

ABUNDANCE OF *ARMILLARIA* WITHIN OLD-GROWTH EASTERN HEMLOCK STANDS IN SOUTH-CENTRAL PENNSYLVANIA

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Abstract—In early summer 2002, 329 soil-sampling pits were dug within an old-growth, eastern hemlock (*Tsuga canadensis* [L.] Carrière) stand in south-central Pennsylvania recently infested with the hemlock woolly adelgid (*Adelges tsugae* Annand). For comparison, 199 similar pits were dug in an adjacent hardwood stand. Rhizomorphs of *Armillaria* (Fr: Fr) Staude were recovered from 8.5 percent of the sample pits in the old-growth hemlock stand, and from 6.0 percent of the pits within the hardwood stand. Average lengths of rhizomorphs per sample pit were 1.8 and 1.6 cm within the hemlock and hardwood stand, respectively. For only pits that contained rhizomorphs, the average length of rhizomorphs per sample pit was 21.5, and 26.6 cm within the hemlock and hardwood stand, respectively. Based on IGS-1 rDNA sequence data, recovered *Armillaria* were either *A. ostoya* or within the *A. gallica/calvescens/sinapina* group. There were no readily apparent differences in *Armillaria* between the two stands.

INTRODUCTION

Root disease caused by *Armillaria* is one of the major problems on tree species worldwide (Kile and others 1991). Infection by *Armillaria* often occurs on hosts weakened by some predisposing factor that reduces overall vigor, compromising host defenses (Wargo and Harrington 1991). Defoliation caused by insects is one of the most common predisposition factors associated with *Armillaria* infections in eastern North America (Horsley and others 2002, Marçais and Wargo 2000, Twery and others 1990). Eastern hemlock is declining throughout much of its range due to infestations by the introduced hemlock woolly adelgid. Some infested hemlock stands have experienced 95 percent tree mortality in the overstory and 90 percent sapling mortality (Orwig and Foster 1998). However, in some cases hemlock mortality may be restricted to 5 percent in infested stands. The reason for this great range in mortality is unknown, but variability in species of *Armillaria* among infested stands may play a role. *Armillaria* species that are more virulent or aggressive may cause greater mortality to adelgid-stressed hemlocks. In contrast, *Armillaria* species that are predominantly saprophytic, or that infect mainly hardwoods, may cause little mortality in an infested hemlock stand. Thus, correct identification of the species of *Armillaria* in a stressed forest may greatly influence forest management plans and procedures. However, precise identification of *Armillaria* species is often problematic (Perez-Sierra and others 2000).

Confusion has surrounded the taxonomy of the genus *Armillaria* for more than a century (Rishbeth 1982). The genus is probably composed of about 40 species worldwide (Watling and others 1991). In North America, *Armillaria mellea sensu lato* is considered to be comprised of ten genetically isolated, biological species (North American Biological Species, NABS) (Anderson 1986, Anderson and Ullrich 1979, Banik and others 1996, Morrison and others 1985). *A. mellea* is the most divergent North American species of *Armillaria* (Anderson and Stasovski 1992) based not only on phylogenetic data but also on morphology (Bérubé and Dessureault 1988) and the lack of clamp connections (Korhonen 1978). *A. ostoyae* and *A. gemina* are more closely related to one another than to the other species of *Armillaria* (Anderson and Stasovski 1992). The remaining species, *A. sinapina*, *A. gallica*, *A. calvescens*, *A. nabsnona*, *A. cepistipes*, and NABS X are considered to be closely related to one another (Anderson and Stasovski 1992). These NABS species-group concepts have been generally supported by other research groups (Coetzee and others 2003, Frontz and others 1998, Piercey-Normore and others 1998, Terashima and others 1998).

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However, there has been increased interest in use of molecular techniques to identify *Armillaria* species (Perez-Sierra and others 2000). The most widely used molecular technique for species identification has been RFLP analysis of the IGS-1 region of the rDNA, pioneered by Anderson and Stasovski (1992). They reported that two groups of *Armillaria* (1. *A. gemina*, *A. ostoyae*, and *A. borealis*; and 2. *A. sinapina*, *A. calvescens*, *A. lutea*, *A. cepistipes*, NABS IX, and NABS X) were more closely related to each other than to *A. mellea* and *A. tabescens*. The two groups could be separated from one another, but interrelationships within the groups were unclear.

The objectives of this research were: 1) determine the abundance of *Armillaria* within two different forest stands in south-central Pennsylvania; and 2) to use IGS-1 sequence data to identify species of *Armillaria* within each stand. One stand was an old-growth, eastern hemlock stand recently infested by the hemlock woolly adelgid, in which we were conducting other research. For comparison, a second-growth, mixed-species hardwood stand that abuts the old-growth hemlock stand was also sampled.

MATERIALS AND METHODS

Study Area

The study area (40°15'N, 77°37'W) is within and adjacent to an old-growth stand (Hemlocks Natural Area, HNA), located within a steep ravine in Perry County, PA, U.S.A. (Pennsylvania Department of Conservation of Natural Resources 1998). Soils are classified as extremely-stony, sandy-loam that are deep, well drained, and are extremely- to strongly-acidic; stones and rocks 0.5-2 m or more in diameter cover about 50 percent of the soil surface (U.S. Department of Agriculture, Soil Conservation Service 1986). Many hemlocks in the stand have been dated, and are 400-500 years old (Cook 1982). The hemlocks have been infested by the hemlock woolly adelgid since the mid-1990s, and hemlock mortality has recently occurred. In addition to eastern hemlock, the HNA contains several old-growth hardwood tree species. The most common associated hardwoods include yellow birch (*Betula alleghaniensis* Britton), sweet birch (*Betula lenta* L.), northern red oak (*Quercus rubra* L.), red maple (*Acer rubrum* L.), and chestnut oak (*Q. prinus* L.).

Although sample sites were initially established within the old-growth HNA, we wanted to compare *Armillaria* species composition and abundance in an adjacent mixed-species hardwood stand. This hardwood stand abuts the HNA in the same ravine, and was likely originally a hemlock stand. However, this stand had been harvested at various times, and is now a second- or third-growth hardwood stand consisting of northern red oak, white oak (*Q. alba* L.), red maple, sugar maple (*A. saccharum* Marsh.), and black gum (*Nyssa sylvatica* Marsh.), as well as a small percentage of young eastern hemlock trees.

Abundance of Soil-Borne Rhizomorphs

If abundant, soil-borne rhizomorphs would serve as an efficient source of material for isolate collection and species identification. Also, a measure of rhizomorph abundance would allow estimation of potential inoculum pressure from *Armillaria* in a forest stand (Wargo and others 1987). However, due to the extremely rocky nature of the soil in the study area, soil samples could not be collected in a systematic manner. Therefore, in areas where surface soil could be sampled due to lack of rocks, 329 soil-sampling points were randomly established in the old-growth HNA, and 199 points randomly established in the adjacent hardwood stand during the early summer of 2002. Wargo and others (1987) reported that 94 percent of *Armillaria* rhizomorph density was contained in the organic soil horizon (fermentation and humus layers) of the forest floor, as opposed to the underlying mineral soil. Therefore, the unincorporated surface litter was removed at each sampling point, and a 15 x 15 x 15 cm cube of soil, consisting mainly of organic matter, was extracted from the forest floor. At times the lower portion of the samples contained a small amount of the upper mineral horizon.

Samples were placed in plastic bags and returned to the lab where they were evaluated for presence or absence of *Armillaria* rhizomorphs. Abundance (presence or absence) of rhizomorphs in each sample was recorded. When present, the total length of rhizomorphs in the sample was measured to the nearest 0.1 cm. The density of rhizomorphs in each stand was calculated by dividing total rhizomorph length

(cm) by number of soil pits. Significant differences ($P = 0.05$) in abundance (percent samples containing rhizomorphs) and density (cm of rhizomorphs per sample) between the two types of stands were evaluated using the two-sample T-test (abundance) and the non-parametric (density) Mann-Whitney test (Zar 1999).

Collection of Isolates for Identification

Initial data from the soil pits revealed that *Armillaria* rhizomorphs were very scarce within the soil of the study area (see Results), and that pit excavation represented an inefficient method to collect isolate material for culturing. Therefore, *Armillaria* rhizomorphs collected from the soil pits were not used for culturing isolates, but only to estimate rhizomorph abundance in the soil. However, during location and excavation of soil pits, we observed that *Armillaria* rhizomorphs commonly occurred on trees, logs, and stumps within the study area. Therefore, during the late summer and fall 2002, and summer 2003, samples of *Armillaria* were collected from tree material during a walk-through the entire study area, including both the HNA and adjacent hardwood stand.

Initial observations revealed very little *Armillaria* on roots and root collars of standing live hemlocks. *Armillaria* was more abundant on roots and root collars of hemlocks that were severely stressed by the hemlock wooly adelgid or that had died from various causes. Therefore, in order to collect a sufficient number of isolates for species identification, sampling was biased towards severely defoliated or dead material. For standing dead trees or snags, bark was removed from the root collar and 2 to 3 large buttress roots were excavated for 1 to 2 m from the trunk with a soil mattock and a soil knife. On stumps and fallen trees, only the root collar was examined. Logs were examined by removing the bark at the butt end.

Armillaria rhizomorphs or mycelial fans were removed from the host material, placed in labeled plastic bags, and returned to the laboratory. Decayed wood was not sampled. A total of 72 samples were collected from standing dead trees, standing live trees, fallen trees or logs, and stumps in the old-growth HNA; 30 similar samples were collected in the adjacent, second-growth hardwood stand. Sample numbers in each stand were related to availability of suitable substrate material.

Isolation Techniques

Rhizomorphs were washed with tap water, surface sterilized with 10 percent Clorox for 5 minutes, rinsed with sterile water, dipped in 95 percent ethanol, flamed, and plated onto 2 percent malt extract agar in Petri plates. Tissue from mycelial fans was directly plated onto malt agar (Maloy 1974, Worrall 1991). Cultures were grown on malt agar in the dark at 23° C within a controlled environment chamber for 4 to 6 weeks.

DNA Extraction, PCR, and Sequencing

Liquid cultures [100ml of liquid MY (20g/L malt extract and 2g/L yeast extract)] were inoculated with 10 1-mm² plugs of agar and mycelium (Anderson and Bailey 1989) and shaken at 100 rpm at ambient room temperature in the dark. Mycelia were harvested on cheesecloth, rinsed with sterile distilled water, and frozen in liquid nitrogen (Terashima and others 1998). Template DNA was extracted using the Qiagen DNeasy Plant Mini Kit (QIAGEN Valencia, CA) following the manufacturer's protocol.

The PCR protocol of Harrington and Wingfield (1995) was adapted to amplify the IGS-1 region of the rDNA. Extracted DNA was used as template in the PCR reactions to amplify the IGS-1 region for the unknown isolates (Coetzee and others 2003). Primers LR12R [5'CTGAACGCCTCTAAGTCAGAA3' (Veldman and others 1981)] and O-1 [5'AGTCCTATGGCCGTGGAT3' (Dushesne and Anderson 1990)], were used for amplification of the IGS-1 region (Frontz and others 1998). PCR products were purified before sequencing with a QIAquick PCR Purification Kit (QIAGEN Valencia, CA). Sequences were generated by The Pennsylvania State University Nucleic Acids Facility with the ABI Hitachi 3100 Genetic Analyzer fluorescence-based capillary electrophoresis unit. Approximately 20 to 40 ng of PCR product was used for sequencing. IGS-1 sequences were obtained with primers LR12R (Veldman and others 1981) and O-1 (Dushesne and Anderson 1990).

DNA sequences were edited in DNASTar (DNA Star Inc., Madison WI) using SeqMan II. The forward and reverse sequences were trimmed to remove non-readable sequences, and base calls were made when necessary. Initial identification of the unknown isolates was based on nucleotide similarity with sequences at GenBank, by using the BLAST search function of the database (Coetzee and others 2003). Unknown sequences were aligned with sequences that showed the highest similarity from GenBank. Sequences were aligned in the Megalign program of DNASTar, using the clustal W method, and the alignment was saved as a PAUP file (<http://www.sinauer.com/detail.php?id=8060#atitle>). The neighbor joining method (using uncorrected p value) was used to generate a tree with *A. nabsnona* defined as the outgroup, since this species falls outside our group of interest (Volk and others 1996). In addition, only six unknown isolates were included in the analysis to eliminate the identical sequences among the unknown isolates that were observed in preliminary distance analyses (data not shown).

RESULTS

Abundance of Soil-Borne Rhizomorphs

There were no statistical differences ($p = 0.05$) in number or length of rhizomorphs collected within the two types of forest stands. Soil-borne *Armillaria* rhizomorphs were recovered from 28 of 329 (8.5 percent) of the sample pits in the old-growth HNA, and from 12 of 199 (6 percent) sample pits within the adjacent hardwood stand. Including soil pits that did not contain rhizomorphs (length = 0 cm), the average lengths (cm) of rhizomorphs per sample pit were 1.8 and 1.6 cm within the HNA and hardwood stand, respectively. When only pits that contained rhizomorphs were analyzed, the average length of rhizomorphs per sample pit was 21.5, and 26.6 cm, within the HNA and hardwood stand, respectively.

Collection of Isolates for Identification

Of the 102 samples collected from trees, 85 (83 percent) yielded positive *Armillaria* cultures. In the HNA, *Armillaria* was recovered from 40 of the 47 (85 percent) old-growth eastern hemlocks sampled; *Armillaria* was also recovered from all three of the dead hemlocks sampled in the hardwood stand. With regard to hardwoods, *Armillaria* was recovered from 23 of the 25 (92 percent) old-growth hardwoods sampled in the HNA, and from 19 of the 27 (70 percent) younger hardwoods sampled in the mixed-hardwood stand.

Within all sampled eastern hemlock trees ($n = 50$), *Armillaria* was recovered from approximately 86 percent of all trees. Within all sampled hardwood trees ($n = 52$), *Armillaria* was isolated from approximately 81 percent of the trees. With regard to living or dead trees in both stands, *Armillaria* was recovered from all ($n = 31$) of the dead hemlocks and 12 of 19 (63 percent) of the living hemlocks. *Armillaria* was recovered from 15 of 23 (65 percent) of the dead hardwoods and 27 of 29 (93 percent) of the living hardwoods.

Species Identification

Of the 85 *Armillaria* isolates obtained from field-collected material, sequence data were usable from 71 of the unknown isolates. Initial BLAST searches revealed that the unknown sequences were similar to either *A. gallica* or *A. ostoyae*. Phylogenetic analysis using neighbor joining trees revealed that unknown sequences were most closely related to two species groups: I) *A. ostoyae*/*A. gemina* and II) *A. gallica*/*A. calvescens*/*A. sinapina*/*A. cepistipes*/NABS X. Of the 71 sequences, 11 (15 percent) of the unknowns were identified as being in group I, whereas the other 60 (85 percent) were identified as in group II (fig. 1). Species identified as being in group I were most closely related to *A. ostoyae* (fig. 1). However, exact species identifications could not be obtained for unknown isolates within group II based on IGS-1 data.

DISCUSSION

Armillaria rhizomorph abundance and lengths from the 528 soil samples within the study area were very low, variability was high, and distribution of rhizomorphs was not uniform among sample plots. *Armillaria* rhizomorphs were recovered from only 8.5 percent of the sample pits in the old-growth HNA, and 6 percent of the sample pits within the adjacent hardwood stand. In contrast, Wargo and others

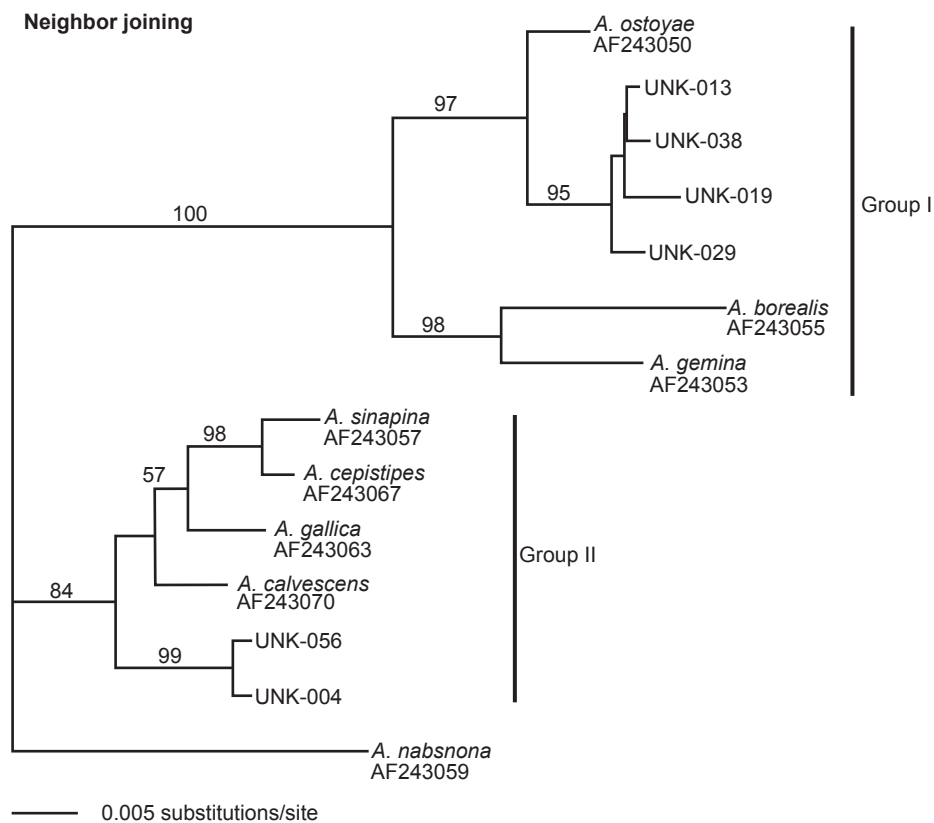


Figure 1—Neighbor-joining tree with 1,000 bootstrap replicates for known and unknown isolates. Horizontal branch length and scale bar correspond to evolutionary distances measured as the proportion of nucleotide substitutions between the sequences. Known isolates are labeled with species names and GenBank accession numbers. *A. nabsnana* was defined as the outgroup. Unknown isolates UNK-013, UNK-019, UNK-029, and UNK-038 are representative of the 11 unknown isolates in group I. Unknown isolates UNK-004 and UNK-056 are representative of the 60 unknown isolates in group II.

(1987) found *Armillaria* rhizomorphs in 95 of 351 (27 percent) of soil pits excavated along a transect in hardwood, transition, and boreal forests that contained declining red spruce (*Picea rubens* Sarg.) in eastern U.S.A. They also reported that rhizomorph density (cm/sample) was 10 times greater in hardwood stands than in transition or boreal stands. Twery and others (1990) also reported high variability in rhizomorph abundance in the soils of mixed oak forests; although comparable data were not given, they reported a direct relationship between rhizomorph abundance and proximity to dead trees, length of time since defoliation, and time of tree death.

The greater abundance of *Armillaria* rhizomorphs found in both of these studies (Twery and others 1990, Wargo and others 1987) may have been related to stand history. Both harvesting and insect defoliation may result in increased numbers of *Armillaria* rhizomorphs in the soil. Stands that have been harvested recently provide abundant dead and dying stumps and roots, food bases that are important for *Armillaria* survival and spread (Pronos and Patton 1978). Since our old-growth stand had not been harvested, and since the adjacent hardwood stand had not been harvested for many decades, it is likely that our stands had less food base. Likewise, the infestation from the hemlock wooly adelgid was just initiating hemlock mortality in our study area, and there were not large numbers of adelgid-killed hemlocks in the stand whose roots would serve as food bases. Although present, dead trees and snags were not common in either stand. It was surprising, however, that both the old-growth hemlock stand and the adjacent hardwood stand had similar numbers of rhizomorphs in spite of differences in stand composition and history.

However, the two stands were contiguous, located in the same steep rocky ravine, and had the same very acidic soil. The lower numbers of rhizomorphs found in this area may be related to the acidic nature of the soil, since some *Armillaria* species may be inhibited by low soil pH (Singh 1983).

In contrast to the limited abundance of *Armillaria* in the soil, as indicated by the limited recovery of *Armillaria* in the soil pits, the fungus was abundant on dead trees and stumps in the two stands, and 83 percent of these samples yielded isolates for identification. Most samples taken were rhizomorphs associated with root collars or roots. Only two samples consisted of mycelial fans, both of which yielded positive *Armillaria* cultures identified as *A. ostoyae*. *Armillaria* was recovered from 86 percent of all eastern hemlocks sampled and from 81 percent of all hardwoods sampled. This was surprising, since rhizomorph production may be greater from hardwood substrates than coniferous substrates (Wargo and others 1987).

Phylogenetic analysis using the neighbor joining method revealed that our unknowns fell within two species groups: I) *A. ostoyae/A. gemina* and II) *A. gallica/A. calvescens/A. sinapina/A. cepistipes*/NABS X. Isolates within group I were more closely related to *A. ostoyae* than *A. gemina*.

The IGS-1 sequences from unknown isolates that cluster within group II are more closely related to each other than to sequences of known isolates from other NABS available in sequence databases (fig. 1). It is therefore unclear what species the unknown isolates in group II may belong to. However, *A. nabsnona*, *A. cepistipes*, and NABS X can be ruled out due to their limited geographic distribution in northwestern North America (Banik and others 1996, Volk and Burdsall 1993, Volk and others 1996). It also appears that these isolates are more closely related to *A. gallica* and *A. calvescens* than to *A. sinapina* based on the distance analysis and bootstrap values that show *A. sinapina* as being very closely related to *A. cepistipes* (fig. 1). This relationship between *A. sinapina* and *A. cepistipes* was previously reported (Terashima and others 1998). However, true biological species definitions cannot be determined within this group using the IGS-1 region of the DNA, since multilocus phylogenetic methods for determining species boundaries have not yet been completed for *Armillaria*.

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