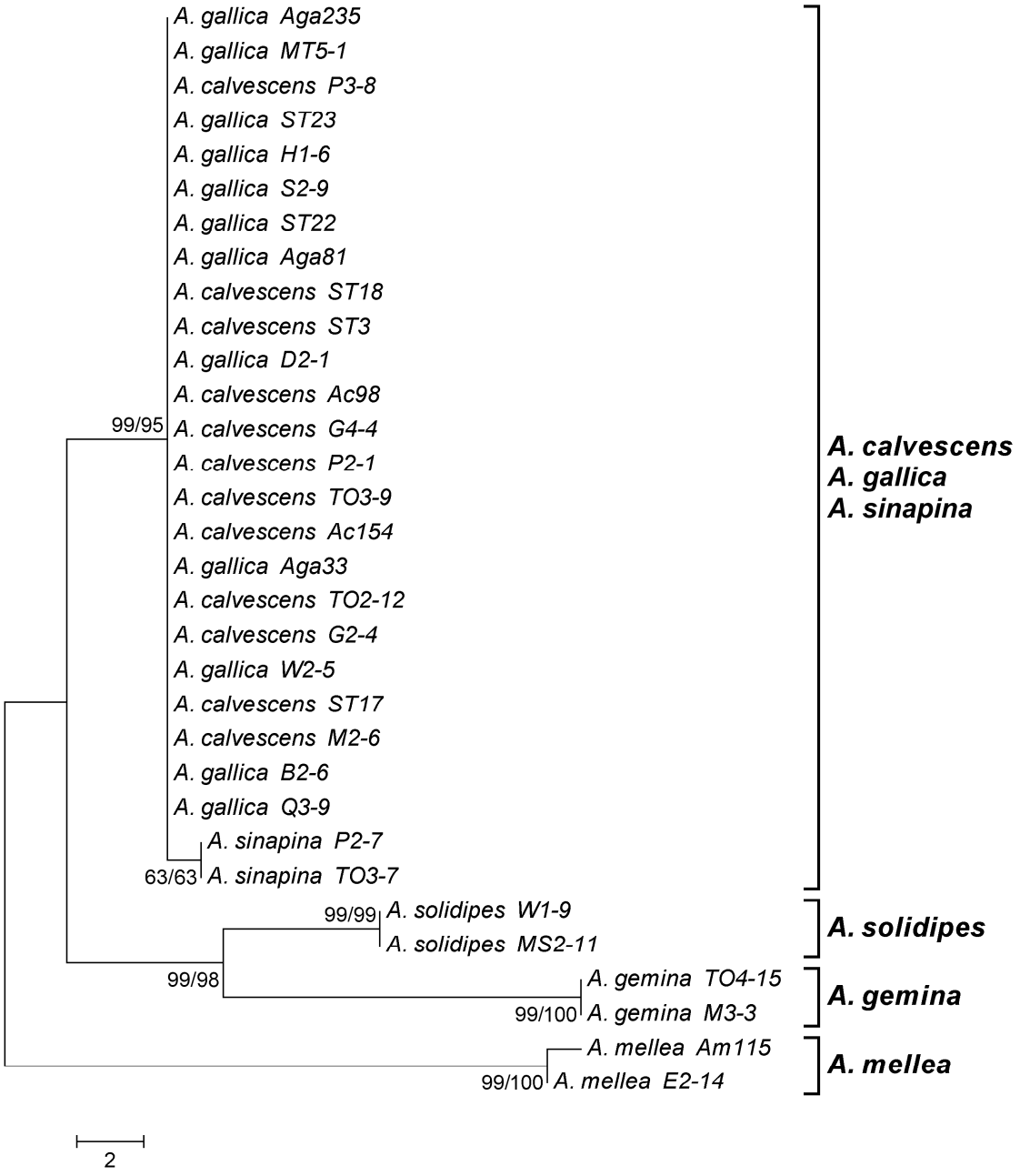

Figure 5.2. Maximum Parsimony (MP) Bootstrap Consensus Tree of *rpb2* Sequences (n = 32) with Gaps and Missing Data Excluded from the Analysis. Bootstrap Support Percentages for Maximum Likelihood and MP (1,000 replicates) with Values Greater Than 50% are Listed Next to the Branches.

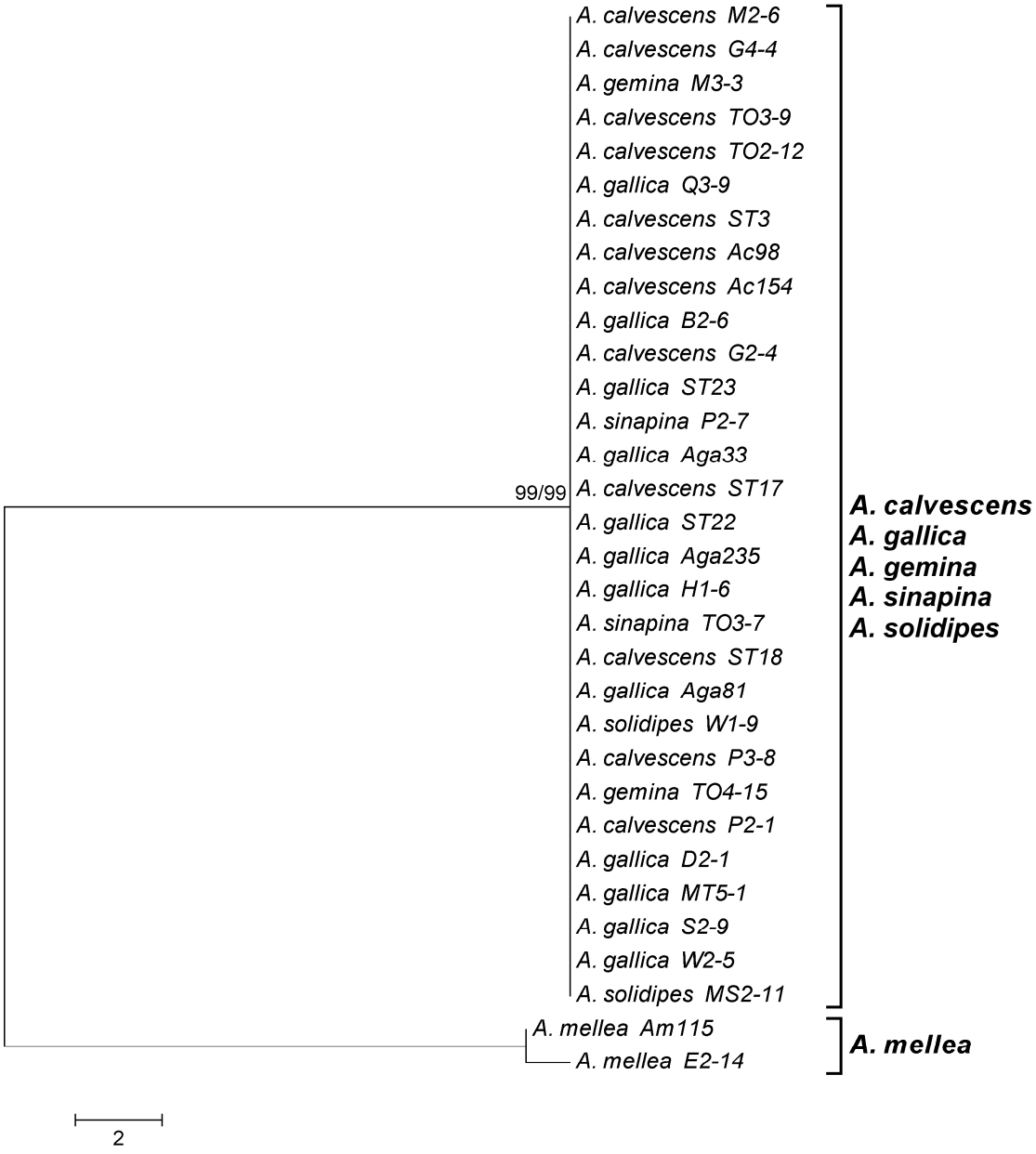

Figure 5.3. Maximum Parsimony (MP) Bootstrap Consensus Tree of nLSU Sequences (n = 32) with Gaps and Missing Data Excluded from the Analysis. Bootstrap Support Percentages for Maximum Likelihood and MP (1,000 Replicates) with Values Greater Than 50% are Listed Next to the Branches.

Concatenated Sequences

Results from the ML and MP analysis using concatenated sequences corroborated results generated from *tef1* sequences. All six northeastern *Armillaria* species grouped into monophyletic clades with greater than 98% BS for each species except *A. gallica*, which generated 95% BS in the ML analysis but only 76% BS in the MP analysis (Figure 5.2). Within the *A. calvescens* clade, a subclade of two isolates (G4-4 and Ac98) was once again present, driven mostly by the sequence diversity present in *tef1* sequences (Figure 5.2). Isolates of *A. calvescens* and *A. gallica* clustered in monophyletic clades with high BS (94/96%), verifying the biological species concept that initially designated these two species from one another. The remaining four species (*A. gemina*, *A. mellea*, *A. sinapina*, and *A. solidipes*) were grouped into strongly-supported monophyletic clades with BS values ≥ 99% (Figure 5.2).

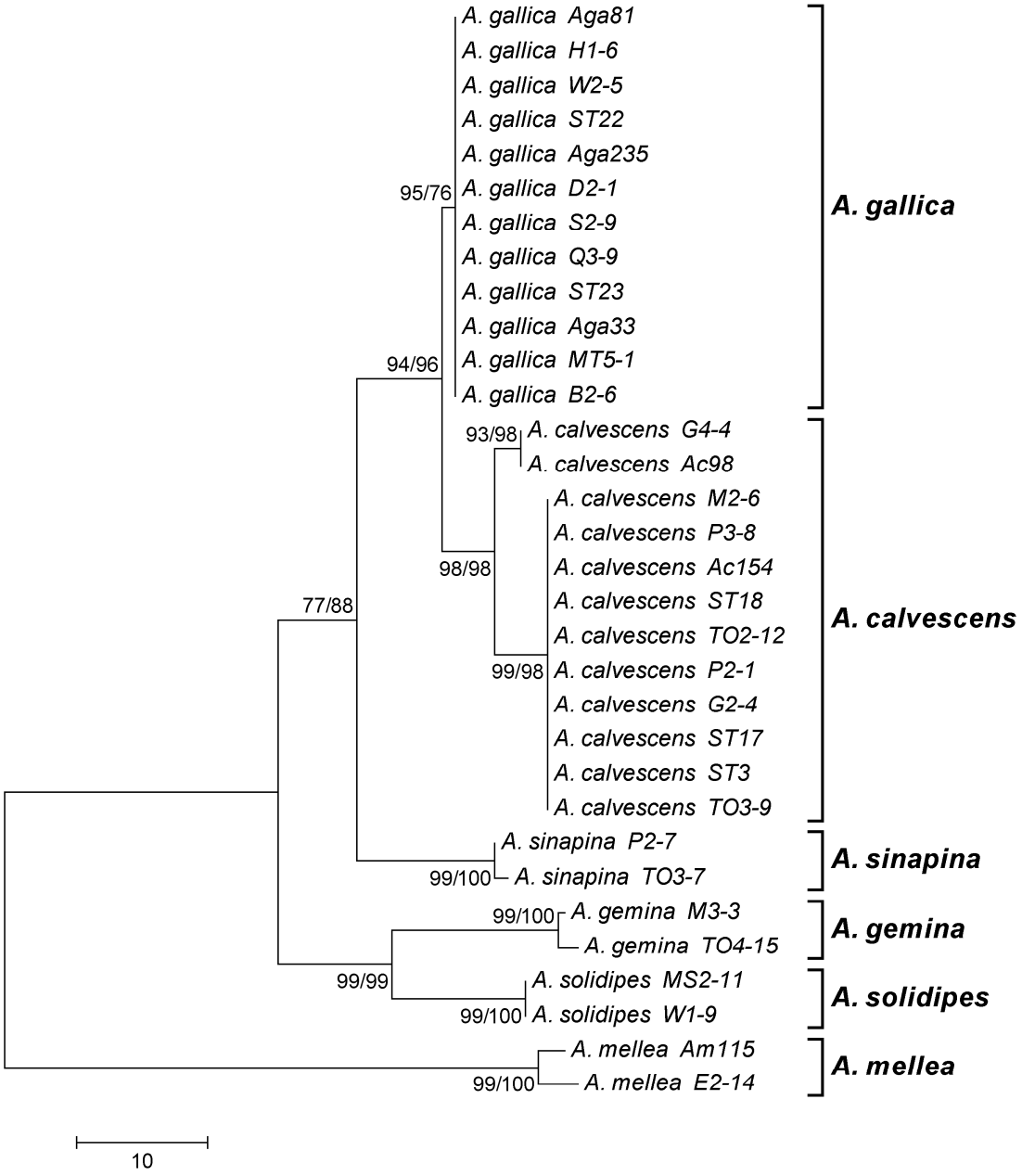

Figure 5.4. Maximum Parsimony (MP) Bootstrap Consensus Tree of Concatenated *tef1*, *rpb2*, and nLSU Sequences (n = 32) with Gaps and Missing Data Excluded from the Analysis. Bootstrap Support Percentages for Maximum Likelihood and MP (1,000 Replicates) with Values Greater than 50% are Listed Next to the Branches.

Discussion

The results from this study show that *tef1* can discriminate all six northeastern *Armillaria* species from one another, and provides another level of testing to verify the identity of field isolates collected from northeastern North America. In addition, *tef1* sequences can distinguish the closely-related biological species, *A. calvescens* and *A. gallica*, a conclusion reached using 12 isolates for each species from a set that originated from a geographically disparate population. While it was not surprising that the phylogenetic reconstruction using nLSU could not discriminate *A. calvescens* from *A. gallica*, it is interesting that the region between domains six and seven of *rpb2* was unable to resolve these two species. Our results show that this region of *rpb2* has utility for discriminating *Armillaria* species in areas of the northeast where *A. calvescens* is absent (see Chapter 2), but the lack of resolution exhibited in this study between the *A. calvescens* / *A. gallica* / *A. sinapina* group should be carefully considered before species identification is attempted with *rpb2*.

Within the *tef1* sequences, the discovery of two *A. calvescens* isolates that formed a separate subclade with significant BS values and that exhibit genetic distances intermediate between the remaining *A. calvescens* isolates and the *A. gallica* clade may support the hypothesis that these two isolates are natural hybrids. However, the *A. calvescens* isolates in question (Ac98 and G4-4) contained all five SNPs that distinguish *A. calvescens* from *A. gallica* and BS values supporting the *A. calvescens* clade were very strong (98/98%; ML/MP). Subclades were also present within the *A. gemina*, *A. solidipes*, and *A. mellea* clades, illustrating the intraspecific variation present in this region of *tef1*. Also, Hasegawa et al. (2010) detected the presence of subclades among *tef1* sequences in several species of *Armillaria* from Japan. Therefore, I believe that *A. calvescens* isolates Ac98 and G4-4 are not cryptic species. Low levels of interfertility between single-spore isolates of *A. sinapina*, *A. cepistipes*, and NABS X has been demonstrated

in vitro (Banik and Burdsall 1998, Bérubé et al. 1996), but natural hybrids of *Armillaria* have never been detected during the identification of field isolates.

It has been shown previously that sequences from the nLSU, IGS-1, and ITS + 5.8S regions within the ribosomal DNA cluster cannot resolve *A. calvescens* from *A. gallica* (Kim et al. 2006). While microscopic features of the fruiting bodies can be used to differentiate these two species (Bérubé and Dessureault 1989), the presence of basidiocarps is unreliable and occurs only for brief periods in the autumn months. Mating tests are difficult to rely on as a sole means of species identification, because fresh haploid testers are constantly required to create the "barrage" zone between incompatible biological species, which even under the best conditions can produce ambiguous results (Baumgartner et al. 2011).

While *A. calvescens* is more common in northern hardwood (beech-birch-maple) forests and *A. gallica* is most abundant in mixed oak forests, there is a large area of range overlap between these two species (Blodgett and Worrall 1992, Brazee and Wick 2009, McLaughlin 2001). The results from this study also corroborates the initial identifications made using the PCR-RFLP protocol designed by McLaughlin and Hsiang (2010), which utilizes sequence diversity in the IGS-2 region to ultimately discriminate *A. calvescens* from *A. gallica*.

The remaining four northeastern *Armillaria* species grouped within monophyletic clades, further confirming the utility of *tef1* in accurately characterizing *Armillaria* species, a claim supported by other studies (Maphosa et al. 2006, Hasegawa et al. 2009, Antonin et al. 2009). This is important for distinguishing between *A. solidipes* and *A. gemina*, which produce fruiting bodies that cannot be distinguished morphologically (Bérubé and Dessurault 1989), and have a very close genetic relationship to one another (Anderson and Stasovksi 1992, Piercey-Normore et al. 1998). While *A. gemina* is generally considered to be uncommon throughout its range and has been collected mostly from hardwood hosts, *A. gemina* has been collected from a coniferous host (eastern hemlock) in stands where *A. solidipes* co-occurs in southern New England (see Chapter 2).

In this study, *tef1* sequences grouped *A. cepistipes* and *A. nabsnona* closely to one another (72/77% BS; Figure 5.1) which is in agreement with results from Maphosa et al. (2006). These two species are not known to occur in eastern North America, and grouped more closely to *A. gallica* than *A. sinapina* in our analysis of *tef1* sequences (Figure 5.1). Previous studies of interfertility among isolates of *A. cepistipes* (also described as NABS XI) have used *A. sinapina* for comparison but not *A. gallica* (Banik and Burdsall 1998, Bérubé et al. 1996). Regardless, our results show the potential utility of *tef1* sequences in discriminating western North American populations of *Armillaria* as well as northeastern populations.

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