MOLECULAR EVOLUTION, INDENTIFICATION AND EPIDEMIOLOGY OF NORTH AMERICAN SPECIES OF A ROOT-INFECTING FUNGUS, ARMILLARIA

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Molecular evolution, identification and epidemiology of North American species of a root-infecting fungus, *Armillaria*.

by

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ABSTRACT

Armillaria (Fr.:Fr.) Staude is a genus of root-infecting fungal pathogens which cause disease in forests and orchard plantations. Biological species of *Armillaria* are identified by sexual incompatibility mating interactions. Epidemiological studies of North American species of *Armillaria* indicated that some are saprotrophic, some mycotrophic, and others are necrotrophic pathogens often producing death of the host. Stress of the host tree is often considered a critical factor in disease development.

Three objectives to this study examined different aspects of the pathogenicity of *Armillaria*. The first was to estimate phylogenetic relationships among North American biological species (NABS) of *Armillaria*. This allowed inference of inheritance of pathogenic traits among species. The second was to develop species-specific molecular markers for NABS *Armillaria*. This would provide a method of identification for pathogenic species. The third was to examine disease development of *Armillaria* inoculum using different types and degrees of stress inflicted on the host tree. Molecular techniques were employed to examine the first two objectives. The third objective involved a field inoculation trial with two Newfoundland isolates of *A. ostoyae*.

This study is consistent with previous phylogenetic hypotheses concerning relationships among species of *Armillaria*. Phylogenetic analysis of randomly amplified DNA regions of unknown function provided strong support for intraspecies clustering. Most NABS *Armillaria* were resolved using four anonymous nucleotide sequences

combined within a single data set. There was stong support for the clustering of NABS I and II, as well as NABS III and VII. Isolates of NABS V showed sequence polymorphism.

Species-specific molecular markers were developed for most NABS *Armillaria*. PCR amplification using a combination of different random primer sets in each of the reaction mixtures, yielded species size-specific bands on an agarose gel for each of NABS I, II and VI. A common band was found specific for NABS III and VII. Although NABS V, IX and X contained less variation, 10 nucleotide primers could be used to confirm their identity. Results from inverse PCR suggested that secondary DNA structure and primer/template competition played a significant role in determining species-specificity with SWAPP 10 nucleotide primers.

Results from the field inoculation trials suggested that two years was sufficient time to allow forest managers to make informed decisions regarding stand management. Host stress appeared to influence *Armillaria* root disease development. The black spruce plantation had more infection than the naturally regenerated stand. There was more disease in the balsam fir thinned stand than the un-thinned stand. Significant correlation between infection and above ground tree symptoms occurred in a very severely defoliated balsam fir sawfly plot only. Well-drained sandy soil seemed to increase the aggressiveness of the *Armillaria* isolate used as inoculum.

The utilization of molecular techniques, combined with knowledge of ecological processes, would greatly enhance the efficiency of forest management.

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LIST OF ABBREVIATIONS

ABI	Applied Biosystems Incorporated
Adj	Adjusted for ties
bp	base pairs
BP	Bootstrap Proportion
°C	degrees Celcius
ca.	calculated
Ca	Calcium
$CaCO_3$	Calcium Carbonate
CI	Consistency Index
cm	centimeters
Ctl	Control
DBH	Diameter at Breast Height
del	deletion
df	degrees of freedom
DNA	Deoxyribonucleic acid
F	fermented layer
g	grams
g/L	grams/Litre
g1	g1 skewness index
gDNA	genomic DNA
H	Humus layer
HI	Homoplasy Index
IGR	Intergenic Region
IGS	Intergenic Sequence
ins	insertion
K	Potassium
Ka	Thousand years ago
Kb	Kilobases
L	Litter layer
m	meters
Ma	Million years ago
20mer	20 nucleotide primer
meq	milliequivalents
Mg	Magnesium
mM	milliMolar
mtDNA	mitochondrial DNA
N	North or Nitrogen
NABS	North American Biological Species
nt	nucleotide
OM	Organic Matter
P	Phosphorus
p	probability

PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
ppm	parts per million
r	Spearman's rank correlation
rDNA	ribosomal DNA
RAPD	Random Amplified Polymorphic DNA
RC	Reverse Compliment
RFLP	Restriction Fragment Length Polymorphism
S	Transitions
SD	Standard Deviation
SWAPP	Sequencing With Arbitrary Primer Pairs
Text	Texture
TBE	Tris Borate EDTA
µL	micro Litres
µM	micro Molar
UV	Ultraviolet
V	Transversions
W	West
3'	3-OH "prime"
5'	5-P "prime"

Chapter 1

GENERAL INTRODUCTION

1.1 Armillaria:

The basidiomycetous fungus, *Armillaria bulbosa* (Barla) Kile and Watling, is one of the largest and oldest living organisms in the world. One individual mycelium, found in northern Michigan, covered 15 hectares and was more than 1500 years old (Smith et al., 1992). The knowledge that a fungus involved in this study has such growth capacity, defies general ideas of fungal habits, and presents an intriguing concept on which to begin a study. Although molecular techniques were used to determine the size of the individual, they can also be used for other purposes.

The genus *Armillaria* (Fr.:Fr.) Staude contains approximately 40 species that are distributed world-wide. Many of these species cause root disease in more than 600 species of woody plants (Raabe, 1962). Some species are necrotrophic pathogens, while others are saprotrophic (Gregory et al., 1991), or form mycotrophic associations with achlorophyllous plants, such as the orchid *Galeola septentrionalis* Reichb. f. (Terashita and Chuman, 1987). Species of the genus *Armillaria* can be divided into annulate and exannulate morphological species, depending on whether an annulus is present on the stipe. Until recently the annulate species were considered a single species, *Armillaria mellea* (Vahl: Fr.) Kummer (*sensu lato*) with a high degree of variation in virulence and host preference (Redfern and Filip, 1991). The biological species concept was applied

to the genus only after Hintikka (1973) described the bifactorial sexual incompatibility system. Sexual compatibility occurs when haploid single spore isolates of the same biological species carry different alleles at two mating type loci. Compatible matings in *Armillaria* produce vegetatively stable diploid mycelia lacking clamp connections. Macroscopic colony morphological differences distinguish between compatible and incompatible matings in the laboratory. A flat crustose mycelium is produced by a compatible mating of two monospore isolates, whereas incompatible matings maintain the fluffly white mycelial appearance of the monokaryon (Hintikka, 1973). Using this criterion seven European species (Korhonen, 1978), five Australasian species (Kile and Watling, 1983), and nine North American biological species (NABS) (Anderson and Ullrich, 1979; Ullrich and Anderson, 1978) of *Armillaria* have been described.

NABS *Armillaria* vary in their degree of pathogenicity. *A. ostoyae* (Romagnesi) Herink (NABS I) is highly pathogenic on conifers (Guillaumin et al., 1989; Gregory, 1985) and distributed throughout the northern hemisphere. *A. gemina* Bérubé and Dessureault (NABS II) has been found to form saprotrophic to weakly pathogenic (Morrison, 1989) associations with hardwoods in south-east Canada (Bérubé and Dessureault, 1989) and eastern United States (Anderson, 1986). *A. calvescens* Bérubé and Dessureault (NABS III) is saprotrophic to weakly pathogenic and associated with hardwoods in the northeastern United States (Proffer et al., 1987), and southeast Canada (Bérubé and Dessureault, 1989), but has been found to be pathogenic on hardwoods in some studies (Harrington et al., 1989; Proffer et al., 1987). *A. lutea* (Synonyms: *A. gallica* Marxmuller and Romagnesi, *A. bulbosa* (Barla) Kile and Watling) (NABS VII)

is saprotrophic to weakly pathogenic and associated with hardwoods throughout the northern hemisphere. *A. sinapina* Bérubé and Dessureault (NABS V) is weakly pathogenic on hardwoods and found throughout North America (Morrison et al., 1985b; Dumas, 1988). *A. mellea* (Vahl: Fr.) Kummer (*sensu stricto*) (NABS VI) is highly pathogenic on hardwoods and found in both northern and southern hemispheres (Guillaumin et al., 1989). *A. nabsnona* Volk and Burdsall (NABS IX) is saprotrophic or weakly pathogenic on hardwoods (Morrison, 1989) and has been collected from a small area in northwestern North America (Morrison et al., 1985b; Volk et al., 1996). NABS X has yet to be named, and is saprotrophic with conifers in southeast British Columbia (Morrison et al., 1985b). Although only some species are pathogenic, all can survive saprotrophically on woody debris in the soil (Redfern and Filip, 1991). There is also a large amount of variation in pathogenicity among individuals within some species.

1.2 Disease:

Pathogenicity refers to the disease-causing characteristic of a genus or species. Virulence refers to an observed ability of an individual of a pathogenic species to cause infection (British Federation of Plant Pathologists, 1973). Rishbeth (1984) suggested that pathogenicity tests should be done using a natural method of infection, and with host material having a moderate degree of resistance. In addition, Koch's postulates must be satisfied to prove pathogenicity of a species (British Federation of Plant Pathologists, 1973).

The first pathogenicity test of *Armillaria* was done by R. Hartig in 1874, who was one of the first to recognize that *Armillaria* caused disease. (Gregory et al., 1991). Root infection occurs when mycelial fans of *Armillaria* grow in the outer bark and penetrate the cambium which causes necrosis of the tissue (Morrison et al., 1991). Plants are predisposed to disease when stress imposes chemical changes so they cannot produce sufficient defense response to maintain a pathogen (Schoeneweiss, 1975). Host stress is an important factor to consider in predisposition of a tree to infection by *Armillaria*, especially in the more resistant host species. Similarly, host stress may create conditions more favourable for the less virulent individuals of *Armillaria* than for the more virulent isolates. Stress may be produced from different sources such as drought, flooding, shading, competition for nutrients, insect damage, pollution, or other foliar diseases (Wargo and Harrington, 1991). Stress may predispose the host to attack by *Armillaria*. Alternatively, *Armillaria* may stress the tree to allow other organisms to attack. *Armillaria* may act as a primary pathogen infecting healthy, vigourous trees, or as a secondary pathogen becoming opportunistic and infecting trees that have been weakened by stress.

1.3 Economic significance:

Because *Armillaria* affects many different host species and is distributed world-wide in boreal, temperate and tropical forests, it is an important consideration in the management of natural and planted forests. *Armillaria* causes endemic disease that is

constantly present within a small area. A balance maintains the coexistance of host and pathogen in natural forests. However, fluctuations in disease levels may occur from changing biological or environmental conditions. It is becoming a common practice to plant seedlings in harvested forest areas in order to replace and improve stands of economically valuable species. Forest management activities may aggravate root disease caused by *Armillaria* by imposing stress on the host plants, and making them more susceptible to fungal attack. Forest harvesting and orchard plantations may provide more opportunities for less virulent *Armillaria* species to cause disease. Hence, a thorough understanding of the pathogen is important for development of management practices and sustainability of the forests.

1.4 General objectives:

The main objective of this study was to investigate pathogenicity of NABS *Armillaria*. Three separate projects provided means to examine important aspects of pathogenicity: 1) To develop a molecular phylogeny of NABS *Armillaria* in North America, in order to allow comparison of inheritance of pathogenic traits among biological species; 2) To develop species-specific molecular markers to provide forest managers with a fast, efficient method to identify pathogenic species; and 3) To examine pathogenicity of two isolates of *A. ostoyae* in inoculation trials with mature conifers in a natural environment.

Chapter 2

Phylogenetic History and Species of *Armillaria*

2.1 INTRODUCTION

2.1a Biological species:

Although interfertility testing is an ideal tool with which to distinguish among biological species, haploid isolates must be paired in culture with tester strains, a process which takes about six weeks. Other techniques used to identify species of *Armillaria* include identification of fruit body or rhizomorph morphology (Marxmüller, 1982; Bérubé and Dessureault, 1988; 1989; Volk et al., 1996; Banik et al., 1996), rhizomorph growth habit in soil (Morrison, 1991), immunological techniques (Lung-Escarment et al., 1985), RFLP with mitochondrial DNA (mtDNA) (Smith and Anderson, 1989), ribosomal DNA (rDNA) (Anderson et al., 1987), without DNA extraction (Harrington and Wingfield, 1995), mitochondrial and nuclear DNA hybridization (Jahnke et al., 1987), isozyme patterns (Morrison, 1982a; Morrison et al., 1985a; Lin et al., 1989; Agustian et al., 1994), clamp connection characters (Larsen et al., 1992) and fractal geometry of rhizomorphs (Mihail et al., 1995). Anderson and Stasovski (1992) compared sequences of the intergenic sequence (IGS) region of rDNA to infer a phylogeny of biological species of *Armillaria*. The relationship between species-specific and phylogenetic characters of a group of species is linked. The phylogenetic history of a group of species is reflected in the species-specific characters of the individuals. Sequence divergence helps to identify

species, but the number and kind of differences among species may serve as an estimate of the phylogeny of the group.

2.1b Molecular techniques:

The utilization of a single 10 nucleotide (nt) primer to amplify species specific bands in various organisms is common, and is correctly referred to as randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McClelland, 1990). A similar technique called SWAPP (sequencing with arbitrary primer pairs) uses two different 10nt primers to amplify fragments (Burt et al., 1994). Since RAPD fragments cannot be sequenced, the use of two different primers permits the fragment to be sequenced, allowing development of more stable 20nt primers. Differences in banding patterns observed when using these techniques may be caused by several mechanisms. RAPD-PCR may produce polymorphisms due to insertion of DNA between two annealing sites producing a fragment too large to be amplified, deletion of DNA containing a primer site resulting in loss of the fragment, nucleotide substitution at the primer site that affects annealing of the primer to the template, or insertion or deletion of a small piece of DNA between primer sites leading to a change in size of the amplified fragment (Weising et al., 1995). Primer/template dynamics may further complicate polymorphisms in DNA banding patterns.

2.1c Phylogeny:

Combined analysis of phylogenetic data has become a popular approach in recent literature. The efficacy of the use of separate or combined approaches has been debated (Hillis, 1987; 1995; Kluge, 1989; Omland, 1994; Miyamoto and Fitch, 1995; de Queiroz et al., 1995; Lutzoni and Vilgalys, 1995). Combined data sets provide a larger number of characters with which to infer a phylogeny of a group of species. Since single gene trees may not accurately reflect species trees (Page and Charleston, 1997), sequences from a larger number of genes incorporated into the gene tree may bring it closer to congruence with the species tree.

On the assumption that nucleotide and protein sequences evolve in a clocklike manner, times of occurrence of branching events in a phylogenetic tree could be determined based on the number of sequence differences that have accumulated within each lineage since divergence from the common ancestor (Wilson et al., 1977). Proteins evolve at different rates depending on the amount of constraint on the molecule, and non-coding DNA typically evolves more rapidly than coding regions (Ohta, 1992).

2.1d Fungal Genomes:

Fungal DNA is organized into chromosomes similar to other eukaryotes (Taylor, 1986). Mitochondrial DNA inheritance may occur through a uniparental mode (either maternally or paternally), biparentally, or with recombination between chromosomes. Animal mtDNA genomes are small (16-19 Kbp), whereas fungal mitochondrial genomes

are more variable in size (18.9-176 Kbp) (Taylor, 1986). The size of the mtDNA genome in *Armillaria* is approximately 80-100 Kbp (Jahnke et al., 1987).

Fungal nuclear genomes are small ($2.0x10^4$ to $8x10^4$ Kbp) compared with other eukaryotes such as insects ($2x10^5$ to $6x10^6$ Kbp), birds (10^6 Kbp), and flowering plants ($2x10^5$ to $2x10^8$ Kbp) (Primrose, 1995). Fungi contain a small amount of repetitive DNA (< 20%) (Arthur et al., 1982; Timberlake, 1978; Hamer et al., 1989; Morton et al., 1995). Nuclear gene intron size of fungi range between 55 to 80 nucleotides in length (Radford, 1993), and the [GC] content lies between 43% and 61% (Storck and Alexopoulos, 1970; Arthur et al., 1982; Jahnke et al., 1987). The [GC] content for *Armillaria* genome is approximately 46% to 48% (Jahnke et al., 1987).

2.1e Objectives:

The objectives of Part A of this study were to determine the effectiveness of combining data sets of four different conserved fragments to estimate a phylogeny, and to infer phylogenetic relationships among NABS *Armillaria* so as to place NABS *Armillaria* into an historical framework.

The purpose of Part B was to develop species-specific primers for NABS *Armillaria* which could be used to rapidly identify isolates collected from diseased trees, and secondarily to examine primer/template dynamics.

Part C discusses observations on repetitive DNA banding patterns shown to be species-specific.

2.2 MATERIALS AND METHODS

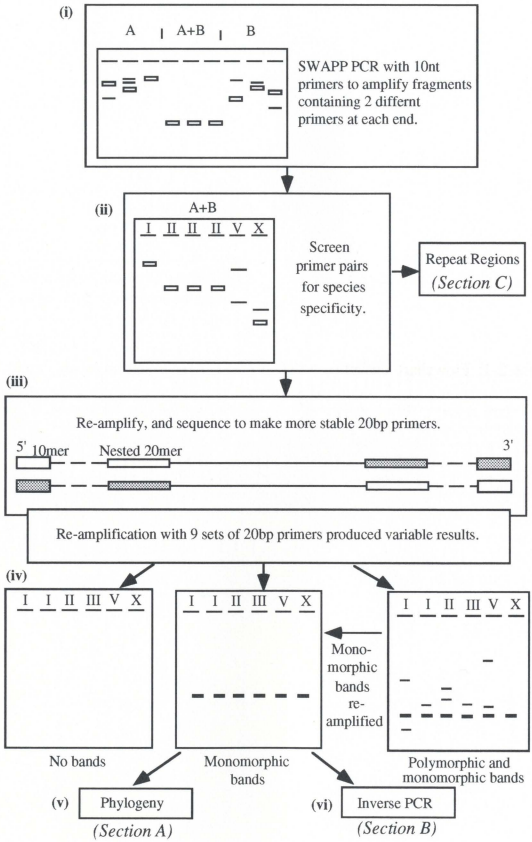
2.2a Procedural summary:

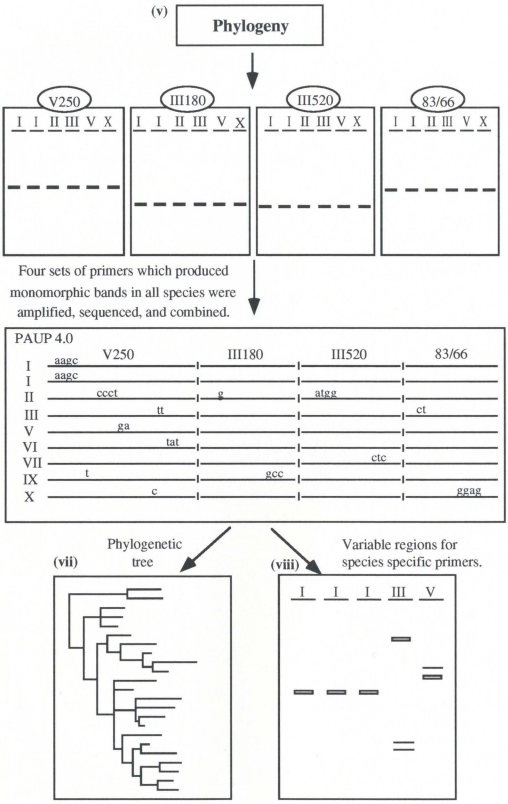
Polymerase chain reaction (PCR) was used to screen single 10nt primers and combinations thereof in order to produce bands with a different primer at each end (Figure 1, i). These primer pairs were then screened for species-specificity (Figure 1, ii). Some primer pairs amplified species-specific 3-band clusters, which is discussed in Part C. Species-specific bands were excised, re-amplified and sequenced to develop more stable, nested 20nt primers from regions located downstream from the 10mer sites (Figure 1, iii). Monomorphic bands were re-amplified (Figure 1, iv) and two separate techniques were employed to produce a phylogeny (Figure 1, v; Part A) and to investigate species-specific sites in the DNA template by inverse PCR (Figure 1, vi; Part B).

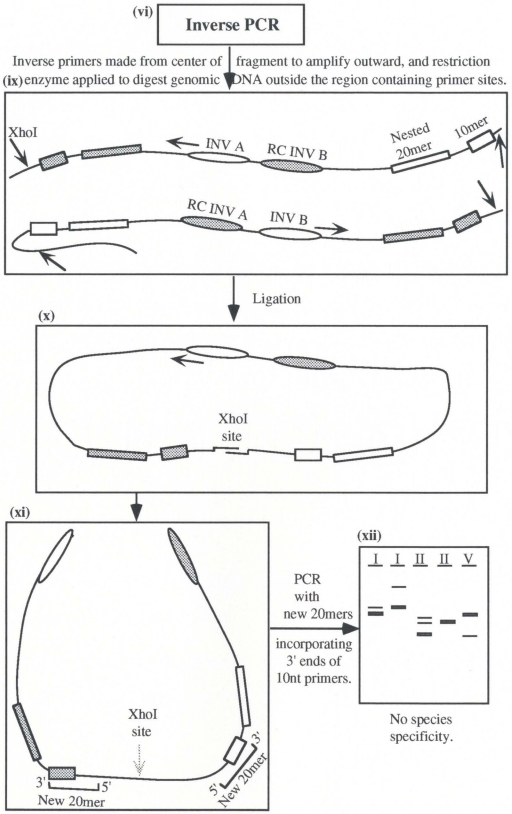
The phylogenetic analysis involved combining monomorphic fragments from four sets of primers to produce a single phylogenetic tree (Figure 1, vii). Variable regions within these sequences were then used to develop species-specific primers (Figure 1, viii).

Inverse PCR was performed on fragments specific to NABS V to locate the 10nt primer sites on template DNA (Figure 1, vi). Twenty nt primers were made from the center of the template DNA in an inverted orientation, so that direction of extension was outward, encompassing the nested 20mers and the species-specific 10mers (Figure 1, ix). DNA digestion was performed using a restriction enzyme with a recognition site upstream from all primer sites on the template DNA, followed by ligation (Figure 1, x) and PCR using inverse primers (Figure 1, xi). The inverse fragment was sequenced to obtain all

Figure 2-1: Flowchart outlining procedure for experiments involving molecular techniques. See text for explanation.


(i)
A
A+B
B
SWAPP PCR with 10nt primers to amplify fragments containing 2 differnt primers at each end.
(ii)
A+B
I II II II V X
Screen primer pairs for species specificity.
Repeat Regions (Section C)
(iii)
Re-amplify, and sequence to make more stable 20bp primers.
5' 10mer
Nested 20mer
3'
Re-amplification with 9 sets of 20bp primers produced variable results.
(iv)
I I II III V X
I I II III V X
I I II III V X
Mono-morphic bands re-amplified
No bands
Monomorphic bands
Polymorphic and monomorphic bands
(v)
Phylogeny
(Section A)
(vi)
Inverse PCR
(Section B)


(v)
Phylogeny
V250
III180
III520
83/66
I I II III V X
Four sets of primers which produced monomorphic bands in all species were amplified, sequenced, and combined.
PAUP 4.0
V250
III180
III520
83/66
I aagc
I aagc
II cect
g
atgg
III tt
ct
V ga
VI tat
VII ctc
IX t
gcc
X c
ggag
(vii)
Phylogenetic tree
(viii)
Variable regions for species specific primers.
I I I III V


(vi)
Inverse PCR
Inverse primers made from center of fragment to amplify outward, and restriction
(ix) enzyme applied to digest genomic DNA outside the region containing primer sites.
XhoI
INV A
RC INV B
Nested 20mer
10mer
RC INV A
INV B
Ligation
(x)
XhoI site
(xi)
XhoI site
3'
5'
New 20mer
5'
3'
New 20mer
PCR with new 20mers
incorporating 3' ends of 10nt primers.
(xii)
I I II II V
No species specificity.

primer sites and new 20nt primers were made from these sites incorporating 10 bases of the 10nt primer for the 3' end, and an additional 10 bases upstream to encompass the 5' end of the primer. These primers were then used to amplify genomic DNA (gDNA) (Figure 1, xii).

2.2b Material examined:

North American biological species of *Armillaria* are indicated by Roman numerals (Anderson and Ullrich, 1979). Fungal material was all haploid and obtained from various sources for the phylogeny (Table 2-1). Material screened for species-specific research included 87 strains of *Armillaria*, and seven non-*Armillaria* species. Isolates were determined as biological species by the source, using interfertility tests with standard tester strains.

2.2c DNA extraction:

Crude genomic DNA from *Armillaria* was extracted from approximately 200mg lyophilized fungal tissue using a modified 2X CTAB procedure loosely based upon the protocol of Zolan and Pukkila (1986). DNA was extracted with chloroform:isoamyl alcohol, precipitated with isopropanol, and resuspended in TE-8 buffer.

2.2d Polymerase Chain Reaction:

PCR reaction components included 0.1 μM each oligonucleotide primer (Table 2-

2), 2mM of each of dATP, dCTP, dGTP, and dTTP, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, and 0.4 units Taq DNA polymerase (Promega). For each 20µL reaction, 2µL of 1:10 dilution of approximately 10ng genomic DNA was added. Five µL of purified DNA was added to each 100µL reaction. One drop of light white mineral oil was placed in each tube to prevent evaporation. Amplification occurred on a Perkin Elmer Cetus 480 Thermal cycler. PCR cycle conditions changed depending on the nature of the primer used. All PCR cycles began with 1 minute preheat at 94°C, and ended with link to 4°C soak. Thirty-five temperature cycles for the 20nt primers consisted of denaturation at 94°C for 1 minute, annealing from 45°C to 50°C (depending on the primer used) for 1 minute, and extension at 72°C for 1 minute with a 50 second ramp time. Amplification with the 10nt primers consisted of 42 cycles of the same temperatures and times, except annealing occurred at 38°C. Two different 10nt primers were used in each reaction to allow for sequencing of the fragments. This method was described by Burt et al. (1994) as sequencing with arbitrary primer pairs (SWAPP). The 10nt primers were obtained from Regional DNA Synthesis Lab, Calgary, Alberta. Twenty nt primers were synthesized by Laboratoire d'Analyse et de Synthèse d'Acides Nucléiques, Université Laval, Québec, Canada. Primer sequences are provided in Table 2-2 for "Phylogeny", and Appendix A for "Species-Specific Markers".

2.2e Phylogenetic Analysis:

Crude sequence alignments were performed on a MacIntosh computer using

Table 2-1: Strains of *Armillaria* showing name of collector, host species on which it was collected, and location of collection.

Sample	Collector	Host species	Location
I DMR 20	T. Harrington	*Abies balsamea*	Wildcat Mt., NH, USA
I BOW PK	J. Bérubé	Undetermined wood debris	Bowring Park, St. John's, NF
I JB 08	J. Bérubé	*Acer saccharum*	Oka, Ottawa River Valley, QC
I JB 09	J. Bérubé	*Betula populifolia*	Sainte-Therese, north Montreal, QC
I JB 13	J. Bérubé	*Betula papyrifera*	Chicoutimi, Lake St-Jean, QC
I GG 12D	J. Bérubé	*Pinus banksiana*	Chicoutimi, Lake St-Jean, QC
I NOF 1076	Y. Hiratsuka	*Pinus contorta*	Hinton, Alberta
I NOF 830	F.J.Emond	*Pinus contorta*	Cow Lake, Alberta
II JB 38	J. Bérubé	*Acer saccharum*	Duchesnay, near Quebec City, QC
II JB 39	J. Bérubé	*Acer saccharum*	Duchesnay, near Quebec City, QC
II 160-8	J. Anderson	Undetermined	Smuggler's Notch, VT, USA
II JB 85	J. Bérubé	*Acer saccharum*	Bromont, Eastern Township, QC
III JB 56	J. Bérubé	*Acer saccharum*	St.-Ange, Beauce, QC
III JB 61	J. Bérubé	unknown	Donnaconna, near Quebec City, QC
V 83 91 1	C. G. Shaw III	unknown	Petersburg, Alaska
V 48-3	J. Anderson	unknown	Ithaca, NY,USA
V JB 75	J. Bérubé	*Acer saccharum*	St-Odilon, Beauce, QC
V JB 66	J. Bérubé	*Acer saccharum*	St-Odilon, Beauce, QC

V Bow Pk	J. Bérubé	American Mountain Ash	Bowring Park, Newfoundland
V JB 07	J. Bérubé	*Acer saccharum*	Bromont, Eastern Townships, QC
V JB 72	J. Bérubé	*Acer saccharum*	St.-Odilon, Beauce, QC
V JB 19C	J. Bérubé	*Pinus strobus*	St.-Jean, Vianney, Lake St.-Jean, QC
VI PD 37	J. Bérubé	*Quercus alba*	Oka, Ottawa River Valley, QC
VI KJS-6	T. Harrington	*Acer rubrum*	New Market, NH, USA
VI GB 898	D. Bills	unknown / Hardwoods	Augusta Co, VA, USA
VI 97-1	J. Anderson	*Acer rubrum*	Provincetown, MA, USA
VII 90-10	J. Anderson	*Fraxinus americana*	Burlington, VT, USA
VII HHB 11912	H.H. Burdsall	dead *Ulmus*	Madison, WI, USA
IX TJV 179-1	T. Volk	*Picea sitchensis*	Jefferson Co, WA, USA
IX TJV 200	T. Volk	*Acer* sp.	Olympic national Park, Jefferson County
IX TJV 188-4	T. Volk	*Acer macrophyllum*	Olympic national Park, Jefferson County
IX 121-2	J. Anderson	*Acer macrophyllum*	Vancouver, BC
IX 139-1	J. Anderson	soil surface	Moscow, Idaho
X SP812015	D. Morrison	conifer stump	South of Nelson, BC

Table 2-2: Primer sequences used to obtain monomorphic fragments in eight NABS *Armillaria*.

Primer	**Primer sequence (5'-3')**
V 250a	CGA ACT GAT CGT CGT CGA
V 250b	GTT TCG AAC GCG AAT ATG CTC
III 180a	ACC ACA TCC TTG TCG CCG AG
III 180b	GTG GTT GAT GAG ATT GTT CG
III 520-1	CAT GGT CGC TAC TTA CTC TGA TAA CGG
III 520-2	GAG TTG ACG TAG ACT AC
83	GGG CTC GTG G
66	GAG GGC GTG A

SeqEd, version 1.0.3 (Applied Biosystems) followed by manual adjustment. The phylogeny was produced with PAUP version 4.0d52 and by permission of D. Swofford, using NABS VI as the outgroup. The following PAUP parameters were used: random stepwise addition sequence, nearest neighbour interchange branch swapping algorithm, characters had equal weights, 10 heuristic replicates for each of the 100 bootstrap replicates, and 50% majority rule consensus with maximum parsimony. Due to technical limitations a constraint was placed on analysis of the data set produced by III180. No more than 90,000 trees were saved with a length ≥ 20, rather than saving all optimal trees.

2.2f Molecular Clock:

A distance matrix from aligned sequences for the combined data set was produced using SeqEd, version 1.0.3 (ABI), and manually corrected for missing data. Divergence times were calculated using the methods described in Berbee and Taylor (1993), with minor modifications. Substitution rates for groups of lineages were not normalized as they were in Berbee and Taylor (1993). Hence, divergence times represent actual distances.

2.2g Inverse PCR:

Inverse PCR was employed to obtain DNA regions flanking the nested 20nt primer sites. Inverted 20nt primers were made from the center of the sequence amplified with primers 17 and 127, specific for NABS V and X. Genomic DNA was quantified on

Hoefer TKO 100 Fluorometer (Hoefer Scientific Instruments) and 0.5μg/μL gDNA was digested with the appropriate restriction enzyme. Restriction enzymes with recognition sites within the sequence to be amplified were not used in the digestion. Digestion reactions contained 0.2-1.0 μg/μL gDNA in a volume of 20μL. Sterile distilled water was mixed with DNA to give a volume of 18μL. Two μL of the appropriate restriction enzyme buffer was mixed with the solution before 2 units of the enzyme was added. Reaction components were incubated at the appropriate temperature for 4 hours (Manniatis et al., 1982). The reaction was stopped by adding 0.5M EDTA (pH 8.0) to a final concentration of 10mM. Digested DNA was purified by extraction with phenol:chloroform (1:1) (modified from Manniatis et al., 1982). Dessicated DNA was resuspended in a Ligation buffer to a concentration of 0.2μg/mL (modified from Ochman et al., 1990). Ligation reaction was initiated by adding 1 unit/uL t4 DNA ligase. Reaction components were incubated at 15°C for 16 hours, followed by precipitation with salt and ethanol. PCR components were identical to those in regular PCR, but changes in the cycle consisted of 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 50°C, and 2 minutes extension at 70°C.

2.2h Purification of PCR product:

PCR product was visualized on 1% NuSieve (FMC) 2% agarose (Sigma) gel in 0.5X TBE buffer by staining with ethidium bromide and fluoresced with UV light. Agarose plugs were removed from bands and DNA was resuspended in 100μL water, and

heated to 70°C for 30 minutes. DNA was purified with Magic PCR Preps DNA Purification system (Promega), and re-amplified in 3 volumes of 100μL reactions. Three 100μL reactions were pooled for each sample, and again purified with the Magic PCR purification kit.

2.2i Sequencing:

Purified DNA was quantified on Hoefer TKO 100 Fluorometer (Hoefer Scientific Instruments), and subjected to electrophoresis on agarose gels as described in "Purification of PCR product". DNA was sequenced on an Automated 373A DNA Sequencer (ABI) using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (ABI). Cycle sequencing reaction involved 25 or 30 temperature cycles (depending on the primers used) of denaturation at 98°C for 1 second, annealing at 45°C or 50°C (depending on the primer used) for 15 seconds, and extension at 60°C for 4 minutes.

Sequences were deposited in Genbank with accession numbers corresponding to samples in Table 2-3; AFO13777 to AFO13797 (for V250), AFO14514 to AFO14533 (for 83/66), AFO14534 to AFO14556 (for III180), and AFO14557 to AFO14579 (for III520). Sequence alignment is shown in Appendix G.

2.3 SECTION A: Phylogeny

2.3A RESULTS

2.3A.a *Analysis of individual fragments:*

The V250, III520 and 83/66 fragments were more variable and produced a larger number of phylogenetically informative sites than the III180 fragment (Figure 2A-1). The data set for III180 contained a larger number of constant characters than the other three data sets (Figure 2A-1). Hence, this data set produced a large number of unresolved taxonomic groups (Figure 2A-2B).

Different groups of taxa were identified by each of the four data sets. Sample sets are listed in Table 2-3. Monophylies for NABS II, III and VII were produced by primer set V250. Primer III520 identified a significant monophyletic clade for NABS IX, and 83/66 produced weakly supported monophylies for NABS I, III and IX (Figures 2A-2 and 2A-3).

The data set from V250 had the largest number of characters (Figure 2A-1) and produced the highest degree of resolution of taxa (Figure 2A-2). The III180 data set contained a larger number of characters than the III520 or 83/66 data sets, yet had a lower Consistency Index (CI) (Table 2-4), indicating that the type of characters within the data set were affecting the outcome of the analysis.

2.3A.b *Phylogenetic signal and congruency:*

The data sets were tested for phylogenetic signal by analysis of frequency

Table 2-3: *Armillaria* isolates amplified by the primer sets indicated at the top of each column. Isolate names correspond to those in Table 2-1. This table illustrates the layout of the combined data set in the NEXUS file for PAUP. Isolate names in the first column (V250) were used to label all branch termini in the topologies.

V-250	III-180	III-520	83/66
I DMR 20	I DMR 20	I DMR 20	I NOF 1076
I Bow Pk	I Bow Pk	I Bow Pk	I Bow Pk
I JB 08	I JB 08	I JB 09	I JB 09
I GG 12D	I GG 12D	I GG 12A	I JB 13
II JB 39	II JB 38	I JB 38	II JB 38
II 160-8	II 160-8	II 160-8	Missing
II JB 85A	II JB 85A	II JB 85B	II JB 39
III JB 56	III JB 56	III JB 56	III JB 56
III JB 61D	III JB 61D	III JB 61D	III JB 61D
I NOF 830	I NOF 830	I NOF 830	I NOF 830
V 83621	V 48-3	V 48-3	V 48-3
V JB 75B	V JB 75B	V JB 75B	V JB 66
V Bow Pk	V Bow Pk	V JB 72	V Bow Pk
V JB 07	V JB 07	V JB 19	V 83911
VI PD 37C	VI PD 37C	VI PD 37C	VI GB 898
VI KJS-6	VI KJS-6	VI KJS-6	VI 97-1
VII 90-10	VII 90-10	VII 90-10	VII 90-10
VII HHB 11912	VII HHB 11912	VII HHB 11912	VII HHB 11912
IX TJV 179-1	IX TJV 179-1	IX TJV 179-1	IX TJV 179-1
IX TJV 200-9	IX TJV 200-9	IX TJV 200-5	Missing
Missing	IX TJV 188-4	IX 121-2	IX TJV 188-4
Missing	IX 139-1	IX 139-1	IX TJV 188-4
X SP812015	X SP812015	X SP812015	X SP812015

Figure 2A-1: Character information content for each of the nucleotide data sets amplified by the four primer sets, V250, III180, III520 and 83/66, comparing total number of characters, number of constant characters, number of phylogenetically uninformative characters and number of phylogenetically informative characters.

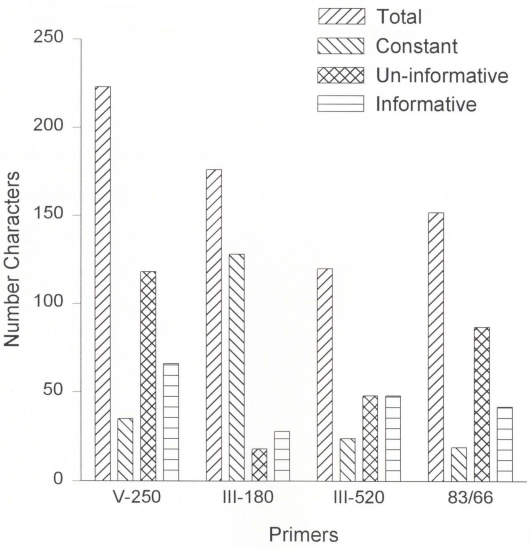

Total
Constant
Un-informative
Informative
250
200
150
100
50
0
Number Characters
V-250
III-180
III-520
83/66
Primers

Figure 2A-2: Phylograms based on nucleotide sequences amplified by **A:** V250 for 224 base pairs, showing highest degree of resolution of the four data sets, and **B:** III180 for 177 base pairs, showing lowest degree of resolution, for eight of the NABS *Armillaria*, using two isolates of NABS VI as outgroup. Numbers indicate bootstrap proportions from 100 bootstrap replicates. Horizontal branch length is proportional to distance and tree was obtained using maximum parsimony.

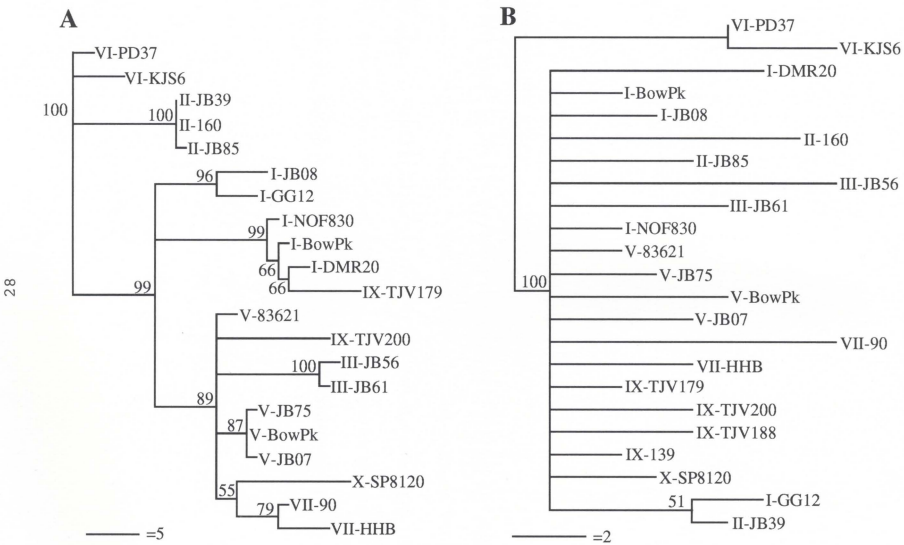

A
VI-PD37
VI-KJS6
II-JB39
II-160
II-JB85
I-JB08
I-GG12
I-NOF830
I-BowPk
I-DMR20
IX-TJV179
V-83621
IX-TJV200
III-JB56
III-JB61
V-JB75
V-BowPk
V-JB07
X-SP8120
VII-90
VII-HHB
100
100
96
99
66
66
99
100
89
87
55
79
=5
B
VI-PD37
VI-KJS6
I-DMR20
I-BowPk
I-JB08
II-160
II-JB85
III-JB56
III-JB61
I-NOF830
V-83621
V-JB75
V-BowPk
V-JB07
VII-90
VII-HHB
IX-TJV179
IX-TJV200
IX-TJV188
IX-139
X-SP8120
I-GG12
II-JB39
100
51
=2

Figure 2A-3: Phylograms based on nucleotide sequences amplified by **A:** III520 for 122 base pairs, and **B:** 83/66 for 150 base pairs, both showing moderate degrees of resolution, for eight of the NABS *Armillaria*, using two isolates of NABS VI as outgroup. Numbersindicate bootstrap proportions from 100 bootstrap replicates. Horizontalbranch length is proportional to distance and tree was obtained using maximum parsimony.

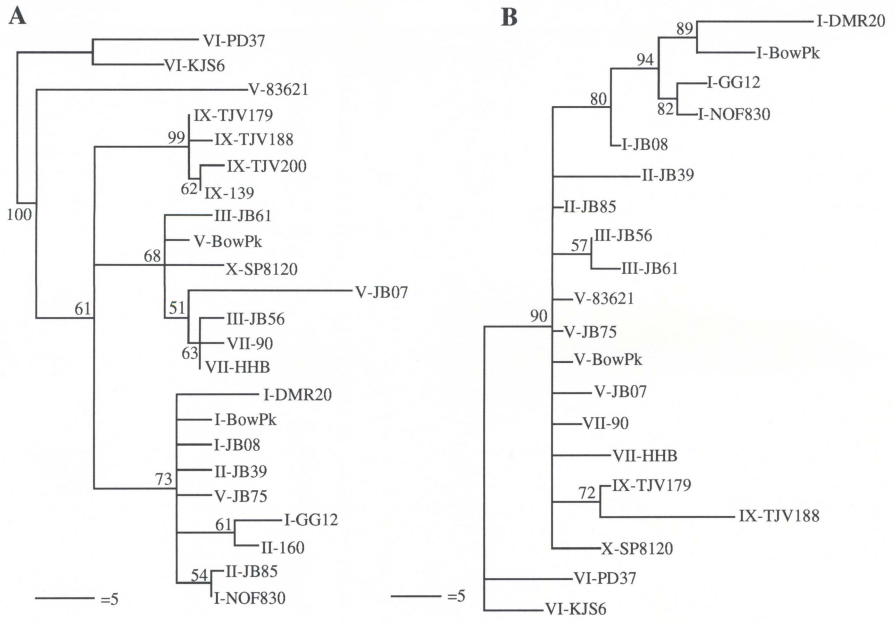

A
VI-PD37
VI-KJS6
V-83621
IX-TJV179
99
IX-TJV188
IX-TJV200
62
IX-139
100
III-JB61
V-BowPk
68
X-SP8120
V-JB07
61
51
III-JB56
VII-90
63
VII-HHB
I-DMR20
I-BowPk
I-JB08
II-JB39
73
V-JB75
I-GG12
61
II-160
II-JB85
54
I-NOF830
=5
30
B
I-DMR20
89
I-BowPk
94
I-GG12
82
I-NOF830
80
I-JB08
II-JB39
II-JB85
III-JB56
57
III-JB61
V-83621
90
V-JB75
V-BowPk
V-JB07
VII-90
VII-HHB
IX-TJV179
72
IX-TJV188
X-SP8120
VI-PD37
=5
VI-KJS6

Table 2-4: Evaluation of frequency distributions of 5000 randomly sampled tree lengths for phylograms of four primer sets, V-250, III-180, III-520, 83/66 and the combination data set (four primers) showing consistency index (CI), homoplasy index (HI), actual tree length (parsimony), mean tree length (random sample), standard deviation (SD) and g1 (skewness index).

Primer set	CI	HI	Actual tree length (parsi-mony)	Mean tree length (random)	SD	g1
V-250	0.795	0.205	307	448	11.4	-0.89
III-180	0.537	0.463	108	124	3.2	-0.69
III-520	0.694	0.306	206	302	9.5	-0.55
83/66	0.774	0.226	217	266	4.9	-0.95
Combined four primers	0.716	0.284	790	1141	21.3	-0.62

distributions of 1000 random trees. Frequency distributions were all skewed to the left as shown by negative g1 values (Table 2-4), and length of each most parsimonious tree of four of the five data sets was at least one standard deviation shorter than the shortest of the random trees (Table 2-4). Shape of the frequency distributions is indicated by the g1 values, the range, and the number tree lengths less than the mean of the random tree lengths (Table 2-4). The length of the most parsimonious III180 tree was within the range of random tree lengths ($p=0.15$), but the g1 value was negative (Table 2-4).

Congruence of the four data sets was measured by applying Kashino and Hasegawa's (1989) likelihood test. Because each data set contained slightly different taxa due to missing data, topologies of the corresponding combined trees were compared (Figure 2A-4). The V250 tree was the only tree congruent with the combined tree (Table 2-5). However, due to the insufficient resolution of each individual tree, the four data sets were combined to produce a final tree which was supported by existing hypotheses.

2.3A.c *Phylogenetic analysis:*

The combined data set separated the eight species into three clades; NABS I, II and all other NABS *Armillaria* (Figure 2A-5). Three isolates of NABS I were separated by large geographical distances but remained very similar with a bootstrap value of 99%. Variability within NABS I was indicated by relatively weak bootstrap support of 67% for the entire clade of five isolates. NABS II formed a strong monophyletic group supported by 99% bootstrap value (Figure 2A-5).

Figure 2A-4: Phylograms from 100 bootstrap replicates of the four combined data sets corresponding to: **A:** V250-Two isolates of NABS IX were removed so taxa would match those in V250 data set, **B:** 83/66-One isolate of NABS II and two isolates of NABS IX were removed to match taxa in 83/66 data set, and **C:** III520, III180, and combined set - Nothing was removed since taxa in III520, III180, and combined set were all the same. This tree is identical to the phylogram in Figure 2A-5. The topology among A, B and C is more similar than the topology between V250 (Figure 2A-2) and the combined set (Figure 2A-5). V250 and the combined set were statistically congruent (Table 2-5).

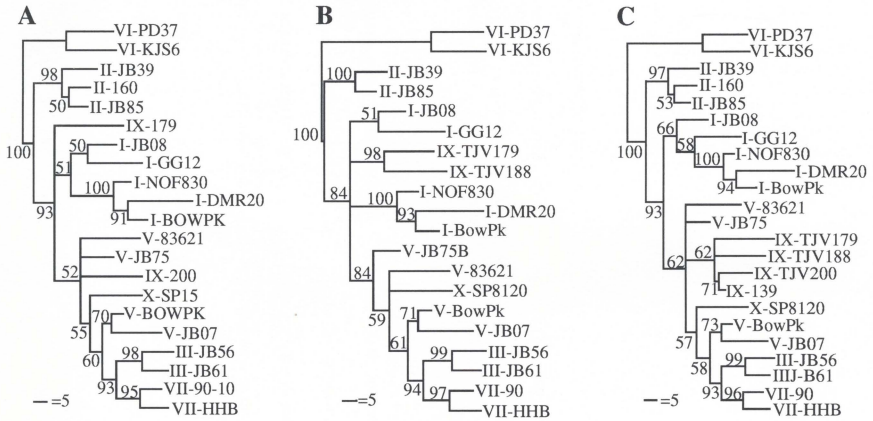
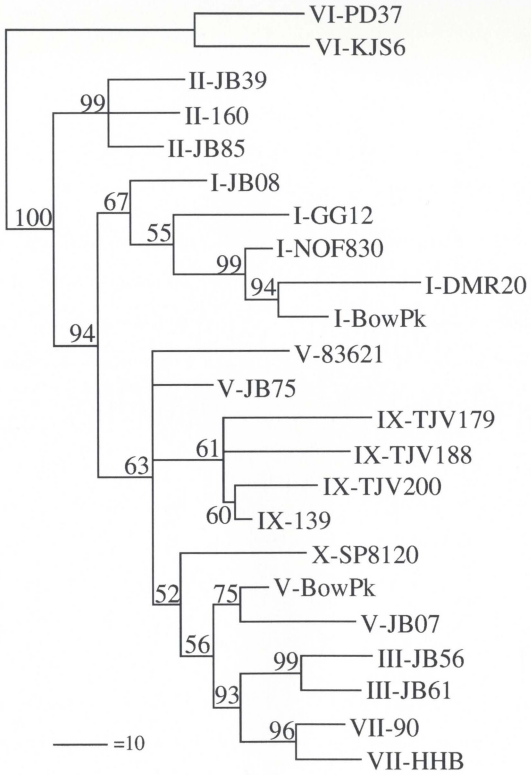

A
VI-PD37
VI-KJS6
II-JB39
II-160
II-JB85
IX-179
I-JB08
I-GG12
I-NOF830
I-DMR20
I-BOWPK
V-83621
V-JB75
IX-200
X-SP15
V-BOWPK
V-JB07
III-JB56
III-JB61
VII-90-10
VII-HHB
=5
B
VI-PD37
VI-KJS6
II-JB39
II-JB85
I-JB08
I-GG12
IX-TJV179
IX-TJV188
I-NOF830
I-DMR20
I-BowPk
V-JB75B
V-83621
X-SP8120
V-BowPk
V-JB07
III-JB56
III-JB61
VII-90
VII-HHB
=5
C
VI-PD37
VI-KJS6
II-JB39
II-160
II-JB85
I-JB08
I-GG12
I-NOF830
I-DMR20
I-BowPk
V-83621
V-JB75
IX-TJV179
IX-TJV188
IX-TJV200
IX-139
X-SP8120
V-BowPk
V-JB07
III-JB56
IIIJ-B61
VII-90
VII-HHB
=5

Table 2-5: Results from Kashino and Hasegawa (1989) likelihood tests comparing each individual tree with the tree from the corresponding combined set for congruency. Maximum parsimony trees from 100 bootstrap replicates of the modified combined sets (V250 and 83/66) are shown in Figure 2A-4. Each individual tree is considered significantly worse than the tree produced from the combined data set in explaining the combined sequences, when the difference of log likelihood is more than twice the standard deviation.

Trees	**Log Likelihood**	**Difference of Log Likelihood**	**Standard Deviation**	**Significantly worse?**
*V250 Combined tree (omitting appropriate taxa to match V250)	-3416.87 -3396.17	20.71 (Best)	21.67	No
III180 III520 Combined tree	-4364.82 -3879.77 -3516.37	848.45 363.40 (Best)	102.39 61.41	Yes Yes
*83/66 Combined tree (omitting appropriate taxa to match 83/66)	-3681.31 -3324.42	356.89 (Best)	53.29	Yes

*Combined set was modified to correspond with the taxa present in the individual sets for likelihood comparisons.

Figure 2A-5: Phylogram based on combined nucleotide sequences from four primer sets for 23 taxa of eight NABS *Armillaria* using two isolates of NABS VI as outgroup. A single most parsimonious tree from 100 bootstrap replicates was produced. Horizontal branch length is proportional to distance,and tree was obtained using maximum parsimony.


VI-PD37
VI-KJS6
II-JB39
II-160
II-JB85
I-JB08
I-GG12
I-NOF830
I-DMR20
I-BowPk
V-83621
V-JB75
IX-TJV179
IX-TJV188
IX-TJV200
IX-139
X-SP8120
V-BowPk
V-JB07
III-JB56
III-JB61
VII-90
VII-HHB
99
100
67
55
99
94
94
63
61
60
52
75
56
99
93
96
=10

NABS III, V, VII, IX and X formed the third clade in the phylogenetic tree (Figure 2A-5). NABS III and VII formed a monophyletic group with strong bootstrap support (93%). NABS IX formed another monophyletic group but bootstrap support was weak (61%). The resolution of isolates of NABS V was poor and indicated polymorphism within the sequences examined. NABS X was weakly associated with NABS III, VII, and part of V. The combined data set contained missing sequences and ambiguity at some character positions (unpublished data).

Although minimum-length networks from each of the four separate data sets were very similar to the trees produced by maximum parsimony (Figures 2A-2 and 2A-3), there were minor differences in 83/66 data set, and there was a much higher degree of resolution in the III180 network (Figure 2A-6B) than the corresponding parsimony tree (Figure 2A-2B).

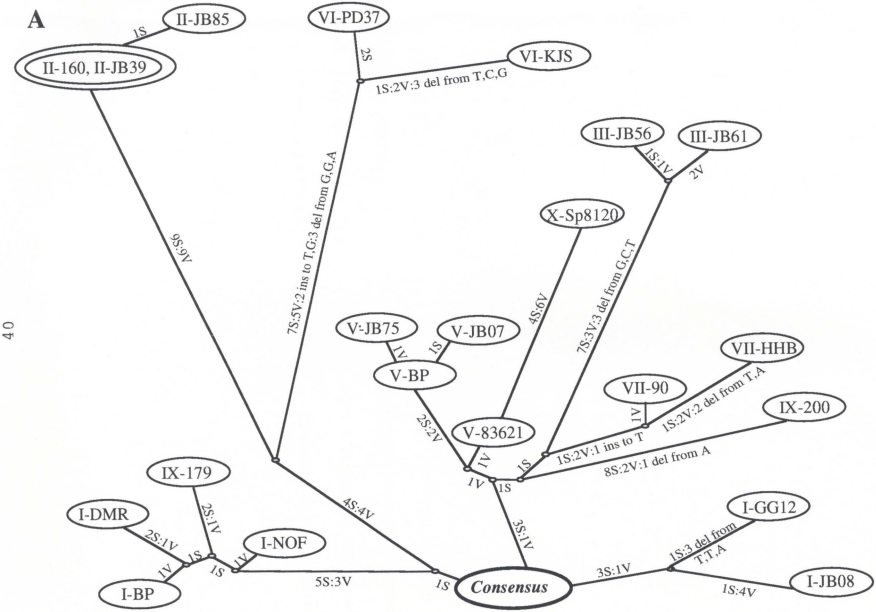
Neighbour joining (NJ) analysis inferred a phylogeny (Appendix B) that was consistent with phylogenetic relationships produced by maximum parsimony.

Comparison of each of the sequences with those in Genbank, using the search algorithm BLAST, showed very little similarity with known sequences.

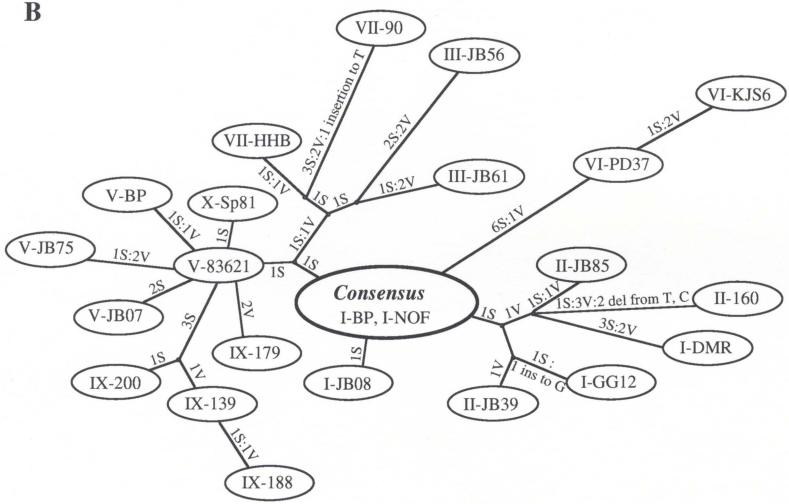
2.3A.d Molecular clock:

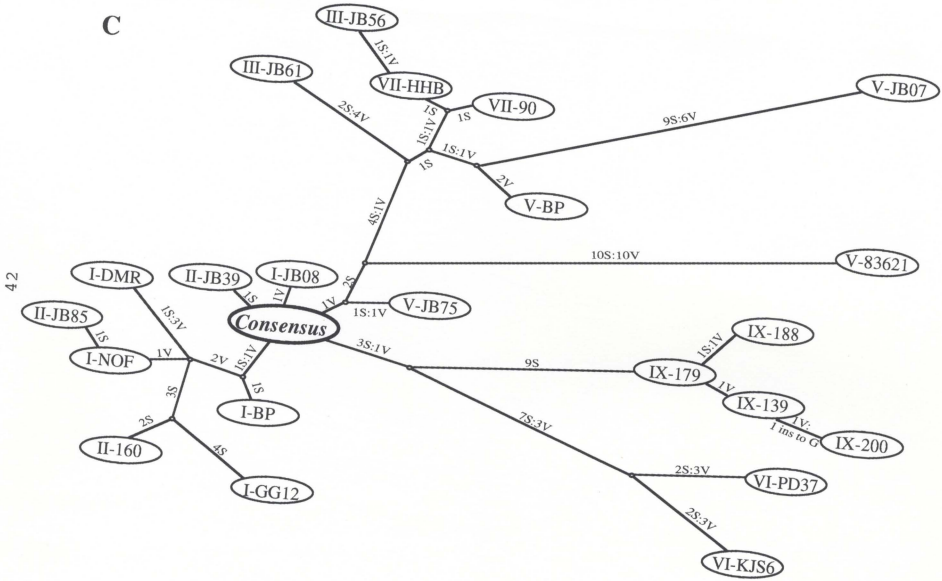
NABS VI was placed at 30 million years (Ma) based on fossil evidence from closely related Agaricales (Hibbett et al., 1995). The phylogeny was superimposed on the molecular clock (Figure 2A-7) based on a calibration point of 30 Ma.

Figure 2A-6: Minimum-length network diagrams based on nucleotide sequences of **A:** V250, **B:** III180, **C:** III520, and **D:** 83/66 comparing variation to a consensus sequence. Substitutions were determined manually to illustrate all substitution changes among taxa. Length of branches correspond to number of substitutions. Transition : transversion ratios are indicated on each branch. S=transition, V=transversion, del=deletion, and ins=insertion. Taxa epithets correspond to those in Figure 2A-5.


A
II-JB85
1S
II-160, II-JB39
VI-PD37
2S
VI-KJS
1S:2V:3 del from T,C,G
III-JB56
1S:1V
III-JB61
2V
X-Sp8120
9S:9V
7S:5V:2 ins to T,G;3 del from G,G,A
4S:6V
7S:3V:3 del from G,C,T
V-JB75
1V
V-JB07
1S
V-BP
2S:2V
V-83621
VII-HHB
VII-90
1V
1S:2V:2 del from T,A
IX-200
1S:2V:1 ins to T
8S:2V:1 del from A
1V
1S
IX-179
2S:1V
I-DMR
2S:1V
1S
I-NOF
1V
1V
1S
I-BP
4S:4V
5S:3V
1S
Consensus
3S:1V
3S:1V
1S:3 del from T,T,A
I-GG12
I-JB08
1S:4V

B


VII-90
III-JB56
VI-KJS6
3S:2V:1 insertion to T
2S:2V
1S:2V
VII-HHB
VI-PD37
1S:1V
1S
1S
1S:2V
III-JB61
V-BP
X-Sp81
6S:1V
1S:1V
1S
1S:1V
V-JB75
1S:2V
V-83621
1S
1S
II-JB85
Consensus
I-BP, I-NOF
1S
1V
1S:1V
1S:3V:2 del from T, C
II-160
2S
3S
2V
3S:2V
I-DMR
V-JB07
IX-179
1S
1V
1S:
1 ins to G
IX-200
IX-139
I-JB08
1S
1V
I-GG12
II-JB39
1S:1V
IX-188


C
III-JB56
1S:1V
III-JB61
VII-HHB
2S:4V
1S
VII-90
1S:1V
1S
1S:1V
9S:6V
V-JB07
2V
V-BP
4S:1V
10S:10V
V-83621
I-DMR
II-JB39
I-JB08
2S
1S
1V
1V
1S:1V
V-JB75
II-JB85
1S
Consensus
1S:3V
I-NOF
1V
2V
1S:1V
3S:1V
9S
IX-188
1S:1V
IX-179
1V
IX-139
1V
1 ins to G
IX-200
3S
1S
I-BP
2S
II-160
4S
I-GG12
7S:3V
2S:3V
VI-PD37
2S:3V
VI-KJS6
42

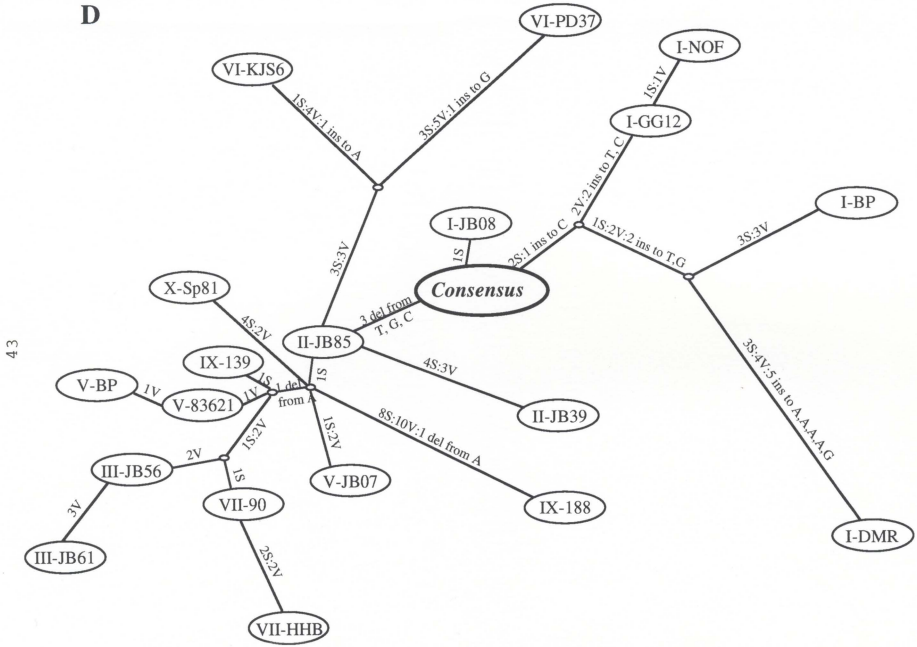
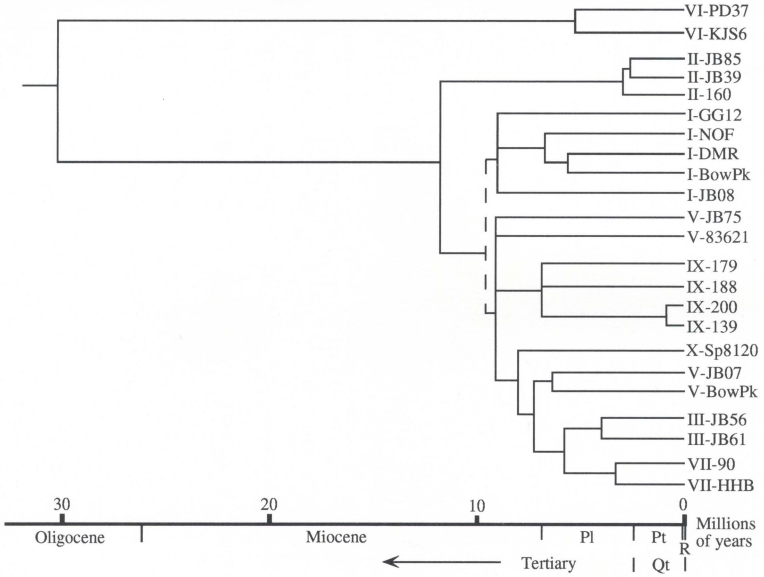

D
VI-PD37
VI-KJS6
1S:4V:1 ins to A
3S:5V:1 ins to G
3S:3V
I-NOF
1S:1V
I-GG12
2V:2 ins to T,C
I-BP
I-JB08
1S
2S:1 ins to C
1S:2V:2 ins to T,G
3S:3V
Consensus
X-Sp81
4S:2V
3 del from T,G,C
II-JB85
3S:4V:5 ins to A,A,A,A,G
IX-139
1S
1 del from A
4S:3V
V-BP
1V
V-83621
1V
II-JB39
1S:2V
1S:2V
8S:10V:1 del from A
III-JB56
2V
1S
V-JB07
IX-188
3V
VII-90
III-JB61
2S:2V
I-DMR
VII-HHB

Figure 2A-7: Divergence times of NABS *Armillaria* estimated from phylogeny (Figure 2A-5) showing branch lengths proportional to the average percent nucleotide substitutions (Table 2-6). Dashed line indicates a large amount of variation around the divergence. Pl is Pliocene, Pt is Pleistocene, Qt is Quaternary, and R is Recent in the time scale.


VI-PD37
VI-KJS6
II-JB85
II-JB39
II-160
I-GG12
I-NOF
I-DMR
I-BowPk
I-JB08
V-JB75
V-83621
IX-179
IX-188
IX-200
IX-139
X-Sp8120
V-JB07
V-BowPk
III-JB56
III-JB61
VII-90
VII-HHB
30
20
10
0
Millions of years
Oligocene
Miocene
Pl
Pt
Qt
R
Tertiary

2.3B DISCUSSION

2.3B.a Analysis of individual fragments:

Sequences of fragments obtained with V250, 83/66 and III520 were more variable, and provided a larger number of phylogenetically informative sites than the III180 sequences (Figure 2A-1). Each of the four data sets resolved different groups of species, yet produced a resolution from the combination of the four data sets that was supported by other studies (Anderson and Stasovski, 1992). The V250 tree resolved NABS II, III and VII supported by bootstrap values of 100%, 100% and 79% respectively. Interestingly, NABS IX-TJV179 formed a significant monophyly with three isolates of NABS I for this primer set only (Figure 2A-2). This isolate of NABS IX was the only one collected from a softwood host, forming a synapomorphy with NABS I, which was also from softwood hosts. The connection between NABS I and IX may be coincidental since sample size was limited, and only two kinds of host were examined, softwood and hardwood. Although primer III180 alone did not resolve any groups (Figure 2A-2), the network diagram and skewness index ($g1=-0.69$) indicated there was phylogenetic information present in the sequence. Primer III520 resolved NABS IX with bootstrap support of 99%, and 83/66 resolved NABS I, III and IX with bootstrap support of 80%, 57% and 72% respectively (Figure 2A-3). The combined tree resolved all NABS except NABS V, which was partially resolved (Figure 2A-5). Separate data sets also failed to resolve all isolates of NABS V.

The numbers and types of characters present in each of the four sequence data sets

yielded insight into the relative contribution of each type of character to the amount of phylogenetic information resolved (Figure 2A-1). The total number of characters in the V250 fragment was high and the number of constant characters was moderate to low compared to the other three fragments (Figure 2A-1). Analysis of this V250 fragment produced a phylogenetic tree yielding the highest resolution, as indicated by the highest consistency index and lowest level of homoplasy (Table 2A-4), topology of the tree (Figure 2A-2A) and the network (Figure 2A-6).

Analysis of the data set containing the second longest fragment (III180) produced the tree yielding the least information of the four fragments (Figure 2A-2B) which is also reflected in the character information content. Fragment III180 had a large number of constant characters compared to the other three fragments (Figure 2A-1) indicating the fragment was more conserved than the others. The actual number of informative characters was the lowest of the four fragments which may have resulted in unresolved intraspecific clustering (Figure 2A-2B), a low consistency index and a high homoplasy index (Table 2A-4). Although maximum parsimony produced an unresolved tree (Figure 2A-2B) the network for III180 formed clusters of related groups sharing substitutions including complete resolution for NABS III and VII, as well as the group of NABS I and II (Figure 2A-6).

The remaining two data sets, from III520 and 83/66, contained smaller number of total characters, and the number of informative characters, HI, and CI were intermediate between those in V250 and III180. The information gained from the branching pattern of

phylogenetic trees of III520 and 83/66 was also intermediate. The network for III520 was in agreement with the phylogenetic tree, but the network for 83/66 produced varied results. It resolved NABS VII, and the clade NABS III and VII, but dissolved NABS IX. The high degree of variation within this sequence was evident in the low bootstrap values for the monophylies (Figure 2A-3B).

By comparing the character information content with the branching patterns of the phylogenetic trees, the actual number of informative characters within a sequence seems to be the most important factor influencing the information presented by the branching pattern of the phylogenetic tree. The number of constant characters may be a measure of the degree of conservation of the fragment within the genus. Generally, the longer the sequence the greater the chance there will be more informative characters present. This notion was not supported by the information content and the length of fragment III180 (Figure 2A-2B).

2.3B.b Phylogenetic signal and congruency:

The fragments amplified by each of the four primer sets were the same length, and were present in most isolates of all NABS, indicating that the regions amplified were well conserved and therefore good candidates for a phylogenetic study. Frequency distributions of tree lengths, homoplasy and consistency indices were used to test whether the data contain more phylogenetic signal than would be expected purely by chance (Table 2-4). Frequency distributions of tree lengths of each of the individual trees as well

as the combined tree were all skewed to the left, as indicated by the negative g1 values (Sokal and Rohlf, 1981) (Table 2-4), indicating presence of phylogenetic signal in the data set (Hillis and Huelsenbeck, 1992). The lengths of the parsimony trees were located at least one standard deviation below the shortest in the frequency distributions (except III180) (Table 2-4), indicating the tree length obtained by maximum parsimony was significantly shorter than a tree produced with random data ($p<0.05$). Since $p=0.15$ for the tree produced by III180, the most parsimonious tree cannot be considered significantly different from a tree produced from random data. However, the negative g1 value supports inclusion of this data set in the combined analysis. Although the function and location of the fragments within the genome was unknown, the left-skewed frequency distributions of tree lengths and the location of the tree length obtained by maximum parsimony outside the 95% confidence intervals, supports the use of these fragments in a phylogenetic study. Consistency indices (CI) were 0.49 to 0.71, and homoplasy indices (HI) were 0.28 to 0.50. Archie (1989) critically evaluated the CI and reported that the CI was insensitive in data sets where the number of trees outnumber the characters, and when the absolute levels of homoplasy increase. This may be occurring in the III180 data set. The CI of the combined data set was more or less an average of the four separate sets (ca. 0.65). Since the combined set produced the most highly resolved tree, and CI was lower than in each of the best trees from the individual sets, then it would seem appropriate to conclude that the CI is not a reliable measure to compare "best" trees among different data sets. However the CI may be used as a general measure of

consistency within a data set, comparing the fit of the most parsimonious tree to that data set.

Congruence among independent data sets and their combinations provides strong evidence for a phylogenetic hypothesis (Hillis, 1987). Kluge (1989) argued for "total evidence" in a phylogeny. He suggested that it was not necessary for individual results to be consistent with the combined result, since the explanatory power of the data in the combined analysis would then be maximized. The use of more than one data set would increase the size of the sequence data available so that the trees obtained may converge toward the one true tree (Quicke, 1993). However, Miyamoto and Fitch (1995) believed that individual analyses should be performed on the data sets because separate analyses may provide insights into evolution of the separate data sets. The insights for the separate data sets in this study were discussed with reference to analysis of individual fragments, but the individual fragments provided insufficient resolution to justify using them individually. The combined analysis of these data sets emphasizes the value of Kluge's (1989) "total evidence" concept to maximize the explanatory power of the data. In this study results obtained from the combined data set were in agreement with a previous phylogenetic hypothesis (Anderson and Stasovski, 1992) and morphological groups (Bérubé and Dessureault, 1989).

It was not possible to test likelihoods of trees containing different numbers and kinds of taxa, so taxa were removed in the combined set corresponding to the missing taxa in the individual sets, and the individual trees were tested against the corresponding

combined set. Since statistical analysis could not be performed on each of these combined trees, Figure 2A-4 provides a comparison of topology. The topology among these trees is more similar than that between the V250 tree and the combined tree which are considered congruent by the likelihood test (Table 2A-5).

The Kashino and Hasegawa (1989) likelihood test was applied to the four separate data sets in this study. The question addressed was whether the most parsimonious of each of the individual trees was significantly worse in explaining the data for the combined analysis, than the tree produced from the combined data set. The V250 tree was not significantly worse, but the other three trees were significantly worse than the tree from the combined data set, suggesting that the data sets were not congruent and do not readily support combining data sets. However, if different data sets are samples of the same species history, yet by themselves they incompletely resolve the phylogenetic history, it would seem appropriate to combine them in the same way that longer nucleotide sequences would improve resolution or a larger number of morphological characters would improve resolution. Since each of the four fragments were chosen from random segments of the DNA, with the only criterion that they be conserved within the genus, then the final result is that the length of DNA sequence has been increased to a size sufficient to resolve a phylogenetic history.

A parsimony based incongruence test, ILD (Farris et al., 1995) was applied to the individual data sets in this study. Cunningham (1997) showed that the ILD was the best of three tests to distinguish degree of incongruence between genes. Interpretation of the

p-value ($p=0.001$) warrents discussion of the phylogenetic trees. Individual trees resolved specific groups leaving others unresolved. Farris et al. (1995) suggested that the ILD is large when groups that are well supported by one tree conflict with groups well supported by another tree. Discrepancy among groups in this study was not a result of incongruent fully resolved trees, but rather because different components of each data set were resolving different groups, leaving a lack of information available to form the remainder of the tree.

Sequences did not match any entries from Genbank. Possible identifications of each of the fragments may have been an unused protein-coding sequence such as a pseudogene, or an intron, or non-coding DNA since start and stop codons were present throughout the sequences (unpublished data). All transition/transversion (S/V) ratios were greater than one except in the 83/66 fragment (Figure 2A-6). Transitions are more common than transversions in protein coding genes (Mason, 1991). Since the fragments in this study were chosen randomly and were of unknown function and location, they may be part of pseudogenes or introns in nuclear DNA with lower S/V ratios than mtDNA, yet evolving fast enough to accumulate more transitions than transversions (except 83/66). The 83/66 fragment may contain more non-coding DNA undergoing less evolutionary constraint, or has been a pseudogene for a longer period of time than the other three fragments, and hence transversions could accumulate at the same rate as transitions. More transitions were apparent in the shared substitutions in the networks, and transversions became more common near taxa differentiation (Figure 2A-6), indicating that

transversions signify changes in amino acids responsible for taxa differentiation. Alternatively, if transitions accumulate at a constant rate, and the terminal branches become saturated with transitions, it would appear that there were fewer transitions than transversions.

2.3B.c Phylogeny of Armillaria:

Both parsimony and distance analyses indicated similar relationships among NABS *Armillaria*. Most of the sequence variation was between rather than within species. Initial observations of the combined phylogenetic tree of NABS *Armillaria* (Figure 2A-5) indicated that one group of species, NABS III, V, VII, IX and X, were more similar to one another than to NABS I, II and VI. This observation was also supported by similarity analysis (Appendix B).

NABS VI is a temperate and tropical species with a wide distribution found in both Northern and Southern hemispheres, and is parasitic on hardwood hosts. It was therefore chosen as the outgroup. It has been considered the most divergent of the North American species (Anderson and Stasovski, 1992) based on morphological features (Bérubé and Dessureault, 1988), the lack of clamp connections on the basidia (Korhonen, 1978), and a larger rDNA repeat, placing it in an rDNA class of its own (Anderson et al., 1989), and mtDNA digests (Anderson and Smith, 1988). Preliminary analyses using sequences of *A. tabescens* as the outgroup always placed NABS VI basal to the rest of NABS *Armillaria* (unpublished data). Isolates of NABS VI grouped together with a large

number of common base substitutions in the networks which were based on direct similarity to consensus sequences (Figure 2A-6), further supporting the choice of VI as outgroup.

NABS III and VII. The close phylogenetic relationship hypothesized by Anderson and Stasovski (1992) and Smith and Anderson (1989), between NABS III and VII, was supported by this study. NABS III and VII formed a monophyletic cluster with a significant bootstrap proportion of 93% in both parsimony and neighbour joining cladograms (Figure 2A-5). NABS III and VII were both found in the same rDNA class (Anderson et al., 1989) based on restriction maps of rDNA. They show similarity in cluster analyses of RFLPs of mtDNA (Smith and Anderson, 1989), in fruit body morphology (Bérubé and Dessureault, 1989), and are considered closely related species based on intergenic (IGR) sequences of rDNA (Anderson and Stasovski, 1992). They are also found as weak pathogens or saprotrophs on hardwood hosts. NABS III and VII are both distributed in eastern North America, but the European counterpart of VII, *A. lutea*, is distributed widely in Europe and far east Asia. Consequently, NABS VII may have given rise to the more narrowly distributed NABS III.

Anderson et al. (1989) placed the European species, *A. cepistipes*, in the same rDNA class as III and VII. *A.cepistipes* exhibits very low interfertility with NABS V (Bérubé et al., 1996) and is partially interfertile with NABS X (Anderson et al., 1980). It is not surprising in this study that isolates of NABS V and NABS X formed a paraphyletic group with the clade containing NABS III and VII in both cladograms

(Figure 2A-5), which implies a close relationship between the monophyletic clade, NABS III and VII, and the more variable clade, NABS V, IX and X. This is consistent with IGR sequences in which V, IX and X form a close common ancestry with III, VII and *A. cespistipes* (Anderson and Stasovski, 1992).

NABS V, IX and X. In this study four isolates of NABS IX formed a monophyletic clade weakly supported with 61% bootstrap value in the phylogram (Figure 2A-5). Missing data in three of 16 fragments may explain the low bootstrap value in this study (Table 2A-3). NABS IX may have diverged from the same common ancestor that gave rise to NABS V and X. This is consistent with phylogenetic analysis of IGR sequences of rDNA of *Armillaria* placing NABS IX more distantly related to V and X (Anderson and Stasovski, 1992). Anderson et al. (1989) showed that NABS V, IX, and X formed the same rDNA class 4. Further evidence supporting their relatedness was given in Miller et al. (1994) who were unable to resolve relationships among III, VII, V, IX and X based upon DNA reassociation values. However, Harrington and Wingfield (1995) were able to distinguish NABS IX from other NABS *Armillaria* using RFLP-PCR. NABS V is distributed across temperate North America and Japan and is found on hardwoods. Both NABS IX and X are small populations found on the west coast of North America but IX generally colonizes hardwoods (Volk et al., 1996) and X colonizes conifers (Anderson et al., 1980; Morrison et al., 1985b). NABS IX and X may be derived from the more widely distributed NABS V, a hypothesis consistent with shared morphological features of NABS V and IX such as absence of scales, small black hairs present on the surface

of the pileus and a more orange coloration than other species (Bérubé and Dessureault, 1988; Volk et al., 1996).

Two isolates of NABS V (VBowPk and VJB07) were collected a long distance (more than 2000 Km) apart, in Newfoundland and Quebec, respectively. The phylogenetic position of the other two isolates of NABS V, V83621 and VJB75, collected from New York and Quebec respectively, was unresolved, but both groups of NABS V remain within the clade containing NABS X and a clade of NABS IX. The paraphyly of isolates of NABS V indicated a high degree of polymorphism within the sequences relative to the other species (Figure 2A-5). Isolates of NABS V also seem to be variable with respect to pathogenicity (Mallett, 1990). This variation may be an indication of a recent sympatric speciation of NABS V, IX and X which was discussed by Anderson et al. (1989) and Miller et al. (1994) as occurring between NABS I and II. The phylogenetic position of NABS X was unclear since only a single isolate with ambiguous sequence was used in this study.

NABS I and II. Despite the large geographical separation of the isolates, IDMR20, IBowPk and INOF830, they remained phylogenetically similar with a significant bootstrap value of 99% (Figure 2A-5). The addition of two more isolates , IJB08 and IGG12, reduced the bootstrap value to 67% for the clade containing NABS I. Host species for the five isolates consisted of a mixture of softwoods and hardwoods (Tables 2A-1 and 3) which may partially explain the variation. NABS I has also been considered variable in its degree of pathogenecity (Rishbeth, 1982; Guillaumin et al., 1983) and in fruit body

morphology (Bérubé and Dessureault, 1988).

This study indicated that there was less variation in DNA of the narrowly distributed NABS II (Bootstrap proportion (BP)=99%) than that of the widely distributed NABS I (BP=67%), but there was a distinct separation between NABS I and NABS II based on parsimony results (Figure 2A-5). This may be explained in several ways. First, the NABS II clade may be an artifact resulting from inherent characteristics of the samples collected and the collection sites, suggested by the high bootstrap value associated with three isolates of NABS II forming an ancestral position to NABS I (Figure 2A-5). Second, these four sequences may indeed represent divergence between NABS I and II, since the high bootstrap value (94%) uniting the clade NABS I with the other clade containing NABS III, VII, V, IX and X, significantly excludes NABS II from the cluster. Nevertheless, the high bootstrap value (100%) connecting NABS II with all other NABS reflects the strong relationship between NABS II and all other NABS *Armillaria* (excluding NABS VI). Thirdly, NABS I and II shared substitutions in the III520 and III180 sequences, but substitutions were shared by NABS VI and II in V250 and 83/66 sequences (Figure 2A-6). In contrast, Miller et al. (1994) showed that isolates of NABS I and II phenetically clustered tightly together based on DNA reassociation measures. But the present study, based on four sequences, may more accurately reflect other differences between these two species such as differences in virulence and rhizomorph morphology (Korhonen, 1978; 1980). Consequently, the synplesiomorphy of occupying a hardwood host, as well as sequence similarity among isolates of NABS II

and VI in this study, may provide conditions sufficient for the NABS II monophyly to appear ancestral to NABS I. NABS II, being limited in distribution, may have diverged from the widely distributed NABS I as supported by networks of fragments III180 and III520 (Figure 2A-6). This type of sympatric speciation was suggested by Anderson et al. (1989) and supported by Anderson and Stasovski (1992). Alternatively, sequences from V250 and 83/66 may have represented paralogous genes from NABS II and VI.

2.3B.d Molecular Clock:

Calculation of substitution rates. Rates of substitution vary among lineages of *Armillaria* (Appendix C). Since two isolates of NABS VI were used as the outgroup, and their distances were similar to one another, calculation of all relative rates was determined using isolate VIPD37. The distance from VIPD37 to present was determined by calculating the midpoint between lowest and highest dissimilar values in the distance matrix, rather than the average of dissimilarity values, or dissimilarity with consensus sequence. A midpoint would eliminate any bias toward short or long branches. However, the midpoint calculated as 14.60% was very similar to the average of 14.33%. In contrast to Berbee and Taylor (1993), substitutions for lineages were not normalized, and relative rates were based on distance. Since the use of genetic distance assumes equal amounts of homoplasy across taxa, Mindell and Thacker (1996) discouraged the use of genetic distance in relative rate tests. However, topologies between the neighbour joining distance (Appendix B) and parsimony based (Figure 2A-5) trees were very similar in this

study. Rate variation between isolates, indicated by the staggered end points of the terminal branches (Figure 2A-5), would also affect molecular clock estimates of divergence times. Divergence times of basal branches were calculated using two of the highest and two of the lowest dissimilarity values to obtain an average to represent the divergence. Divergences occurring near terminal branches were calculated as an average of all possible rates for each divergence.

Berbee and Taylor (1993) provided molecular evidence that the Basidiomycetes emerged 200 to 300 Ma at the beginning of the Mesozoic, and that mushrooms appeared about 130 Ma, coinciding with the radiation of the Angiosperms. The origin of Agaricales in the Mesozoic era is suggested by their present day involvement in ectotrophic symbioses leading to the evolution of the Pinaceae (Pirozynski,1976). Divergence of mushroom groups, *Athelia* and *Spongipellis*, occurred approximately 120 Ma (Berbee and Taylor, 1993), and the divergence of *Coprinus* was even more recent (Bruns et al., 1992).

The oldest fossil gilled mushroom was found in the mid-Cretaceous (90-94 Ma). A more recent finding was dated 25-30 Ma (Hibbett et al., 1995) revised from an earlier estimate of 40 Ma (Poinar and Singer, 1990). This mushroom, *Coprinites dominicana*, was considered to be a member of the Agaricales, and had many similarities with the present-day genus *Coprinus* (Poinar and Singer, 1990). Although accelerated morphological divergence has been observed among mushroom genera (Bruns et al., 1989), and rare recessive alleles can produce extensive morphological change in the

basidiocarp (Hibbett et al., 1994), Hibbett et al. (1995) suggested that extant morphologies such as gilled mushrooms, may be of ancient origin. However, the difficulties of relating extinct fossil mushrooms to extant genera, suggest that present-day genera may be fairly recent. Assuming NABS VI is as old as *Coprinites*, the calibration point in this study was 30 Ma for NABS VI with 14.60% substitution in that time. Consequently, NABS VI may be younger than 30 Ma, but this calibration point was used as an estimate based on limited available fossil evidence. The objective of this section was an attempt to put the evolutionary divergence of NABS *Armillaria* into an historical framework. This nucleotide substitution rate of 48.7% per 100 Ma for anonymous gene sequences in *Armillaria* was higher than the 1% per 100 Ma used for 18S ribosomal gene sequences (Berbee and Taylor, 1993). Since sequences used in this study were conserved only within the genus *Armillaria*, and rRNA genes are found in all fungi, a faster rate of evolution would be consistent with the degree of conservation of the genes.

Co-evolution of fungus and host. Angiosperms were thought to have evolved from Gymnosperm ancestors. Coniferous Gymnosperms appeared about 300 Ma, but Angiosperms did not appear until 140 Ma (Stewart and Rothwell, 1993). Meeuse (1975) and Hughs (1977) suggested that Angiosperms evolved from a heterogeneous Gymnosperm ancestry displaying a combination of characters, some found only in Angiosperms, some only in Gymnosperms, and some that fit neither group. The rapid evolution and diversification of the Angiosperms in the Cretaceous may account for the establishment of ancestral *Armillaria* species as pathogens of Angiosperm hardwoods.

If this early *Armillaria* ancestor had been exposed to heterogenetic traits of conifers from the Carboniferous, an already established group related to the Angiosperms, perhaps present-day *Armillaria* retained its variable genetic traits allowing the pathogen compatibility with the genetic diversity of its host. The variation in soft and hardwood host pathogenicity displayed by the extant genus *Armillaria* may be reflected in exposure of its ancestor to host heterogeneity. Basidiomycetes that radiated in the Mesozoic are considered the most aggressive wood rotters in present environments (Robinson, 1990). *Armillaria* can subsist as a saprotroph on decaying wood debris in the soil until a suitable host becomes available. The ancestor of NABS VI may have been one of those wood rotters, with extant species maintaining both saprotrophic and pathogenic modes of life.

Major climatic changes occurred about 50 to 60 Ma (Hopkins et al., 1971). Radiation of the more modern type of Angiosperms occurred, while diversity of the Gymnosperm flora decreased (Stewart and Rothwell, 1993). Major continental uplifts and climatic cooling occurred during the Miocene, which was characterized by more mesophytic vegetation in which broad-leaf evergreens were limited to lower latitudes, and present-day forest associations became established (Stewart and Rothwell, 1993). If the ancestor of NABS VI contained genetic diversity suitable for colonization of both soft and hardwoods, then diversification of the ancestor would occur as the Angiosperms radiated 50 Ma, and remained successful for 30 million years. Dramatic climatic fluctuations occurred during the tertiary, although it was characterized mainly by tropical forest (Wolfe, 1971). The proposed separation of two lineages of *Armillaria* during these

changes is supported by the present world-wide distribution of NABS VI, and the radiation of eight other more recent NABS *Armillaria* throughout the Northern Hemisphere. Phylogenetic study of *Pleurotus* demonstrated that early evolving species are presently broadly distributed, whereas more recently evolved species are restricted in their geographic distributions (Vilgalys and Sun, 1994). Perhaps the widely distributed NABS VI continued to colonize the successful hardwood hosts, and the other less widely distributed ancestral group colonized both soft and hardwood hosts.

Vegetation was adapted to drier, cooler environments in the Pliocene (Heusser and King, 1988), which included softwood conifers of the present-day boreal forest. Since conifers began to invade the habitat already occupied by *Armillaria* in the Miocene, a coniferous niche was opened which required adaptations of the pathogen to a different host. The second lineage of *Armillaria* diverged into 2 major groups as environmental changes occurred (Figure 2A-7). One group became highly pathogenic on conifer hosts (NABS I) and the other group formed five species neither entirely pathogenic nor saprotrophic, and colonizing both hard and softwood hosts (NABS III, V, VII, IX and X).

Isolates of NABS I were successful in the Miocene, and presently colonize the Northern Hemisphere. Although two data sets in this study supported similarity between NABS I and II (Figure 2A-3A, and Figure 2A-6B and C), it also demonstrated synplesiomorphies present between NABS II and VI (Figure 2A-2A and Figure 2A-6A and D). These synplesiomorphies affected the combined phylogenetic tree placing NABS II basal to all NABS *Armillaria* including NABS I, with significant bootstrap support of

94% (Figure 2A-5). This in turn affected the placement of NABS II in the molecular clock making it appear ancestral to NABS I. However, NABS I and II have been shown to be very similar based on morphological characters (Bérubé and Dessureault, 1989) and molecular characters (Anderson et al., 1989; Anderson and Stasovski, 1992; Miller et al., 1994), with the exception of host preference and base substitutions from anonymous sequences in this study (Figure 2A-6A and D). NABS I is presently distributed throughout the Northern Hemisphere, while NABS II occupies a limited area east of the Great Lakes Region. Literature evidence is in favour of NABS II being sympatrically derived from NABS I, but this study suggests that NABS II is ancestral to NABS I.

As the Laurentide ice sheet receded from the Great Lakes Region about 13 thousand years ago (Ka), the area was first invaded by conifers such as spruce and fir (Davis and Jacobson, 1985), and later hardwoods such as sugar maple and chestnuts followed (Davis, 1981). Other species present in this area today include beech, yellow birch, basswood, ash and oak making these forests highly diversified. If previous studies prove correct then perhaps the phylogenetic species of *Armillaria* had gone through a bottleneck from the more primitive character of colonizing a hardwood host in NABS VI, to a more recent character of colonizing a softwood host in NABS I, and reverting back to colonizing a hardwood again in NABS II. The opening of the hardwood niche by the retreat of the Laurentide ice sheet provided a habitat in which these primitive characters, retained from the ancestral genotype, could re-surface in a sympatric speciation event creating NABS II which presently colonizes hardwoods (Table 2A-1). Base substitutions

in the sequences in this study reflect independent characters common to NABS II and VI. Alternatively, if this study proves correct, then perhaps NABS I had diverged from NABS II, rapidly colonizing the Northern hemisphere and causing NABS II to recede toward the Great Lakes area. In addition, perhaps NABS II resulted from hybridization between NABS I and VI, retaining the ancestral polymorphism apparent in the sequences.

The divergence producing NABS I, specializing as pathogens of softwood hosts, also produced a second lineage, an ancestral form which was not quite as specialized, colonizing both soft and hardwoods and sometimes producing disease, but mostly obtaining nutrients saprotrophically (NABS III, V, VII, IX and X). All members from this second lineage, as well as NABS II, produce monopodial branched rhizomorphs which are typical of less virulent *Armillaria*. NABS I retained an ancestral character from NABS VI in that both produce dichotomously branching rhizomorphs which are typical of more virulent forms. The ancestor to NABS III and VII diverged about 10 Ma, and was probably more similar to the more widely distributed NABS VII than the more narrowly distributed III. The remaining non-specialists include NABS V, IX and X. The high degree of diversity in NABS V in this study has also been shown by Harrington and Wingfield (1995). The consistency in variation may have been overlooked and explained as being an unresolved group, whereas the variation may be evidence that speciation is occurring in this group. The wide distribution of NABS V throughout Asia and North America may represent the ancestral state which produced local outgrowths forming the more narrowly distributed monophyletic NABS IX and NABS X.

2.4 SECTION B: Species-Specific Markers

2.4A RESULTS

2.4A.a Species-specific SWAPP markers with 10nt primers:

A set of 200 10nt primers were examined and 42 pairs of these were screened for bands specific to a species or group of species. Electrophoresis of SWAPP PCR product on 3.0% agarose gels showed species specificity for several pairs of 10nt primers. Primers 83 and 147 produced a 600bp fragment for NABS III and VII (Figure 2B-1A). Primers 83 and 66 produced three bands at 115, 135 and 170bp for NABS VI (Figure 2B-1B). Primers 17 and 127 produced a 390 bp fragment for NABS V and X, and a 525bp fragment for NABS VII (Figure 2B-1C). Primers 29 and 122 produced a 380bp fragment for NABS IX (Figure 2B-1D). Primers 151 and 159 produced an 850bp fragment for most NABS I and II. Primers 34 and 122 produced three bands for NABS III at 240, 300 and 360 bp.

2.4A.b Species specific 20mer markers:

Monomorphic fragments produced by three nested 20nt primer sets, V250, III180, III520, and one 10nt primer set, 83/66, yielded sequences containing short regions which were specific to species. These fragments were the same fragments used in the phylogeny. Primers were made from these regions and re-amplification from gDNA produced bands showing species-specificity (Figure 2B-2). Approximately 30 isolates were screened against each species specific primer. Primers 5a/b amplified a 120bp band in six isolates

Figure 2B-1: SWAPP-PCR amplification with 10nt primer pairs showing bands specific to a species or group of species of NABS *Armillaria*. **A**: Primer set 83/147 produced a 600bp fragment (arrow) in isolates of NABS III and VII. **B**: Primer set 83/66 produced a 3-band repeat, 115bp, 135bp and 170bp (arrows), in isolates of NABS VI. **C**: Primer set 17/127 produced a 390bp fragment in isolates of NABS V and X, and a 525bp fragment (arrows) in NABS VII. **D**: Primer set 29/122 produced a 275bp fragment (arrow) in NABS IX. Far left lanes represent 123bp size ladder in A and 1Kb size ladder in B, C, and D, NABS *Armillaria* are indicated by Roman numerals, At is *A. tabescens*, and Ctl is negative control.

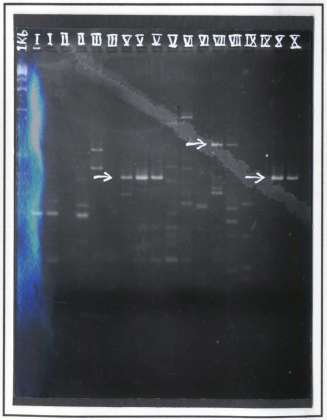
A


123
I
II
III
IV
V
VI
VII
VIII
IX

B


Kb
I
II
III
IV
V
VI
VII
VIII
IX
X
At
Ctl

C

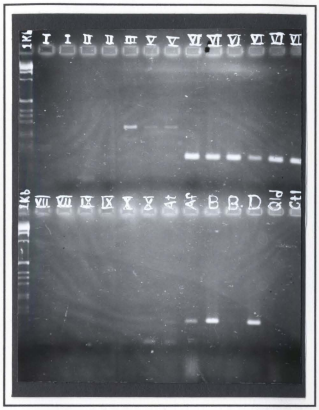

Kb
I
II
III
IV
V
VI
VII
VIII
IX
X

D

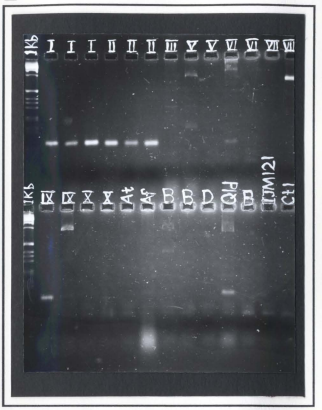

Kb
I
II
III
IV
V
VI
VII
VIII
IX
X
At
Ctl
Kb

Figure 2B-2: PCR amplification of species specific fragments showing **A**: 120bp band in NABS VI amplified by 5a/b, **B**: 80bp band in NABS I and II amplified by 10/12, **C**: 220bp band in NABS II amplified by 1a/b, **D**: 180bp band in NABS III, VII, X, and European B amplified by 9a/b. Roman numerals at top of gel represent species of NABS *Armillaria*, Roman letters indicate European species, At is *Armillaria tabescens*, Af is *A. fumosa*, Qld is Qld8 an isolate from Australia, IJM121 is a European isolate of *A. ostoyae*, Ctl is negative control, and 1Kb is the 1Kb DNA size ladder used to indicate size of bands.

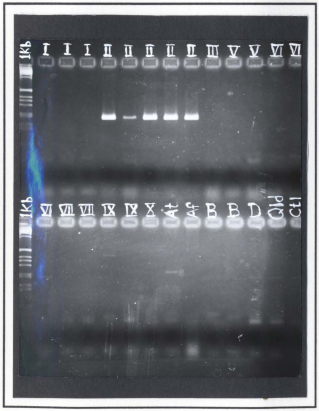
A


1Kb
I I II II III V V VI VII VII IX IX X X At Af B B D Pld Ct1

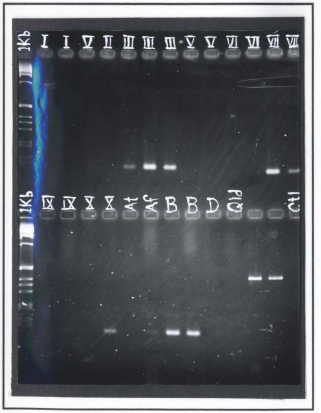
B


1Kb
I I I II II III V V VI VII VII IX IX X X At Af B B D Pld B TM121 Ct1

C


1Kb
I I I II II II II III V V VI VI VI VII VII IX IX X X At Af B B D Pld Ct1

D


1Kb
I I V II III III III V V VI VII VII IX IX X X At Af B B D Pld Ct1

of NABS VI as well as some European species. Primers 10/12 amplified an 80bp fragment in NABS I and II, primers 1a/b amplified a 220bp band in NABS II. Primers 9a/b amplified a 180bp band in NABS III, VII, X and some European species (Figure 2B-2). Primers 6a/b amplified a 140bp band in NABS III, V, VII, IX, X and European species. Utilization of these 20bp primers, as well as some 10bp primers for confirmation, allowed identification of most NABS *Armillaria* (Figure 2B-3).

2.4A.c Development of nested 20nt primers:

Since 10nt primers are easily denatured from the template strand, more stable 20nt primers were developed from the sequences obtained from the species specific 10mers representing each species or group of species. Sequences of two to five isolates were aligned before a consensus sequence was chosen (For example, Figure 2B-4). Sequences from the 10nt primers which were used to design the 20nt primers are enclosed in parentheses in Table 4.

Amplification of the DNA using the nested 20nt primers produced a variety of results. One set of primers, IX-250a/b, produced no bands (Figure 2B-5A). Primer set VII-520-1/2 produced bands only within NABS III and VII but the PCR product was weak (Figure 2B-5B). Primer set V-250a/b produced monomorphic bands in all species (Figure 2B-5C). Seven of the 9 primer sets developed produced many bands, some were more variable (ie. I-850) than others (ie. v-250), but there was a monomorphic band across most species tested within each primer set (Figure 2B-5D). An additional *Armillaria* genus-

Figure 2B-3: Key for identification of NABS *Armillaria* using molecular characters obtained by 10 and 20nt primers. Suggested amplification reaction conditions and cycles, and electrophoresis conditions are outlined in "Materials and Methods".

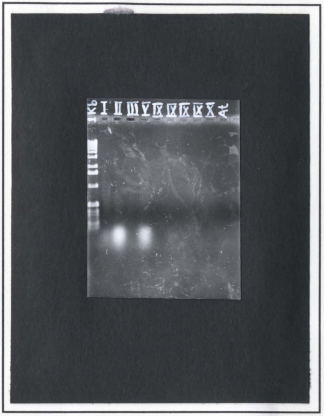
```
1. PCR with primers 6a/b:
      a) 140 bp band = NABS III, V, VII, IX or X.....Go to 4.
      b) No band = NABS I, II or VI.....Go to 2.

   2. PCR with primers 1a/b and 5a/b:
         a) 120bp band = NABS VI.
         b) 220bp band = NABS II.
         c) No band.....Go to 3.

      3. PCR with primers 10/12:
            a) 80bp band = NABS I.
            b) No band.....Go to 4.

4. PCR with primers 9a/b:
      a) 180bp band = NABS III, VII or X.....Go to 5.
      b) No band = NABS V or IX.....Go to 7.

   5. PCR with VII-520-1/2:
         a) 70bp band = NABS III or VII.....Go to 6.
         b) No band = NABS X.....Go to 6.

6. PCR with 10bp primers:
a) 34/122 = 3/band repeat = NABS III.
b) 17/127 = 525bp band = NABS VII.
          = 390bp band = NABS X.

7. PCR with 10bp primers:
a) 29/122 = 350bp band = NABS IX.
b) 17/127 = 390bp band = NABS V.
c) 17/82 = 390bp band = NABS V.
```

Figure 2B-4: Alignment of nucleotide sequences of 390bp fragments amplified by SWAPP 10nt primers 17/127 for NABS V and X (from Figure 2B-1C), showing regions of DNA from which more stable nested 20nt primers (V250a/b) were developed (underlined). Sequences are written in 5' to 3' direction. RC is reverse complement. Inverse primer sites are shown at center of sequence. Dashes indicate no sequence.

```
1 X-Sp12    TCAATGTGTTGCCAGTTTTGTTGGAAGAGCGTCGTGCGCCAGATCGATTTTGTG
2 X-sp15    TC-AYGTGTTGCCAGTTTTGTTGGAAGAGCGCCGTGCGCCAGATCGATTTTGTG
3 V-83621   TCAATGTGTTGCCAGTTTTGTTGGAAGAGCGTCGTGCGCCAGATCGATTTTGTG
4 V-83911   TCAATGTGTTGCCAGTTTTGTTGGAAGAGCGTCGTGCGCCAGATCGATTTTGTG
5 V-NOF891 TCAATGTGTTGCCAGTTTTGTTGGAAGAGCGTCGTGCGCCAGATCGATTTTGTG

          (V/X250b)
1 TTTCGAACGCGAATATGCTCAAAATGCGCGAGCAAGCAGTGAAGTATTCGGCTCACTTCTTTT
2 TTTCGAACGCGAATATGCTCAAAATGCGCGAGCAAGCAGYGAAGTATTCGGCTCACTTCTTTT
3 TTTCGAACGCGAATATGCTCAAAATGCGCGAGCAAGCAGTGAAGTATTCGGCTCACTTCTTTT
4 TTTCGAACGCGAATATGCTCAAAATGCGCGAGCAAGCAGTGAAGTATTCGGCTCACTTCTTTT
5 TTTCGAACGCGAATATGCTCAAAATGCGCGARCAAGCAGTGAAGTATTCGGCTCACTTCTTTT

                    (RC INV250b)
1 TGGCATAGTACTCATACGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA
2 TGGSATAGTACTCATACGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA
3 TGGCATAGTACTCATGCGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA
4 TGGCATAGTACTCATGCGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA
5 TGGCATAGTACTCATACGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA

                    (INV250a)
1 CCAGCTCACTTTGATCRCGACGCTCTTACCTACTAAAACATTGTCAAGTATGTTTCATGTCTT
2 CCAGCTCACTTTGATCACGACGCTCTTATCTACTAAAACATTGTCAAGTATGTTTCATGTCTT
3 CCAGCTCACTTTGATCACGACGCTCTTACCTACTAAAACGTTGTCAAGTATGTTTCATGTCTT
4 CCAGCTCACTTTGATCACGACGCTCTTACCTACTAAAACGTTGTCAAGTATGTTTCATGTCTT
5 CCAGCTCACTTTGATCACGACGCTCTTACCTACTAAAACGTTGTCAAGTATGTTTCATGTCTT

                          (RC V250a and X250a)
1 TTCTCACCGAGAGAGCAGATGCTGAATCGACGACGATCAGTTCGCCGGCGTGGATGCCTACCC
2 TTCTCACCGMGAGAGCAGATGCTGAATCGACGACGATCAGTTCG-CGGCGTKGATGCCTACCC
3 TTCTCACCGAGAGAGCAGATGCTGAATGGACGATGATCAGTTCGCCGGCGTGGATGCCTACCC
4 TTCTCACCGAGAGARCAGATGCTGAATGGACGATGATCAGTTCGCCGGCATGGATGCCTACCC
5 TTCTCACCGAGAGANCAGATKCTGAATGGACGATGATCAGTTCGCCGGCGTGGATGCCTACCC


1 CTTCAATATTTACGAGAAGAATTC
2 CTYCAATATTTACGAGAAGAATTC
3 CCTCRATA----------------
4 CTTCAATATTTACGAGAAGAATTC
5 CTTCAATATTTACGAGAAGAT---
```

Figure 2B-5: PCR amplification of NABS *Armillaria* using nested 20nt primers developed from sequences of SWAPP 10nt primers showing the variable results obtained. **A**: Primer set IX250a/b produced no bands. **B**: Primer set VII520-1/2 produced bands found in NABS III and VII and European species B. **C**: Primer set V250a/b produced 250bp fragments found in all NABS *Armillaria*. **D**: Primer set I850a/b produced polymorphic bands with some degree of monomorphism, found in all species of *Armillaria*. NABS *Armillaria* are indicated by Roman numerals, At is *A. tabescens*, Ae is *A. ectypa*, Ah is *A. hinula*, Roman letters indicate European species, Ctl is negative control, and 1Kb size ladder is in far left lane.

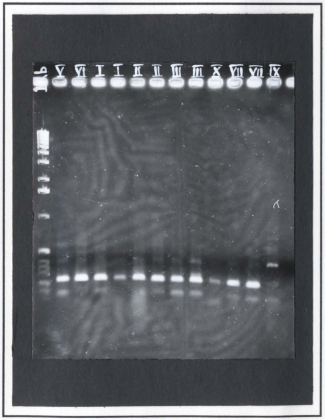
A


Kb
I
II
III
IV
V
VI
VII
VIII
IX
X
XI

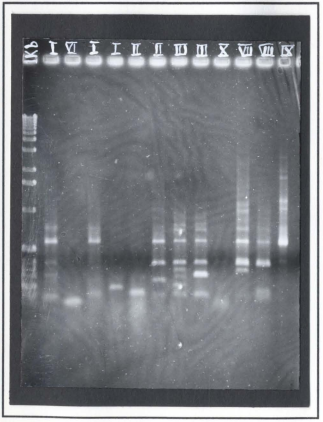
B


Kb
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
BU

C


Kb
V
VI
I
I
II
II
III
III
X
VII
VIII
IX

D


Kb
I
VI
I
I
II
II
III
III
X
VII
VIII
IX

specific 220bp band was found with two 10nt primers, 29 and 34. 'Gene walking' was used to search for the species specific 10nt primer site using a 20nt and a 10nt primer pair. Results produced variable bands (Figure 2B-6).

2.4A.d Inverse PCR:

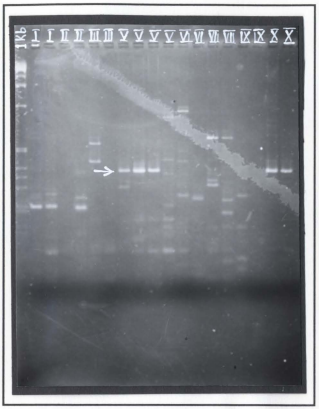
Inverse PCR was employed to locate the 10nt primer sequences amplified by V250 and I850. Fragments amplified by 10nt primers, 17/127, produced 390bp bands present in NABS V and X (Figure 2B-7A and 2B-8). Nested 20nt primers were made downstream from these 10nt primer sites and re-amplification with the nested 20nt primers produced monomorphic bands in all NABS *Armillaria* (Figure 2B-7B and 2B-8). Six restriction enzymes were tested for inverse PCR and a 1Kbp band was amplified in NABS V using restriction enzyme XhoI (Figure 2B-7C and 2B-8). The sequence of this 1Kb band was used to locate the 10nt primer sites from which more stable post inverse 20nt primers were developed. These 20nt primers (v-17 and v-127) produced no species specificity (Figure 2B-7D and 2B-8). Twelve restriction enzymes were tested for the I850 but none produced PCR product larger than I850 fragment. The fragment for the I850 (400bp) was longer than that for v250, which increases the chance for internal recognition sites. Results from inverse PCR on *Armillaria* strains such as NABS V-83621, III-JB56, V-NOF891, VII-90-10, IX-TJV200 and I-JB13 showed numerous mismatches in the 10base primer-template duplex (Figure 2B-9).

Figure 2B-6: Banding pattern observations from **A**: a genus specific 220bp band with 10nt primers 29/34, and **B**: PCR with 10 and 20nt primers showing numerous bands when the "gene walking" technique was applied. Roman numerals indicate NABS *Armillaria*, Af is *A. fumosa*, Ctl is negative control, and the far left lane contains the 1Kb size ladder. Numbers in **B** represent primers used in amplification reactions.

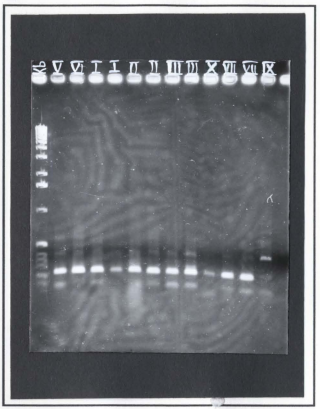
A
1Kb
I
II
III
IV
V
VI
VII
Af
Ch
B
1Kb
VI
V
IV
III
II
17
500a/17
500a
1Kb
VI
V
IV
III
II
34
500b/34
500b

Figure 2B-7: PCR amplification showing results obtained at each of four stages to locate the SWAPP 10mer sites. **A**: Amplification of 390bp fragment (arrow) in NABS V and X using SWAPP 10nt primer pair 17/127. **B**: Amplification of 250bp band in all NABS *Armillaria* using nested 20nt primer pair, V250a/b, developed from sequences amplified by SWAPP 10nt primers 17/127, which were chosen to be specific for NABS V. **C**: Inverse PCR amplification of ligated product of NABS II and V from each of four restriction enzymes, using inverse 20nt primers, INV250a/b, showing the 1Kbp band (arrow) in NABS V cut by restriction enzyme XhoI. **D**: PCR amplification of genomic DNA using new 20nt primers incorporating SWAPP 10mer sites, V-17 and V-127, developed from sequences in **C**. Roman numerals indicate NABS *Armillaria*, At is *A. tabescens*, Ctl is negative control, and the 1KB size ladder is in the far left lanes.

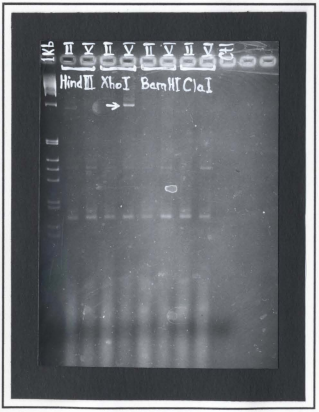
A



B



C



D

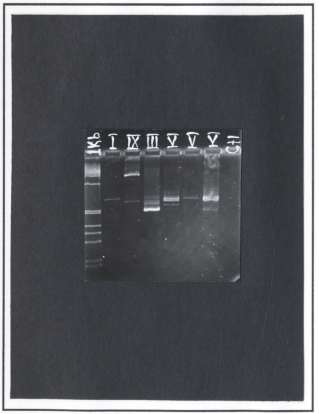


Figure 2B-8: Alignment of nucleotide sequences obtained from the four stages corresponding to those in Figure 2B-7. Sequence "17/127" was produced from SWAPP 10nt primers 17/127 (Figure 2B-7A). Sequence "V250a/b" was produced from nested primers V250a/b (Figure 2B-7B), showing similarity with SWAPP 17/127 sequence. Sequence "INV250A" was amplified from inverse primers INV250a/b (Figure 2B-7C), and shows similarity with the latter half of sequences from V250 and 17/127 from which the primer was developed. The sequence continued past the end of the V250 fragment showing all the primer sites. Sequence "V-17/V-127" (Figure 2B-7D) was produced from 20nt primers developed from the 10nt primer sites, showing no similarity with the other sequences. All four sequences were from NABS V isolate V83621. Primer sites are underlined and names in parentheses. RC is reverse complement, and dashes indicate no sequence.


1 17/127 TCAATGTGTTGCCAGTTTTGTTGGAAGAGCGTCGTGCGCCAGATCGATTTTG
2 V250a/b ----------
3 INV250A ----------
4 V-17/V-127 ATCGAGKKACATSCACSAGCKGGCACTGCTGGCMSGACTCMACGASRCTSAT
(V250b)
1TGTTTCGAACGCGAATATGCTCAAAATGCGCGAGCAAGCAGTGAAGTATTCGGCTCACTTCTTT
2------GAACGCGAATATGCTCAAAATGCGCGAGCAAGCAGTGAAGTATTCGGCTCACTTCTTT
3----------
4RTACCGATYCCGAAKSTGASCCGACTGCGTCGTCTAMGAGGGATCCKGTTCAGWGTCGTAGTAG
(RC INV250b)
1TTGGCATAGTACTCATGCGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA
2TTGGCATAGTACTCATGCGTCCACACAGTGTTGTGTCTCAACCAGAGGTAGATGCCAATGAAGA
3----------
4ATYCACTGCACGAGGSTGAATCCTCTACGGATGGGTATKTACAGTGAATNGTGAGGTACAAAWT
(INV250A)
1CCAGCTCACTTTGATCACGACGCTCTTACCTACTAAAACGTTGTCAAGTATGTTTCATGTCTTT
2CCAGCTCACTTTGATCACGACGCTCTTACCTACTAAAACGTTGTCAAGTATGTTTCATGTCTTT
3----------TACTACARCGTYGCYAAGTATGTTTCAWGTCTKC
4CACCTTGGAKYGASGATTCATCGSWGGTATWGRMTCAGTCAACAGTTGGGTCARCAAAACAGCG
(RC V250a)
1TCTCACCGAGAGAGCAGATGCTGAATGGACGATGATCAGTTCGCCGGCGTGGATGCCTACCCCC
2TCTCACCGAGAGAGCAGATGCTGAATCGACGACGATCAMAR----------
3TCTCACCGAGAGAGCAGATGCTGARTCGACGACGAKYAKKCYSACRGTGCGGGWGCAAGCCCCT
4ACGATGCTRTSAACCTTCTKGCTGKGKGACTAKCTCAGRGATACTCACACCGACAGTTCATGAN
1TCRATA----------
2----------(RC #17)----------
3ACGTTTTCGGCTCACTGCTTTCGGGGAGGGCGMKSATMCGTCCATATAGTTTCGWGACTAATSM
4GGCGTCGTCGTGTNGGGTANCACANTNNTCGNNTCANCATNNANTCTCNNANCCNAATCAANTG
1----------
2----------(RC #17)----------
3AGTGGTRKTYAYYARTRAGGACCASWTCACKNNNYNCACGAYGCTCTCASCGWMTGAATAGYMS
4NACCCNNCGCAGCNTCCCGTAGTCCNCTTCCCCTGAGGAGGNAAATCGCCACGNNTNTNTNNTN
1----------
2----------
3ACAATYTWWTCCAGCTTCTTCTSTCASMGTCCAC----------
4TTGAAGNTTCCCCCTTGNCNACANANNAGNNCNCCNNNCANCATAANNCNTGNNACNNACACCN

Figure 2B-9: Primer/template alignments for the sequences amplified by inverse primers **A**: INV250a and **B**: INV250b showing sequence of primer(top) and template (bottom), for species of NABS *Armillaria*. Dashes represent continuation of template sequence with no primer site. Dots in primer sequence indicate a match, and mismatches are indicated by bases. Ambiguity in template sequence is shown in parentheses with the two possible bases. RC is reverse complement. A: All possible primer sites are indicated by A-1 to A-5 from INV250a, and B: B-1 to B-4 from INV250b. A-1 and B-1 sites show alignment for 20nt primers. All other sites show 10nt primer alignments. Isolates used in amplification reactions are indicated at left of each sequence.

INV250A

A-1 RC v-250A:

```
                 ....  c    g  ....  t  c.g.tcg
V-83621           tcga(a/c)(c/g)acga(g/t)tattctc----------------

V-NOF891
   t    c    g  ..  g    a    c  ...  c    a    g  .  t  .  g
-(g/t)(g/c)(a/g)ac(a/g)(a/c)(c/g)gat(c/t)(a/t)(g/t)t(c/t)c(a/g)-

                 .... ..acg..ca.t..g
III-JB56          tcgaacgcgaatatgctca--------------------------------

                 ....  c  ...  g  ......  t  ..
VII-90-10         tcga(c/g)gac(a/g)atcagt(c/t)cg--------------------

                 ....c  g  ac...c.g  t  tcg
IX-TJV200         tcgaa(c/t)gtgattat(g/t)atc-------------------------
```

A-2 RC #17

```
       ..  g    g  c.cagg            g  .  g    g  c...  g    g
V83621 ga(g/t)(a/g)gcgctc---------(a/t)a(a/g)(g/t)acca(c/g)(a/t)

        ..g  g  c.c  a  gg            ..  g    g    c  c c.g  g
VNOF891 gac(g/c)tcn(a/c)cc-----------ga(a/g)(a/c)(c/g)g gac a/c)

          .  a  ....c  a  gg                    ....  c  cc.  g  g
VII90-10  g(a/g)ggccg(a/g)cc-----------------gagg(g/c)ata(g/t)a

                                        .  a  g . c    c  c.  g  .
IXTJV200  --------------------------g(a/g)ag(a/c)(c/t)ta(c/t)g

          g  ...  c  c.agg           ...g.c.ag.
IJB13    (g/c)agg(c/a)tctca---------gagacgcccg----------------
```

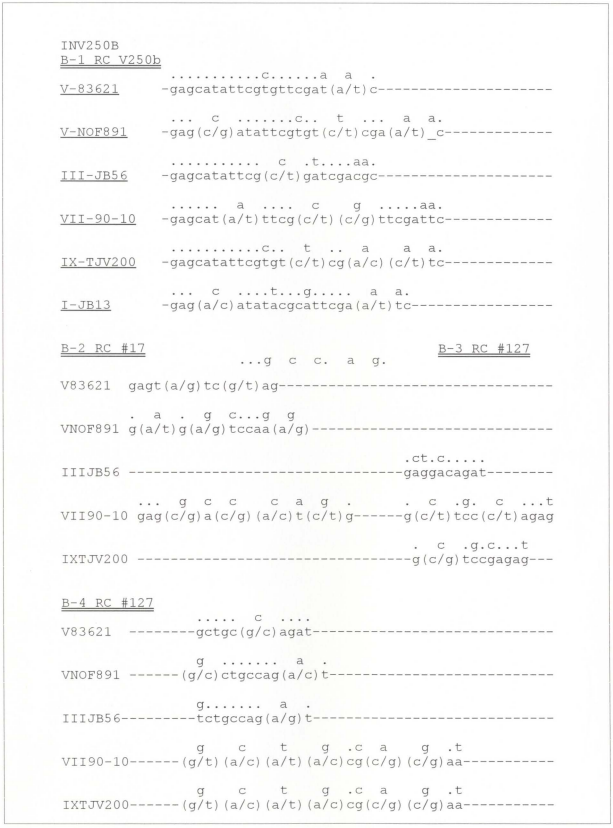
A-3 RC #17

A-4 RC #17

```
          ....cc..g  g
IXTJV200   gagggtcaa(c/t)
```

A-5 RC #17

```
             g    a  gg..  c    a  g.
VII90-10    (g/t)(g/t)ntcc(c/g)(a/c)tg
```


INV250B
B-1 RC V250b
V-83621
V-NOF891
III-JB56
VII-90-10
IX-TJV200
I-JB13
B-2 RC #17
B-3 RC #127
V83621
VNOF891
IIIJB56
VII90-10
IXTJV200
B-4 RC #127
V83621
VNOF891
IIIJB56
VII90-10
IXTJV200

2.4A.e Primer/template dynamics:

The sequence amplified by primer INV250A showed primer sites for the reverse complement (RC) V250a at position A-1 (80bp downstream from INV250A)(Figure 2B-10), RC #17 at positions A-2 (145bp downstream from INV250A), A-3 (200bp downstream from INV250A), A-4 (235bp downstream from INV250A), A-5 (290bp downstream from INV250A) and A-6 (350bp downstream from INV250A) (Figure 2B-10). The sequence for NABS I showed no similarity to primer V250a at A-1, III-JB56 showed 10 mismatches in 20 bases of the primer, IXTJV200 showed 9/20 mismatches, VII90-10 and VNOF891 matched perfectly with the primer, and V83621 showed 5 mismatches. Most mismatches were near the 5' end of the primer.

The sequence amplified by primer INV250B showed primer sites for RC V250b at position B-1 (80bp downstream from INV250B), RC #17 at B-2 (160bp downstream), and RC #127 at B-3 (180bp downstream) and B-4 (290bp downstream). The sequences for I-JB13 and III-JB56 showed 3 mismatches in 20 bases for V250b, and IX-TJV200, VII-90-10, V-83621, and V-NOF891 showed 2/20 mismatches each. Most mismatches were near the 5'end of the primer.

Summary of occurrence of PCR bands in agarose gels for each group of primers, SWAPP 10nt primers, nested 20nt primers, and species-specific primers, is presented in Appendices D, E, and F.

2.4A.f Significance of the 10base primer sites in specificity:

Sequences amplified from the SWAPP 10nt, nested 20nt, and inverse primers aligned with one another (Figure 2B-8), but the sequence obtained by the post inverse 20nt primers incorporating the 10base primer sites did not align with the others (Figure 2B-8) indicating that the sequence produced from the site incorporating the 10nt primers was not homologous to the initial species specific sequence. Amplification of the DNA using post inverse 20nt primers made from the 3' ends of the 10bp primer sites and an additional 10 base pairs on the 5' end of the sequences produced by primers 17 and 127 for NABS V, produced variable bands (Figure 2B-7D). Distances between suggested primer sites (Figure 2B-10) did not agree with length of electrophoresis product (Figure 2B-7). A stem loop structure was proposed to explain the discrepancies (Figure 2B-11).

Figure 2B-10: Line drawing of sequences amplified from inverse primers INV250a/b of six isolates of *Armillaria* showing location and 5' to 3' orientation of all possible primer sites. RC is reverse complement. Primer sites A-1 to A-5 and B-1 to B-4 correspond to sites in Figure 2B-9. Scale is 2 inches = 100bp in length.

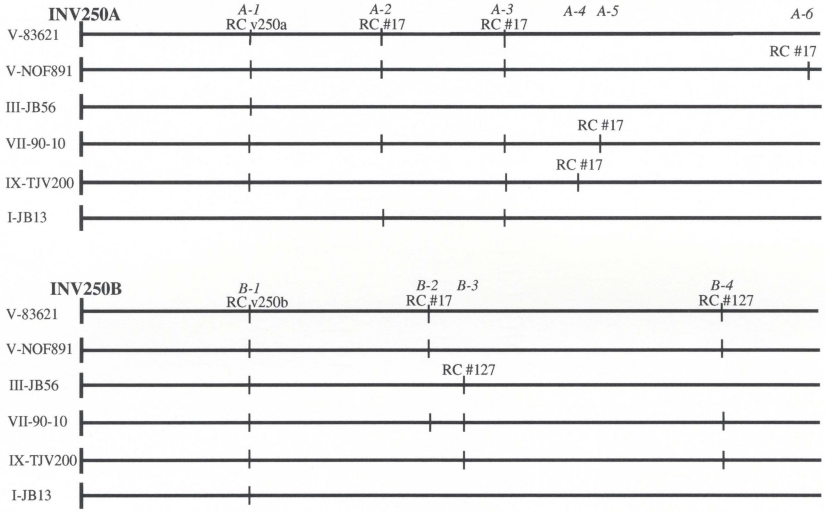
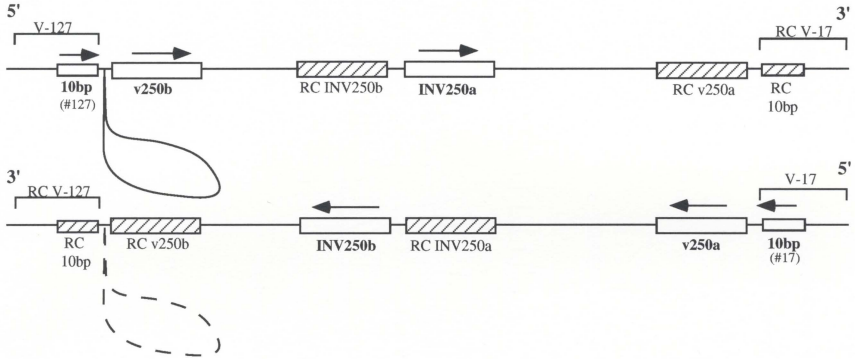

INV250A
A-1
RC v250a
A-2
RC #17
A-3
RC #17
A-4
A-5
A-6
V-83621
V-NOF891
RC #17
III-JB56
VII-90-10
RC #17
IX-TJV200
RC #17
I-JB13
INV250B
B-1
RC v250b
B-2
RC #17
B-3
B-4
RC #127
V-83621
V-NOF891
III-JB56
RC #127
VII-90-10
IX-TJV200
I-JB13

Figure 2B-11: Diagrammatic representation of location and orientation of SWAPP 10nt and 20nt primer sites and the hypothesized stem loop structure in genomic DNA showing the fragment bordered by primers 17/127. Shaded boxes represent reverse complemented (RC) primer sites, and clear boxes represent the active primer sites. Arrow represents direction in which extension occurs. The 20nt primers incorporating the 10bp primer sequences at the 3' ends are shown as V-127 and V-17. Diagram is not drawn to scale.


5'
V-127
3'
RC V-17
10bp
(#127)
v250b
RC INV250b
INV250a
RC v250a
RC
10bp
3'
RC V-127
5'
V-17
RC
10bp
RC v250b
INV250b
RC INV250a
v250a
10bp
(#17)

2.4B DISCUSSION

2.4B.a Species-specific SWAPP markers with 10nt primers:

Amplification with SWAPP 10nt primer pairs produced bands present within a species or group of related species (Figure 2B-1). False negatives were present in two isolates of NABS III (Figure 2B-1A) and one isolate of NABS V (Figure 2B-1C). These false negatives may be a result of lack of sufficient template DNA to be amplified or insufficient template match at the primer site. Weising et al. (1995) suggested that 95% of RAPD markers were amplified from nuclear DNA. Since much of nuclear DNA is present in low copy numbers, and the primers used in this study were the same as RAPD primers, then insufficient template DNA may not produce enough amplification product to be visible on an agarose gel. Although the genomes of relatively few fungi have been investigated, nuclear genomes of fungi are smaller than those of other eukaryotes (Primrose, 1995), and contain < 20% repetitive DNA (Arthur et al., 1982; Timberlake, 1978). However, complex RAPD fragment patterns found from fungal DNA (Weising et al., 1995) may be explained by mismatch and primer competition. Primers prefer to bind to sites with a higher degree of similarity (Williams et al., 1993).

If each of the 10 bases in the template DNA primer sites were not sufficiently conserved to be exactly the same among isolates of the same species, then amplification of the fragment may not occur in some isolates. Caetano-Anollés et al. (1992a) showed that it was necessary for the first eight nucleotides from the 3' end of the primer to match the template in order for the amplifications to be consistent. Single base changes within

this region produced variable amplification patterns. However, Sommer and Tautz (1989) showed that a 20nt primer required only three matching nucleotides at the 3' end of the primer for successful amplification of the same band. Williams et al. (1990) showed that a single base change at any position on a 10nt primer produced changes in the amplified product. A change near the 3' end produced a more dramatic change in product than one near the 5' end. Nevertheless, if the match at the 3' end of the 10nt primers in this study were less than three, then amplification would be weak or nonexistent.

Isolates of NABS VI produced a three band marker with primers 83/66. However, the 300bp fragment present in all remaining NABS *Armillaria* was also present in NABS VI but much weaker (Figure 2B-1B). Since the 3-band character of NABS VI was shorter in length than the 300bp fragment it was likely outcompeting the 300bp fragment. Hence, the "context effect" described by Welsh and McClelland (1993) seemed to have occurred. The 300bp band in NABS VI was not visible in the gel even though it may not have contained a mutation but rather the degree of template matching the primer sequence for the 3-band character was greater than the match for the 300bp fragment. Similarly, Rieseberg (1996) discussed the effects of competition among RAPD fragments. DNA regions poorly matched with the primers would have weak amplification. If these poorly matched fragments were homologous among taxa, then competition may occur with other fragments that represent a better match with the primer. This would result in the conclusion that the fragments were not homologous among taxa in question, when they were indeed homologous but containing a smaller degree of primer/template

complementarity. This may explain the weak presence of the 300bp fragments in NABS VI (83/66) (Figure 2B-1B).

2.4B.b Species-specific 20mer markers:

Specificity of markers. It was not surprising that NABS I and II produced the same marker for 10/12, since NABS I and II have been shown to be very similar based on fruit body morphology (Bérubé and Dessureault, 1988; 1989), DNA reassociation values (Miller et al., 1994), rDNA (Anderson et al., 1989; Anderson and Stasovski, 1992) and RFLP patterns (Harrington and Wingfield, 1995). Primers 10/12 specific for both NABS I and II also showed an 80bp band in Qld8, an isolate from Australia (Figure 2B-2B) (Table 2B-5). Similarly, IXTJV179 produced an 80bp band (Table 2B-5). This was the only NABS IX isolate collected from a softwood host. Individuals of NABS I also inhabit softwood hosts. Interestingly, an *A. ostoyae* isolate from Europe (ie. NABS I, IJM121) produced no band (Table 2B-5). This indicated that the 80bp marker for NABS I and II may be specific to North American isolates only. The same isolate did not amplify when tested for the 850bp band in 151/159 (10nt primers amplified NABS I and II) (Table 2B-3). Neither of two markers produced a band in European IJM121 suggesting either insufficient DNA, or a large difference between European and North American isolates of *A. ostoyae*.

Primer 5a/b amplified a 120bp band in isolates of NABS VI including the isolate from Europe (VI-JM122) (Figure 2B-2A) (Table 2B-5). Similarly, this isolate produced

a NABS VI-specific 3-band repeat with 83/66 (Table 2B-3). The primers specific for NABS VI also produced a band in European B and D, *A. fumosa*, NABS V, X, and European IJM121. However, intensity of the band in NABS V and X were low compared with NABS VI (Figure 2B-2A). The other isolates were not North American isolates, so this marker may be considered specific for NABS VI.

The marker produced by 1a/b, specific to NABS II, was not present in any other isolate tested (Figure 2B-2C). This marker appeared to be strongly specific to NABS II.

All primer sets were examined on closely related non-*Armillaria* species (Appendices D, E and F), and produced amplification product for isolates within the genus only.

Dynamics. Isolates showing the presence of a band with species-specific primers, may not always show presence of a band with the more conserved nested primers (Tables 2B-3 and 4). Primer 5a/b amplified a 120bp band in VI97-1, but there was no band produced from VI97-1 with V250, the primer sequence from which 5a/b was developed. It is interesting that 220bp 1a/b band was present in all isolates of NABS II tested including IIJB38 (Table 2B-5), but the V250 band was missing from IIJB38 (Table 2B-4). Sample IX139 produced the 175bp specific band with primers 6a/b, which were designed from V250 (Appendix G), but the V250 band was not present in IX139. RAPD-PCR may produce polymorphisms from deletion of a primer site, or mutation within the primer site preventing the fragment from being amplified (Weising et al., 1995). The nested 20nt primers may undergo similar processes as was suggested by this study.

Species specificity. A 180bp marker amplified by 9a/b was found in NABS III, VII, X, European B, *A. fumosa* and one isolate of NABS IX. NABS III, VII and B were shown to have a low level of interfertility (Anderson et al., 1980), similar nuclear rDNA (Anderson and Smith, 1988; Anderson et al., 1989) and similar DNA reassociation values (Miller et al., 1994). The band in NABS X was weak and was present in only one of the two isolates tested, and IX was one of 5 isolates tested. NABS IX and X were found to be paraphyletic to NABS III and VII (Figure 2A-5). The weak amplification may be explained by a small amount of DNA present in the sample, or that the primer/template complementarity may have been insufficient to produce strong amplification with these reaction conditions, reflecting a lower level of specificity of the primer for NABS III and VII.

Primer set 6a/b amplified bands in NABS III, V, VII, IX and X and some European and Australian species but not in NABS I, II and VI. Consequently, this marker was the first step in the key used to separate the two clades for identification (Figure 2B-3). NABS V, IX and X belonged to the same rDNA class (Anderson and Smith, 1988). NABS III, V, VII, IX and X also formed a single clade using four anonymous sequences (Figure 2A-5). However, identification of NABS V could be inferred if PCR with 6a/b produced 175bp band, and subsequent PCR with 9a/b produced no band.

NABS V was the only pathogenic species in the first clade which may require identification by foresters, whereas both I and VI in the second clade are very pathogenic hence were identified at the beginning of the key. Steps 2 and 3 in the key differentiated

between and confirmed identification of each of NABS I, II and VI. Further identification among NABS III, V, VII, IX and X involved amplification using various other primers. There was more variation in species-specific 20bp fragments for NABS III, V, VII, IX and X, than for NABS I, II and VI, making the former less reliable as markers than the latter.

Weak amplification or none at all may not necessarily mean that the sample was not the species for which the marker was designed, but rather that too little DNA resulted in insufficient amplification. It may be necessary to include a positive control for the sample such as amplification with a general fungal rDNA primer to indicate presence of DNA. Alternatively, if the primer/template complementarity is not 100%, or the primer site is missing, or insertion of a large piece of DNA between sites has occurred, then amplification may also be weak or non-existent, indicating that the region used to develop the primer was not present in that particular isolate, and therefore was not highly conserved within the species.

Primer sequence mismatches. The primer sequence chosen to differentiate NABS II from I (1a/b) was the same in both NABS I and II except the third and first positions at the 3' terminus of NABS II (Appendix G). Primer 1a/b strongly differentiated between NABS I and II probably due to the purine-purine mismatch in third position from the 3' end, two substitutions at the 3' end, and the stringent annealing temperature of 60°C.

Primer 5a (specific to NABS VI) contained 4 base changes within 6 positions of 3' end of the selected primer (Appendix G). The three changes nearest the 3' end were

pyrimidine-pyrimidine having little effect on extension. However the sixth change from the 3' end resulted in a purine-purine mismatch having a significant effect on extension (Kwok et al., 1990). The strong differentiation between NABS II and VI was probably due to the combination of 4 substitutions at the 3' end one of which was purine-purine, two additional non-deleterious mismatches from NABS II at 11 and 13 bases from the 3' end, and the stringent annealing temperature. Primer 6a (specific for NABS III and VII) contained two mismatches in six positions from the 3' end which were non-deleterious, and therefore specificity was not strong.

Results may have been complicated by the presence of coding regions within some of the anonymous sequences from which the primers were designed. If the region coded for a protein, then a base change at the first position of the codon would be less likely to occur than one at the third position, a silent substitution. For example, if 'g' in the third position from the 3' terminus in 1a and 5a was at the first position of the codon, this base would be highly conserved and therefore a good choice for a marker. However, if it was in the third position of the codon, then silent substitutions could be common, and the substitution would be a poor choice for a marker.

The type of substitution, transition or transversion, in a protein coding region may also play a role in the ability for the primer to differentiate among taxa. Two of the 3 primers which differentiated among taxa (1 and 5) contained a transversion from the consensus sequence within 3 bases at the 3' end of the selected primer sequence. The third primer (10/12) contained a 3-base insertion at the 3' end. The insertion itself was further

strengthened by the 'g' and 'c' contained within it. The remaining primers contained no transversion near the 3' ends, and neither did they differentiate taxa at the same resolution as 1a/b, 5a/b and 10/12. A transversion in a sequence produces a purine-purine mismatch which was shown to significantly affect extension (Kwok et al., 1990). If the region is a protein coding region, then a transversion mismatch would indicate that the amino acid was different. Consequently, the primer would have a strong capacity to differentiate among taxa.

2.4B.c Development of nested 20nt primers:

Welsh and McClelland (1993) recommended the use of 20nt primers where context effects were a problem. Since a template match of 20 bases would be more stable than a match of 10 bases, nested 20nt primers were developed from the sequences produced by the SWAPP 10nt primers (Figure 2B-1). Sequences were aligned and 20nt primers were chosen from regions downstream from the 10bp sites (Figure 2B-4). Since 290bp sequences were present in only NABS V and X (Figure 2B-1A) then sequencing and subsequent alignment could only be performed on these samples. Therefore the only criterion that could be imposed was that the primer sequences be specific to the species for which they would be developed. For example, the primer sequence for NABS V contained a 'c' and 'a' at positions 2 and 8 from the 3' end, whereas NABS X contained two 'g's at these positions (Figure 2B-4). This should have been sufficient to produce specificity when amplified since the changes were located within the 8 nucleotide domain

at the 3' end of the primer (Caetano-Anollés et al., 1992a; Sommer and Tautz, 1989). However, amplification with V250 and X250 produced monomorphic bands in all NABS *Armillaria* (eg. Figure 2B-5C). The nine primer sets developed, using the same method, produced variable results; no bands (Figure 2B-5A), partial species specificity (Figure 2B-5B), monomorphic bands in all species (Figure 2B-5C), and polymorphic bands showing some degree of monomorphism (Figure 2B-5D). It was unexpected that a high proportion of bands showing species specificity with 10nt primers (Figure 2B-1), actually contained regions downstream of the primer sites that were highly conserved within the genus *Armillaria* and produced monomorphic fragments (Figure 2B-5). If the internal sequence and nested 20nt primer sites were so highly conserved, what was responsible for the species specificity shown by the 10nt primers in the banding patterns on the agarose gel? Since species specificity was produced by amplification with 10nt primers then the region of DNA responsible for delineating species must have been the matching template sequence to the 10nt primer sequence. Alternatively, since the banding pattern contained few bands, competition among primer sites on the template DNA or interference by secondary structure, may have played a role in species specificity (Welsh and McClelland, 1993). Investigation of the phenomenon required that the 10base primer sites be located, sequenced, and used to develop 20mers. These 20mers would then be used in PCR of genomic DNA to test for species specificity.

2.4B.d Inverse PCR:

Inverse PCR was employed to locate the 10bp sites on the template DNA and develop more stable post inverse 20nt primers using the 10bp site as the 3' end of the 20nt primer, so that species specificity could be retained by maintaining the 10 base 3' terminal primer/template match and by eliminating competition among primer sites. Inverse PCR of V250 for NABS V and X resulted in polymorphic bands in all species (Figure 2B-7D).

Sequences obtained with nested 20nt and 10nt primers aligned with one another indicating they were homologous (Figure 2B-8). The fragment bordered by nested 20nt primers was more conserved than the entire region bordered by 10mers, but this conserved region lay within the species specific region. The sequence obtained from inverse PCR using restriction enzyme XhoI and the inverse primer INV250 was homologous to the latter half of both fragments amplified by V250 and 17/127 (Figure 2B-8) indicating the choice of band obtained by XhoI (Figure 2B-7C) was correct. However, the 20nt primers, incorporating 10bp sites at the 3' end, chosen from this sequence did not produce species specificity. The sequence obtained by INV250 was more ambiguous further from the primer site, which made choice of primer bases uncertain. The significance of primer/template dynamics and proposed primer sites will be discussed (Section 2.4B.g).

Genus specific band and cloning. PCR with two 10mer primers, 29 and 34, produced 220bp monomorphic band in all species of *Armillaria*, making the fragment suitable to include in a phylogenetic study (Figure 2B-6A). Attempts to sequence the

220bp band resulted in ambiguous sequences, appearing as though two or more fragments of the same length were present in the sequencing reactions. It was thought that blunt-ended cloning of PCR product with PUC 18 in DH5-α *Escherichia coli* cells (Sambrook et al., 1989) would provide individual fragments which could then be amplified and sequenced. However, good progress was being made with four other monomorphic fragments to be used in the phylogeny. Since these four fragments produced a phylogenetic history of the genus *Armillaria* (2.3 Section I: Phylogeny) cloning of the 29/34 band was discontinued.

Gene walking. The search for the species specific RAPD primer site was ongoing. A technique employed to locate the site, described by Parker et al. (1991), was called "targeted gene walking" PCR. The technique can produce amplification of unknown DNA sequence adjacent to a known sequence. It uses a sequence specific primer with a nonspecific "walking" primer. In this study the 20mer was the sequence specific primer and the 10mer was the "walking" primer. If the portion of the same fragment containing the 20mer could be amplified, then the sequence would produce the missing species specific 10mer site from which a more stable primer could be made. However, PCR product showed numerous and ambiguous bands for each sample (Figure 2B-6B). Many bands were very close together and smears were common.

Touchdown PCR. When final results from Inverse PCR technique revealed that the 10mer sites were not responsible for species specificity of the RAPD bands, numerous attempts were made to refine the PCR cycle of these final primers. The initial cycle was

94°C in 1 second for 1 minute, 45°C in 1 second for 1 minute, and 72°C in 50 seconds for 1.5 minutes for 35 cycles. The annealing temperature was changed to 50°C and extension time to 1 minute, then the annealing temperature was changed to 55°C, then to 60°C, 58°C, 63°C, magnesium was increased from 2mM to 4mM and annealing temperature at 58°C. 'Touchdown PCR' (Don et al., 1991) was tried with the intentions of eliminating non-specific annealing of the primer to template DNA. In contrast, another technique was used to increase amplification of non-specific products. Neither cycle produced ideal results. Although the 20mer primers were made from the 3' ends of the RAPD sites, there were sites in other parts of the genome which produced a better match, as indicated by the unknown bands (Figure 2B-7D). PCR with nonspecific 20mers could produce variable bands in the same way that RAPDs could. Similar results with polymorphic bands were found from other techniques such as the nested 20nt primers (Figure 2B-5D) and inverse PCR with 20mers (Figure 2B-7D).

2.4B.e Why would 20mers produce numerous polymorphic bands?

Unless the 20mer is perfectly matched to a region of DNA, as a target specific primer, the 20 bases in that primer provide room for mismatching. Caetano-Anolles et al. (1992a) showed that it was necessary for the first eight nucleotides from the 3' end of the primer to match the template in order for amplifications to be consistent. Sommer and Tautz (1989) showed that a 20mer required only 3 matching nucleotides at the 3' end of the primer for successful amplification. Assuming the 3 nucleotides at the 3' end were

matching, then the remaining 5 bases (of the 8) could be randomly distributed (maintaining their order) among the remaining 17 bases of the 20mer. The number of combinations of 17 distinct bases (n) taken 5 at a time (r) would be:

$${}_n C_r = n!/r!(n-r)!$$ (Huntsberger and Billingsley, 1981)

$$= 17!/5!(17-5)!$$

= 6,188 different combinations.

Factors influencing primer/template binding would ultimately affect the success of amplification. However, assuming all influences to be minimal, the addition of a non-specific 20mer to a PCR reaction would theoretically be equivalent to adding a maximum of 6,188 different 8mer primers to the reaction. Since primer/template binding and amplification is influenced by many chemical and physical attributes of primer and DNA, this number would be considerably reduced. Nevertheless, the addition of more than one primer to the reaction would increase the number of successful amplifications and visible bands on the agarose gel. This would explain the occurrence of many bands when 20mers were used in this study. Another explanation for longer primers producing more amplification product than shorter primers was described by Caetano-Anollés et al. (1992b). Shorter primers are less stable than longer primers before extension begins, and if a hairpin loop located adjacent to the primer annealing site contains high 'gc' content

creating strong bonds, then the 10base primer may not be sufficiently stable to cause denaturation of the loop. Therefore, less amplification product is produced with shorter primers.

2.4B.f Primer/template dynamics:

Sequences amplified from inverse primers (INV250a/b) revealed primer sites with variable degrees of complementary base matching (Figure 2B-9). The 20nt primers matched the template better than the 10nt primers. Primer V250b formed a better match than V250a to all the sample sequences (Figure 2B-9) indicating that the sequence incorporating V250b was more conserved than that encompassing V250a. Absence of a primer site for V250a at position A-1 in I-JB13 (Figure 2B-10) was confirmed (Appendix E). Since there was no primer site present, there was no amplification product. However, the internal sequence was present since INV250 amplified a sequence for IJB13. Presence of the primer site was confirmed for V83621, IIIJB56, VII90-10, and IXTJV200 (Table 2-9), producing a 250bp band, but VNOF891 was not tested. Since size changes in RAPD amplification product are rarely observed (Weising et al., 1995), the absence of the 250bp band in I-JB13 may reflect the recessive condition for that marker. About 95% of RAPD markers act as dominant markers, and less than 5% act codominantly, while the absence of the fragment represents a recessive allele (Williams et al., 1990). Although these fragments are technically not RAPD fragments, they were obtained by 10nt primers and a PCR cycle similar to RAPD-PCR. Therefore they would be expected to exhibit

characteristics of RAPD-PCR.

Kwok et al. (1990) found that a single g:t mismatch at the terminal 3' end amplified as efficiently as a fully complementary primer/template duplex. This 3' terminal g:t mismatch was present in 3 non-ambiguous and 6 ambiguous duplexes (Figure 2B-9). Efficient extension of DNA also occurs for pyrimidine-pyrimidine and purine-pyrimidine mismatches, whereas purine-purine mismatches do not extend efficiently (Kwok et al., 1990). Purine-purine mismatches are present in 11 10mers and 8 20mers in non-ambiguous positions.

Primer set V250 and X250 did not distinguish between NABS V and X because there was one mismatch in the last four 3' positions, and the 3' terminus was a 't'. Kwok et al. (1990) found that the combination of a 3' terminus 't' as well as a mismatch at the penultimate position would produce efficient amplification. Differentiation between NABS V and X seem to have required more rigorous mismatches in primer design.

2.4B.g Significance of 10base primer sites in specificity:

Since the alignment between homologous portions of sequences from V250a/b and 17/127 contained 97.8% similarity (Figure 2B-8), then they were indeed homologous, but the ends of the sequences between V250 and 10bp sites, contained variation which was evident from inverse PCR (Figure 2B-8). By summing distances between proposed primer sites (Figure 2B-10) a comparison between sequence length and size of electrophoretic bands could be made. The distance between V250a and V250b was 225bp

(add 40bases between inverse primers) (Figure 2B-10). This corresponded to the size of the band on the agarose gel (Figure 2B-7B). However, calculation of the shortest distance between proposed primer sites 17 and 127 for NABS V (Figure 2B-10) resulted in a much longer fragment (540bp) than the band found in the agarose gel (390bp) (Figure 2B-7A). Although one of the bands obtained from V-17/V-127 from inverse PCR was ambiguous, it was approximately 530bp long (Figure 2B-7D) and closer in length to the calculated distance of 540bp if A-3 and B-3 were the selected sites (Figure 2B-10).

A stem loop structure may explain the discrepancy in the distance between the proposed primer sites (Figure 2B-10) and the length of the amplified product (Figure 2B-7A). Since the sequence for RC #127 at B-4 in NABS V, was a perfect match (0/10 mismatches) then confidence could be placed in the location of this primer. Because #127 was located outside V250b, then #17 must have been located on the opposite side of V250a. Possible locations for #17 and #127 would be those presented in Figure 2B-10. A-3 was the likely site for primer #17 in NABS V because the two samples have 0 and 1 purine-purine mismatches in non-ambiguous positions, whereas the A-2 site had 3 and 3 purine-purine mismatches in non-ambiguous positions (Figure 2B-9). The other species also indicate more favourable extension at the A-3 site. Assuming primer #17 was located at the A-3 site, and primer #127 at B-4, the sequence would be 540bp long. One explanation would be that a loop of 150bases (540-390bp) was formed between V250 and a 10nt primer in order to make the size of amplified product (Figure 2B-7A) agree with distance between proposed primer sites (Figure 2B-10). DNA loop structures are not

easily denatured by short primers (Caetano-Anollés et al., 1992b). The longer the complementary regions within the loop structures, the more stable the loop becomes. Secondary structure of introns in the nuclear rRNA genes in *Hymenoscyphus ericae* contain stem loop structures with a high proportion of 'gc' pairing in the stem portion of the loop (Egger et al., 1995). Hairpin loops with high 'gc' content have been described in inverted repeats of bean (Xodo et al., 1991). If the 10nt primer site was near the terminal stem portion of the loop, there may not have been sufficient extension to produce enough stability to denature the complementary regions of the loop, so extension proceeded past the base of the loop to produce a shorter PCR fragment (Figure 2B-11). Alternatively, a process similar to the replication of palindromic base sequences at telomeres (Cavalier-Smith, 1974) may have occurred without the endonuclease activity. If the formation of a hairpin loop of palindromic ends occurred in early stages of PCR, the 3' ends of the loop would act as a primer for extension of the DNA. The final product would then be twice as long as the original DNA. A third process which may account for the increased sequence length would involve PCR jumping (Innis et al., 1990). If the template DNA was not long enough to act as template for the primers, the primers may have amplified shorter fragments during the first few cycles of PCR. The 3' ends of the shorter fragments would then overlap, and a longer fragment would subsequently be amplified. However, since the fragment in this study was found in more than one sample, and the DNA was in good condition, the likelihood of the occurrence of PCR jumping would be low.

Results from this study suggest that the species specific fingerprints obtained by 10nt primer amplification was likely a result of competition among primer/template binding sites and secondary DNA structure, rather than the presence of discrete 10nt sites specific to a species. Secondary structure has the potential to interfere with amplification and produce intact PCR fragments which span portions of DNA that are actually found some distance apart.

2.4B.h Qualitative primer/template mismatch:

Since there was a high degree of ambiguity in the primer/template sequences obtained from inverse PCR, two viewpoints will be taken to discuss variation in complementarity of the primer sites with template DNA; liberal and conservative. The liberal viewpoint imposed no restraint on the choice of base in the ambiguity code in template DNA. It assumed that the nucleotide in the primer was consistent with the matching base in the ambiguity code of the template DNA, ultimately giving the least number of mismatches. The conservative viewpoint uses caution when matching bases in the primer with those in the ambiguity code of the template DNA. It assumed that nucleotides in the primer were not the matching bases in the ambiguity codes, resulting in the largest number of mismatches. A consensus viewpoint combined the two approaches for analysing PCR dynamics.

Liberal. A 390bp band was present in V83621 using 17/127 (Table 2B-3), and potential sites for primer 17 were present at two locations, A-2 and A-3, downstream from

INV250A primer site (Figure 2B-10). The A-2 site contained 5/10 mismatches, with the 3 near the 5' end being purine-purine mismatches indicating that they would have an effect on extension (Kwok et al., 1990). All mismatches were within 6 bases of the 5' terminus, having less of an affect on extension than if they were near the 3' terminus (Kwok et al., 1990). In contrast A-3 site had 3/10 mismatches which were (possibly 2 were purine-purine) at positions 1, 5 and 10 from 3' terminus. One possible purine-purine was at the 3' terminus. If the nucleotide at position 1 from 3' end was 'a' then this A-3 site with 80% gc content would outcompete A-2. If the position were 't' then it would be more likely that A-2, with 80% gc content would outcompete the A-3 site. Competition would be high between these two sites.

Although VNOF891 was not tested with 17/127, speculation can be made on the success of its amplification. There were 4/10 mismatches in A-2 site (80% gc content) with three purine-purine and one purine-pyrimidine, whereas the 5/10 mismatches in the A-3 site (80% gc content) contained a mixture of purine-purine (3) and purine-pyrimidine (2) mismatches. The purine-purine in the A-2 site was third from 3' end, but in A-3 site it was fifth from 3' end. Since purine-purine mismatches are deleterious to extension (Kwok et al., 1990), the mismatches at A-3 site were less hazardous to extension than those at A-2 site. Consequently, A-3 site would most likely outcompete A-2 site.

There was no 390bp band for IIIJB56 using 17/127 (Table 2B-3), and the inverse PCR results indicate there was no #17 primer site located within the vicinity with INV250A (Figure 2B-9). Therefore inverse PCR sequences supported results from

electrophoresis banding pattern.

The remaining samples contradict results from inverse PCR. However, fragments other than those amplified from sites located in the inverse sequences may be outcompeting those presented here. Although A-2 and A-3 sites in VII90-10 have the same number of mismatches (3/10), A-3 site (80% gc content) would be more probable for amplification because it consisted of non-deleterious mismatches, whereas A-2 site (80% gc content) consisted of two purine-purine mismatches at the 5' terminus. Competition may be high since the purine-purine mismatch was outside the 8 nucleotide domain necessary for amplification (Caetano-Anollés et al., 1992a).

The A-3 primer site in IXTJV200 contained 80% gc, with only two mismatches, was a likely site for amplification since both mismatches were purine-pyrimidine. Site A-2 in IJB13 contained 80% gc content and all four mismatches were within five positions from the 5' terminus, with two being purine-purine. The A-3 site also had two purine-purine mismatches three positions from the 5' but it had 70% gc content. Therefore, the A-2 site would probably outcompete the A-3 site for IJB13.

All of the 4/10 mismatches and 80% gc content in A-4 for IX-TJV200 were non-deleterious producing strong amplification.

Both mismatches for VII-90-10 at site A-5 were purine-purine. The primer, with 80% gc content, may produce amplified product since deleterious mismatches were 4 and 9 from the 3' end.

The B-2 site for primer #17 in sample V-83621 contained 5/10 mismatches, 1 was

purine-purine, and 80% gc content, indicating this was poor site. Since B-4 site for primer 127 contained no mismatches and had 60% gc content, amplification most likely occurred from this site. B-4 site was also the only site downstream from INV250A which could code for primer 127.

Although B-2 site for VNOF891 had 80% gc content, and contained 2 non-deleterious mismatches, it seems probable amplification could occur from this site. The B-4 site contained no mismatches and had 60% gc content which represented a good binding site for primer 127.

The B-3 site for IIIJB56 (60% gc content) had three non-deleterious mismatches. However the B-4 site for the same sample and same primer #127, contained one purine-purine mismatch at the 3' terminus. Competition would be high.

The B-2 site for VII90-10 contained 3/10 mismatches and 80% gc content, with two purine-purine mismatches near the 5' end may amplify product. For the same sample the B-3 site contained 2/10 mismatches, with one being purine-purine, and 60% gc content, whereas the B-4 site contained 1/10 pyrimidine-pyrimidine mismatches and 60% gc content. Consequently the B-4 site would most likely outcompete B-3 for primer 127.

Both B-3 and B-4 site for 127 for sample IXTJV200 contained 60% gc content. B-3 site had 3/10 mismatches and B-4 had 4/10 mismatches. Since one of the mismatches for B-3 was purine-purine, then B-4 may have outcompeted B-3.

Conservative. The A-3 site for sample V83621 was more likely the binding site for #17 than A-2 site. A-2 contained 7/10 mismatches, four were purine-purine with on

at the third position from the 3' end, and 80% gc content. The A-3 site (80% gc content) contained 6/10 mismatches, four were purine-purine and 2 of these were within four positions from 3' end.

The A-2 site was less likely the binding site for #17 for sample VNOF891 than A-3 since it contained 7/10 mismatches, 5 were purine-purine, and 80% gc content, whereas A-3 site also contained 7/10 mismatches but the fourth from the 3' end was a possible purine-purine and 2 at the 5', end and 80% gc content.

A-3 site for primer 17 for sample VII90-10 might outcompete the A-2 site since both have 50% mismatch, but 2 of A-2 mismatches were purine-purine, both were near the 5' end, and the one A-3 purine-purine mismatch was second from the 5' terminus. Both sites contained 80% gc content.

The A-3 site for sample IXTJV200 was a good binding site for primer 17 since it contained 80% gc content and only 1 of its 6/10 mismatches was purine-purine and located second from the 5' end.

The A-3 site may have outcompeted the A-2 site for primer #17 in sample IJB13 since both sites contained 80% gc content. Three of the 6/10 mismatches in A-2 was purine-purine with two near the 5' terminus and one at the 3' terminus. Two of the 4/10 mismatches in A-3 was purine-purine both near the 5' end.

Amplification may occur from the A-4 site in sample IXTJV200 containing 4/10 mismatches and 80% gc content. Its one non-ambiguous purine-purine mismatch was at the 5' terminus.

The poor A-5 binding site for primer 17 in sample VII90-10 contained 7/10 mismatches and 80% gc content with six purine-purine mismatches.

The B-2 binding site for #17 in V83621 contained 5/10 mismatches and 80% gc content. One purine-purine mismatch was fourth from the 3' end, indicating that extension may be difficult. The B-4 site for #127 contained a single pyrimidine-pyrimidine mismatch and 60% gc content, indicating it was an efficient site.

One of the 5/10 mismatches for B-2 site for 17 in VNOF891 was purine-purine in the second position from the 3' end, and the 80% gc content indicated it was a poor site. The B-4 site for primer 127 was a good binding site containing 60% gc content and 2/10 purine-purine mismatches, one located at the 3' end.

The B-3 site for primer #127 in IIIJB56 contained 3/10 non-deleterious mismatches near 3' end and 60% gc content, probably producing weak PCR product. Whereas one of the two mismatches in B-4 was purine-purine and at the 3' terminus and 60%gc content, producing weak or no PCR product.

Weak product was probably also produced by B-2 and B-3 sites in VII90-10 with 6 and 4/10 mismatches, and 80% and 60% gc content. The B-4 site for 127 was a poor site with four of its 8/10 mismatches purine-purine, one at the 3' end, 60% gc content.

B-3 and B-4 sites for 127 in IXTJV200 would produce weak amplification since they contained 4, with one being purine-purine, and 8/10 mismatches, with three being purine-purine, and both have 60% gc content. The B-3 site may outcompete the B-4 site since it would produce a shorter fragment, since smaller fragments are more efficiently

amplified than larger ones.

Consensus viewpoint. Since true primer/template complementarity and subsequent amplification probably exists somewhere between liberal and conservative viewpoints, consensus primer/template complementarity was extropolated from the level of agreement between the two viewpoints.

The A-3 site would likely outcompete the A-2 site for primer #17 in V-83621 and V-NOF891. However, competition would be high, and if mismatch dynamics become equal, the shorter fragment will outcompete the longer fragment. The A-3 site was also better than the A-2 site for #17 for NABS VII, IX and I. The A-4 was good but the A-5 site was considered poor.

B-2 site for primer 17 in V-83621, V-NOF891 and VII-90-10 were considered poor. However, primer 17 at this site was oriented in the wrong direction and too close to primer 127 to produce a fragment. Site B-3 for primer 127 in VII-90-10 and IX-TJV200 was also poor, whereas B-4 in V-83621 and V-NOF891 and VII-90-10 formed a very good binding site, but not in IX-TJV200. This would explain presence of the 390bp fragment in NABS V isolates. There was also a 525bp fragment in VII-90-10, but there was no homology between 390bp in V and the 525bp in VII. Evidence supported the location of the primer sites for 390bp fragment in NABS V to A-3 for #17 and B-4 for #127.

2.5 SECTION C: Repetitive DNA

2.5A RESULTS

Three nearly equidistant bands were visible in the agarose gel for NABS III (240bp, 300bp, and 360bp) with primers 34/122, NABS VI (115bp, 135bp, and 170bp) with primers 83/66, and NABS VII (160bp, 170bp, and 200bp) with primers 83/66 (Figure 2C-1A and C). Stairstep banding patterns were produced when each band was excised and re-amplified (Figure 2C-1B and D).

2.5B DISCUSSION

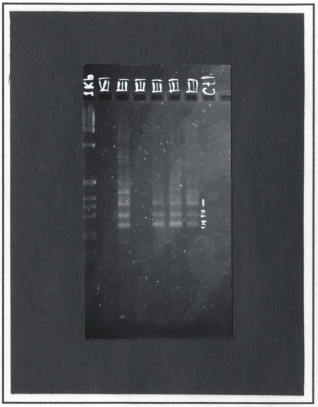
The length of the fragments in NABS III was approximately 240bp, 300bp, and 360bp (Figure 2C-1A), with the repeat unit being 60bp long. Each of the three bands were excised and re-amplified separately, producing a stair step banding pattern (Figure 2C-1B) indicating the presence of internal primer annealing sites on the longer fragments.

Similarly, the three fragments in NABS VI were 115bp, 135bp, and 170bp long, making the repeat units 20 and 35bp long (Figure 2C-1C). The three fragments in NABS VII were 160bp, 170bp, and 200bp long, making the repeat units 10 and 30bp long. Both sets of three bands in NABS VI and VII produced stairstep banding patterns indicating that internal annealing sites were present in the longer bands (Figure 2C-1D). Minisatellites range in size from a few hundred to several Kbp, and the repeat units vary from 10 to 60bp. The repeat units in this study fell within this range.

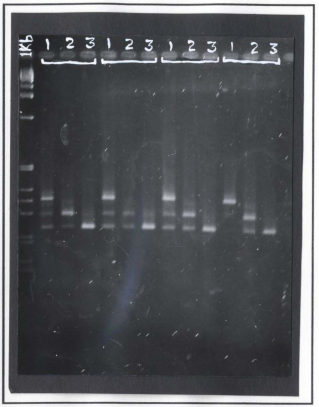
Variation among repeat sequences are reflected in length differences and base composition. All three bands for NABS III showed equal intensity on the original gel.

Figure 2C-1: PCR amplification with SWAPP primers showing repetitive banding pattern found in separate species (A and C), and the stair-step banding pattern produced when each band was excised and re-amplified separately (B and D). **A:** Three bands present in NABS III (1 at 360bp, 2 at 300bp, and 3 at 240bp) with primers 34/122. **B:** Excised bands from A shows re-amplified product from separate bands, 1, 2, and 3, for each sample in A. **C:** Three bands present in NABS VI (1 at 170bp, 2 at 135bp, and 3 at 115bp), and NABS VII (4 at 200bp, 5 at 170bp, and 6 at 160bp) with primers 83/66. **D:** Stair-step banding patterns produced from re-amplified excised bands in C.

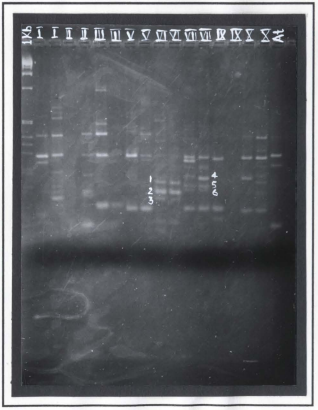
A



B



C



D

However, in each stair step pattern the intensity of the shorter 1 or 2 bands was less than the topmost longer band. Morton et al., (1995) reported differences among sub-repeat sequences of oomycetes. The longer band contained a considerable amount of DNA since it was excised and resuspended from the original gel. Since PCR product of the top band was already present in the reaction tube in large quantities, this product had a head start in the second reamplification and used more reagents, making fewer reagents available to produce smaller bands, appearing weak on the agarose gel. These weak bands occurred in all three species.

The three amplified bands in each of the three species may have been part of a longer repeat region. Equidistant bands were present above the 3-band repeat in NABS III at 400bp, 440bp, and 480bp (Figure 2C-1A), suggesting a longer array. These repeat units were 40bp long. However, because the 3 shorter bands were smaller, competition among reagents in the amplification reaction favoured shorter fragments. Primer sites may have differed slightly among regions creating variation in strength among amplification product. Similarly, a longer fragment at 240bp was present above the 3-band cluster in NABS VII.

Repetitive DNA sequences are present in all eukaryotic and some prokaryotic organisms (Singer and Berg, 1991) varying from 2bp to several thousand bp. The repeat regions in this study appeared to be species-specific to NABS III, VI and VII. Microsatellite identification of sperm whales (Richard et al., 1996), brook charr (Angus and Bernatchez, 1996) and tomato cultivars (Rus-Kortekaas et al., 1994) produced

variation. Rus-Kortekaas et al. (1994) found that RAPD DNA was more conserved than microsatellite DNA in tomato. Since the repeat regions in this study were found using 10nt primers (ie. RAPD primers) then it would be expected that they be conserved such as within a species or group of species.

Chapter 3

Pathogenicity of *Armillaria ostoyae* in Newfoundland

3.1 INTRODUCTION

Armillaria ostoyae (Romagnesi) Herink is considered a highly pathogenic root pathogen (Wargo and Shaw, 1985) commonly found associated with many coniferous tree species (Guillaumin et al., 1989). *A. ostoyae* is distributed throughout North America and Asia, and completes a northern circumboreal distribution with its European counterpart, *A. obscura* (Secretan) Herink (Guillaumin et al., 1991). Two species of NABS *Armillaria* occur in Newfoundland. *A. ostoyae* (NABS I) is commonly found in soils throughout the island (Singh, 1981b), and *A. sinapina* (NABS V) occurs as an isolated collection from a hardwood, Mountain ash, in a city park (Bérubé, personal communication). *A. ostoyae* has been associated with root disease of black spruce and balsam fir, causing mortality (Blodgett and Warrall, 1992), and is considered one of the two major diseases of conifer seed plantations in Canada (Côté and Dessureault, 1994). The root system of black spruce and balsam fir is shallow (upper 30cm) with wide spreading lateral roots growing in the same soil layer as the rhizomorphs of *Armillaria* (humus and 0-15cm deep) (Singh, 1975). Trees growing in plantations in Newfoundland are generally planted as 2-year old seedlings started in cone shaped containers. Spreading of the roots as the tree grows is restricted by the container so that roots of a mature tree remain deformed, affecting

nutrient uptake and predisposing the tree to *Armillaria* root disease (Livingston, 1990).

Trees weakened by *Armillaria* are more susceptible to other invaders that can overcome defense reactions of the host (Mallett, 1995; Mallett and Volney, 1990; Cobb, 1989), making *Armillaria* a primary pathogen. Alternatively, trees weakened from biotic or abiotic stress tend to be more vulnerable to *Armillaria* (Wargo and Shaw, 1985; Singh, 1975; Filip, 1989) making it a secondary pathogen. Singh (1983) reported that because trees growing in Newfoundland soils are growing in unfavourable soil conditions, they are stressed and more susceptible to *A. mellea* (*sensu lato*). Many forest insect pests also cause damage and stress to trees, providing an opportunity for secondary pests or pathogens to attack. If the insect infestation is low, the tree may be able to withstand the attack and ward off secondary invasions, surviving for many years and possibly recovering if conditions are unfavourable for the invaders. However, if the insect attack is severe, and one or several secondary invaders attack, the tree may succumb and die within one or two growing seasons. The dead tree would then act as an inoculum source for *Armillaria* to increase biomass and attack surrounding living trees.

Two reviews (Filip, 1989; Wargo and Harrington, 1991) and numerous studies have been carried out on the relationship between insect defoliation and *Armillaria* root disease (Mallett and Volney, 1990; James and Goheen, 1981; Raske and Sutton, 1986; Wargo, 1977; Kulhavy et al., 1984). Different species of insect pest feed in a variety of ways causing various amounts of stress on a tree. The hemlock looper (*Lambdina fiscellaria fiscellaria* (Guen.), (Lepidoptera: Geometridae)) has undergone four major

outbreaks since 1912 (Carroll, 1956) and poses a threat to the forest industry when outbreaks begin. The insects feed mainly on new foliage from early spring as a first instar larva, through to late summer when pupation occurs. They may feed on old foliage later in the season if all new foliage has been consumed. The looper is termed a 'wasteful' feeder in that it will take several bites from one needle and move on to the next (Carroll, 1956). The needles senesce and turn brown after the feeding has damaged them. The balsam fir sawfly (*Neodiprion abietis* Harris) (Hymenoptera: Diprionidae) is also a defoliator, but this insect feeds on old foliage and consumes entire needles before moving on to the next (Ives and Wong, 1988). It may defoliate a tree leaving the trunk and bare branches standing with only the current year's foliage present. The balsam woolly adelgid (*Adelges piceae* (Ratz.) (Homoptera: Adelgidae)) is also a common pest in Newfoundland, but it generally causes little tree mortality. Feeding of this aphid causes deformation and stunting of the branches, sometimes causing defoliation. It is believed that the formation of galls by this insect decreases water flow to the crown (Hollingsworth and Hain, 1994) placing water stress on the tree. Other studies have investigated the incidence of *Armillaria* root rot in adelgid infested balsam fir stands (Hudak and Singh, 1970; Hudak and Wells, 1974).

Depending on the type of foliage damage, the degree of stress placed on a tree may vary. Mined leaves were consistently avoided by herbivores, whereas chewed and artificially defoliated leaves were preferred (Hartley and Lawton, 1987). Defoliation from insect feeding may induce a response from the tree by increasing leaf phenolic levels by

25%, whereas mechanical damage from artificial defoliation may elicit an increase in phenolic levels by only 9% (Hartley and Lawton, 1987). Consequently, the type of insect damage may induce different chemical changes in foliage. Defoliation is also known to cause chemical changes in root bark of trees (Parker and Houston, 1971).

Inoculation trials with *Armillaria* have been commonly done on greenhouse seedlings or young potted plants (Mallett and Hiratsuka, 1988; Mugala et al., 1989; Singh, 1983, Rishbeth, 1982, 1984; Shaw et al., 1981; Anselmi et al., 1994). Few field inoculation trials have been performed on mature established trees (Davidson and Rishbeth, 1988; Wargo and Houston, 1974; Wilbur et al., 1972). Incidence of *Armillaria* in Newfoundland has been extensively studied (Singh, 1981a, 1981b; Warren and Singh, 1970; Singh and Raske, 1983; Singh and Carew, 1983), however field inoculation of mature trees has never been done in a boreal forest.

The purpose of this study was to examine the pathogenicity of *Armillaria ostoyae* on black spruce and balsam fir trees undergoing various types and degrees of stress by inoculating roots with two Newfoundland isolates. Several objectives were proposed. The first objective was to determine whether different methods of insect feeding would affect rate of infection by *A. ostoyae*. A second objective was to examine effects of artificial defoliation on infection rate of *A. ostoyae*. Thirdly, a comparison was made between pathogenicity of *A. ostoyae* in a plantation and in a naturally regenerated black spruce stand. The last objective examined the effects of the degree of insect and artificial damage to the tree on the infection rate by *A. ostoyae*.

3.2 MATERIALS AND METHODS

3.2a Source of inoculum:

Two isolates of *Armillaria ostoyae*, AS14-2R-2 and AS11-4H-1, were used as inoculum, and were collected from Crabbes River and Big Cooks Pond areas respectively, by G. Warren in 1991. AS14 was isolated from recently killed black spruce trees in a plantation and AS11 from birch stumps left from a hardwood cutover, and grown on 3% Malt extract agar.

3.2b Inoculation of trees:

Rye kernals were soaked in water with $CaCO_3$ (21.3g/L) and distributed in bags, and autoclaved twice for 45 minutes at 24 hour intervals. Whole cultures of *Armillaria* were mashed in a blender with water, and each autoclaved jar was inoculated with 10mL of the liquid inoculum, and incubated for 3-5 weeks. Inoculum blocks, consisting of autoclaved white birch twigs (3cm x 20cm), were placed in the inoculated mason jars and incubated to allow mycelium to grow beneath the bark layer. These blocks were then placed against the long axis of a major root with one side of the mycelial fan exposed to the root, and secured in place by two pieces of wire. The upper forest layer of soil or moss was replaced to cover the root.

3.2c Experimental design:

The experiment was manipulative with completely randomized design, and was

conducted from July, 1994, when host trees were inoculated with *Armillaria*, to July, 1996, when the inoculum was removed and results recorded. Experimental units were defined as inoculated trees within a plot, and each tree was undergoing one of four stress levels. The stress level was the treatment variable, and the treatments were randomly assigned to experimental units (ie. inoculated trees in discrete physical locations). Ten trees were inoculated per fungal isolate for each treatment variable, and the area on which trees were located for that treatment was referred to as a plot. Survival of inoculum on the birch twigs was imperative for examination of the effects of *Armillaria* on host trees. If lab inoculation of birch twigs was not successful, then fewer than 10 trees were inoculated for each treatment group. Infection was determined by observing presence of mycelium on the inoculum block and on or in the root, by listing six levels of pathogenicity:

1 = Mycelium *absent* on block, *absent* on root, No infection.

2 = Mycelium *absent* on block, *present* on root, Foreign *Armillaria*.

3 = Mycelium *present* on block, *absent* on root, No infection.

4 = Mycelium *present* on block, *present* **ON** root, Superficial colonization.

5 = Mycelium *present* on block, *present* **IN** root, Cambial infection.

6 = Mycelium *absent* on block, *present* **IN** root, Foreign *Armillaria*.

Root response was determined by the following criteria:

1 = No response, healthy root.

2 = Superficial swelling, necrosis, or lesion on the root.

3 = Resinosis, callus tissue, or necrosis present below the bark.

4 = Lesion located away from inoculum block, indicating foreign *Armillaria*.

5 = Reaction to wire damage.

Tree health was used as an indication of above ground tree symptoms with the following criteria:

1 = Healthy, 0% defoliation from beginning of study.

2 = Lower and inner needles yellow, chlorotic, or pale green.

3 = Needles green but sparse.

4 = Dead branches present in half the tree.

5 = Recovering from adelgid with new growth on affected branches.

3.2d Definiton of treatment groups:

Two species of conifer, balsam fir (*Abies balsamea*) and black spruce (*Picea mariana*), were inoculated with both isolates of *Armillaria*. Treatments involving artificial defoliation were defined as low when 30% of the branches were removed at each node of the tree; as moderate when 50% of the branches were removed; and as severe when 80% of the branches were removed from each node.

Different definitions were required for levels of defoliation for each type of insect. Hemlock looper defoliation levels took into account 1992, 1993, 1994 and percent crown defoliation, but were based mainly on the 1993 percent defoliation levels. Low levels were defined as 20 - 50% 1993 needles absent; moderate levels were 50 - 80% 1993

needles absent; and severe defoliation was 80 - 100% 1993 needles absent from the branches.

Two levels of defoliation were defined for the balsam fir sawfly. Severe levels consisted of ≥ 70% needles missing from the crown, and moderate levels were anything less than 70% needles missing from the crown.

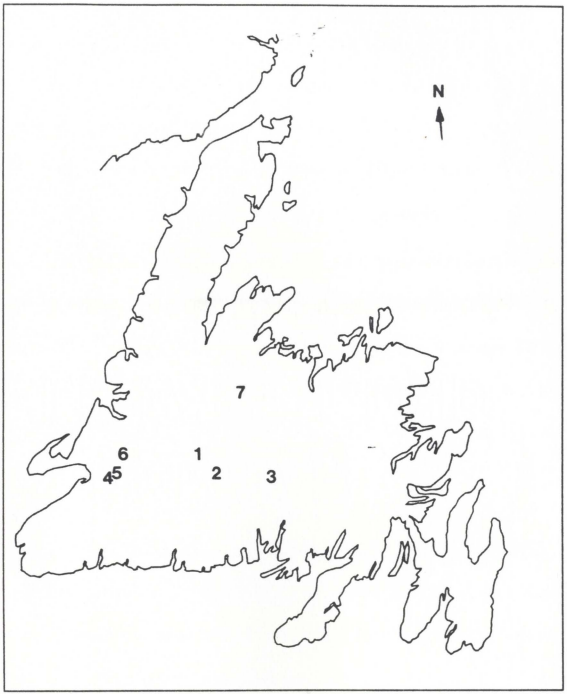
Three levels of adelgid damage were defined for the thinned site, and two levels (low and severe) for the unthinned site. Low level of damage included trees with less than 5% foliage missing and containing swollen nodes. The moderately defoliated trees were those with 5 - 30% foliage missing, swollen nodes, and they may or may not have stunted branches. The severely defoliated trees were those with stunted branches, swollen nodes, and more than 30% foliage missing, chlorotic and/or necrotic.

Control trees were inoculated in the same manner as the treatment groups, except the birch segments were sterile and autoclaved, containing no fungal growth. In artificially defoliated plots the control trees were healthy with no branches removed. However, in the insect defoliated plots, the control trees were chosen without prior knowledge of the level of defoliation. Consequently they consisted of three different levels of defoliation, but no fungal inoculum was applied to the root.

3.2e Site Descriptions:

Locations of the seven plots are shown on the map of Newfoundland (Figure 3-1). The Hungry Hill site (plot 1), located in central Newfoundland (48° 42.33' N and

Figure 3-1: Map of the island of Newfoundland showing location of the seven plots. Plot 1 was black spruce natural regeneration, plot 2 was balsam fir hemlock looper, plot 3 was balsam fir on a sandy site, plot 4 was adelgid thinned, plot 5 was adelgid un-thinned, plot 6 was balsam fir sawfly defoliation, and plot 7 was black spruce plantation.


N
7
6
1
4
5
2
3

56° 28.25' W), was a dry to moist natural regeneration black spruce stand with trees spaced from 0.25 to 3 meters apart. The upper part of the site was well drained and the lower portion was very wet. *Betula papyrifera* was heavily browsed. Flora was dominated by *Picea mariana* (70%), and other species included *Abies balsamea*, *Picea glauca*, *Betula papyrifera* (0.6 m high), *Alnus* sp. (2 meters high), *Cornus canadensis*, *Vaccinium angustifolium*, *Kalmia angustifolia*, *Maianthemum canadense*, *Clintonia borealis*, *Gaultheria hispidula*, *Trientalis borealis*, *Linaea borealis*, *Hieracium aurantiacum*, *Taraxacum officinale* and *Epilobium angustifolium*. Bryophytes consisted of *Hylocomnium splendons*, *Dicranum* sp., *Pleurozium shreberi*, and *Sphagnum* sp.

The Snowshoe Pond site (plot 2) near Douglass Lake, (48° 26' N and 56° 44.5' W) was a wet balsam fir site defoliated by the hemlock looper in 1993. Trees were 10 to 12 m high, spaced about 2.5 meters apart, and the forest floor was moss covered with 15% fallen logs. The upper part of the site was moist to wet. The understory consisted of *Abies balsamea* (< 0.3 m high), *Betula papyrifera* (< 0.3 m high), *Moneses uniflora*, *Cornus canadensis*, *Gaultheria hispidula*, *Clintonia borealis*, *Maianthemum canadense*, ferns, and scattered lichens and mushrooms. Bryophytes included *Ptilium crista-castrensis*, *Hylocomium splendons*, and *Pleurozium shreberi*.

Site 3 was artificially defoliated balsam fir (plot 3) also near Douglass Lake (48° 26'N and 55° 44.5'W). Trees were growing on a well drained ridge at the edge of a gravel pit. The upper part of the site was well-drained sandy material, and the lower part was moist with bryophytes. The forest floor consisted of compact dry soil overlain with

many loose brown needles. The dominant tree species was *Abies balsamea*, with small amounts of *Picea mariana*, and understory *Betula papyrifera*. Other plants included *Cornus canadensis*, *Rubus idaeus*, *Maianthemum canadense*, *Kalmia angustifolia*, and *Vaccinium angustifolium*. Bryophytes were *Pleurozium shreberi*, *Dicranum spp.* and *Hylocomium splendons*.

Sites 4 and 5, near Crabbes River in southwestern Newfoundland, were located adjacent to one another (48° 21.5'N and 58° 43.5'W). One plot was thinned (plot 4) and the other was not thinned (plot 5). Both were dominated by *Abies balsamea* which was infested with the woolly adelgid (*Adelges piceae*). Site 4 was open canopy, thinned, dry to moist and covered with many dry bare stumps and twigs (40%). Black spruce and white birch was present before thinning as evidenced by the fallen logs. Other plants included *Cornus canadensis*, *Linaea borealis*, *Betula papyrifera* (< 0.3 m high), *Solidago rugosa*, *Clintonia borealis*, *Rubus idaeus*, *Anaphalis margaritaceae*, *Gaultheria hispidula*, *Prunus pensylvanica*, *Acer* seedlings and ferns. Bryophytes included *Pleurozium shreberi*, and *Rhytidiadelphus loreus*. Site 5 was closed canopy, flat and moist with some decaying stumps and logs, and many brown needles and decaying birch leaves on the ground. Understorey flora included *Prunus pensylvanica* (< 0.6 m high), *Betula papyrifera*, *Abies balsamea* and ferns were present only where light reached the floor. Other plants were *Linaea borealis*, *Maianthemum canadense*, *Cornus canadensis*, some *Clintonia borealis*, *Ribes glandulosum*, and bryophytes such as *Hylocomium splendons*, *Dicranum sp.* and *Ptilium crist-castrensis*. Mushrooms such as *Hydnum, Lactarius, Rusula*, *Mycena* and

Cantharellus were abundant.

The Caribou Lake site (plot 6), was dominated by *Abies balsamea* infested with balsam fir sawfly, and was located on a south facing slope (48° 34.5'N and 58° 14'W). The upper main site was moist to dry with *Pinus strobus*, *Sorbus* sp., *Clintonia borealis*, *Solidago rugosa*, *Betula papyrifera* (0.5 to 1 m high), *Rubus idaeus, Linaea borealis*, *Cornus canadensis*, *Epilobium angustifolium*, *Kalmia angustifolia*, ferns, and bryophytes such as *Sphagnum spp.*, *Polytrichum sp.*, *Ptilium crista-castrensis*, and *Hylocomium splendons*. The lower site consisted of a moist lumpy terrain with moss covered logs and stumps overlain with a carpet of *Linaea borealis* and *Cornus canadensis*. Plants growing on the forest floor included *Linaea borealis, Cornus canadensis, Solidago rugosa, Rubus idaeus*, and understory *Abies balsamea* and *Betula papyrifera* (1 m high).

Plot 7 was a container planted black spruce plantation near South Pond, central Newfoundland (49° 21.75'N and 56° 08.75'W). The site was flat, dry to moist with *Alnus* sp. (2 meters high), *Picea glauca*, *Betula papyrifera*, *Cornus canadensis*, *Vaccinium angustifolium*, *Maianthemum canadense*, *Epilobium angustifolium,* and lichens and stumps.

3.2f Statistical analysis:

Non-parametric Kruskal-Wallis and Spearmans Rank Corrrelation tests (Sokal and Rohlf, 1981) were performed in Minitab Release 7.2, Standard version (Minitab, Inc.) and graphs were generated in Sigmaplot version 2.01 (Jandel Corporation). Treatments within

each plot were replicated 5 to 10 times for each isolate, but it was not feasible to replicate entire plots with similar physical characteristics, so two pairs of plots were compared using statistical tests. These plots were black spruce naturally regenerated stand (plot 1) and plantation (plot 7), and the balsam fir adelgid thinned (plot 4) and un-thinned (plot 5) stands. Comparison among the three insect defoliated plots were discussed biologically (as opposed to statistical analysis) with reference to major differences confounding the comparisons.

The six levels of pathogenicity were ranked in four levels for statistical comparisons. Level 1 remained as absence of inoculum, level 2 was omitted, level 3 was described as no infection, level 4 was superficial colonization, level 5 was cambial infection, and level 6 was omitted. This resulted in levels 1, 2, 3 and 4.

3.3 RESULTS

Most rank correlations were less than 0.500 indicating poor relationship between variables. The highest correlation between isolate and level of pathogenicity (r=-0.409), and also root response and pathogenicity (r=+0.700), was with the balsam fir sawfly plot, the most severely defoliated plot. The highest correlation between presence of rhizomorphs and pathogenicity (r=+0.630) occurred in the black spruce natural regeneration stand (Table 3-1).

There was a significant difference in virulence among the isolates. Both isolates produced significantly more disease than controls in all plots except the un-thinned adelgid plot (no disease) and the black spruce plantation ($H_{0.05[2]}$=4.76, p=0.093, df = 2, r=-0.202) (Figure 3-2). The only significant effect of treatment on pathogenicity occurred in the black spruce naturally regenerated stand, in which healthy and low level artificial defoliation had more root disease than high levels of defoliation ($H_{0.05[3]}$=17.54, p=0.001, df=3, r=-0.162) (Table 3-1). There was significantly more root response with pathogenicity in all sites except both adelgid plots (Table 3-1). The only site showing a significant relationship between tree health and pathogenicity was the sawfly plot ($H_{0.05[2]}$=6.86, p=0.033, df=2) (Table 3-1). Presence of rhizomorphs produced significantly more pathogenicity in plots 1, 2 and 3 only (Table 3-1).

The black spruce naturally regenerated stand (plot 1) and the black spruce plantation (plot 7) were similar with regards to height, diameter at breast height (DBH) and age (Figure 3-3). The adelgid infested balsam fir sites, thinned (plot 4) and un-thinned

Table 3-1: Effects of treatment, isolate, root response, tree health and presence of rhizomorphs on occurrence of disease in each of seven plots using Kruskal-Wallis and Spearman's Rank Correlation tests.

Factor	H	df	p (α = 0.05)	Rank Correlation (r)	Interpretation
Plot 1					
Treatment	15.48 17.54	3 3	0.002 0.001 (Adj.)	-0.162	Healthy and 30% artificial defoliation had more disease than controls, 50%, or 80% defoliation.
Isolate	9.57 10.84	2 2	0.009 0.005 (Adj.)	-0.321	Isolate AS-14 was more virulent than isolate AS-11 or controls.
Root Response	13.87 15.70	1 1	0.000 0.000 (Adj.)	+0.423	Tree roots with necrosis had more virulence than roots with no response.
Tree Health			P>0.05		
Rhizomorphs	30.67 34.70	1 1	0.000 0.000 (Adj.)	+0.630	There was more disease when rhizomorphs were present.
Plot 2					
Treatment			P > 0.05		
Isolate	3.31 5.21	1 1	0.069 0.023 (Adj.)	-0.302	Isolate AS-14 was more virulent than AS-11.
Root Response	5.67 9.19	3 3	0.017 0.001 (Adj.)	+0.436	There was more disease on necrotic roots than on roots with no necrosis.
Tree Health			P > 0.05		
Rhizomorphs	2.60 4.09	1 1	0.107 0.043 (Adj.)	+0.268	There was more disease when rhizomorphs were present.
Plot 3					
Treatment	16.15 18.30	5 5	0.007 0.003 (Adj.)	-0.546	All levels of artificial defoliation showed more virulence than controls.
Isolate	17.25 19.54	2 2	0.000 0.000 (Adj.)	-0.660	Both isolates were more virulent than autoclaved controls.
Root Response	16.22 18.67	2 2	0.000 0.000 (Adj.)	+0.714	Root necrosis and cambial infection showed more virulence than no response or wire damage.
Tree Health			P > 0.05		
Rhizomorphs	5.97 6.77	1 1	0.015 0.009 (Adj.)	+0.433	There was more disease when rhizomorphs were present.

Plot 4					
Treatment			P > 0.05		
Isolate	4.51 5.23	1 1	0.034 0.022 (Adj.)	-0.371	Isolate AS-11 was more virulent than controls.
Root Response			P > 0.05		
Tree Health			P > 0.05		
Rhizo-morphs			P > 0.05		
Plot 5					No tests performed since there was no infection.
Plot 6					
Treatment			P > 0.05		
Isolate	4.19 4.86	1 1	0.041 0.028 (Adj.)	-0.409	Isolate AS-11 was more virulent than autoclaved controls.
Root Response	11.9 13.84	2 2	0.003 0.001 (Adj.)	+0.700	Root necrosis and cambial infection showed more virulence than no response.
Tree Health	5.91 6.86	2 2	0.053 0.033 (Adj.)	+0.486	Trees with needles missing or dead branches had more infection than healthy trees
Rhizo-morphs			P > 0.05		
Plot 7					
Treatment			P > 0.05		
Isolate	4.01 4.76	2 2	0.135 0.093 (Adj.)	-0.202	Both isolates were more virulent than autoclaved controls.
Root Response	5.75 6.93	1 1	0.017 0.009 (Adj.)	+0.319	Root necrosis was more virulent than no response.
Tree Health			All trees in one health category.		
Rhizo-morphs	3.21 3.82	1 1	0.073 0.051	+0.239	There was more disease when rhizomorphs were present.

Figure 3-2: Proportion of trees in each plot showing degree of pathogenicity for each of the two inoculum isolates, AS-14 and AS-11, and control blocks. Numbers represent combination of superficial colonization and cambial infection.

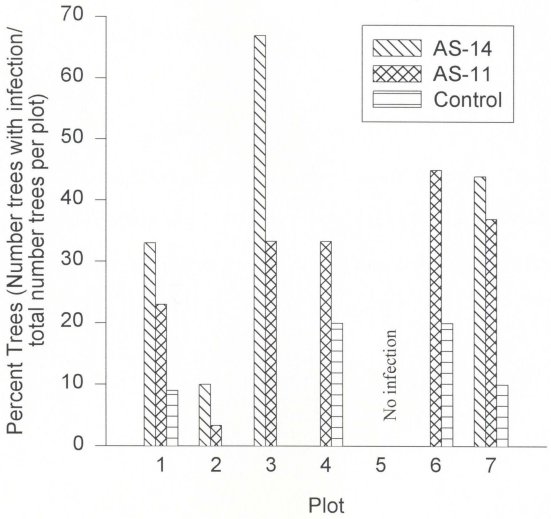
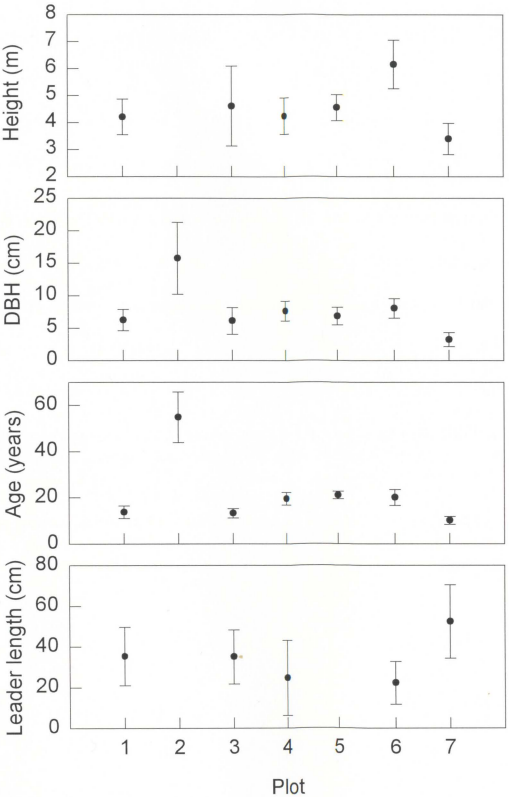

Percent Trees (Number trees with infection/ total number trees per plot)
70
60
50
40
30
20
10
0
AS-14
AS-11
Control
No infection
1
2
3
4
5
6
7
Plot

Figure 3-3: Comparison of physical factors of trees (y-axis) in each of seven plots (x-axis) showing mean and standard deviation for each plot. **A**: Height of trees (meters) estimated from soil surface to apical tip. Trees in plot 2 were not measured and were estimated to be 10 to 12 m high. **B**: Diameter at breast height (DBH) (cm) was taken approximately 1.3 meters above soil level. **C**: Tree age (years) was counted from stem cores at DBH. **D**: Leader length (cm) was estimated from the highest whorl of branches to the apical tip, representing the last year of growth. Trees in plot 2 were too high to estimate leader length, and those in plot 5 were too dense. Sample sizes for plots 1 through 7 are 93, 70, 30, 40, 20, 30 and 70 trees respectively.


Height (m)
8
7
6
5
4
3
2
DBH (cm)
25
20
15
10
5
0
Age (years)
60
40
20
0
Leader length (cm)
80
60
40
20
0
1
2
3
4
5
6
7
Plot

(plot 5) were also similar with regards to height, DBH and age (Figure 3-3), as well as soil profile (Figure 3-4). However, the looper defoliated balsam fir site (plot 2) was taller, older, and had a larger DBH than any other site (Figure 3-3). Although the artificially defoliated balsam fir (plot 3) was similar in tree measurements, the soil profile was very different with less than 2 cm organic layer, and high sand composition (Figure 3-4, Table 3-2). The sawfly defoliated site (plot 6) was taller than the other balsam fir sites, but was similar in age and DBH (Figure 3-3).

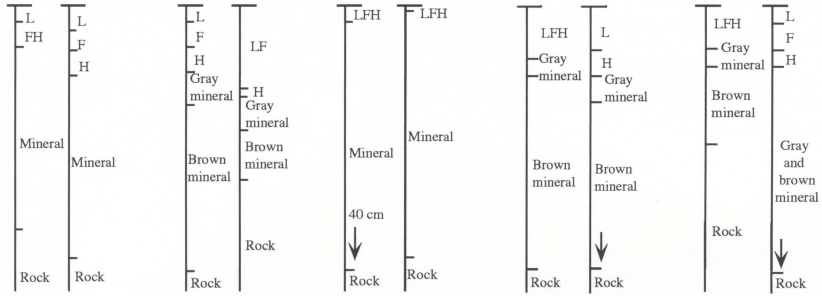
Virulence was defined according to incidence, superficial colonization of mycelium on the root, and intensity, its entry into root cambial tissue. There was 4% cambial infection in the total 350 trees in the study, but there was an average of 25% both cambial infection and superficial colonization combined (Table 3-3).

The highest level of infection occurred on the balsam fir sandy site (Table 3-3, Figure 3-5) corresponding to the highest level of root response (Figure 3-6). But the highest proportion of rhizomorphs was found associated with roots in the black spruce natural regenerated stand (Figure 3-7). However, rhizomorphs were also abundant on the balsam fir sandy site and black spruce plantation. The balsam fir sandy site contained a large proportion of sand content and a higher pH than all other sites (Table 3-2).

Within the plots pathogenicity corresponded to root response (Figures 3-8 and 3-9). Root response increased with disease incidence and vice versa. The significant relationship between treatment and pathogenicity in the black spruce natural regeneration site was unexpected in that low levels defoliation had higher pathogenicity than higher

levels defoliation (Figure 3-8).

Figure 3-4: Soil profiles for six plots showing depth of LFH and mineral layers. LFH represents the upper organic layer consisting of litter (L), fermented layer (F), and humus (H). Mineral layers consist of gray and brown layers. Nutrients have leached out of the gray layer and into the brown layer producing a color difference. The mineral layer contains both gray and brown layers with no color differentiation. Rock represents the parent material from the bedrock. Two sites per plot were sampled except plots 4 and 5.


L
FH
Mineral
Rock
Site 1
L
F
H
Mineral
Rock
Site 2
Black spruce
Natural regeneration
Plot 1
L
F
H
Gray
mineral
Brown
mineral
Rock
Site 1
LF
H
Gray
mineral
Brown
mineral
Rock
Site 2
Balsam Fir
Hemlock looper
Plot 2
LFH
Mineral
40 cm
Rock
Site 1
LFH
Mineral
Rock
Site2
Balsam Fir
Artificial defoliation
Plot 3
LFH
Gray
mineral
Brown
mineral
Rock
Site 1
L
H
Gray
mineral
Brown
mineral
Rock
Site 2
Balsam Fir
Woolly adelgid
Plots 4 and 5
LFH
Gray
mineral
Brown
mineral
Rock
Site 1
L
F
H
Gray
and
brown
mineral
Rock
Site 2
Balsam Fir
Sawfly
Plot 6

Scale: —— = 5 cm

Table 3-2: Comparison of soil properties for six plots, natural regeneration black spruce (1), balsam fir looper defoliation (2), balsam fir artificial defoliation (3), balsam fir adelgid and thinned (4), balsam fir adelgid and un-thinned (5), and balsam fir sawfly defoliation (6). Samples were taken from two sites within each plot (1,2), and analysis was done on the upper LFH soil layer (a) and the lower mineral layer (b). Soil properties included pH (in water), organic matter (OM) (%), nitrogen (N) (%), phosphorus (P) (ppm), potassium (K) (meq/100g), calcium (Ca) (meq/100g), magnesium (Mg) (meq/100g), silt (%), clay (%), sand (%) and texture (Text).

Plot	pH-1a	pH-1b	pH-2a	pH-2b	OM-1a	OM-1b	OM-2a	OM-2b	N-1a	N-1b	N-2a	N-2b
1	3.78	3.98	4.10	4.90	64.96	5.78	57.54	16.11	1.24	0.10	0.74	0.21
2	3.64	3.75	3.38	3.84	92.32	5.16	86.17	8.33	1.19	0.06	1.19	0.14
3	4.85	5.05	5.42	5.02	22.9	4.38	15.33	3.97	0.47	0.06	0.38	0.05
4	-	-	3.81	4.54	-	-	90.12	7.24	-	-	1.59	0.14
5	3.86	4.17	-	-	86.4	5.70	-	-	1.44	0.14	-	-
6	-	4.23	3.70	4.23	-	5.24	92.13	5.15	-	0.11	1.83	0.10

Plot	P-1a	P-1b	P-2a	P-2b	K-1a	K-1b	K-2a	K-2b	Ca-1a	Ca-1b	Ca-2a	Ca-2b
1	25	12	14	2	2.13	0.13	1.75	0.11	6.94	1.97	8.94	0.97
2	60	31	20	5	2.48	0.05	1.16	0.06	9.09	0.28	1.19	0.15
3	17	45	25	17	0.66	0.05	0.93	0.03	6.35	0.28	7.09	0.18
4	-	-	30	5	-	-	2.26	0.08	-	-	18.34	0.93
5	50	22	-	-	1.78	0.06	-	-	16.23	0.79	-	-
6	-	2	41	3	-	0.30	1.89	0.02	-	0.54	18.65	0.30

Plot	Mg-1a	Mg-1b	Mg-2a	Mg-2b	Silt-1	Silt-2	Clay-1	Clay-2	Sand-1	Sand-2	Text-1	Text-2
1	2.47	0.51	2.45	0.19	68	49	10	15	22	36	Silty loam	Loam
2	3.45	0.12	2.93	0.09	51	46	6	10	43	44	Loam	Loam
3	1.33	0.05	1.08	0.03	28	13	6	5	66	82	Sandy loam	Sandy loam
4	-	-	7.09	0.39	-	48	-	8	-	45	-	Silty loam
5	5.75	0.36	-	-	39	-	7	-	55	-	Sandy loam	-
6	-	0.25	7.05	0.11	47	55	11	6	43	39	Loam	Silty loam

Table 3-3: Degree of infection caused by each isolate of *A. ostoyae* used as inoculum showing percentages per plot and total percentages with and without controls. Since plots 3 and 5 had no controls, a comparison was made among all plots with and without controls. Values are percent of the total for each isolate with sample size indicated in parentheses. Dashes indicate no samples taken.

Isolate	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7
AS-14	33 (42)	10 (30)	67 (15)	-	-	-	44 (25)
AS-11	23 (40)	3.3 (30)	33 (15)	33 (30)	0 (20)	45 (20)	37 (35)
Control	9 (11)	0 (10)	-	20 (10)	-	20 (10)	10 (10)
Total (with controls)	26 (93)	5.7 (70)	50 (30)	30 (40)	0	37 (30)	36 (70)
Total (without controls)	28 (82)	6.7 (60)	50 (30)	33 (30)	0	45 (20)	40 (60)

Figure 3-5: Proportion of trees at each pathogenicity level within plots. Percent trees was calculated as number of trees infected according to each level of pathogenicity, divided by the total number of trees within each plot. Levels of pathogenicity are as follows: 1 = mycelium absent from inoculum block and absent from root; 2 = mycelium absent from block, present on root (foreign *Armillaria*); 3 = mycelium present on block, absent from root (no infection); 4 = mycelium present on block, present **ON** root (superficial colonization); 5 = mycelium present on block, present **IN** root (cambial infection); 6 = mycelium absent from block, present **IN** root (foreign *Armillaria*).

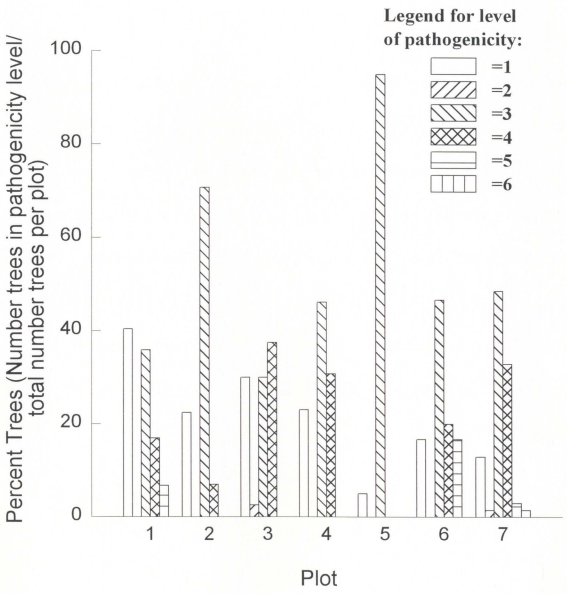

Legend for level of pathogenicity:
=1
=2
=3
=4
=5
=6
Percent Trees (Number trees in pathogenicity level/ total number trees per plot)
100
80
60
40
20
0
1
2
3
4
5
6
7
Plot

Figure 3-6: Proportion of trees showing each type of root response to infection within plots. Percent trees was calculated as number of trees exhibiting each type of root response per plot, divided by the total number of trees within each plot.

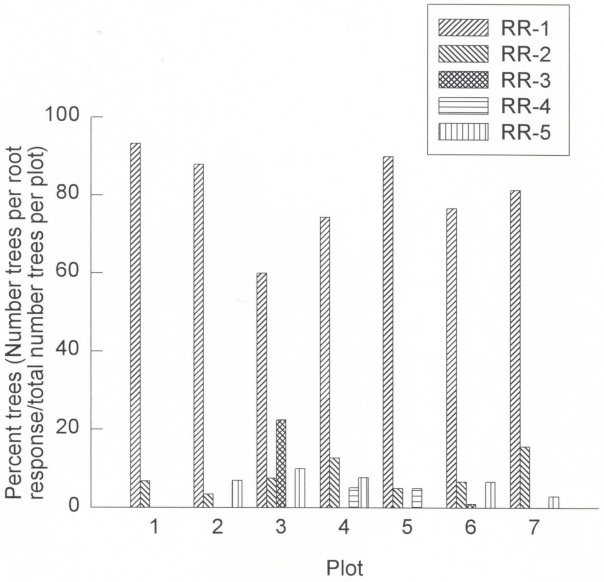

RR-1
RR-2
RR-3
RR-4
RR-5
100
80
60
40
20
0
Percent trees (Number trees per root response/total number trees per plot)
1
2
3
4
5
6
7
Plot

Figure 3-7: Proportion of trees in each plot containing roots associated with rhizomorphs as an indication of disease potential.

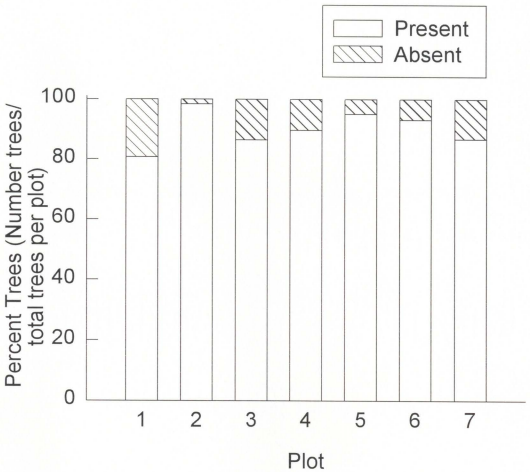

Present
Absent
Percent Trees (Number trees/ total trees per plot)
100
80
60
40
20
0
1
2
3
4
5
6
7
Plot

Figure 3-8: Relationship between level of defoliation of the host (x-axis) and infection by *A. ostoyae*. Percent trees (y-axis) was calculated as number of trees per pathogenicity level per level of defoliation (treatment), divided by the total number of trees in each defoliation level. Pathogenicity levels are indicated as follows; ---o--- is no mycelium on inoculum block and none on root; ---□--- is mycelium on block but none on root; ---△--- is mycelium on block *and* on root, and; ---▽--- is mycelium in cambial tissue. **A**: Black spruce natural regeneration (plot 1), **B**: Balsam fir hemlock looper (plot 2), **C**: Balsam fir artificial defoliation (plot 3), **D**: Balsam fir adelgid, thinned (plot 4), **E**: Balsam fir adelgid, un-thinned (plot 5), **F**: Balsam fir sawfly (plot 6), and **G**: Black spruce plantation (plot 7).

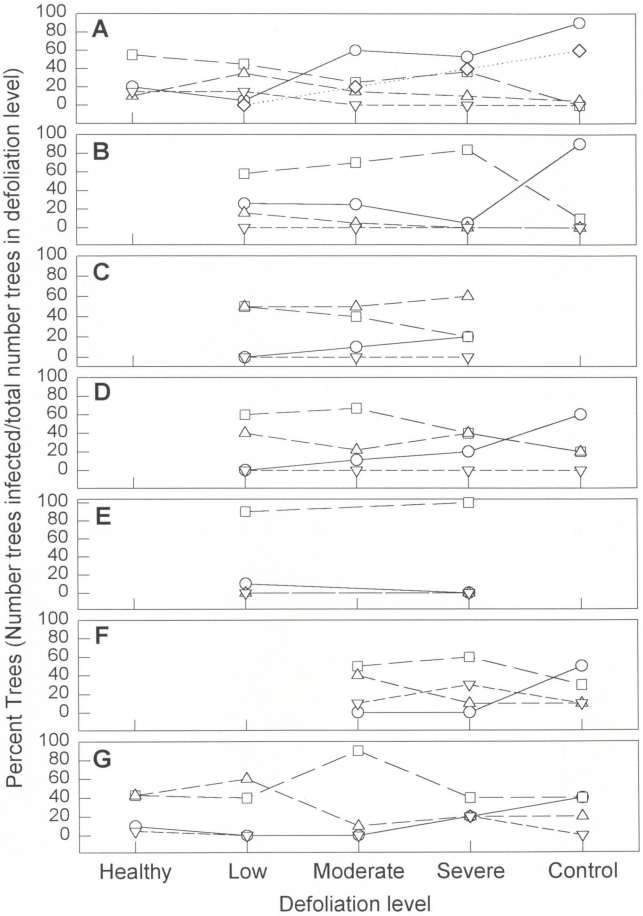
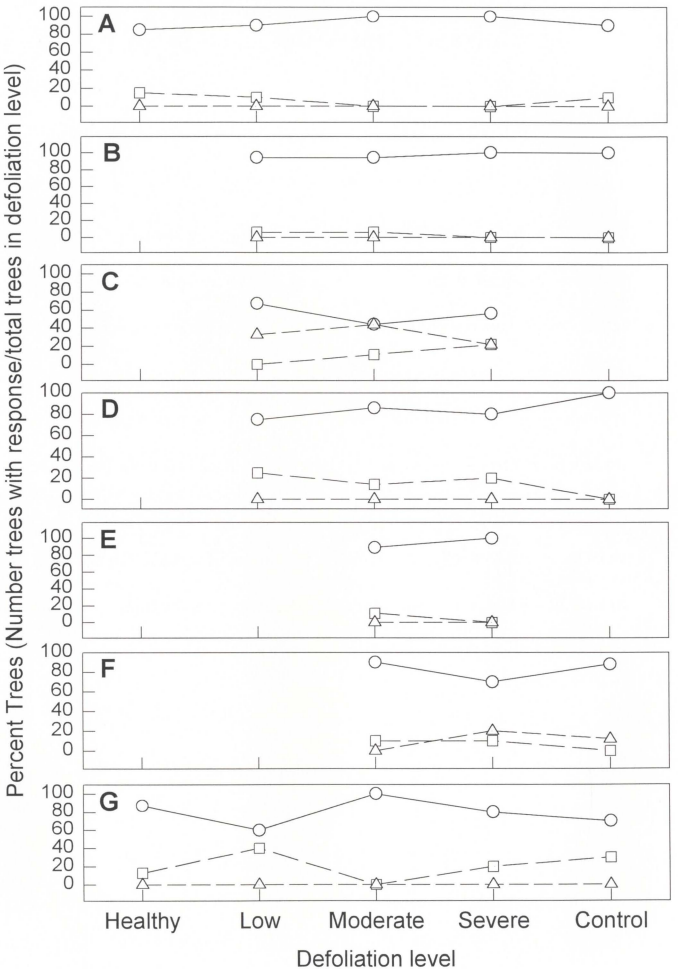

A
B
C
D
E
F
G
100
80
60
40
20
0
Percent Trees (Number trees infected/total number trees in defoliation level)
Healthy
Low
Moderate
Severe
Control
Defoliation level

Figure 3-9: Relationship between level of defoliation of the host (x-axis) and root response measured as three levels for each plot. Root response was calculated as number of trees per response type at each level of defoliation (treatment), divided by total number of trees in each defoliation level. Root responses are indicated as follows;---o--- is no response, healthy root; ---□--- is superficial colonization of mycelium on root; ---△--- is cambial infection of mycelium under bark of root. **A**: Black spruce natural regeneration (plot 1), **B**: Balsam fir hemlock looper (plot 2), **C**: Balsam fir artificial defoliation (plot 3), **D**: Balsam fir adelgid, thinned (plot 4), **E**: Balsam fir adelgid, un-thinned (plot 5), **F**: Balsam fir sawfly (plot 6), and **G**: Black spruce plantation (plot 7).


A
B
C
D
E
F
G
100
80
60
40
20
0
Percent Trees (Number trees with response/total trees in defoliation level)
Healthy
Low
Moderate
Severe
Control
Defoliation level

3.4 DISCUSSION

3.4a Disease causing agent:

The underlying assumption in this study was that the *A. ostoyae* isolates used for inoculum were the disease causing agents. This was verified by the use of controls. Controls were included which consisted of securing an autoclaved sterile wooden block to tree roots, undergoing the same stress levels as experimental trees, in the same manner that the inoculated blocks were secured to experimental trees. If occurrence of disease in controls was as high as in the experimental trees, then analysis of the experiment would be invalid. However, in most testable cases isolate 1 was significantly more virulent than isolate 2, and/or isolate 2 was significantly more virulent than controls (Table 3-1 and Figure 3-2), justifying further analysis of the study. In plots 1 and 2 isolate AS-14 was more virulent than AS-11 and controls. In plot 7 both isolates were more virulent than controls (Table 3-1) though statistically significant only at $\alpha = 0.10$, with a p-value of 0.093. Because this site was a plantation, a large amount of foreign *Armillaia* may have been present. However, a p-value of 0.093 may be considered biologically important. Isolate AS-14 was collected from an area several kilometers away from the adelgid plot (plot 4 and 5), and isolate AS-11 was collected from Big Cookes Pond outside Corner Brook. It was not unexpected that isolate AS14, collected from a conifer, was more virulent on conifers than AS11 which was collected from a hardwood. Control trees tested for presence and effects of foreign *Armillaria* on the trees. There were two superficial infections in controls of plot 4, 1 superficial and 1 cambial presence in controls of plot

6, and 1 superficial in controls of plot 7. This indicated that foreign *Armillaria* was present in the soil, however inoculum may have escaped into the soil and travelled some distance attacking the root at a weakened point.

3.4b Pathogenicity:

Average total infection (25%) was lower than that reported by Mallett and Hiratsuka (1988) on Lodgepole pine. However they used 2 year old seedlings in a greenhouse environment whereas this study used mature trees (Figure 3-3). Rishbeth (1982) reported 12% pine seedlings killed by inoculation with *A. ostoyae*, and Singh (1980) inoculated 2 to 4 year old black spruce seedlings in a greenhouse with *A. mellea* (*sensu lato*) producing 21% infection. A larger amount of superficial colonization than cambial infection in all the plots (Figure 3-5) would suggest that two years was not sufficient time for *A. ostoyae* to establish root infection on mature trees in Newfoundland, but the pathogen was beginning its attack by moving onto the root. Although the rate of spread of *A. ostoyae* varies with climate and location, van der Kamp (1993) reported that the time from tree response to tree death of 110 year old firs was 6 years, much longer than the time permitted by this study. The oldest stand in this study (plot 2) was 45 to 70 years old and demonstrated low infection (Table 3-3, Figure 3-5). Older trees tend to be more resistant to *Armillaria* root disease than younger trees (Buckland, 1953; Johnson et al., 1972; MacKenzie, 1987), and would explain the slower rate of disease progression. All other plots contained trees ranging between 10 and 25 years old which were more

capable of producing root response to resist infection. Callus formation in conifers increased between 5 and 20 years of age (Johnson et al., 1972). Whitney (1988) found that 6 to 7 year old black spruce trees were more susceptible to *Armillaria* than older 10 to 20 year old trees in plantations. The two isolates used as inoculum were collected in 1991 and cultured in the lab for three years before field inoculation, possibly losing some aggressiveness during that time. Proportions of infected trees in this study varied considerably from plot to plot (Figure 3-5 and Table 3-3).

Rhizomorph growth across all plots did not appear to be positively correlated with pathogenicity of *Armillaria* suggesting that soil conditions (Redfern, 1973, 1978), and possibly *Armillaria* isolate (Gregory, 1985; Mallett and Hiratsuka, 1988), may interactively play roles in the growth of rhizomorphs. Singh (1981a) showed that a well-drained loam site favoured rhizomorph growth, over moist and wet sites. Similarly, Whitney (1978) reported poor rhizomorph growth in wet sites. Non-rhizomorph root to root infection has been observed with *A. obscura* (Whitney, 1988). Plots with the largest number of roots containing rhizomorphs corresponded with plots containing the largest amount of superficial colonization by *Armillaria* (Plots 1, 3, 4 and 7) (Figures 3-5 and 3-7). However, Omdal et al. (1995) reported that the ability of *A. ostoyae* to produce disease was highly correlated with rhizomorph production.

3.4c Foreign Armillaria:

Isolates were still alive after two years on the inoculum blocks in all cambial

infected roots except one, occurring in plot 7, which may have been a foreign *Armillaria* isolate already in the soil, or the isolate from the inoculum block before it died. Otherwise records of cambial infection, with inoculum block still harbouring the pathogen suggested infection was caused by the inoculum itself, and occurred in sites 1, 6 and 7. Similarly, the absence of the inoculum on the block, but surface colonization of the root might imply that a foreign *Armillaria* was invading the tree. Small amounts of suspected foreign *Armillaria* occurred in the balsam fir artificial defoliation and the severely infested balsam fir sawfly plots (Figure 3-5). These incidents were negligible compared with the infection associated with *Armillaria* remaining on the inoculum block.

Measures of root response also indicated that foreign *Armillaria* was affecting the trees. A response by the root away from the inoculum block indicated that a foreign *Armillaria* had attacked the root. Similarly, root damage caused by the wire used to secure the inoculum block to the root, created an opening for any organism to invade the root and cause it to respond, or response may have been caused by mechanical injury. One or both responses (5 and 7) were present in all plots except the naturally regenerated black spruce (plot 1) which also did not exhibit pathogenicity levels 2 or 6 (Figure 3-5). Despite this, plot 1 had the largest proportion of roots associated with rhizomorphs (Figure 3-7), suggesting that if the rhizomorphs were from foreign *Armillaria*, the trees were able to prevent the pathogen from invading the root.

3.4d Black spruce plantation and natural regeneration:

The black spruce plantation had more colonization and cambial infection (40%) ($H_{0.05[1]}$=10.58, p=0.001, df=1, r=+0.259), than the naturally regenerated black spruce plot (28%) (Table 3-3), supporting implications by Buckland (1953) and Livingston (1990) who reported that root deformities from container planting pre-disposed spruce to *Armillaria* root disease.

Both plots had more infection in healthy and 30% artificially defoliated trees than in more severely defoliated trees supporting results from Parks et al. (1994) who refuted the long standing paradigm that defoliation contributed to enhanced disease in conifers, by reporting that defoliated seedlings had less disease than undefoliated seedlings. In contradiction to this finding, seedlings grow fast relative to mature trees, hence seedlings would allocate more carbon to growth than to defense compounds (Entry et al., 1991b). Seedlings are believed to be more susceptible to disease than mature trees (Whitney, 1988). However in this study, low levels of defoliation may have been sufficient to minimize any effects from water stress but not enough for defense production and so *Armillaria* attacked the roots. The lack of infection in the moderately defoliated trees may have resulted from insufficient foliage re-growth to re-route all the carbon allocation to re-growth, but stressed enough to induce production of defense compounds. Infection decreased in the naturally regenerated stand with severity of defoliation, but in the plantation infection increased as defoliation increased (Figure 3-8) supporting the findings of Wargo and Harrington (1991). The 80% defoliation alone in the naturally regenerated

stand was not enough stress to allow *Armillaria* to attack, since any water stress was minimized by removing 80% of the foliage, and the roots were spread out to maximally absorb water. But in the plantation 80% defoliation combined with root deformities from the planting method which may have minimized water uptake, and with an increased necessity to replace lost foliage, insufficient amounts of defense compounds may have been produced allowing *Armillaria* to attack. Consequently these trees would be infected by *Armillaria* before the slower growing trees at the lower levels of defoliation. Root response in both plots coincided with pathogenicity (Figure 3-9), emphasizing progression of the disease. Both sites were dry to moist (see site descriptions), but the lower portion of the naturally regenerated site was very wet and not suitable for growth of *Armillaria* (Singh, 1981a; Whitney, 1978), possibly accounting for less infection in that site (Figure 3-8). In addition, the plantation contained more decaying stumps than the naturally regenerated stand, serving as substrate for both inoculum isolates and foreign isolates. Whitney (1988) reported an association between *Armillaria*-killed trees and below ground inoculum from old growth stumps in Ontario. Disease may have also been complicated by the presence of foreign *Armillaria* as indicated by infection in controls (Figure 3-8).

3.4e Thinned and Un-thinned stands with adelgid:

There was more infection by *A. ostoyae* in the thinned (33%) (plot 4) than the un-thinned plot (0%) (plot 5) (Figure 3-5, Table 3-3) ($H_{0.05[1]}$=6.36, p=0.012, df=1), which was also reflected in the above ground health of the tree. The un-thinned plot had

significantly better health than the thinned plot ($H_{0.05[1]}$=4.32, p=0.038, df=1). In contrast, Filip and Goheen (1995) showed that there was no difference in tree mortality due to *Armillaria* root disease between thinned and un-thinned plots. Similarly, Entry et al (1991b) reported lower infection in an un-thinned Douglas Fir control stand than a thinned stand with *A. ostoyae*, although differences were not significant. Another study showed significantly more infection by *A. ostoyae* in light limited conifer seedlings (indicative of an un-thinned site) than light and nitrogen balanced seedlings (indicative of thinned site) (Entry et al., 1991a). However in this study additional stress was placed on the thinned and un-thinned stands by the woolly adelgid, accounting for some discrepancies in results.

Isolate AS-11, the only isolate used in this plot, was significantly more pathogenic than autoclaved control blocks (Table 3-1 and Figure 3-2) indicating that the higher incidence of pathogenicity from inoculum blocks was more than by chance alone. However, controls contained 20% infection compared with 33% in experimental trees (Table 3-3), indicating that foreign *Armillaria* was present and active in the soil. There may also have been an effect from the large number of dead and decaying stumps in the area serving as inoculum source for both blocks and foreign *Armillaria*. An additional carbon source, such as decaying stumps, must be present for fungi to degrade the defensive compounds of the host (Kirk, 1981). After thinning, the additional carbon source (stumps) and drier soil (possibly increasing the aggressiveness of the pathogen (Whitney, 1995)) became available to the fungus, so the biomass of the fungus increased. Stress imposed on trees by the adelgid may have caused an increase in glucose and

fructose in the bark of the root (Wargo, 1972; Wargo et al., 1972). Glucose increases fungal growth in the presence of phenolic compounds (Wargo, 1980). Armed with an increase in biomass from the carbon source, *Armillaria* could utilize root glucose and degrade any phenolic compounds which may have present in the root of the host. Trees that are growing extremely fast or are highly stressed may not allocate carbon for production of defense compounds (Entry et al., 1991a). The greater light conditions and less competition from thinning would provide an opportunity for trees to grow faster. If additional carbon source increased the biomass of the inoculum, both in the surrounding stumps and in the inoculation blocks, then defense compounds produced by the stressed trees would be inadequate to prevent *A. ostoyae* from entering the roots. If foreign *Armillaria* was already mounting attack on other roots, the trees would be stressed more than they appeared to be from the adelgid alone. There were very few decaying stumps in the unthinned stand and therefore little inoculum source. Singh (1970) reported that most of the dead and chlorotic trees infected with *A. mellea* (*sensu lato*) were located near infected stumps.

The thinned stand contained more silt and more soil nutrients than the un-thinned stand, such as Mg, Ca, K and organic matter, but N and P were no higher than other sites (Table 2). Since *Armillaria* can absorb minerals from soil (Morrison, 1982b), the higher incidence of rhizomorphs in the thinned stand (Figure 3-7), (probably from increased aeration (Morrison, 1976), loss of moisture from the thinning process, and increased nutrients (Morrison, 1975)), would improve the pathogenic capacity of the fungus.

Redfern (1973) suggested that soil disturbance, such as thinning a stand, may stimulate fresh rhizomorph development. Similarly, Singh (1975) found more disease in cutover sites with moist or dry well-drained soils and suggested that well-drained sites have more rhizomorphs and a higher incidence and intensity of root disease. In addition, the water stress in the crown of the tree, caused by reduction in sap flow from the aphid galls (Hollingsworth and Hain, 1994), would have been compounded by the lowering of the soil moisture from the thinning process. However the extra minerals and light, and less competition from the thinning process, would also improve tree vigour. Though not significant there was a higher degree of root response in the thinned than in the un-thinned plots to both pathogen and wire damage (Figure 3-6). Soil pH was more favourable for *Armillaria* in the mineral layer of the thinned stand (4.54) but was lower in the un-thinned stand (4.17). Dichotomously branching rhizomorphs, such as those of *A. ostoyae*, prefer acidic soils (Morrison, 1974; Singh, 1975).

There was more colonization present in low and severe adelgid than in moderate adelgid infestations in the thinned plot (Figure 3-8). Superficial necrosis was high in low and severely infested trees (Figure 3-9) coinciding with high levels of colonization by *Armillaria* (Figure 3-8) indicating that the trees were responding to the presence of *Armillaria*. The un-thinned plot had no *Armillaria* on or in the root at either level of adelgid infestation, yet *Armillaria* survived on the inoculum block (Figure 3-7). This might imply that trees were not as stressed as those in the thinned site and able to prevent entry of the pathogen by producing defensive compounds, or the wet un-thinned site, with

little carbon source from stumps, was unsuitable for fungal growth and disease progression was slower to occur. In addition, the level of photosynthesis was probably lower than in the thinned site due to limited light conditions and hence less carbohydrate storage in the roots, producing little fungal biomass. Hudak and Wells (1974) reported that disease incidence in adelgid damaged stands increased significantly with severity of damage, but the total amount of disease was higher (ca. 56%) than in this study (33%).

3.4f Above ground tree symptoms:

Above ground tree symptoms positively correlated with infection in the sawfly infested site only ($H_{0.05[2]}$=6.95,p=0.031, df=2, r=+0.489). This site also had the largest amount of cambial infection (Figure 3-5), and the second largest total infection (Table 3-3). At the time of inoculation this was the site with the most severe level of needle defoliation compared with all other plots. Above ground symptoms on Douglas Fir trees infected with *A. ostoyae* are not obvious until mycelium grew close to the root collar (Bloomberg and Morrison, 1989). Live spruce can be infected with *A. ostoyae* without showing noticable symptoms (Whitney et al., 1989; Livingston, 1990). Defoliation in this plot involved the slow chewing of the needles by sawfly larvae until the branches were bare, whereas artificial defoliation involved removal of entire branches at the nodes. Haukioja and Neuvonen (1985) found that insect damage was a more effective inducer of changes in birch foliage than mechanical damage. Defense reactions are not induced by artificial defoliation to the same degree as they are by pathogens because of the

absence of a stimulus such as saliva (Hartley and Lawton, 1987). An insect bites the same needle many times adding saliva with each bite. If many insects on the same tree steadily consume many needles, adding saliva with each bite, the tree is being triggered to produce defense reactions, using elicitors produced by both the insect saliva as well as cell wall fragments of the host (Yamada, 1992), in many places and over a prolonged period of time. Manual defoliation, by removing a large amount of plant tissue without saliva, would cause the plant to respond to elicitors produced by the plant itself, in one small area on the plant, and at a single moment in time.

The severe nature of the sawfly attack may have pre-disposed this site to attack by *A. ostoyae*. There was also evidence of root collar weevil (*Hylobius* sp.) and woolly adelgid (*Adelges piceae*), contributing to the degree of stress inflicted on the tree. Raske and Sutton (1986) reported that incidence of root collar weevil increased with increasing defoliation of black spruce in Newfoundland. The degree of stress on the other sites may not have been severe enough to pre-dispose the trees to infection. Trees in the other sites as well as this one could still produce defense reactions (Figure 3-9). Although this site had the highest level of cambial infection (Figure 3-5), there was no higher level response to the infection such as resinosus or production of callus tissue to compartmentalize the fungus. If the trees were low vigour and severely weakened, such as by insect removal of photosynthetic tissue, then resinosis would be uncommon or absent (Buckland, 1953). Plot 6 contained fewer rhizomorphs but a larger amount of cambial infection (Figure 3-5). This may be explained by infection from a larger amount of root to root contact, or in this

case inoculum block to root contact, due to the severity of the balsam fir sawfly attack on the trees, and the seemingly faster rate of infection in this stand than in others.

3.4g Low level pathogenicity:

Plots 2 and 5 had the lowest number of rhizomorphs (Figure 3-7), and showed the lowest pathogenicity levels (Figure 3-5), yet *Armillaria* was alive on the inoculum blocks without spreading to the roots in the largest proportion of trees of any site, as indicated by pathogenicity level 3 (Figure 3-8). Since root response and pathogenicity was low in both these sites, then soil and/or tree conditions were unsatisfactory for the growth and pathogenicity of *A. ostoyae* but allowed *Armillaria* to survive on the blocks. The looper defoliated balsam fir trees in plot 2 were 8 to 10 m high, much taller than trees in any other plot. They were also much older and had a larger DBH than any others (Figure 3-3). Detrimental effects of *Armillaria* root disease generally decreases with increasing age of the tree (Buckland, 1953; Johnson et al., 1972; MacKenzie, 1987). Resin production in Lodgepole pine increased until 50 years of age, but decreased in 91 to 120 years of age (Shrimptom, 1973). Trees in plot 2 were 45 to 70 years old, more capable of defense against root infection. In contrast, Whitney (1995) reported no significant difference in infection by *A. ostoyae* between different ages of balsam fir.

Soil pH was lower in these two plots than in the others, ranging from 3.4 to 3.8 in plot 2 (balsam fir hemlock looper site), and 3.9 to 4.2 in plot 5 (balsam fir un-thinned, adelgid site), whereas the others ranged from 3.7 to 5.4 (Table 3-2). Rhizomorph growth

may be encouraged by a higher pH (Singh, 1970), however if tree condition is severely weakened then direct contact may be sufficient to spread infection. Although the sawfly site had a pH similar to the adelgid un-thinned site, but more infection than the adelgid site, the trees in the sawfly site appeared to be more severely stressed with less foliage.

There was no significant effect of treatment on virulence in plot 2 (Table 3-1), but low level defoliation had more superficial colonization than moderate or high level defoliation (Figure 3-8). An increase in root response by swelling and superficial necrosis coincided with surface colonization (Figure 3-9) indicating that the trees were responding to early *Armillaria* attack. Nutrient levels in the old growth looper plot were similar to those of other plots, but soil pH was lowest in this plot (Table 3-2). Entry and Emmingham (1995) reported that nutrient concentrations in mineral soil were equal for all forest ages. Trees in this site may have been recovering from the insect attack, lessening stress, and allowing the tree to respond to fungal attack.

3.4h Balsam fir on a sandy site:

Balsam fir growing on a well-drained sandy site (plot 3) appeared to be more prone to attack by *A. ostoyae* as indicated by the high level of superficial colonization on the root (Figure 3-5). However it also appeared to be more capable of defending itself as indicated by more severe root response (Figure 3-6), and would explain the absence of cambial infection. Redfern (1978) reported the highest incidence of infection by *A. mellea* (*sensu lato*) on an acid (pH 4.9) sandy soil, supporting the findings in this study (Table

3-3). Similarly, Whitney (1984) found that *Armillaria* root rot was more severe on coarse-textured sandy soils than silty soils.

Low levels of artificial defoliation were associated with superficial colonization of *Armillaria* on the root in plot 3. Necrotic resinosus (response 4) was more prevalent than superficial necrosis in the low levels of defoliation (Figure 3-9) indicating the trees were in good health and able to respond to the attack. Singh (1980) showed that conifer species with less infection produced more resinosis on the root. Buckland (1953) showed more resinosus occurred on the more vigorous host. Also *Armillaria* may be in a more optimal environment in the sandy site and therefore more aggressive. Superficial colonization by *Armillaria* was high at 30 and 50% defoliation levels, but slightly increased at 80% defoliation. This difference was not significant, but they were significantly different from controls (Table 3-1). All artificially defoliated trees required more growth to replace the tissue removed, with more required in the 80% than the 50 or 30% defoliation. This increase in growth may have been sufficient to allocate carbon to the production of sugar and cellulose for growth rather than for production of defense compounds (Entry et al, 1991a). Trees with lowered resistance are more susceptible to disease (Gregory et al., 1991). The lack of any cambial infection in this site may have been a reflection on the ability of the host to produce sufficient defense compounds in light of the re-growth process. Good quality soil conditions (Table 3-2) was beneficial to the host as well as the pathogen.

Rhizomorphs from *A. mellea* tend to grow in the surface 5 cm of soil on moist

sites and are absent from that layer on dry sites (Morrison, 1976), corresponding to the layer (top 10 cm) which contains the highest concentration of nutrients (Entry and Emmingham, 1995). Soil profiles in this study showed that the top 5 cm of soil consisted of organic matter (LFH) in all sites sampled except both sites in plot 3 (Figure 3-4) which contained <1 and 2 cm LFH above 30 to 40 cm of sandy, mineral, dry soil. Blodgett and Warrall (1992) reported that *A. ostoyae* grew well in spruce-fir sites with soils high in sand, but low in silt and clay content. Plot 3 also had a large amount of rhizmorphs present around the root (Figure 3-7) and the highest amount of root response to pathogen attack (Figure 3-6), as well as the largest amount of superficial root colonization (Figure 3-5) indicating that *A. ostoyae* was pathogenically active in that site. However good drainage and aeration in the sandier soil of plot 3 (Table 3-2) may have been beneficial for growth of both *Armillaria* (Morrison, 1976) and the host tree (Redfern, 1978). Excellent tree health in plot 3, supported by the highest proportion of healthy trees of all the balsam fir plots, and the longest leaders of all balsam fir plots measured (Figure 3-3), suggested that soil conditions were near optimal for growth of balsam fir. Low organic matter in plot 3 in both LFH and mineral layers (Table 3-2) suggested that soil nutrient conditions for growth of rhizomorphs was not optimal since Morrison (1982a) showed that soils high in organic matter supplied more nutrients. Blodgett and Warrall (1992) showed that organic matter in the mineral layer was 13.8%, similar to that found in the organic layer in this study (Table 3-2). However soil pH of plot 3 was higher than that of the other sites (Table 3-2) and closer to the optimal pH for growth of *Armillaria* rhizomorphs

according to Singh (1970), but was too high according to Singh (1983) (3.8 was better than 4.8) and Singh (1981a) (3.5-4.3 was better than 4.0 to 4.9). Plot 3 had large amounts of P in the mineral layer (Table 3-2). However, Shields and Hobbs (1979) found soil phosphorus levels to be quite variable. An association between low soil nitrogen and high incidence of root disease in conifer stands has been reported (Shields and Hobbs, 1979; Singh, 1970). Plot 3 contained the lowest nitrogen of all the plots in both soil layers (Table 3-2). High soil nitrogen inhibits rhizomorph growth (Kirk, 1981), especially if it takes the form of nitrate nitrogen over ammonium nitrogen (Li et al., 1967). Good rhizomorph growth for plot 3 (Figure 3-6) was probably a result of optimal pH (Singh, 1970), decreased N (Kirk, 1981; Li et al, 1967) and increased aeration and less moisture from sandier soil. Whitney (1995) reported higher infection in black spruce and balsam fir by *A. ostoyae* on drier sites. The high level of root colonization in this site may have been a reflection of good conditions for rhizomorph growth.

In contrast with the natural regeneration black spruce plot, virulence on balsam fir increased with severity of defoliation, but virulence on black spruce decreased with severity of defoliation (Figure 3-8), reflecting greater susceptibility in balsam fir than in black spruce. Rizzo and Harrington (1988) reported a higher incidence of root and butt rot in fir than in spruce. However, Redfern (1978) showed more infection by *A. mellea* (*sensu lato*) in *Picea abies* and *P. sitchensis* than in *Abies grandis*.

3.4i Agent of predisposition - insect or pathogen?:

Debate has arisen as to whether *Armillaria* root disease predisposes a stand to insect attack, or if the insect infestation predisposed the stand to root disease (Mallett, 1995; Mallett and Volney, 1990). Warren and Singh (1970) reported the incidence of conifer trees containing both *Hylobius* weevil damage and *Armillaria* root rot was low (and approximately the same as those containing *Armillaria* alone) compared to trees with weevil only. This might imply that the weevil infested the trees before *Armillaria*. Other studies suggested that the weevil provided infection courts for *Armillaria* (Whitney, 1961; Smerlis, 1961). Hudak and Wells (1974) concluded that aphid damage was a main factor influencing root disease. However absence of disease in the un-thinned site in this study indicated that the adelgid alone was not predisposing the host to root disease, until soil conditions changed. James and Goheen (1981) reported more Douglas fir and subalpine fir trees infected with *A. mellea* (*sensu lato*) than bark beetles or wood borers, implying that *Armillaria* pre-disposed these trees to beetles and borers. Cobb (1989) suggested that root rot fungi predisposed trees to bark beetle attack. Beetles and borers require that trees be stressed to reduce defense reactions before they can attack. However, on white fir and Engelmann spruce the opposite occurred, emphasizing host species differences. Mallett and Volney (1990) showed more *Armillaria* root disease in dead and top-killed jack pine following a jack pine budworm infestation. They suggested that since *Armillaria* was present in the roots at the time of defoliation, then *Armillaria* may have predisposed the trees to insect attack. Hertert et al. (1975) reported that root disease was an important

factor in predisposing Grand Fir to attack by bark beetles. Raske and Sutton (1986) interpreted *Armillaria* as being a secondary pathogen in spruce-budworm defoliated stands. Preliminary investigations for this study showed that *Armillaria* was present in all sites before the study began. Singh (1981b) showed that *Armillaria* was ubiquitous in Newfoundland soils, however at the time of inoculation it was not infecting any of the major roots in this study.

The adelgid infestation seemed to impose the least amount of stress on the host trees. However, when thinning was implemented it caused a change or disturbance in site conditions, which affected both host and pathogen. The pathogen became more aggressive, and the host trees relinquished defense reactions for faster growth.

The hemlock looper damage occurred on older, more resistant trees, and at the time of inoculation the stand was scheduled to be sprayed for hemlock looper. Inadvertently the experiment in this stand was complicated by the trees being older and more resistant to root disease, as well as recovery from looper damage. Hence very little infection occurred from inoculation.

The sawfly damaged stand, the stand suffering from a moderate amount of infection, was actually the most severely infected of the three types of insect damage. This stand had previously been infested with the hemlock looper, and currently by the balsam fir sawfly. Trees in this stand were devastated with sawfly damage leaving bare branches containing tufts of current years foliage at the tips. It was difficult to find moderately damaged trees and impossible to find low level damage in this stand. There

was also evidence of *Hylobius* weevil and woolly adelgid which further depressed the host defense system.

In the moist wet soils of Newfoundland *A. ostoyae* may act as a secondary pathogen waiting for other stress agents to reduce the quality of host defense reactions, or as a primary pathogen with reduced virulence due to unsuitable soil conditions. However, in drier, sandier soils in Newfoundland, *A. ostoyae* may act as a primary pathogen, becoming aggressive and predisposing the host tree to agents of suppression. Depending on the aggressiveness of the isolate, soil conditions, and the type of insect defoliation, either insect or fungus may predispose the tree to attack. It was suggested that several consecutive dry years, followed by a wet year, would trigger insect development and infestations. If the top soil layer becomes dry, such as with the thinned adelgid site, *Armillaria* may become more active and attack the roots.

Chapter 4

CONCLUSIONS

4.1 Phylogeny:

Combining data sets from four conserved fragments is an effective way to estimate a phylogeny. DNA regions examined in this study represented regions conserved within species and were variable among species, containing a level of variation suitable for studies in phylogeny and species specificity. The use of SWAPP PCR is a promising technique to locate regions of DNA suitable for phylogenetic studies. SWAPP regions tend to be short, randomly selected, and numerous which would increase the sample size and improve the "total evidence" (Kluge, 1989) of the phylogenetic history of a group of organisms.

Short conserved fragments can be found throughout the nuclear genome. With no prior knowledge of the function or specific location of the fragment, the choice is more random than choosing the fragment based on a known history. The use of "Goodness of Fit" statistics, such as those used in this study, confirm presence of phylogenetic signal and congruency for anonymous sequences. Combination of more than one fragment incorporates a larger part of the whole organism into a molecular phylogeny, and can be valuable for identifying missing pieces of a phylogenetic history. The phylogeny presented here compared anonymous sequence characters with biological species of *Armillaria*. The sequences represented a small aspect of the relationship among species

used to assemble an evolutionary history of *Armillaria*. Similarly, the consistency of the disjunct group of NABS V in previous and present studies has been considered problematic. However, the variability of NABS V may act as a clue to resolve the evolutionary history of the genus. Nevertheless, the study illustrated the effect that different characters can have on the topology of a species tree, and that more study is required to resolve the evolutionary history of closely related species of *Armillaria*.

Phylogenetic relationships were successfully inferred from the four data sets, and the combined phylogenetic tree supported existing literature hypotheses. NABS III and VII formed a significant monophyletic cluster. Five isolates of NABS I formed a monophyly. NABS II was ancestral to NABS I, and NABS V, IX and X exhibited variation in clustering patterns.

The sequences may have consisted of non-coding DNA since start and stop codons were present and randomly located throughout the sequences, and transition : transversion ratios were generally greater than one. It has been shown that fungi contain introns (Radford, 1993), and sub-repeat sequences (Morton et al., 1995).

Most of the phylogenetic information obtained was found in fragment V250, and the least in III180, even though both were longer than the other two fragments. Hence, the notion that longer fragments provide more information may not always be accurate.

Lineages containing large geographic distances among collection sites of isolates of NABS I and small distances among those of NABS II produced significant bootstrap values. Similar results occurred with NABS V. Therefore, geographic distance appeared

to have no correlation with sequence polymorphisms in biological species of *Armillaria*, and suggested that similarities or differences among isolates was not a result of geographic isolation.

A molecular clock placed NABS *Armillaria* in an historical framework by correlating the co-evolution of the pathogen with its host. The calibration point was calculated from the discovery of a fossil mushroom, *Coprinites dominicana*, from 30Ma. Broad climatic changes and diversification of host Angiosperms were speculated to be connected with the divergence of NABS VI from the remaining NABS *Armillaria*. NABS II appeared ancestral to NABS I in this study. However, in order to assimilate evidence from this study with that of current literature, it was hypothesized to have gone through a bottleneck from colonization of a hardwood host, to a softwood host, and reverting back to the hardwood host again. Alternatively, NABS I may have indeed diverged from NABS II and rapidly colonized North America, causing NABS II to recede toward the Great Lakes region. Another explanation may be that NABS II was a result of hybridization between NABS I and VI.

4.2 Species-Specific Markers:

This study was successful in developing species-specific markers for most NABS *Armillaria*. Twenty nt markers were more reliable for NABS I, II and VI than for NABS III, V, VII, IX and X. However, the 10nt markers initially developed could be used to confirm the more variable species such as NABS III, V, VII, IX and X. In addition, other

methods of identification such as interfertility tests, basidiome morphology, geographic distribution, and RFLP-PCR patterns from other studies may be used for confirmation of identification. The entire process including DNA extraction, PCR, and electrophoresis could be completed within 10 hours. This would make it possible for forest managers to determine species of *Armillaria* in two working days as opposed to two months with mating tests.

Primer/template dynamics and PCR were examined in terms of the sequences amplified by species-specific SWAPP 10nt primers which were used to develop more stable nested species-specific 20mers. However the primer sequences chosen were highly conserved within the genus, *Armillaria.* This, in turn, raised a question regarding the mechanism underlying the species-specificity of the 10nt SWAPP primers. What was responsible for specificity; the template sequence of 10 bases complementary to the SWAPP primer, or competition among primer sites and DNA secondary structure? Results suggested that secondary structure and competition did indeed play a role in determining species markers. A stem-loop theory was presented which provided an explanation for discrepancies between primer site distances and band sizes.

Results from Inverse PCR demonstrated the potential value of the technique to locate flanking sequence regions and secondary structure, and to explain some of the anomalies present in molecular studies. Context effects were evident between the 3-band repeat and the 390bp band for NABS VI with primers 83/66. It was hypothesized that a recessive allele was represented by the absence of a nested 20nt primer site where the

SWAPP PCR product (which included the nested 20mers) was present. Similar results were obtained from the species-specific primers in which PCR product was produced, but nothing was amplified from the corresponding nested primers. If protein coding regions were present, silent substitutions, transitions, and transversions could play a large role in stability and specificity of the primer for template DNA. The type and number of primer base mismatches with template DNA could also play a role in determining extension efficiency of the primer regardless if the template DNA was protein or non-coding.

Two or more conserved fragments of the same length may present problems for studies such as this one, however, the occurrence of this phenomenon seemed to be uncommon.

Gene walking with long and short primers produced numerous bands. Similarly, results from nested 20mers and Inverse PCR produced multiple bands in a single sample. This was explained in terms of calculating the number of 8nt primers that could be made from random base selection of a 20nt primer.

Weak amplification may have been due to variation in PCR primer/template dynamics or from insufficient amount of genomic DNA. This could be avoided by using a positive control for these samples using primers from a multi copy gene such as rDNA.

It was hypothesized that primer/template dynamics operate at the primary nucleotide sequence level while influenced by secondary DNA structural levels. Both levels of complexity may be important in taxa differentiation with nucleotide sequence having base composition and order specific to taxa, and the secondary structure having

structural convolutions and loops specific to taxa. Both levels of complexity may play a role in PCR and the production of the banding pattern. The significance of a "molecular ecos" is that variation in results among taxa may be occurring at several different levels of genome complexity, rather than the misconception that PCR product always results from amplification of a single continuous piece of DNA with well-defined perfectly matched primer sites at either end.

4.3 Repetitive DNA:

Repetitive DNA sequences were found with 10nt SWAPP primers that appeared to be species-specific. Sequencing of the shortest repeat unit with subsequent inverse PCR to locate flanking sequences, could potentially provide species-specific markers.

4.4 Pathogenicity:

Interaction among many factors complicated results for *Armillaria* root disease development. Among the genetic components, host species may determine degree of susceptibility to the pathogen. Within a species genetic variation may play an important role such as the "sink competition hypothesis" (Larson and Whitham, 1997) in the adelgid infested trees. Similarly, the aggressiveness of the inoculum source used, as well as developmental changes with host age, may have influenced disease occurrence. Planting method, soil conditions, and stand thinning appeared to influence the pathogenicity of *A. ostoyae*. Both artificial and insect defoliation occurred at varying intensities, and results

were complicated by other factors influencing the ability of the tree to respond to attack.

The influence of stress from artificial defoliation was different from that of insect defoliation. Within the plots, statistically significant differences were apparent in the black spruce naturally regenerated stand only, in which more infection occurred in low level defoliation rather than high levels. The method of defoliation may have inadvertently acted to alleviate water stress in the high levels of defoliation by removing excess transpiring tissue so the host could sufficiently produce defensive reactions to the inoculum. Artificial defoliation in black spruce plantation and balsam fir on a sandy site were biologically examined. Infection occurred at all levels of artificial defoliation but results were confounded by the added stress of the planting method in the black spruce plantation, and the optimal soil conditions for growth of the pathogen in the balsam fir sandy site.

A drawback in the experimental design involved the method of treatment selection. The treatment, being insect species and degree of insect defoliation, was chosen based on pre-existing infestations in the plots. If the tree was genetically controlling the level of susceptibility to insect defoliation, then the measure of disease would not necessarily be a reflection of the influence of the insect on the tree. However, if the insect was determining the level of susceptibility of the tree, which in turn determined the degree of insect defoliation, then the measure of disease would indeed be a reflection of the influence of the insect defoliation on the tree. For example, the "sink competition hypothesis" (Larson and Whitham, 1997) explained how plant architecture affected

resistance to aphid galling. Resistant genotypes had a larger number of natural sinks (buds) relative to sources (stem volume) than susceptible genotypes. Similarly, Scarr (1995) reported green, non-defoliated jack pine trees in a stand severely infested with jack pine budworm, suggesting that the green trees were resistant to defoliation.

Two years appeared to be sufficient time to produce results which would allow forest managers to assess the progression of *Armillaria* root disease, and make well-informed decisions regarding stand management. However, more than 2 years would be required to complete an inoculation study in Newfoundland and produce results on disease causing death of the host. Comparison of trees within plots were statistically justified, but comparison between plots would have been committing pseudoreplication (Hurlbert, 1984). Larger sample sizes and replicated plots might improve resolution and statistically support the study.

4.5 General Conclusions and Future Directions:

Armillaria has been present in the forest ecosystem remaining in a state of balance and sometimes creating openings to allow new species to colonize. As this balance becomes displaced by the increasing forest and orchard industries, and the horticultural enthusiasm for ornamental plants, *Armillaria* can overcome the lowered resistance of host plants. The increasing attention placed on *Armillaria* root disease has become noticeable regarding plantations and other re-cultivated disturbed areas, and has developed into an economic concern.

The distinction among biological species of *Armillaria* is the foundation on which all three studies were developed. All species of *Armillaria* have the capacity to be pathogenic, however some are more aggressive pathogens than others. The ability to distinguish among these pathogenic species is essential to understanding the epidemiology of the genus. The species specific marker study was successful in developing markers for the highly pathogenic species. Although it was also successful for the weakly pathogenic species, there was more variability among them, and more work is required to distinguish among closely related species.

The phylogenetic study attempted to examine the history of the genus to shed light on the development of pathogenicity through time, and to relate the evolution of the species to evolution of host plants. A more accurate account of geographic distributions would greatly improve resolution of the phylogeny.

The habitat in which *Armillaria* is found is influenced by environmental changes. Attempts to understand these influences began with inoculation of greenhouse plants and young trees in plantations. These studies allow strict control of environmental influences, and have provided and will continue to provide valuable information on environmental effects. However, inoculation studies on naturally regenerated stands are equally as valuable to understand epidemiology. These stands can provide insight into conditions in which *Armillaria* does not express pathogenic activity. More of these studies are required in order to understand epidemiology of *Armillaria*.

Pathogenic variation within a species is another level of variation which should

also be addressed. Establishment of biological species provides a foundation on which pathogenicity could be inferred, and an indication of the amount of concern to be applied to the occurrence of the species. However, wide variation in pathogenicity within a species provides no pre-existing knowledge on which to base forest management practices. It would be necessary to monitor disease progression on a continuous basis for foresters to determine seriousness of disease in a species with wide variation in pathogenicity. Future research would be required to allow more predictive measures of disease detection such as an investigation of pathogenic genotypes rather than species. Molecular techniques have the capacity to provide fast efficient probes for virulence genes. This technique, in combination with knowledge of environmental influences, would provide more ammunition with which foresters could maintain their industry.

Chapter 5

REFERENCES

Anderson, J. B. 1986. Biological species of *Armillaria* in North America: Redesignation of groups IV and VIII and enumeration of voucher strains for other groups. Mycologia. 78:837-839.

Anderson, J. B. and Smith, M. L. 1988. Variation in ribosomal and mitochondrial DNAs in *Armillaria* species. pp. 60-71. *In:* Morrison, D. J. (Ed). Proceedings of the Seventh International Conference on Root and Butt Rots. August 9-16, 1988. IUFRO Working Party S2.06.01. Vernon and Victoria, British Columbia.

Anderson, J. B. and Stasovski, E. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria*. Mycologia. 84:505-516.

Anderson, J. B. and Ullrich, R. C. 1979. Biological species of *Armillaria mellea* in North America. Mycologia. 71:402-414.

Anderson, J. B., Bailey, S. S. and Pukkila, P. J. 1989. Variation in ribosomal DNA among biological species of *Armillaria*, a genus of root-infecting fungi. Evolution. 43(8):1652-1662.

Anderson, J. B., Petsche, D. M. and Smith, M. L. 1987. Restriction fragment polymorphisms in biological species of *Armillaria mellea*. Mycologia. 79:69-76.

Anderson, J. B., Korhonen, K. and Ullrich, R. C. 1980. Relationships between European and North American Biological species of *Armillaria*. Experimental Mycology. 4:87-95.

Angus, B. and Bernatchez, L. 1996. Usefulness of heterologous microsatellites obtained from brook charr, *Salvelinus fontinalis* Mitchill, in other *Salvelinus* species. Molecular Ecology 5:317-319.

Anselmi, N., Cellerino, G. P. and Nicolotti, G. 1994. Investigations on forest trees resistant to *Armillaria* species. pp. 17-25. *In:* Johanssen, M. and Stenlid, J. (Eds). Proceedings of the Eigth International Conference on Root and Butt Rots. August 9-16, 1993. Wik, Sweden and Haikko, Finland. IUFRO Working Party S-750 07, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Archie, J. W. 1989. Homoplasy excess ratios: new indices for measuring levels of homoplasy in phylogenetic systematics and a critique of the consistency index. Systematics Zoology 38:253-269.

Arthur, R., Herr, F., Straus, N., Anderson, J. and Horgen, P. 1982. Characterization of the genome of the cultivated mushroom, *Agaricus brunnescens*. Experimental Mycology. 7:127-132.

Augustian, A., Mohammed, C., Guillaumin, J. J. and Botton, B. 1994. Discrimination of some African *Armillaria* species by isozyme electrophoretic analysis. 1994. New Phytology 128:135-143.

Banik, M. T., Volk, T. J. and Burdsall Jr., H. H. 1996. *Armillaria* species of the Olympic Peninsula of Washington State, including confirmation of North American biological species XI. Mycologia. 88:492-496.

Berbee, M. L. and Taylor, J. W. 1993. Dating the evolutionary radiations of the true fungi. Canadian Journal of Botany. 71:1114-1127.

Bérubé, J. A. and Dessureault, M. 1988. Morphological characterization of *Armillaria ostoyae* and *Armillaria sinapina* sp.nov. Canadian Journal of Botany 66:2027-2034.

Bérubé, J. A. and Dessureault, M. 1989. Morphological studies of the *Armillaria mellea* complex: Two new species, *A. gemina* and *A. calvescens*. Mycologia. 81:216-225.

Bérubé, J. A., Dessureault, M., Berthelay, S. and Guillaumin, J. J. 1996. Interfertility between *Armillaria cepistipes* and *A. sinapina*. Phytoprotection. 77:67-74.

Blodgett, J. T. and Worrall, J. J. 1992. Site relationships of *Armillaria* species in New York. Plant Disease 76:170-174.

Bloomberg, W. J. and Morrison, D. J. 1989. Relationship of growth reduction in Douglas-fir to infection by *Armillaria* root disease in southeastern British Columbia. Phytopathology 79:482-487.

British Federation of Plant pathologists. 1973. A guide to the use of terms in plant pathology. Phytopathological Papers. 17:1-55.

Bruns, T. D., Fogel, R., White, T. J. and Palmer, J. D. 1989. Accellerated evolution of a false-truffle from a mushroom ancestor. Nature. 339:140-142.

Bruns, T. D., Vilgalys, R., Barns, S. M., Gonzalez, D., Hibbett, D. S., Lane, D. J., Simon, Luc, Stickel, S. Szaro, T. M., Weisburg, W. G. and Sogin, M. L. 1992. Evolutionary relationships within the fungi: Analysis of nuclear small subunit rRNA sequences. Molecular Phylogeny and Evolution 1:231-241.

Buckland, D. C. 1953. Observations on *Armillaria mellea* in immature douglas fir. Forestry Chronicle 29:344-347.

Burt, A., Carter, D. A., White, T. J. and Taylor, J. W. 1994. DNA sequencing with arbitrary primer pairs. Molecular Ecology. 3: 523-525.

Caetano-Anollés, G., Bassam, B. J. and Gresshoff, P. M. 1992a. Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. Molecular and General Genetics. 235:157-165.

Caetano-Anollés, G., Bassam, B. J. and Gresshof, P. M. 1992b. DNA amplification fingerprinting with very short primers. *In*: Applications of RAPD technology to plant breeding. Crop Science Society of America.

Carroll, W. J. 1956. History of the hemlock looper, *Lambdina fiscellaria fiscellaria* (Guen.), (Lepidoptera: Geometridae) in Newfoundland, and notes on its biology. Canadian Entomologist. 88:587-599.

Cavalier-Smith, T. 1974. Palindromic base sequences and replication of eukaryote chromosome ends. Nature. 250:467-470.

Cobb, F. W. Jr. 1989. Interactions among root disease pathogens and bark beetles in coniferous forests. pp. 142-148. *In*: Morrison, D. J. (Ed). Proceedings of the Seventh International Conference on Root and Butt Rots. IUFRO Working Party S2.06.01. Forestry Canada, Pacific Forestry Center, Victoria, B. C.

Côté, E. and Dessureault, M. 1994. Effects of site factors on the occurrence of *Armillaria* root rot disease in black spruce (*Picea mariana* Mill. (B. S. P.)) seed orchards. pp. 17-25. *In*: Johanssen, M. and Stenlid, J (Eds). Proceedings of the Eigth International Conference on Root and Butt Rots. August 9-16, 1993. Wik, Sweden and Haikko, Finland. IUFRO Working Party S-750 07, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Cunningham, C. W. 1997. Can three incongruence tests predict when data should be combined? Molecular Biology and Evolution. 14:733-740.

Davidson, A. J. and Rishbeth, J. 1988. Effect of suppression and felling on infection of oak and Scots pine by *Armillaria*. European Journal of Forest Pathology. 18:161-168.

Davis, 1981. Quaternary history and the stability of forest communities. *In* West, D. C., Shugart, H. H. and Botkin, D. B. (Eds.) Forest Succession: Concepts and Application. Springer Verlag, Berlin. 517pp.

Davis, R. B. and Jacobson, Jr., G. L. 1985. Late glacial and early holocene landscapes in northern New England and adjacent areas of Canada. Quaternary Research. 23:341-368.

de Queiroz, A., Donoghue, M. J. and Kim, J. 1995. Separate versus combined analysis of phylogenetic evidence. Annual Review of Ecology and Systematics. 26:657-681.

Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K. and Mattick, J. S. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research. 19: 4008.

Dumas, M. T. 1988. Biological species of *Armillaria* in the mixed wood forest of Northern Ontario. Canadian Journal of Forest Research. 18:872-874.

Egger, K. N., Osmond, G. and Goodier, J. L. 1995. Sequence and putative secondary structure of group I introns in the nuclear-encoded ribosomal RNA genes of the fungus *Hymenoscyphus ericae*. Biochimica et Biophysica Acta. 1261:275-278.

Entry, J. A. and Emmingham, W. H. 1995. Influence of forest age on nutrient availability and storage in coniferous soils of the Oregon Coast Range. Canadian Journal of Forest Research. 25:114-120.

Entry, J. A., Cromack Jr., K., Hansen, E. and Waring, R. 1991a. Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. Phytopathology. 81:89-94.

Entry, J. A., Cromack Jr., K., Kelsey, R. G. and Martin, N. E. 1991b. Response of douglas fir to infection by *Armillaria ostoyae* after thinning or thinning plus fertilization. Phytopathology. 81:682-689.

Farris, J. S., Källersjö, M., Kluge, A. G. and Bult, C. 1995. Testing significance of incongruence. Cladistics. 10:315-319.

Filip, G. M. 1989. Interactions among root diseases and agents of defoliation. pp. 149-155. *In:* Proceedings of the Seventh International Conference on Root and Butt Rots. Morrison, D. J. (Ed). IUFRO Working Party S2.06.01. Forestry Canada, Pacific Forestry Center, Victoria, B. C.

Filip, G. M. and Goheen, D. J. 1995. Precommercial thinning in *Pseudotsuga*, *Tsuga*, and *Abies* stands affected by *Armillaria* root disease: 10-year results. Canadian Journal of Forest Research. 25:817-823.

Gregory, S. C. 1985. The use of potato tubers in pathogenicity studies of *Armillaria* isolates. Plant Pathology. 34:41-48.

Gregory, S. C., Rishbeth, J. and Shaw III, C. G. 1991. Pathogenicity and virulence. *In* *Armillaria* Root Disease. Shaw III, C. G. and Kile, G. A. (Eds.) Agriculture Handbook No. 691. Forest Service. United States Department of Agriculture, Washington, D. C.

Guillaumin, J. J., Anderson, J. B. and Korhonen, K. 1991. Life cycle, interfertility, and biological species. In: *Armillaria* Root Disease. Edited by Shaw III, C. G. and Kile, G. A. pp. 10-20. Agriculture Handbook No. 691. Forest Service. United States Department of Agriculture. Washington, D.C.

Guillaumin, J. J., Berthelay, S. and Savin, V. 1983. Étude de la polarité sexuelle des *Armillaries* du groupe *mellea*. Cryptogamie Mycologie. 4:301-319.

Guillaumin, J. J., Mohammed, C. and Berthelay, S. 1989. *Armillaria* species in the Northern temperate hemisphere. pp. 27-43. *In:* Proceedings of the Seventh International Conference on Root and Butt Rots. Morrison, D. J. (Ed). IUFRO Working Party S2.06.01. Forestry Canada, Pacific Forestry Center, Victoria, B. C.

Hamer, J. E., Farrall, L., Orbach, M. J., Valent, B. and Chumley, F. G. 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proceedings of the National Academy of Science. (USA)86:9981-9985.

Harrington, T. C., Worrall, J. J. and Rizzo, D. M. 1989. Root and butt rots and other disturbance agents in montane spruce-fir forests of New Hampshire. pp. 257-266. *In:* Proceedings of the Seventh International Conference on Root and Butt Rots. Morrison, D. J. (Ed). IUFRO Working Party S2.06.01. Forestry Canada, Pacific Forestry Center, Victoria, B. C.

Harrington, T. C. and Wingfield, B. D. 1995. A PCR-based identification method for species of *Armillaria*. Mycologia. 87:280-288.

Hartley, S. E. and Lawton, J. H. 1987. Effects of different types of damage on the chemistry of birch foliage, and the responses of birch feeding insects. Oecologia 74:432-437.

Haukioja, E. and Neuvonen, S. 1985. Induced long-term resistance of birch foliage against defoliators: Defensive or incidental? Ecology 66:1303-1308.

Hertert, H. D., Miller, D. L. and Partridge, A. D. 1975. Interaction of bark beetles (Coleoptera: Scolytidae) and root-rot pathogens in Grand fir in northern Idaho. Canadian Entomologist. 107:899-904.

Heusser, L. E. and King, J. E. 1988. North America, with special emphasis on the development of the Pacific Coastal Forest and Praire/Forest boundary prior to the last Glacial Maximum. In Huntley, B. and Webb III, T. (Eds.). Vegetation History. Kluwer Academic Publishers, London. 803pp.

Hibbett, D. S., Grimaldi, D. and Donoghue, M. J. 1995. Cretaceous mushrooms in amber. Nature. 377:487.

Hibbett, D. S., Tsuneda, A. and Murakami, S. 1994. The secotoid form of *Lentinus tigrinus*: Genetics and development of a fungal morphological innovation. American Journal of Botany. 81:466-478.

Hillis, D. M. 1987. Molecular versus morphological approaches to systematics. Annual Review of Ecology and Systematics. 18:23-42.

Hillis, D. M. 1995. Approaches for assessing phylogenetic accuracy. Systematic Biology. 44:3-16.

Hillis, D. M. and Huelsenbeck, J. P. 1992. Signal, noise and reliability in molecular phylogenetic analyses. Journal of Heredity. 83:189-195.

Hintikka, V. 1973. A note on the polarity of *Armillariella mellea*. Karstenia. 13:32-39.

Hollingsworth, R. G. and Hain, F. P. 1994. Balsam woolly adelgid (Homoptera: Adelgidae): effects on growth and abnormal wood production in Fraser fir seedlings as influenced by seedling genetics, insect source, and soil source. Canadian Journal of Forest Research. 24:2284-2294.

Hopkins, D. M., Matthews, J. V., Wolfe, J. A. and Silberman, M. L. 1971. A Pliocene flora and insect fauna from the Bering Strait Region. Palaeogeography, Palaeoclimatology, Palaeoecology. 9:211-213.

Hudak, J. and Singh, P. 1970. Incidence of *Armillaria* root rot in balsam fir infested by balsam woolly aphid. Canadian Plant Disease Survey. 50:99-101.

Hudak, J. and Wells, R. E. 1974. *Armillaria* root rot in aphid damaged balsam fir in Newfoundland. Forestry Chronicle. 50:74-76.

Hughs, N. F. 1977. Palaeo-succession of earliest angiosperm evolution. Botanical Review. 43:105-127.

Huntsberger, D. V. and Billingsley, P. 1981. Elements of Statistical Inference. 5th edition. Allyn and Bacon, Inc. Boston.

Hurlbert, S. H. 1984. Pseudoreplication and the design of ecological field experiments. Ecological Monographs 54:187-211.

Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (Ed.) 1990. PCR Protocols. Academic Press, London.

Ives, W. G. H. and Wong, H. R. 1988. Tree and Shrub Insects of the Prairie Provinces. Information Report NOR-X-292. Northern Forestry Center, Canadian Forestry Service.

Jahnke, K.-D., Bahnweg, G. and Worrall, J. J. 1987. Species delimitation in the *Armillaria mellea* complex by analysis of nuclear and mitochondrial DNAs. Transactions of the British Mycological Society. 88: 572-575.

James, R. L and Goheen, D. J. 1981. Conifer mortality associated with root disease and insects in Colorado. Plant Disease. 65:506-507.

Johnson, A. L. S., Wallis, G. W. and Foster, R. E. 1972. Impact of root rot and other diseases in young Douglas Fir plantations. Forestry Chronicle. 48:316-319.

Kashino, H. and Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. Journal of Molecular Evolution. 29:170-179.

Kile, G. A. and Watling, R. 1983. *Armillaria* species from South-eastern Australia.

Transactions of the British Mycological Society. 81:129-140.

Kirk, T. K. 1981. Toward elucidating the mechanism of action of the ligninolytic systems in basidiomycetes. pp.131-148. *In*: Hollaender, A. (Ed). Trends in the biology of fermentations for fuels and chemicals. Basic Life Sciences. Volume 18. Plenum Press. New York.

Kluge, A. G. 1989. A concern for evidence and phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). Systematic Zoology. 38:7-25.

Korhonen, K. 1978. Interfertility and clonal size in the *Armillaria mellea* complex. Karstenia. 18:31-42.

Korhonen, K. 1980. Origin of clamped and clampless basidia in *Armillariella ostoyae*. Karstenia. 20:23-27.

Kulhavy, D. L., Partridge, A. D. and Stark, R. W. 1984. Root diseases and blister rust associated with bark beetles (Coleoptera: Scolytidae) in western white pine in Idaho.

Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C. and Sninsky, J. J. 1990. Effects of primer - template mismatches on the polymerase chain reaction: Human immunodefiency virus type 1 model studies. Nucleic Acids Research. 18:999-1005.

Larsen, M. J., Banik, M. T. and Burdsall Jr., H. H. 1992. Clamp connections in North American *Armillaria* species: occurrence and potential application for delimiting species. Mycologia. 84:214-218.

Larson, K. C. and Whitham, T. G. 1997. Competition between gall aphids and natural plant sinks: plant architecture affects resistance to galling. Oecologia. 109:575-582.

Li, C. Y., Lu, K. C., Trappe, J. M. and Bollen, W. B. 1967. Selective nitrogen assimilation by *Poria weirii*. Nature. 213:814.

Lin, D., Dumas, M. T. and Hubbes, M. 1989. Isozyme and general protein patterns of *Armillaria* spp. collected from the boreal mixed wood forest of Ontario. Canadian Journal of Botany. 67:1143-1147.

Livingston, W. H. 1990. *Armillaria ostoyae* in young spruce plantations. Canadian Journal

of Forest Research. 20:1773-1778.

Lung-Escarment, B., Mohammed, C. and Dunez, J. 1985. Nouvelles méthodes de déterminiation des *Armillaires* européens: Immunologie et electrophorèse en gel de polyacrylamide. European Journal of Forest Pathology. 15:278-288.

Lutzoni, F. and Vilgalys, R. 1995. Integration of morphological and molecular data sets in estimating fungal phylogenies. Canadian Journal of Botany. (Supplement 1):S649-S659.

MacKenzie, M. 1987. Infection changes and volume loss in a 19-year old *Pinus radiata* stand affected by *Armillaria* root rot. N. Z. J. Forest Science. 17:100-108.

Mallett, K. I. 1990. Host range and geographic distribution of *Armillaria* root rot pathogens in the Canadian Prairie Provinces. Canadian Journal of Forest Research. 20:1859-1863.

Mallett, K. I. 1995. Relationships among *Armillaria* root disease, jack pine budworm and jack pine forest ecosystems. pp.107-119. In: Volney, W. J. A., Nealis, V. G., Howse, G. M., Westwood, A. R., McCullough, D. R. and Laishley, B. L. (Eds.) Jackpine budworm biology and management. Canadian Forest Service. Northwest Region. Information Report NOR-X-342.

Mallett, K. I. and Hiratsuka, Y. 1988. Inoculation studies of lodgepole pine with Alberta isolates of the *Armillaria mellea* complex. Canadian Journal of Forest Research. 18:292-296.

Mallett, K. I. and Volney, W. J. A. 1990. Relationships among jack pine budworm damage, selected tree characteristics, and *Armillaria* root rot in jack pine. Canadian Journal of Forest Research. 20:1791-1795.

Manniatis, T., Fritsch, E. F. and Sambrook, J. 1982. Molecular Cloning: A laboratory manual. Cold Spring Harbour Laboratory, USA.

Marxmüller, H. 1982. Etude morphologique des *Armillaria* ss. str. à anneau. Bulletin de la Société Mycologique de France. 98:87-124.

Mason, S. F. 1991. Organic Replication and Genealogy. pp 191-232. *In*: Chemical Evolution: Origin of the elements, molecules, and living systems. Clarendon Press. Oxford.

Meeuse, A. D. J. 1975. Floral evolution in the Hamamelididae. Acta Botanica Neerlandica. 24:155-191.

Mihail, J. D., Obert, M., Bruhn, J. N. and Taylor, S. J. 1995. Fractal geometry of diffuse mycelia and rhizomorphs of *Armillaria species*. Mycological Research. 99:81-88.

Miller, O. K. Jr., Johnson, J. L., Burdsall, H. H. Flynn, T. 1994. Species delimitation in North American species of *Armillaria* as measured by DNA reassociation. Mycol. Res. 98:1005-1011.

Mindell, D. P. and Thacker, C. E. 1996. Rates of molecular evolution: Phylogenetic issues and applications. Annual Review of Ecology and Systematics. 27:279-303.

Miyamoto, M. M. and Fitch, W. M. 1995. Testing species phylogenies and phylogenetic methods with congruence. Systematic Biology. 44:64-76.

Morrison, D. J. 1974. Effect of soil pH on rhizomorph growth of *Armillaria mellea*. Bi-monthly Research Notes 30. Canadian Forestry Service:18-19.

Morrison, D. J. 1975. Ion uptake by rhizomorphs of *Armillaria mellea*. Canadian Journal of Botany. 53:48-51.

Morrison, D. J. 1976. Vertical distribution of *Armillaria mellea* rhizomorphs in soil. Transactions of the British Mycological Society. 66:393-399.

Morrison, D. J. 1982a. Effects of soil organic matter on rhizomorph growth by *Armillaria mellea*. Transactions of the British Mycological Society. 78:201-207.

Morrison, D. J. 1982b. Variation among British isolates of *Armilaria mellea*. Transactions of the British Mycological Society. 78:459-464.

Morrison, D. J. 1989. Pathogenicity of *Armillaria* species is related to rhizomorph growth habit. pp. 142-148. *In:* Morrison, D. J. (Ed). Proceedings of the Seventh International Conference on Root and Butt Rots. IUFRO Working Party S2.06.01. Forestry Canada, Pacific Forestry Center, Victoria, B. C.

Morrison, D. J. 1991. Identity of *Armillaria* isolates used in studies of rhizomorph behavior and pathogenicity. Mycological Research. 95:1437-1438.

Morrison, D. J., Chu, D. and Johnson, A. L. S. 1985b. Species of *Armillaria* in British Columbia. Canadian Journal of Plant Pathology. 7:242-246.

Morrison, D. J., Thomson, A. J., Chu, D., Peet, F. G. and Sahota, T. S. 1985a. Isozyme patterns of *Armillaria* intersterility groups occurring in British Columbia. Canadian Journal of Microbiology. 31:651-653.

Morrison, D. J., Williams, R. E. and Whitney, R. D. 1991. Infection, disease development, diagnosis, and detection. In: *Armillaria* Root Disease. United States Department of Agriculture, Forest Service. Agriculture Handbook No. 691. Washington, D. C.

Morton, A., Tabrett, A. M., Carder, J. H. and Barbara, D. J. 1995. Sub-repeat sequences in the ribosomal RNA intergenic regions of *Verticillium alboatrum* and *V. dahliae*. Mycological Research. 99:257-266.

Mugala, M. S., Blenis, P. V., Hiratsuka, Y. and Mallett, K. I. 1989. Infection of lodgepole pine and white spruce by Alberta isolates of *Armillaria*. Canadian Journal of Forest Research. 19:685-689.

Ochman, H., Medhora, M. M., Garza, D. and Hartl, D. L. 1990. p.219-227. Amplification of flanking sequences by inverse PCR. In Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (Eds.). PCR Protocols: A guide to methods and applications. Academic Press, Inc. San Diego, California. 482pp.

Ohta, T. 1992. The nearly neutral theory of molecular evolution. Annual Review of Ecology and Systematics. 23:263-286.

Omdal, D. W., Shaw III, C. G., Jacobi, W. R. and Wager, T. C. 1995. Variation in pathogenicity and virulence of isolates of *Armillaria ostoyae* on eight tree species. Plant Disease. 79:939-944.

Omland, K. E. 1994. Character congruence between a molecular and a morphological phylogeny for dabbling ducks (*Anas*). Systematic Biology. 43:369-386.

Page, R. D. M. and Charleston, M. A. 1997. From gene to organismal phylogeny: Reconciled trees and the gene tree / species tree problem. Molecular Phylogenetics and Evolution. 7:231-240.

Parker, J. and Houston, J. R. 1971. Effects of repeated defoliation on root and collar extractives of sugar maple trees. Forest Science. 7:91-95.

Parker, J. D., Rabinovitch, P. S. and Burmer, G. C. 1991. Targeted gene walking polymerase chain reaction. Nucleic Acids Research. 19:3055-3060..

Parks, C. G., Filip, G. M. and Hansen, E. M. 1994. The influence of water stress and insect defoliation on the development of disease in *Abies grandis* seedlings inoculated with *Armillaria ostoyae.* pp. 52-64 *In:* Johansson, M. and Stenlid, J. (Eds.) Proceedings of the eigth international conference on root and butt rots. August 9-16, 1993. Wik, Sweden and Haikko, Finland. IUFRO Working Party S-750 07. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Pirozynski, K. A. 1976. Fossil Fungi. Annual Review of Ecology and Systematics. 7:237-246.

Poinar, G. O. and Singer, R. 1990. Upper Eocene gilled mushroom from the Dominican Republic. Science. 248:1099-1101.

Primrose, S. B. 1995. Principles of Genome Analysis. Blackwell Science Ltd. London.

Proffer, T. J. , Jones, A. L. and Ehret, G. R. 1987. Biological species of *Armillaria* isolated from sour cherry orchards in Michigan. Phytopathology. 77:941-943.

Quicke, D. L. J. 1993. Principles and techniques of contemporary taxonomy. Blackie Academic and Professional, an imprint of Chapman and Hall. London. 311 pp.

Raabe, R. D. 1962. Host list of the root rot fungus, *Armillaria mellea.* Hilgardia. 33:25-88.

Radford, A. 1993. A fungal phylogeny based upon ortidine 5'-monophosphate decarboxylase. Journal of Molecular Evolution. 36:389-395.

Raske, A. G. and Sutton, W. J. 1986. Decline and mortality of black spruce caused by spruce budworm defoliation and secondary organisms. Information Report N-X-236. Canadian Forestry Service, Newfoundland Forestry Center.

Redfern, D. B. 1973. Growth and behavior of *Armillaria mellea* rhizomorphs in soil. Transactions of the British Mycological Society. 61:569-581.

Redfern, D. B. 1978. Infection by *Armillaria mellea* and some factors affecting host resistance and the severity of disease. Forestry. 51:121-135.

Redfern, D. B. and Filip, G. M. 1991. Inoculum and Infection. In: *Armillaria* Root Disease. United States Department of Agriculture, Forest Service. Agriculture Handbook No. 691. Washington, D.C.

Richard, K. R., Whitehead, H. and Wright, J. M. 1996. Polymorphic microsatellites from sperm whales and their use in the genetic identification of individuals from naturally sloughed pieces of skin. Molecular Ecology. 5:313-315.

Rieseberg, L. H. 1996. Homology among RAPD fragments in interspecific comparisons. Molecular Ecology. 5:99-105.

Rishbeth, J. 1982. Species of *Armillaria* in southern England. Plant Pathology. 31:9-17.

Rishbeth, J. 1984. Pathogenicity tests for *Armillaria*. In: Kile, G. A., Ed. Proceedings of the sixth international conference on root and butt rot of forest trees; 1983 August 25-31; Melbourne, Victoria, and Gympie, Australia, Melbourne, Australia: International Union of Forestry Research Organizations: 131-139.

Rizzo, D. M. and Harrington, T. C. 1988. Root and butt rot fungi on Balsam Fir and Red Spruce in the White Mountains, New Hampshire. Plant Disease. 72:329-331.

Robinson, J. M. 1990. Lignin, land plants and fungi: Biological evolution affecting Phanerozoic oxygen balance. Geology. 15:607-610.

Rus-Kortekaas, W., Smulders, M. J. M., Arens, P. and Vosman, B. 1994. Direct comparison of levels of genetic variation in tomato detected by a GACA-containing microsatellite probe and by random amplified polymorphic DNA. Genome. 37:375-381.

Sambrook, J., Fritch, E. F. and Maniatis, T. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY.

Scarr, T. A. 1995. Phenotypic variation in jack pine susceptibility to jack pine budworm. pp.79-88 In: Volney, W. J. A., Nealis, V. G., Howse, G. M., Westwood, A. R., McCullough, D. R. and Laishley, B. L. (Eds.) Jack pine budworm biology and management. Canadian Forest Service. Northwest Region. Information Report NOR-X-342.

Schoeneweiss, D. F. 1975. Predisposition, stress, and plant disease. Annual Review of Phytopathology. 13:193-211.

Shaw III, C. G., MacKenzie, M., Toes, E. H. A. and Hood, I. A. 1981. Cultural characteristics and pathogenicity to *Pinus radiata* of *Armillaria novae-zelandiae* and *A. limonea*. New Zealand Journal of Forestry Science. 11:65-70.

Shields Jr., W. J. and Hobbs, S. D. 1979. Soil nutrient levels and pH associated with *Armillariella mellea* on conifers in Northern Idaho. Canadian Journal of Forest Research. 9:45-48.

Shrimpton, D. M. 1973. Age- and size-related response of lodgepole pine to inoculation with *Europhium clavigerum*. Canadian Journal of Botany. 51:1155-1160.

Singer, M. and Berg, P. 1991. Genes and Genomes; a changing perspective. University Science Books. California.

Singh, P. 1970. *Armillaria* root rot in a coniferous plantation in Newfoundland. Bi-monthly Research Notes, Department of Fisheries and Forestry, Ottawa, Canada. 26:5-6.

Singh, P. 1975. *Armillaria* root rot: Distribution and severity in softwood plantations in Newfoundland. Acta Phytopathologica Academiae Scientarum Hungaricae. 10:389-406.

Singh, P. 1980. *Armillaria* root rot: Artificial inoculation and development of the disease in greenhouse. European Journal of Forest Pathology. 10:420-431.

Singh, P. 1981a. *Armillaria mellea*: Growth and distribution of rhizomorphs in the forest soils of Newfoundland. European Journal of Forest Pathology. 11:208-220.

Singh, P. 1981b. *Armillaria mellea*: Distribution and hosts in Newfoundland and Labrador. Canadian Plant Disease Survey. 61:31-36.

Singh, P. 1983. *Armillaria* root rot: Influence of soil nutrients and pH on the susceptibility of conifer species to the disease. European Journal of Forest Pathology. 13:92-101.

Singh, P. and Carew, G. C. 1983. *Armillaria* root rot on urban trees: Another perspective to the root rot problem in Newfoundland. Canadian Plant Disease Survey. 63:3-6.

Singh, P. and Raske, A. G. 1983. *Armillaria* root rot in spruce budworm-damaged balsam fir stands. CANUSA Newsletter No. 26.

Smerlis, E. 1961. Pathological condition of immature balsam fir stands of *Hylocomium-Oxalis* type in the Laurentide Park, Quebec. Forestry Chronicle. 37:109-115.

Smith, M. L. and Anderson, J. B. 1989. Restriction fragment length polymorphisms in mitochondrial DNAs of *Armillaria*: identification of North American biological

species. Mycological Research. 93:247-256.

Smith, M. L., Bruhn, J. N. and Anderson, J. B. 1992. The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. Nature. 356:428-431.

Sokal, R. R. and Rohlf, F. J. 1981. Biometry. 2nd ed. San Franscisco: W. H. Freeman.

Sommer, R. and Tautz, D. 1989. Minimal homology requirements for PCR primers. Nucleic Acids Research. 17:6749.

Stewart, W. N. and Rothwell, G. W. 1993. Palaeobotany and the evolution of plants. 2nd edition. Press Syndicate of the University of Cambridge, New York. 521 pp.

Storck, R. and Alexopoulos, C. J. 1970. Deoxyribonucleic acid of fungi. Bacteriological Review. 34:126-154.

Taylor, J. W. 1986. Fungal evolutionary biology and mitochondrial DNA. Experimental Mycology. 10:259-269.

Terashita, T. and Chuman, S. 1987. Fungi inhabiting wild orchids in Japan, IV. *Armillariella tabescens*, a new symbiont of *Galeola septentrionalis*. Transactions of the Mycological Society of Japan. 28:145-154.

Timberlake, W. E. 1978. Low repetitive DNA content in *Aspergillus nidulans*. Science. 202:973-975.

Ullrich, R. C. and Anderson, J. B. 1978. Sex and diploidy in *Armillaria mellea*. Experimental Mycology. 2:119-121.

van der Kamp, B. J. 1993. Rate of spread of *Armillaria ostoyae* in the central interior of British Columbia. Canadian Journal of Forest Research. 23:1239-1241.

Vilgalys, R. and Sun, B. L. 1994. Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. Proceedings of the National Academy of Sciience, USA. 91:4599-4603.

Volk, T. J., Burdsall Jr., H. H. and Banik, M. T. 1996. *Armillaria nabsnona*, a new species from western North America. Mycologia. 88:484-491.

Wargo, P. M. 1972. Defoliation-induced chemical changes in sugar maple roots stimulate

growth of *Armillaria mellea*. Phytopathology. 62: 1278-1283.

Wargo, P. M. 1977. *Armillariella mellea* and *Agrilus bilineatus* and mortality of defoliated oak trees. Forest Science. 23:485-492.

Wargo, P. M. 1980. Interaction of ethanol, glucose, phenolics and isolate of *Armillaria mellea*. Phytopathology. 70:480. Abstract.

Wargo, P. M. and Harrington, T. C. 1991. Host stress and susceptibility. pp.88-101. In Shaw III, C. G. and Kile, G. A. (Eds.) *Armillaria* root disease. United States Department of Agriculture, Forest Service. Agriculture Handbook No. 691.

Wargo, P. M., Parker, J. and Houston, D. R. 1972. Starch content of defoliated sugar maple. Forest Science. 18:203-204.

Wargo, P. M. and Houston, D. R. 1974. Infection of defoliated sugar maple trees by *Armillaria mellea*. Phytopathology. 64:817-822.

Wargo, P. M. and Shaw III, C. G. 1985. *Armillaria* root rot: The puzzle is being solved. Plant Disease. 69:826-832.

Warren, G. L. and Singh, P. 1970. *Hylobius* weevils and *Armillaria* root rot in a coniferous plantation in Newfoundland. Bi-monthly Research notes, Department of Fisheries and Forestry, Ottawa, Canada. Vol. 26:55.

Weising, K., Nybom, H., Wolff, K. and Meyer, W. 1995. DNA fingerprinting in plants and fungi. CRC Press, Inc. 322pp.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research. 18:7213-7218.

Welsh, J. and McClelland, M. 1993. Characterization of pathogenic microorganisms by genomic fingerprinting using arbitrarily primed PCR. In Persing, D. H., Smith, T. F., Tenover, F. C. and White, T. J. (Eds.) Diagnostic Molecular Microbiology: Principles and applications. Mayo Foundation Rochester MN. 641pp.

Whitney, R. D. 1961. Root wounds and associated root rots of white spruce. Forestry Chronicle. 37:401-411.

Whitney, R. D. 1978. Root rot of spruce and balsam fir in northwestern Ontario II. Causal Fungi and site relationships. Great Lakes Forest Research Center, Sault Ste. Marie,

Ontario, Report O-X-284. Canadian Forestry Service, Department of the Environment.

Whitney, R. D. 1984. Site variation in *Armillaria mellea* in three Ontario conifers. pp. 122-130. *In* Kile, G. A. (Ed.)Proceedings of the sixth International conference on Root and Butt Rots of Forest Trees. August 25-31, 1983. <elbourne, Victoria, Gympie, Queensland, Australia, Melbourne: International Union of Forestry Research Organization.

Whitney, R. D. 1988. *Armillaria* root rot damage in softwood plantations in Ontario. Forestry Chronicle. 64:350-351.

Whitney, R. D. 1995. Root-rotting fungi in white spruce, black spruce and balsam fir in northern Ontario. Canadian Journal of Forest Research. 25:1209-1230.

Whitney, R. D., Ip, D. W. and Trwin, R. N. 1989. *Armillaria* infections in symptomless white spruce, black spruce and red pine saplings in Ontario plantations. pp. 546-549. *In:* Morrison, D. J. (Ed). Proceedings of the Seventh International Conference on Root and Butt Rots. IUFRO Working Party S2.06.01. Forestry Canada, Pacific Forestry Center, Victoria, B. C.

Wilbur, W., Munnecke, D. E. and Darley, E. F. 1972. Seasonal development of *Armillaria* root rot of peach as influenced by fungal isolates. Phytopathology 62:567-570.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA ploymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 18:6531-6535.

Williams, J. G. K., Hanafey, M. K., Rafalski, J. A. and Tingey, S. V. 1993. Genetic analysis using random amplified polymorphic markers, Methods in Enzymology. 218:704-740.

Wilson, A. C., Carlson, S. S. and White, T. J. 1977. Biochemical evolution. Annual Review of Biochemistry. 46:573-639.

Wolfe, J. A. 1971. Tertiary climatic fluctuations and methods of analysis of Tertiary floras. Palaeogeography, Palaeoclimatology, Palaeoecology. 9:27-57.

Xodo, L. E., Manzini, G., Quadrifoglio, F., van der Marel, G. and van Boom, J. 1991. DNA hairpin loops in solution. Correlation between primary structure, thermostability and reactivity with single-strand-specific nuclease from mung bean.

Nucleic Acids Research. 19:1505-1511.

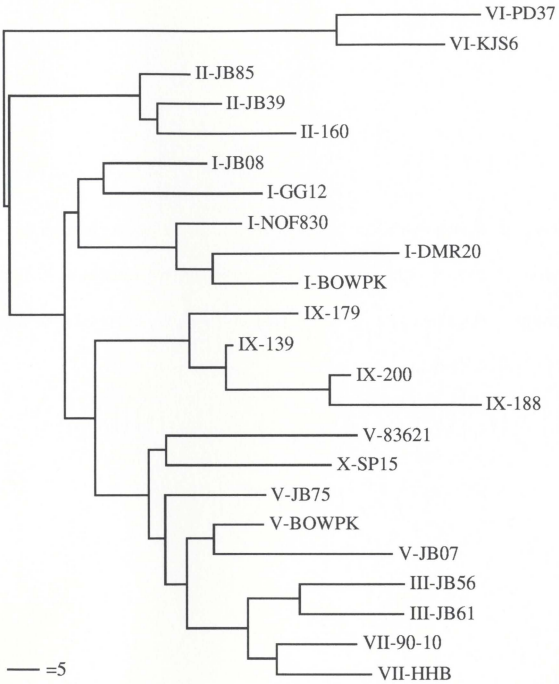
Yamada, T. 1992. Biochemistry of Gymnosperm xylem responses to fungal invasion. pp. 147-164. *In:* Blanchette, R. A. and Biggs, A. R. (Eds). Defense Mechanisms of Woody Plants Against Fungi. Springer-Verlag, Berlin.

Zolan, M. E. and Pukkila, P. J. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Molecular and Cellular Biology. 6:195-200.

Appendix A: List of primers used in all stages of PCR and Inverse PCR to obtain species-specific markers.

Primer	Primer sequence
Species specific 10nt SWAPP primers:	
151	gct gta gtg t
159	gag ccc gta g
17	cct ggg cct c
127	atc tgg cag c
83	ggg ctc gtg g
66	gag ggc gtg a
34	ccg gcc cca a
122	gta gac gag c
147	gtg cgt cct c
29	ccg gcc tta c
Nested 20nt primers developed from SWAPP fragments:	
I-850a	acg agc caa acg atg acc gg
I-850b	gcg cct cga tac agt acg tc
II-850a	gga aga gaa ggt agt aga gg
II-850b	acg cct cga tac agc acg tc
III-520-1	cat ggt cgc tac tta ctc tga taa cgg
III-520-2	gag ttg acg tag act ac
VII-520-1	gtg gcc aaa cac ttt gat ctt cct agg
VII-520-2	agt gtt aga tct aga gt
III-180a	acc aca tcc ttg tcg ccg ag
III-180b	gtg gtt gat gag att gtt cg
V-250a	cga act gat cgt cgt cga
X-250a	cga act gat cat cgt cca
V/X-250b	gtt tcg aac gcg aat atg ctc
VII-500a	agt ctg aag gaa tca tg
VII-500b	ctg gta cat atg cag tca cc
IX-250a	ttc tac acg caa atg acc ag
IX-250b	tcg gtg aag tct tcg aag ac
Inverse primers from center of SWAPP fragments:	
INV250a	gat cac gac gct ctt acc
INV250b	cac aac act atg tgg acg
INV-151	ctc gac ccc aac cgt tcc
INV-159	tgc tga agc tgc tcc aga tcc
Extended 10nt primers:	
V-127	gtc atg gct atc tgg cag c
V-17	gtg gcc cct ggg cct c
20nt species-specific primers:	
1a	gtc gtc gat tta aca tcg gt
1,5,6b	aaa tgc gcg agc aag cag tg
5a	gca gct atc tct gga tgg gt
10	gta gag gtg ctg ctg ctg ct
12	act ttt tta gtc cgg gat tc
6a	ggt ctt cat tgg cat cta ct
7	tgc tga cgt ttt ggc aag ata aaa c
8	cag tat ctt gtg tgg cca gg
9a	tca cat aga ata ggt gat g
9b	ggt ctg aag tta att g

Appendix B: Phenogram based on combined nucleotide sequences from four primer sets for 23 taxa of eight NABS *Armillaria*, using two isolates of NABS VI as outgroup. A neighbour joining algorithm was used, and horizontal branch length is proportional to distance.


VI-PD37
VI-KJS6
II-JB85
II-JB39
II-160
I-JB08
I-GG12
I-NOF830
I-DMR20
I-BOWPK
IX-179
IX-139
IX-200
IX-188
V-83621
X-SP15
V-JB75
V-BOWPK
V-JB07
III-JB56
III-JB61
VII-90-10
VII-HHB
=5

Appendix C: Nucleotide substitutions for divergences (mean percent substitution ± standard deviation (SD)) and lineages (mean percent substitution ± SD) for NABS *Armillaria* (Figure 2A-5).

Event	Divergence of taxa	Percent substitution of divergence (mean ± SD)	Divergence time (Ma ± SD)	Taxa	Percent substitution of lineage (mean)
Radiation of NABS *Armillaria* from *A. mellea*.	* NABS VI - All other NABS.	5.70 ± 1.86	11.71 ± 3.82	VI-PD37	14.33 ± 0.32
	VIPD37 - VIKJS6	2.45 ± 0.42	5.03 ± 0.86	VI-KJS6	13.98 ± 1.45
Origin of NABS II ??	NABS II - All other NABS .	1.45 ± 0.35	2.98 ± 0.72	II-160	2.35
	II160 - IIJB39, IIJB85	1.30 ± 0.36	2.67 ± 0.74	II-JB85	1.13
				II-JB39	1.63
Radiation of all other NABS excluding NABS II and VI.	* All other NABS - NABS II and VI.	4.65 ± 0.89	9.55 ± 1.83	I-JB08	4.22
				I-GG12	4.95
				I-NOF830	4.48
				I-DMR	7.28
Divergence of NABS I from NABS III, V, VII, IX and X.	NABS I - III, VII, V, IX and X.	4.40 ± 1.47	9.04 ± 3.02	I-BowPk	5.68
	IJB08 - IGG12, INOF830, IDMR, and IBowPk.	4.40 ± 1.71	9.04 ± 3.51	III-JB56	7.9
				III-JB61	7.25
	IGG12 - INOF830, IDMR and IBowPk.	3.20 ± 1.59	6.58 ± 3.27	VII-90-10	6.45
				VII-HHB	6.85
	INOF830 - IDMR and IBowPk.	2.60 ± 1.56	5.34 ± 3.21	V-83621	6.5
				V-JB75	4.4
Divergence of predominantly saprotrophic NABS from pathogenic NABS I.	* NABS III, VII, V, IX and X - NABS I	4.50 ± 1.00	9.25 ± 2.05	IX-TJV179	5.75
				IX-TJV188	7.8
				IX-TJV200	7.6
Radiation of monophyly NABS IX.	NABS IX - NABS III, VII, V and X	3.30 ± 2.53	6.78 ± 5.20	IX-139	4.7
	IXTJV179, IXTJV188 - IXTJV200, IX139	3.65 ± 2.32	7.50 ± 4.77	X-Sp8120	6.3
				V-BowPk	6.05
	IXTJV200, IX139 - IXTJV179, IXTJV188	0.35 ± 2.20	0.72 ± 1.48	V-JB07	8.8
The splitting of isolates of NABS V.	* NABS III, VII, X, , VBowPk and VJB07 - all others	3.90 ± 1.21	8.01 ± 2.49		
	NABS III, VII, VBowPk, VJB07 - NABS X	3.50 ± 0.59	7.19 ± 1.21		
	VBowPk, VJB07 - NABS III and VII	3.00 ± 0.95	6.16 ± 1.95		
Divergence of *A. cepestipes* related group.	NABS III and VII - VBowPk and VJB07	2.95 ± 0.44	6.06 ± 0.90		
	NABS III - NABS VII	1.95 ± 0.83	4.01 ± 1.71		
	NABS VII - NABS III	1.65 ± 0.44	3.39 ± 0.90		

Appendix D: Occurrence of PCR bands in 3% agarose specific to species or group of species of NABS *Armillaria* for 10nt primer pairs showing length (bp) of representative band, absence (-) of band when tested, and sample not tested (NT) for the primer set. The three bands indicated in NABS III represent fragments 240, 300, and 360bp long, and in NABS VI represent fragments 115, 135, and 170bp long.

Sample	29/122	83/147	34/122	17/127	83/66	151/159
I-GG12	-	-	NT	NT	300	-
I-JB08	-	-	-	NT	300	850
I-NOF830	-	-	NT	-	300	850
I-JB77	-	-	NT	NT	NT	NT
I-JB40	NT	NT	NT	-	300	850
I-JB14	NT	NT	NT	NT	NT	850
I-Bow Pk	NT	NT	NT	NT	300	-
I-GG13	NT	NT	NT	NT	300	-
I-JB15	NT	NT	NT	NT	NT	850
I-JB87	NT	NT	NT	NT	NT	850
I-JB46	NT	NT	NT	NT	NT	850
I-JB06	NT	NT	NT	NT	300	850
I-JB79	NT	NT	NT	NT	300	850
I-JB97	NT	NT	NT	NT	NT	850
I-JB73	NT	NT	NT	NT	NT	850
I-JB90	NT	NT	NT	NT	NT	850
I-JB96	NT	NT	NT	NT	NT	850
I-JB86	NT	NT	NT	NT	NT	850
I-JB09	NT	NT	NT	NT	300	NT
I-DMR20	NT	NT	NT	NT	300	850
I-Amm9067	NT	NT	NT	NT	NT	850
I-JM121	NT	NT	NT	NT	NT	-
I-PD32	NT	NT	NT	NT	300	NT
I-JB16	NT	NT	NT	NT	300	NT
II-JB85	-	-	NT	NT	300	850

II-JB38	-	-	-	NT	300	850
II-28	-	-	NT	-	-	-
II-35-5	NT	NT	NT	-	300	NT
II-160	NT	NT	NT	NT	300	850
II-JB39	NT	NT	NT	-	300	850
II-JB19	NT	NT	NT	NT	300	850
II-JB102	NT	-	NT	NT	300	NT
III-11	-	520	3-band	NT	300	NT
III-MD52	-	520	-	-	300	NT
III-JB56	-	520	3-band	-	300	NT
III-JB61	NT	520	3-band	NT	300	NT
III-JB55	NT	520	3-band	NT	300	NT
V-JB19	NT	NT	NT	390	-	NT
V-JB66	NT	NT	NT	390	300	-
V-JB72	-	-	NT	NT	NT	NT
V-83911	-	-	NT	390	300	NT
V-NOF891	NT	-	-	NT	NT	NT
V-83621	NT	NT	NT	390	NT	NT
V-JB75	NT	NT	NT	390	300	NT
V-JB07	NT	NT	NT	-	300	NT
V-BOWPK	NT	NT	NT	NT	300	NT
V-86372	NT	NT	NT	NT	300	-
V-JNOF898	NT	NT	NT	NT	NT	850?
VI-GB898	-	-	NT	-	3-band	NT
VI-KJS6	-	-	-	NT	3-band	NT
VI-97-1	-	-	-	-	3-band	850?
VI-49-8	NT	NT	NT	-	3-band	850?

VI-PD37	NT	NT	NT	NT	3-band	NT
VI-JM122	NT	NT	NT	NT	3-band	NT
VI-FM01	NT	NT	NT	NT	3-band	NT
VII-90-10	-	520	NT	525	300	NT
VII-HHB	-	520	-	525	300	-
IX-139	350	-	-	-	300	NT
IX-121	350	-	NT	-	300	NT
IX-180	350	NT	NT	NT	300	-
IX-188	-	NT	NT	NT	300	NT
IX-200	350	NT	NT	-	300	NT
IX-179	350	-	NT	-	300	850
X-SP12	-	-	NT	390	300	NT
X-SP15	-	-	-	390	300	NT
Qld8	NT	NT	NT	NT	300	NT
A.tabescens MB222	-	-	-	NT	NT	NT
A.tabescens CBS198.54	-	-	NT	NT	300	NT
A.tabescens 924852	NT	NT	NT	-	300	NT
A.tabescens CBS139.32	NT	-	NT	NT	300	NT
B8809	NT	NT	NT	NT	300	NT

Appendix E: Occurrence of PCR bands in 3% agarose for each nested 20nt primer pair developed from sequences of SWAPP 10nt primers (Appendix D) for isolates of *Armillaria* showing length (bp) of representative band, absence (-) of band when tested, and sample not tested (NT) for the primer set. Primers in parentheses represent the SWAPP 10nt primer sequence from which the nested 20nt primer was designed.

Sample	III520 (83/147)	VII520 (83/147)	III180 (34/122)	V250 (17/127)	I850 (151/159)
I-JB86	220	-	NT	NT	NT
I-JB08	220	-	180	250	400
I-JB14	220	NT	NT	250	NT
I-JB73	220	NT	NT	-	400
I-BOWPK	220	NT	180	250	400
I-JB13	220	NT	180	-	NT
I-JB40	220	NT	NT	NT	NT
I-JB09	220	NT	NT	NT	400
I-GG12	220	NT	180	250	400
I-MS21	220	NT	180	-	NT
I-PD32	220	NT	NT	-	-
I-JB46	220	NT	180	NT	NT
I-Amm9067	220	NT	180	-	400
I-JB77	NT	NT	NT	250	NT
I-DMR20	NT	NT	NT	250	NT
I-NOF830	NT	NT	180	250	NT
I-JB15	NT	NT	NT	250	NT
II-JB38	220	-	180	-	400
II28	220	NT	NT	NT	NT
II-160	220	NT	180	250	400
II-JB85	220	NT	180	250	400
II-JB39	-	NT	180	250	400
II-JB19	NT	NT	NT	250	400
II-JB102	NT	NT	180	250	NT
II-35-5	NT	NT	180	NT	NT

III-JB55	220	110	180	250	400
III-JB56	220	110	180	250	NT
III-MD52	220	-	NT	250	NT
III-11-1	220	110	NT	NT	NT
III-JB61	220	NT	NT	250	400
V-83621	220	-	NT	250	400
V-BOWPK	NT	NT	180	250	400
V-JB72	220	NT	180	250	400
V-JB19	220	NT	180	250	400
V-48-6	-	NT	NT	NT	NT
V-86372	-	NT	NT	250	NT
NA145D52	220	NT	NT	NT	NT
V-JNOF898	220	NT	NT	NT	NT
V-JB162	220	NT	NT	NT	NT
V-JB164	220	NT	NT	NT	NT
V-JB75	NT	NT	180	NT	NT
GC1121985	220	NT	NT	NT	NT
V-JB07	NT	NT	180	250	NT
VI-97-1	220	-	180	-	-
VI-PD37	NT	NT	180	250	NT
VI-JM122	220	NT	NT	250	NT
VI-KJS6	NT	NT	180	NT	NT
VI-GB898	220	NT	180	250	NT
VII-90-10	220	110	180	250	400
VII-HHB	220	110	180	250	400
IX-139	220	-	180	-	NT
IX-188	220	NT	180	250	400

IX-121	NT	NT	-	-	NT
IX-180	220	NT	180	250	NT
IX-179	200	NT	180	250	400
IX-200	-	NT	180	250	400
X-SP12	-	NT	NT	NT	NT
X-SP15	NT	NT	180	250	NT
A.ectypa	220	-	NT	-	NT
A.hinula	220	-	180	250	NT
B472	220	110	180	250	NT
B82544	NT	110	180	250	-
B850929	220	110	180	250	400
A.tabescens	220	NT	180	-	400
A.fumosa	220	NT	180	NT	400
D-1MD	220	NT	180	250	-
Laccaria	-	NT	-	-	-
Collybia	220	NT	-	-	-
Rickenella	220	NT	NT	NT	NT
A.tabescens CBS 139.32	NT	NT	180	-	NT
Amanita	-	NT	-	-	-
Ascomycete	-	NT	-	-	NT
Russula	-	NT	-	-	NT
Cortinarius	-	NT	-	-	NT
Suillus	-	NT	-	-	NT
A.tabescens 924852	-	NT	180	-	400

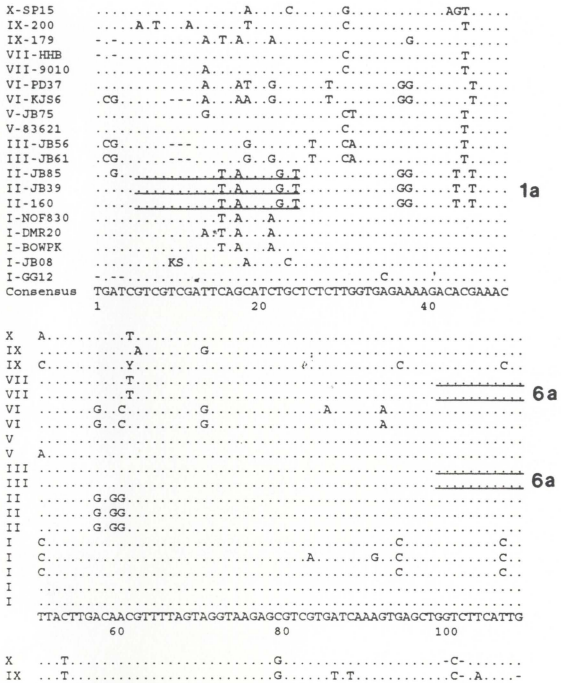
Appendix F: Occurrence of species-specific PCR bands in 3% agarose for isolates of NABS *Armillaria* showing length (bp) of representative band, absence (-) of band when tested, and sample not tested (NT) for each 20nt primer pair (Appendix E). Primers in parentheses represent the sequence from which the species-specific 20nt primer was designed.

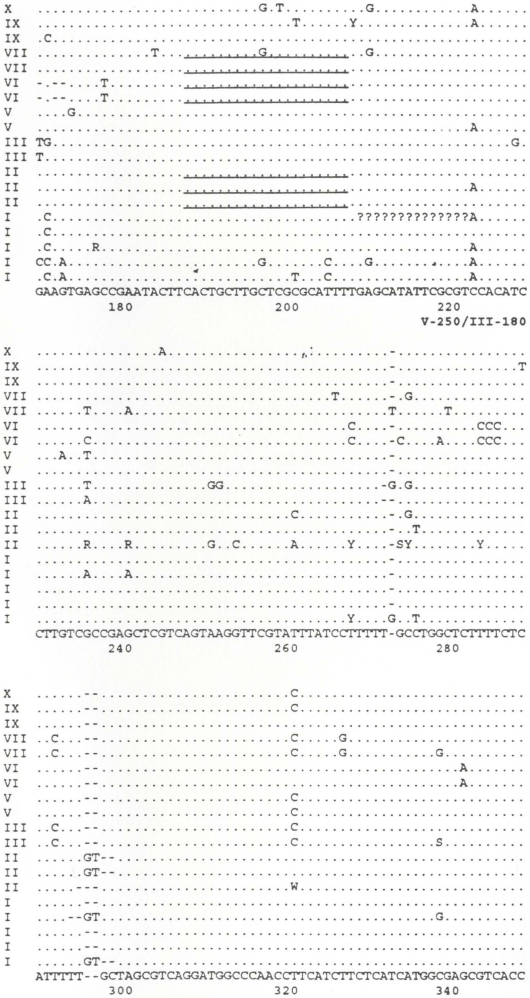
Sample	1a/b (V250)	3a/b (V250)	5a/b (V250)	6a/b (V250)	10/12 (83/66)	9a/b (III520)	7/8 (III520)
I-JB09	-	-	-	-	80	-	-
I-JB16	-	-	-	-	80	-	-
I-JB87	-	-	NT	-	80	-	-
I-JB77	-	-	-	-	80	-	-
I-GG12	-	-	-	NT	80	-	-
I-JM121	-	NT	120	NT	-	NT	-
I-JB13	-	NT	NT	NT	80	NT	NT
I-JB06	-	NT	-	NT	80	-	-
I-JB15	NT	NT	NT	NT	80	NT	NT
I-JB40	NT	NT	NT	NT	80	NT	NT
I-JB90	-	NT	NT	NT	NT	NT	NT
II-JB38	220	-	-	-	80	-	-
II-160	220	NT	-	-	80	-	-
II-JB19	220	-	-	NT	80	-	NT
II-JB39	220	-	-	NT	80	-	-
II-JB85	220	-	NT	NT	80	-	-
III-JB56	-	250	-	175	-	180	70
III-JB61	NT	250	NT	175	NT	180	70
III-JB55	NT	250	-	NT	-	180	70
V-83621	-	250	-	NT	NT	-	70
V-BowPk	NT	NT	NT	175	NT	NT	NT
V-83911	NT	NT	NT	175	NT	NT	NT
V-JNOF898	-	-	NT	-	-	-	NT
V-JB19	-	-	-	175	NT	NT	NT

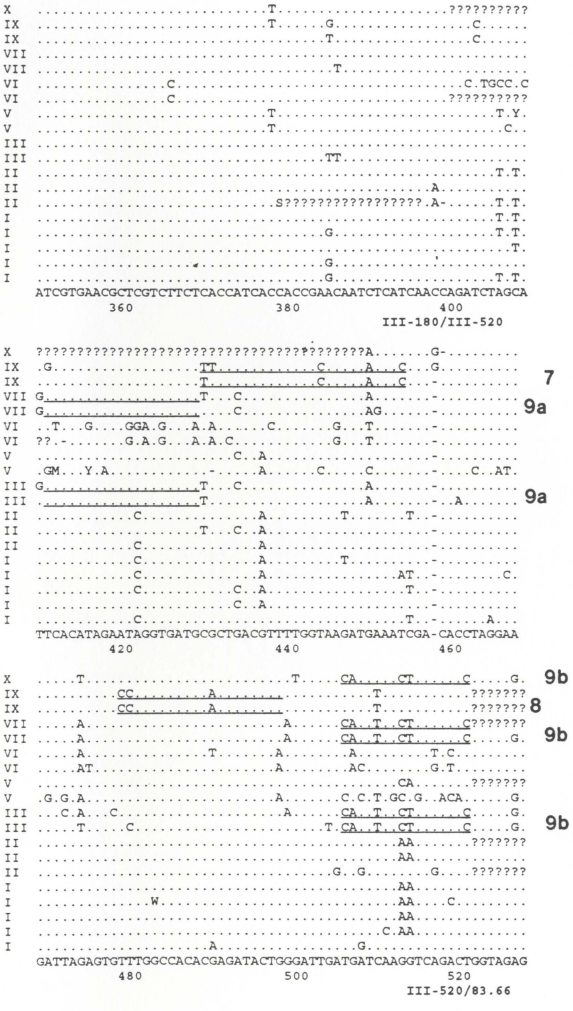
V-86372	-	-	120	175	-	-	-
V-JB07	-	-	-	NT	NT	-	70
V-JB66	NT	NT	NT	175	-	-	70
V-JB72	NT	NT	NT	175	NT	NT	70
VI-KJS6	-	-	120	-	-	NT	NT
VI-GB898	NT	-	120	NT	NT	-	NT
VI-PD37	-	-	120	-	NT	-	-
VI-49-8	-	-	120	-	-	-	-
VI-97-1	-	NT	120	NT	-	-	NT
VI-JM122	NT	NT	120	NT	NT	-	NT
VII-HHB	-	-	-	175	-	180	70
VII-90-10	-	250	-	175	-	180	70
IX-139	-	-	NT	175	NT	-	70
IX-200	-	-	-	175	-	-	-
IX-188	-	-	-	175	NT	180	70
IX-121	NT	NT	NT	NT	NT	NT	70
IX-179	-	-	-	NT	80	-	-
IX-180	-	-	-	NT	NT	-	NT
X-SP12	-	-	-	-	-	-	-
X-SP15	-	-	-	175	-	180	NT
X-POR-100	NT	-	120	NT	NT	NT	-
A.tabescens CBS139.32	-	-	-	-	-	-	NT
A.fumosa	-	-	120	175	-	180	NT
B850929	-	-	120	175	-	180	NT
B880901	-	-	-	175	-	180	NT

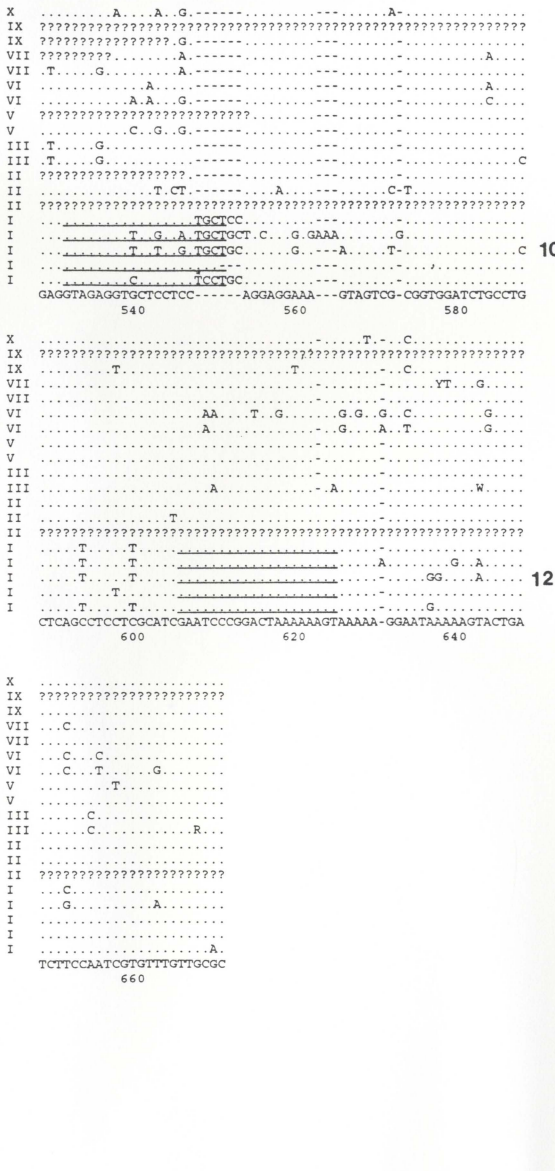
D4MD	-	-	120	-	-	-	NT
Qld8	-	-	-	175	80	-	NT
B472M3	NT	NT	NT	175	-	NT	NT
NA#248	NT	NT	NT	NT	NT	-	NT
B82544	NT	NT	NT	NT	NT	180	NT
A.tabescens 924852	NT	NT	NT	-	-	NT	NT

Appendix G: Combined nucleotide sequences obtained from nested 20nt primers for all NABS *Armillaria*, showing location of species-specific primer sites for 3a (specific for NABS III), 1a (specific for NABS II), 6a (specific for NABS III and VII), 5a (specific for NABS VI), and the complement to primers 1, 3, 5, and 6a which is general for all species. Other primer sites included 9a/b amplifying NABS III, V, VII, and X; 7/8 for NABS IX; and 10/12 for NABS I and II. Dots represent matches with consensus sequence, dashes represent deletions, mismatches are represented by bases, and question marks indicate missing data.

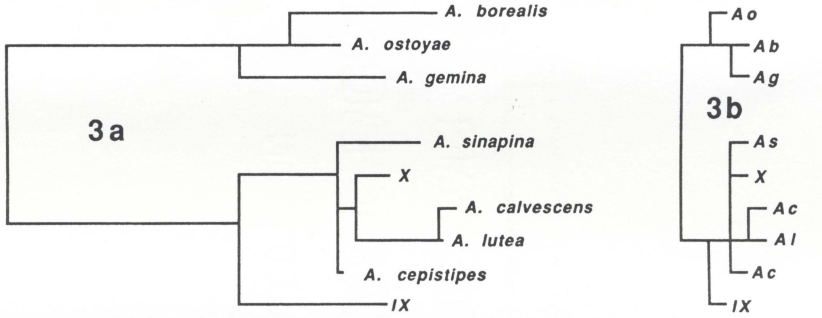

X-SP15
IX-200
IX-179
VII-HHB
VII-9010
VI-PD37
VI-KJS6
V-JB75
V-83621
III-JB56
III-JB61
II-JB85
II-JB39
II-160
I-NOF830
I-DMR20
I-BOWPK
I-JB08
I-GG12
Consensus
TGATCGTCGTCGATTCAGCATCTGCTCTCTTGGTGAGAAAAGACACGAAAC
1
20
40
1a
6a
6a
5a
TTACTTGACAACGTTTTAGTAGGTAAGAGCGTCGTGATCAAAGTGAGCTGGTCTTCATTG
60
80
100
GCAGCTACCTCTGGTTGAGACACAACACTATGTGGACGCATGAGTACTATG--CCAAAAA
120
140
160


1,5,6 b
1,5,6 b
V-250/III-180


ATCGTGAACGCTCGTCTTCTCACCATCACCACCGAACAATCTCATCAACCAGATCTAGCA
360
380
400
III-180/III-520
TTCACATAGAATAGGTGATGCGCTGACGTTTTGGTAAGATGAAATCGA-CACCTAGGAA
420
440
460
GATTAGAGTGTTTGGCCACACGAGATACTGGGATTGATGATCAAGGTCAGACTGGTAGAG
480
500
520
III-520/83.66
7
9a
9a
8
9b
9b
9b


10
12

Appendix H: Unrooted phylogenetic trees based on nucleotide sequences of IGR of nine species of *Armillaria*. Results from these trees can be compared with those in Figure 2A-5. **3A**: One of 22 most parsimonious trees for 106 variable sites. Scale bar equals four substitutions. **3B**: Strict consensus of the 22 most parsimonious trees (branch lengths not drawn to scale). (From Anderson and Stasovski, 1992).


A. borealis
A. ostoyae
A. gemina
3a
A. sinapina
X
A. calvescens
A. lutea
A. cepistipes
IX
Ao
Ab
Ag
3b
As
X
Ac
Al
Ac
IX