Relatedness and Spatial Distribution of *Armillaria* Genets Infecting Red Pine Seedlings

Myron L. Smith, Johann N. Bruhn, and James B. Anderson

First and third authors: Department of Botany, University of Toronto, Erindale College, Mississauga, Ontario L5L 1C6, Canada; and second author: Department of Plant Pathology, University of Missouri, Columbia 65211.

Present address of first author: Department of Botany, Biotechnology Laboratory, University of British Columbia, Vancouver, V6T 1Z4, Canada.

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ABSTRACT

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Genetic similarity among *Armillaria* genets responsible for root disease foci in a northern Michigan red pine plantation was investigated. The study plantation was established in 1984 in a clear-cut portion of a 60-yr-old hardwood forest. *Armillaria* isolates obtained from 87 moribund seedlings were examined. All 87 isolates were identified as *A. ostoyae* on the basis of mitochondrial DNA (mtDNA) restriction fragment patterns and/or mating interactions with voucher isolates. Independent assays of nuclear DNA restriction fragment patterns and somatic incompatibility groups agreed completely in distinguishing genets at the study site. Given knowledge of the stand history and previous estimates of mycelial growth rates, we conclude that several genets occupying large territories must have been established long before the 1984 stand conversion. Genetic similarity estimates were made by examining 83 nuclear DNA restriction fragment markers in 16 *A. ostoyae* genets within the plantation and an additional four *A. ostoyae* genets sampled outside the plantation. Together with data on the distribution of mtDNA types, these nuclear DNA similarity estimates are consistent with the hypotheses that 1) mtDNA (maternal) lineages exist in the local population; 2) members of the population were established by effectively anisogamous sexual mating events; and 3) the breeding population of *A. ostoyae* at this site extends beyond 1 km.

Additional keywords: DNA fingerprinting, forest pathology.

Armillaria root disease can cause considerable mortality in both naturally regenerated forests (21,43) and forest plantations (17,26). How *Armillaria* populations become established in plantations is of considerable interest in forest pathology. In this study, we define the individual unit of *Armillaria* populations as the *genet,* a term with ample precedent with respect to plants (16,19) and more recently to fungi (30). The genet is a mitotic cell lineage established in a mating of two gametic nuclei, which are presumably carried to a site by windborne basidiospores. In *Armillaria,* this mitotic lineage is commonly observed to occupy a spatial territory that may encompass several adjacent woody root systems (12). Since *Armillaria* lacks conidia or other mitotic propagules, genets are not found as collections of highly dispersed ramets. Rather, genets appear to spread only locally from a point of origin by continuous vegetative extension. Within the territory occupied by the genet, however, the spatial limits of the ramet remain unclear. What might constitute a distinct ramet at one time could become subdivided into a number of ramets through famine-induced thinning or mechanical fragmentation of the vegetative network of rhizomorphs and mycelium. Conversely, because all parts of the same genet are somatically compatible, what might be distinct ramets at one time could potentially coalesce during periods of growth caused by an abundance of available food bases.

Evidence from the field for the establishment of populations of genets includes observations of *Armillaria* infection foci within seedling plantations established on previously nonforested sites (32,33) and the detection of multiple mating-type alleles in local populations, indicating genetic diversity (12). Nevertheless, although *Armillaria* fruit-bodies produce vast quantities of basidiospores, monokaryotic mycelia have not yet been detected in nature. One likely explanation is that few *Armillaria* monokaryons survive, and these are quickly converted to diploid mycelia, either by preestablished diploid mycelia or by incoming basidiospores. This process is apparently rapid and efficient for a number of other basidiomycetes (2,45). In addition to the establishment of new genets by sexual mating events, preexisting *Armillaria* genets may become apparent only as disease develops in the plantation (36). In such cases, the estimated age of *Armillaria* genets based on measures of vegetative growth rates would exceed that of the plantation (39).

At the site examined in this study, Armillaria root disease began killing seedlings in the study plantation in 1986, 2 yr after bare root seedlings were planted. By the end of 1992, 17% of the seedling population had been killed, and the spatial pattern of mortality suggested multiple developing disease foci. Because the plantation site had previously supported a mixed hardwood forest, the relationships among the responsible *Armillaria* genets, the spatial arrangement of these genets, and their origin were of interest. The objectives of this study were to 1) identify *Armillaria* species responsible for mortality of red pine seedlings at a study site in northern Michigan; 2) document the distribution of the *Armillaria* genets responsible for the observed disease; and 3) use markers in both mitochondrial and nuclear genomes of these genets to estimate genetic similarity and to study the genetic structure of the local *Armillaria* population.

MATERIALS AND METHODS

Culture collections representing the study site. The study site consisted of a 130- × 60-m red pine (*Pinus resinosa* Aiton) seedling plantation within a 1.2-ha clearing in a northern Michigan

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TABLE 1. Genet identification, source, and characteristics of *Armillaria* isolated from the red pine plantation vicinity

Species Genet	Number of isolates	Host source[x]	SIG[y]	L1-1 pattern	Mating type	mtDNA type
A. ostoyae						
b	18	PR	b	b	C4	3
	2	PR	nt[z]	b	nt	3
	1	sAR	b	nt	C4	3
d	1	PR	d	d	nt	4
e	2	PR	e	e	nt	9
f	17	PR	f	f	nt	3
g	1	PR	g	g	nt	3
	2	PR	nt	g	nt	3
h	1	PR	h	h	nt	6
	1	sPO	h	nt	C3	6
i	8	PR	i	i	nt	4
	2	PR	nt	i	nt	4
j	1	PR	j	j	nt	7
l	1	PR	l	l	nt	5
m	10	PR	m	m	nt	1
	1	sQR	m	nt	C5	1
o	13	PR	o	o	nt	2
	1	PR	nt	o	nt	2
p	1	PR	nt	p	nt	1
r	2	PR	nt	r	nt	1
t	1	PR	nt	t	nt	8
u	1	PR	nt	u	nt	5
v	1	PR	nt	v	nt	8
z	1	PR	nt	z	nt	5
A	2	sPO	A	nt	C6	9
O-1	1	sBP	nt	O-1	nt	4
O-2	1	sBP	nt	O-2	nt	3
O-4	1	sPO	nt	O-4	nt	1
O-5	1	sPO	nt	O-5	nt	5
A. sinapina						
O-3	1	sPO	nt	O-3	nt	V-1

[x]PR, seedling of *Pinus resinosa* Aiton and stump (s) of AR, *Acer rubrum* L.; BP, *Betula papyrifera* Marsh.; PO, *Populus grandidentata* Michx. or *P. tremulae* Michx.; or QR, *Quercus rubra* L.
[y]Somatic incompatibility group.
[z]Not determined.

hardwood forest (lat. 45°58′28″ N, long. 88°21′46″ W). Shortly after site clearing, 3-yr-old bare root red pine seedlings were planted at a density of about 0.3 seedlings per square meter in June 1984. Details of seedling mapping and site characteristics are given by Bruhn et al (9), and analysis of mortality patterns is ongoing (9,10). Moribund chlorotic seedlings were removed to the laboratory for isolation of *Armillaria*. General seedling chlorosis leading to death was consistently associated with Armillaria root disease. Pure cultures, obtained from mycelial fans under the bark in the root collar region of infected seedlings, were maintained on 2% malt extract agar (MEA). Isolates taken directly from infected pine seedlings are referred to below as "mortality isolates."

For this study, 87 mortality isolates were selected (Table 1). Isolates in the south quadrant and arbitrarily selected isolates representing the remaining area of the plantation were included in this population sample (Fig. 1). Additional cultures were obtained from the subhymenium or single basidiospores of five *Armillaria* fruit-bodies collected within the plantation and from five mycelial fan collections taken from aspen (*Populus* sp.) stumps located 400–800 m west of the study site (Table 1). Where monosporous isolates were available, either from fruit-bodies collected in the field or obtained through laboratory-grown fruit-bodies of mortality isolates (13), mating-type alleles were examined, and species were identified on the basis of mating interactions with North American species monosporous voucher isolates (3). Since monosporous isolates were not obtained from all genets at the site, a comprehensive analysis of the distribution of mating-type alleles was not possible. Where monosporous isolates were not available, mycelial isolates were identified to species by overall *Eco*RI restriction fragment patterns of mitochondrial DNAs (mtDNAs) (37) and/or through "diploid-haploid" confrontations (24) with monosporous voucher isolates. Genet identification was carried out independently by both analysis of somatic incompatibility (1,35) and restriction fragment patterns of nuclear and mtDNAs.

Identification of somatic incompatibility groups (SIGs). The 87 mortality isolates (collected during 1986–1989) constitute an arbitrary sample of a much larger collection, which has been

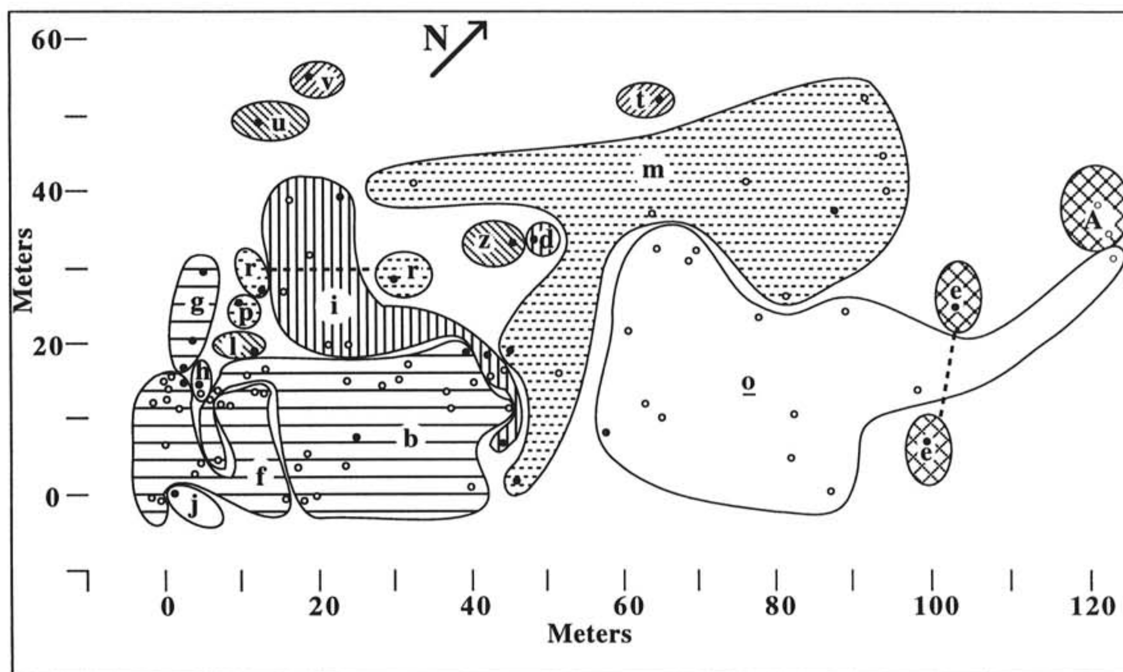

Fig. 1. Map of *Armillaria ostoyae* isolates (circles) from moribund red pine seedlings in plantation. Solid lines enclose isolates of each genet and are not intended to indicate the exact genet boundaries. Distribution of genets are based on both nuclear DNA fragment patterns with probe L1-1 and somatic incompatibility groups. Genets are distinguished by lowercase letters. The isolates represented by solid circles were used in detailed analysis of nuclear DNA fragment patterns (Table 2). Genets with identical mitochondrial DNA (mtDNA) types are indicated by unique shading patterns. Absence of genet shading indicates that the mtDNA type is unique in the population sample.

classified to 18 *A. ostoyae* (Romagnesi) Herink genets by somatic incompatibility tests. Mycelial confrontations of paired mycelial isolates were made and analyzed by procedures similar to those previously described (1). Inocula (1 mm^3) were placed approximately 2 mm apart at the center of petri plates containing 3% MEA. After incubation at room temperature for 3–4 wk, plates were examined for the presence of demarcation lines between the paired mycelia. Demarcation lines are evident as reduced growth and/or dark pigment deposition along the zone of interaction. Presence of a demarcation line indicates somatic incompatibility between mycelia of different genetic constitution. Absence of a discernible demarcation line indicates very similar or identical genetic constitution of the paired isolates. Because a complete set of mycelial interactions would have required $[n(n-1)]/2$ individual tests (3,741 pairings for $n = 87$ isolates), a strategy requiring fewer tests was adopted. In 1987, all isolates derived from each third of the plantation were tested with each other and classified into genets. Genets that overlapped plantation thirds were then consolidated by testing at least two representatives of each genet with representatives of each genet from the remaining two plantation thirds. From then on, the genet identity of each newly acquired isolate was determined by pairing it with a set of tester isolates representing all known genets from the plantation.

Identification of nuclear phenotypes by DNA restriction fragment pattern analysis. Techniques employed for extraction of genomic, nuclear, and mitochondrial DNAs from freeze-dried mycelia, agarose gel electrophoresis, Southern transfers, DNA-DNA hybridizations (62 C, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.5% sodium dodecyl sulfate), and autoradiography were as described previously (37). Genomic target DNAs from mortality isolates digested with *Eco*RI (BioCan, Mississauga, Ontario, Canada) were hybridized with radiolabeled L1-1 nuclear DNA probe. Mortality isolates were considered to belong to the same genet if they shared a common DNA fingerprint evident as the pattern of fragments hybridizing to L1-1. Probe L1-1 was selected from a genomic library of *A. ostoyae* constructed in lambda EMBL3 (4) because of its unusually strong hybridization signal with total radiolabeled nuclear DNA but weak signals

TABLE 2. Binary presence-absence data matrix[y] of *Eco*RI nuclear DNA fragment patterns for 30 isolates[z] representing 21 *Armillaria* genets

Probe	i			g			b		m			r		e																
Size	1	2	3	1	2	3	1	2	1	2	3	1	2	1	2	p	h	j	f	o	u	v	z	t	d	O-1	O-2	O-4	O-5	O-3
p2																														
1.8					9				1	1	1											1								
p8																														
8.7																9	1								1		1			1
7.9				1	1	1			1	1	1	1	1	1	1	1					1	1	1	1						
7.6									1			1	1			9											1			
7.4				1	1	1										9					1									
7.1												1	1									1					1			
6.8				1	1	1															1									
6.6																				1										
6.2									1	1	1															1				
6.1				1	1	1													1					1				1		
5.8									1	1	1															1				
5.4	1		1			1		1	1	1				1	1	1			1	1	1		1		1	1	1	1	1	1
5.2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	
4.9																			1	1	1									1
4.7				1	1	1	1	1												1	1									
4.6	1	1	1																						1	1		1		
4.4							1	1																						
4.3									1	1	1													1						
4.2		1																			1	1			1					
4.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
3.7																														1
3.6																														1
3.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
p7																														
5.5	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1
4.9	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3.2	9	1	1	1	1	1	1	1				1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3.0	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
p8																														
14.0	9																	1									1		1	1
11.5	9	1	1	1	1	1	1	1	1	1	1			1	1	1		1	1	1	1	1			1	1	1			
10.5	9	1	1				1	1				1	1			1			1											
8.6	9																1										1			
7.9	9															1	1		1	1					1	1				
7.3	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1		1	
5.7	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1		1	
5.3	9			1	1	9											1													1
4.8	9	1	1	1	1	9	1	1	1	1	1	1	1				1	1	1					1	1				1	
4.5	9					9	1	1	1	9	1									1	1	1	1	1	1					
p9																														
5.5	1		1			1		1	1	1				1	1	1			1	1	1		1	1	1		1	1	1	1
p10																														
1.8	1	1	1	1	1	1						1	1	1	1				1		1	1		1		1			1	1
1.3	1	1	1	1	1	1	1	1	1	1	1					1	1	1	1	1	1	1	1	1	1	1	1	1	1	

(*continued on next page*)

[y] *Eco*RI fragments (present [1], absent [blank], or missing data [9]) are identified by the hybridizing probe and fragment size in kilobase pairs. Underlined areas within the matrix indicate differences between isolates of the same genet.

[z] *Armillaria* genets are as designated in column 1 of Table 1. Arabic numerals indicate multiple isolates for a given genet. Four isolates of *A. ostoyae*, O-1, O-2, O-4, and O-5, and one isolate of *A. sinapina*, O-3, were from 400–800 m west of the plantation.

with mtDNA and nuclear ribosomal DNA. A preliminary study indicated that probe L1-1 hybridized to 17–25 distinct fragments and that overall fragment patterns were unique with different isolates of *A. ostoyae*.

Distribution of mtDNA types within and among genets. The mtDNA type of each *Armillaria* isolate was determined by probing *Eco*RI-digested genomic DNAs with radiolabeled *A. ostoyae* mtDNA as described by Smith and Anderson (37). MtDNAs from isolates with identical *Eco*RI fragment patterns but distinct L1-1 nuclear markers were subsequently examined for mtDNA restriction fragment polymorphisms with the enzymes *Bam*HI, *Bgl*II, *Pst*I, and *Pvu*II (BioCan).

Estimating genetic similarity of genets. A phenetic comparison of 21 *Armillaria* genets was made as follows. Isolates were arbitrarily selected to represent 16 of the 18 *A. ostoyae* genets detected in the red pine plantation and an additional four *A. ostoyae* genets and one *A. sinapina* Bérubé & Dessureault genet sampled at locations 400–800 m west of the plantation. Hybridization signals produced by each of 16 pUC18 plasmids containing anonymous nuclear DNA inserts from *A. gallica* Marmüller & Romagnesi (syn. *A. bulbosa* (Barla) Kile & Watling) isolate 453 were examined (39). Nine of these pUC18 probes and lambda probe L1-8 selected from *A. ostoyae* as described above hybridized to at least one polymorphic restriction fragment from the representative *Armillaria* isolates. *Eco*RI fragments of similar size detected by a given probe were scored as present (1) or absent (blank) in each isolate (Table 2). Comparisons across hybridization membranes were simplified by including multiple isolates of the same genet at different positions across the gel to monitor minor irregularities in fragment position. For example, three isolates of genet g were positioned in lanes 5, 8, and 17. In addition, fragment patterns were consistent, except where indicated in Table 2, in replicate experiments involving all probe-genet combinations. Pairwise Jaccard's similarity coefficients (S_J) (41) were computed for cluster analysis from the 83 consistent fragments coded in Table 2. Average linkage clustering (unweighted paired group method with arithmetic means [UPGMA]) (41) was used to cluster isolates by computing the relative average similarities of groups

TABLE 2. (*continued from preceding page*)

Probe	i			g			b		m			r		e																
Size	1	2	3	1	2	3	1	2	1	2	3	1	2	1	2	p	h	j	f	o	u	v	z	t	d	O-1	O-2	O-4	O-5	O-3
p18																														
9.2																														1
8.6	1	1	1	1	1	1	1	1									1									1				
7.9																														1
7.2																												1		
7.0				1	1	1										1				1	1								1	1
6.1														1	1															1
5.4																1	1	1	1	1	1	1	1	1	1	1	1			1
4.4	1	1	1	1	1	1	1	1	1	1	1	1	1			1	1		1					9	1	1		1	1	
3.7	1	1	1	1	1	1	1	1						1	1	1	1		1	1	1				1	1	1	1	1	
3.3									1	1	1	1	1	1	1	1	1	1					1	1						
3.1									1	1	1							9						1						
2.9																		9	1			1								
2.6	1	1	1	1	1	1	1	1	1	1	9	1	1	1	1		1	9	1						1	1		1	1	
p22																														
10.4	9	1	1						1	1	1					1		1				1							1	
9.6	9			1	1	1	1	1				1	1	1	1						1	1		1	1					
9.1	9																1						1							
8.8	9																											1		
7.7	9																			1										
6.1	9	1	1																											
5.8	9																													1
5.2	9																							1		1				
4.4	9			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1		1		1	1	1	
4.1	9																									1				
p23																														
7.7																														1
7.3																														1
7.2	1	1	1				1	1	1	1	1	1	1	1	1			1	1	1	1	1	1		1		1	1	1	
7.0				1	1	1										1	1													
6.1							1	1														1		1			1			
5.8				1	1	1										1					1					1				
5.6																				1										
L1-8																														
28.0																9			1					9						
18.5	9	1	1				1	1				1	1			9								9	1		1	1	1	
16.0	1	1	1				1	1	1	1	1			1	1	9	1	1	1	1	1	1	1	9		1	1	1	1	
14.0												1	1			9								9						
11.5				1	1	1					9			1	1	9	9				1			9						1
8.6											9					9	9							9						1
7.9				1	1	1					9		9		9	9								9						1
7.3													9		9	9								9						1
7.1				1	1	1							9		9	9			1	1	1			9						
6.3												1	9	9		9								9		1	1			
6.0	1	1	1	1	1	1	1	1	1	1	1		9	9	1	9	1	1		1	1	1	1	9	1	1	1	1	1	
5.5													9	9		9			1					9				1	1	
4.9	9		9	1	1	1							9			9					1			9					1	
4.7	9	1	9	1	1	1	1	1	1	1	1	1	9	1	1	9	1	1	1	1	1	1	1	9		1	1	1		1
4.6	9											1	9			9								9						
4.4	9								1	1	1		9			9				1		1		9	1					
3.9	9												9			9								9	1					

of isolates (Fig. 2). Jaccard's coefficients and cluster analysis were computed with the Corr and Cluster modules, and skewness and kurtosis of histograms were tested with Stats/Statistics in SYSTAT version 5.0 (44).

RESULTS

Identification and characterization of genets and species. Nuclear markers were examined in *Eco*RI-digested genomic DNAs of the 87 *Armillaria* isolates taken from moribund red pine plants at the study site. On the basis of DNA fingerprints revealed by probe L1-1, 17 distinct nuclear types were identified within the plantation (Table 1). An additional genet, A, was detected only as a distinct SIG and was not tested for nuclear restriction fragment length polymorphisms (RFLPs). These 18 nuclear types represent as many genets.

All 18 genets from within the plantation and four of the five genets (O-1, O-2, O-4, and O-5) from west of the plantation had mtDNA *Eco*RI fragment patterns that were greater than 60% similar to those of voucher isolates for *A. ostoyae*. MtDNA restriction fragment similarity values of greater than about 40% generally indicate conspecificity (37). Genet O-3 was tentatively identified as *A. sinapina* by virtue of an overall *Eco*RI mtDNA fragment pattern that was about 60% similar to voucher specimens 48-6 and 205-4. The species identifications based on mtDNA fragment patterns were consistent with sexual mating interactions and/or diploid-haploid interactions with monosporous voucher isolates. Species identifications were also consistent with colony morphology characteristics (14).

Restriction fragment patterns associated with five restriction enzymes indicated that only nine unique mtDNA types occur in the 22 *A. ostoyae* genets from the plantation vicinity (Table 1). On the basis of a mtDNA restriction site map of *A. ostoyae*

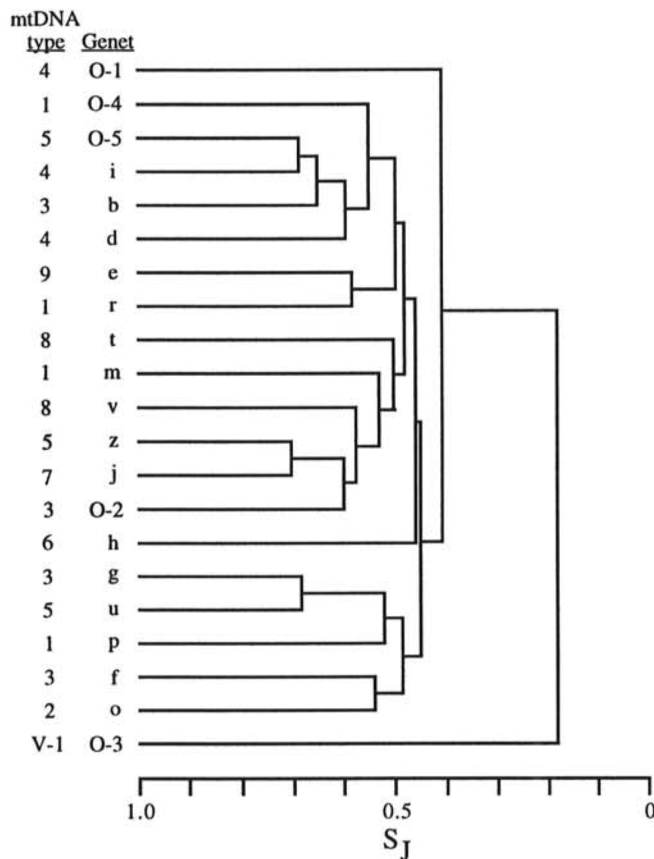

Fig. 2. Tree diagram constructed by average linkage clustering of Jaccard's similarity coefficients computed from *Eco*RI nuclear DNA fragment patterns of representative *Armillaria* isolates (Table 2). At left are genet designations as in Table 2 and mitochondrial DNA types as in Table 1.

(38), these unique types are the result of length mutations at several different regions of the mtDNA. Three of the nine mtDNA types (numbers 1, 3, and 5) were each associated with four distinct nuclear types; one mitochondrial type (number 4) with three nuclear types; two mitochondrial types (numbers 8 and 9) with two nuclear types; and three mitochondrial types (numbers 2, 6, and 7) with one nuclear type. As in a previous study (40), only a single mtDNA type was detected among all isolates of a genet.

Somatic incompatibility tests among the plantation mortality isolates were also used to identify genets. Any evidence of a demarcation line between confronted isolates resulted in their placement into different SIGs. For the 73 mortality isolates for which comparisons were possible (Table 1), there was perfect agreement between groupings on the basis of somatic incompatibility reactions and L1-1 nuclear types. On the basis of all data, there appears to be very little spatial overlap among genets of *A. ostoyae* in the plantation, and genet boundaries have been drawn accordingly in Figure 1. The *A. ostoyae* genets responsible for seedling mortality range in size from less than about 5 m (genets d, h, p, and l), to over 70 m in their longest dimension. The largest genets (m and o) occupy at least 0.14 and 0.15 ha, respectively.

Estimation of genetic similarity. For detailed analysis of nuclear DNA fragment patterns, one or more representative isolates were selected from 16 of the 18 *A. ostoyae* genets within the plantation in addition to the four *A. ostoyae* isolates and one *A. sinapina* isolate obtained from mycelial fans 400–800 m west of the plantation. Ten anonymous nuclear DNA fragments cloned from *A. bulbosa* and *A. ostoyae* provided 87 marker fragments in the set of 30 *Armillaria* isolates (Table 2). Aside from probe p10, which hybridized to only two size classes of fragments, and p2, which hybridized to two monomorphic fragments in addition to the polymorphism presented in Table 2, all other probes hybridized to multiple fragments (>10). Because of the complexity of fragment patterns, genetic interpretations were not attempted. In most cases, only fragments between 3 and 14 kilobase pairs (kbp) in size were included in the analysis. Beyond this size range, signal intensities were suboptimum for consistent scoring in some lanes. Multiple isolates from six genets were included to examine fragment pattern variations within genets and to standardize the scoring of fragments within and between gels. Fragment mobility was measured as the distance from the origin (sample well) to the nearest 0.5 mm, which is equivalent to about ±1.5% of the fragment size in the 4,000- to 9,000-base pair range. Fragments of similar mobility were scored as present in constructing the binary matrix (Table 2). As indicated (underlined) in Table 2, four fragments were not consistently represented throughout some genets. In particular, variation is evident at 5.4 kbp with probe p4 and at 5.5 kbp with probe p9. In an independent experiment (not shown) with probe p4 and DNAs from 5, 5, and 11 isolates of genets i, m, and b, respectively, the fragments at 5.4 kbp did not always show up in repeated tests with the same isolate. The basis for these minor fragment polymorphisms within *Armillaria* genets was not determined. Because of a lack of reproducibility, these four fragments were not included in subsequent analysis of genetic similarity.

The relative similarities of *Armillaria* genets (based on nuclear DNA fragment patterns) are presented as a dendrogram (Fig. 2) and as a composite frequency histogram (Fig. 3). The histogram distinguishes sets of pairwise S_J comparisons made between and within species. Comparisons between genets with identical mtDNA types are also identified in Figure 3.

DISCUSSION

Concepts of fungal individualism have intrigued biologists for over 50 yr. The "unit mycelium" concept (11,29) proposed that the "individual" basidiomycete might consist of a genetic mosaic. By this concept, a physiologically integrated individual, or unit mycelium, might incorporate a large number of different nuclear genotypes. Studies using genetic markers to delimit individuals

do not support this concept. Rather, basidiomycete individuals are observed to maintain discrete territories (30,31) and can be genetically stable over long periods of time (39). In this regard, they behave in much the same way as distinct genetic individuals in other taxa for which the physical boundaries of individuals are more easily defined. The distinct boundaries between adjacent genets of *Armillaria* are probably caused, at least in part, by the prevalence of somatic incompatibility in local populations.

We have identified *Armillaria* genets by two methods, somatic compatibility testing and assay of RFLP markers in nuclear DNA (Table 1). Identification of genets was completely consistent for all 73 *A. ostoyae* isolates with which both methods were used. Such close agreement is expected where there is sufficient polymorphism with respect to the two criteria and where mating in the population approximates a random pattern. Where these conditions are not met, disagreement between the two criteria in the identification of genets is expected to become more frequent. Indeed, cases where members of one SIG show polymorphism for other markers and where members of two SIGs show no polymorphism for other markers are well known (18,34). One continuing source of uncertainty with respect to the reliability of somatic compatibility reactions in identifying genets is that the genetic basis of somatic incompatibility in *Armillaria* is unknown. In *Heterobasidion annosum* (Fr.:Fr.) Bref., at least three or four loci appear to control somatic incompatibility, and multiple alleles occur at least at one locus (15).

The apparent sizes of genets depicted in Figure 1 are probably conservative. Genets that occur along a plantation edge may well extend beyond the plantation. In a sense, the plantation seedlings have served as baits to map genet distributions in the plantation. Therefore, what proportion of genets in the plantation could have originated between the time the plantation was established (1984) and the end of the sampling period (1989)? Growth rate estimates for *Armillaria* species range up to 2.0 m/yr in temperate climates (35). Growth rates at the Michigan study site are estimated to be lower, about 0.2 m/yr for *A. gallica* (39). Growth rates for the *A. ostoyae* genotypes at the site have not been determined.

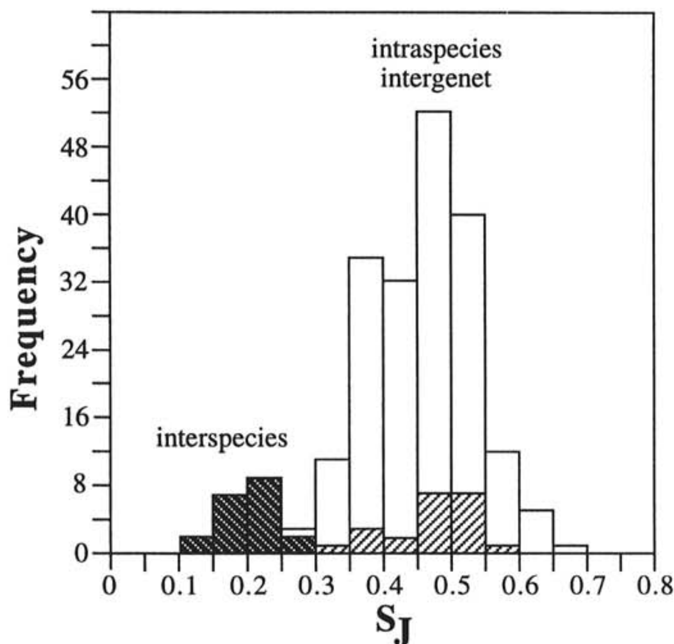

Fig. 3. Frequency histogram of Jaccard's similarity coefficients for pairwise comparisons of *Armillaria* genets represented in the binary data matrix (Table 2). Interspecies comparisons were made for the single isolate of *A. sinapina* with one isolate of each *A. ostoyae* genet. Intraspecies-intergenet comparisons are among *A. ostoyae* genets only. The subset of these comparisons, for genets with identical mitochondrial DNA types, is represented by the inset crosshatched area within the intraspecies-intergenet histogram.

With a radial growth rate of 1.0 m/yr, a genet would achieve a maximum size of 10 m in its longest dimension over 5 yr of unimpeded growth. This is within the size range of the smallest genets, d, h, l, p, and z, within the plantation. Given the same average growth rate, we would conclude that the five largest genets, b, f, i, m, and o, which occupy the most of the plantation area, must have been established prior to 1984. For these genets to have been established after 1984, growth rates in excess of 7 m/yr would be required to explain the existing spatial territories. From Figure 1, it also appears that at least the large genets tend to occupy a territory to the exclusion of others. This would mean that the presence of one genet might impede the progress of another as the mycelia meet and interact in roots. Therefore, a genet's actual rate of expansion may be much lower than its potential rate along borders with neighboring genets. If so, then estimates of genet age based on unimpeded growth are likely to be low.

Levels of genetic similarity among 21 *Armillaria* genets in the plantation vicinity were estimated through scoring the presence or absence of anonymous nuclear DNA fragments. The S_J values presented in Figure 2 occur in two main clusters. First, *A. sinapina* genet O-3 links into the cluster of *A. ostoyae* genets below a coefficient of 0.2. The relatively low S_J value between the two species is expected, given previous evidence for genetic divergence among North American *Armillaria* species (5,6,37). The second cluster of S_J values in Figure 2 ranges from 0.4 to 0.7 and represents comparisons between genets of *A. ostoyae*.

On the basis of DNA fingerprint patterns, mitotic and meiotic lineages of higher fungi are usually easily distinguished (8,22,23, 25,27,28). Occasionally in these studies, isolates are encountered that exhibit only minor differences in fragment pattern. It is difficult to determine whether such isolates represent mitotic lineages that contain somatic mutations or closely related genets that arose through sexual reproduction. In this study, isolates that were unambiguously identified as belonging to the same genet in the initial analysis with the L1-1 probe and on the basis of somatic incompatibility testing (Table 2) showed minor fragment differences in subsequent tests with other probes. Three observations indicate that these *A. ostoyae* isolates are from the same mitotic cell lineage. First, the intragenet variation was evident in only four fragments. It was observed that the presence-absence state of one of these variable fragments (at 5.4 kbp with probe p4) was not consistent in an independent experiment with isolates from genets b, m, and i. Although not determined here, it is possible that minor variation of this sort is caused by partial digestion or persistent chemical modification of enzyme recognition sites (46). Second, aside from these occasional differences in fragment pattern, isolates from the same genet were identical for the majority of fragments examined, including those that were rare in the population sample at large. For diploid organisms such as *A. ostoyae,* the possibility that unrelated genets are similar by chance alone can be excluded, provided that fragment frequencies in the population sample are low and approximate those of the effective breeding population. Third, this minor variation occurs among somatically compatible isolates that are located close to each other at the site.

The repeated observations in this and other studies (20,24,34, 40,42) that nuclear genotypes are not discontinuously distributed is inconsistent with the movement of mitotic propagules of *Armillaria* and suggests that a diploid genotype spreads mainly by mitotic growth and radial extension. There is evidence, however, that genets at the site were initiated by sexual mating events. Among the 22 *A. ostoyae* genets sampled in the pine plantation vicinity, only nine unique mtDNA types were detected. On the basis of a restriction site map and a survey of 17 unique mtDNAs from across North America (38), differentiation of more than 1,300 distinct mtDNA types may be possible for *A. ostoyae*, assuming that size variants identified at different regions along the molecule can occur together in all combinations. In all, 13 mtDNA types were evident in a sample of 13 *A. ostoyae* isolates examined from Newfoundland, Vermont, Ontario, Washington, and British Columbia. Evidently, mtDNA repeat frequency

increases within a local area, characteristic of locally distributed mtDNA lineages (7).

The intraspecies-intergenet nuclear similarities represented in Figures 2 and 3 can be examined with reference to both mtDNA types and the spatial distribution of genets. Each of the 10 *A. ostoyae* genets for which multiple isolates were examined had only one characteristic mtDNA type. In agreement with previous work (40), this indicates that the mtDNA of *A. ostoyae* is functionally inherited from a single parent in nature. Given the uniparental mode of inheritance, mtDNA lineages also represent "maternal" genet lineages. Within these maternal lineages, nuclear marker similarity coefficients range from 0.33 to 0.58, indicating considerable mixis of nuclear markers (Fig. 2) and supporting a view that *A. ostoyae* genets originated at the site through sexual mating events.

The distribution of S_J values for pairwise comparisons among genets containing identical mtDNA types can be examined through a frequency histogram (Fig. 3). Although the number of comparisons of genets containing identical mtDNA types is small and the frequency distribution does not significantly differ from that of the intraspecies-intergenet comparisons (Fig. 3), it is interesting that this component of the histogram is skewed ($g_1 = -0.660$) toward similarity values higher than those of the overall intraspecies-intergenet histogram ($g_1 = -0.015$). It is also interesting that the genet comparisons giving the highest similarity values did not contain identical mtDNA types. Although it is expected that, on average, individuals of a given mtDNA lineage should be more closely related than those with different mtDNA types, the effects of outbreeding may obscure these trends within relatively few generations.

In Figure 3, the frequency distribution of intergenet S_J values among *A. ostoyae* is platykurtic ($g_2 = -0.292$). Nevertheless, the mean ($\pm$SD) S_J value from comparisons between *A. ostoyae* genets outside and inside the plantation (0.441 ± 0.079) is not significantly different from the mean S_J for comparisons of genets found within the plantation (0.468 ± 0.075). Therefore, the breeding population at the Michigan site likely extends beyond a distance of 1 km. This may also be inferred from the distribution of mtDNAs in the area. For example, mtDNA type 5 occurs in genet O-5, which is separated by about 800 m from genets l, u, and z, which also contain mtDNA type 5. Additional samples from distances in excess of 1 km would be required to determine the extent of the local breeding population. Rizzo and Harrington (34) also found no distinction in similarity values when isozyme patterns were compared among *A. ostoyae* SIGs within and between study plots located 10 km apart. These observations are consistent with the possibility that *Armillaria* breeding populations are panmictic over a considerable area. However, it may be that definitive nuclear markers, such as sequences of short, variable regions, need to be developed to provide the fine resolution required for studies on *Armillaria* population structure.

In summary, *Armillaria* isolates infecting red pine seedlings after stand conversion of a northern Michigan hardwood forest were examined. All pathogenic isolates on red pine seedlings were identified as *A. ostoyae.* On the basis of estimates of size and shape, it can be concluded that at least some of the detected genets were already established at the time of stand conversion. The largest *A. ostoyae* genets (which are therefore responsible for most mortality) probably predate even the existing hardwood forest, which replaced a forest of mostly pine after a 1928 fire. It should be noted that while individual *A. ostoyae* genets were found to infect seedlings several meters apart, rhizomorphs collected from soil in the immediate vicinity were all identified as *A. gallica,* a much less aggressive pathogen also represented throughout the plantation by a nonoverlapping mosaic of genets. The spatial overlap between *A. ostoyae* and *A. gallica* may be explained by resource partitioning through niche specialization. Similarly overlapping species mosaics have been reported in New Hampshire (34).

That distinct mtDNA types were shared within several groups of *A. ostoyae* genets within the local area was unexpected because mtDNA is highly polymorphic in *A. ostoyae.* It is likely that each of these groups represents a portion of a mtDNA (maternal) lineage and that many genets within this population originated from locally distributed basidiospores. Clearly, analysis of mtDNA fragment patterns should not be used as the sole criterion for distinguishing genets at the population level. Considerable mixis of nuclear genomes was evident in all maternal lineages examined at the site. To account for the observations of mtDNA distributions, we hypothesize that sex in *Armillaria* involves functionally anisogamous gametes, whereby a newly established "female" monokaryon is fertilized by an incoming compatible basidiospore or germling. The cytoplasmic input of this "male" gamete would be minimal and likely lost from the resultant fertile mycelium. Finally, the distribution of both nuclear and mtDNA markers is consistent with the possibility that the local breeding population of *A. ostoyae* around the study site extends beyond 1 km. It is anticipated that a comparative analysis of populations beyond this distance would provide estimates of local inbreeding levels and the geographic range of breeding populations.

LITERATURE CITED

1. Adams, D. H. 1974. Identification of clones of *Armillaria mellea* in young-growth Ponderosa pine. Northwest Sci. 48:21-28.
2. Adams, T. J. H., Williams, E. N. D., Todd, N. K., and Rayner, A. D. M. 1984. A species-specific method of analysing populations of basidiospores. Trans. Br. Mycol. Soc. 82:359-361.
3. 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.
4. 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 43:1652-1662.
5. Anderson, J. B., Petsche, D. M., and Smith, M. L. 1987. Restriction fragment polymorphisms in biological species of *Armillaria mellea.* Mycologia 79:69-76.
6. Anderson, J. B., and Stasovski, E. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria.* Mycologia 84:505-516.
7. Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A., and Saunders, N. C. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst. 18:489-522.
8. Brown, J. K. M., O'Dell, M., Simpson, C. G., and Wolfe, M. S. 1990. The use of DNA polymorphisms to test hypotheses about a population of *Erysiphe graminis* f. sp. *hordei.* Plant Pathol. 39:391-401.
9. Bruhn, J. N., Mihail, J. D., and Pickens, J. B. 1994. Spatial dynamics of *Armillaria* genets in *Pinus resinosa* plantations established on hardwood sites in northern Michigan. Pages 437-446 in: Proc. Int. Conf. Root Butt Rots, 8th. J. D. Stenlid, ed. International Union of Forestry Research Organisations (IUFRO) Working Party S2.06.01., Swedish Agricultural University, Uppsala.
10. Bruhn, J. N., Pickens, J. B., and Mihail, J. D. 1992. Epidemiology of Armillaria root disease in red pine plantations. (Abstr.) Phytopathology 82:1152.
11. Buller, A. H. R. 1931. Researches on Fungi. Vol 4. Hafner, New York.
12. Guillaumin, J.-J., Anderson, J. B., and Korhonen, K. 1991. Life cycle, interfertility, and biological species. Pages 10-20 in: Armillaria Root Disease. C. G. Shaw III and G. A. Kile, eds. U.S. Dep. Agric. For. Serv. Agric. Handb. 691.
13. Guillaumin, J.-J., Berthelay, S., and Savin, V. 1983. Étude de la polarité sexuelle des Armillaires du groupe *mellea.* Cryptogam. Mycol. 4:301-319.
14. Guillaumin, J.-J., Mohammed, C., and Berthelay, S. 1989. *Armillaria* species in the northern temperate hemisphere. Pages 27-43 in: Proc. Int. Conf. Root Butt Rots, 7th. D. J. Morrison, ed. International Union of Forestry Research Organisations (IUFRO) Working Party S2.06.01., Forestry Canada, Victoria.
15. Hansen, E. M., Stenlid, J., and Johansson, M. 1993. Genetic control of somatic incompatibility in the root-rotting basidiomycete *Heterobasidion annosum.* Mycol. Res. 97:1229-1233.
16. Harper, J. L. 1977. Population Biology of Plants. Academic Press, New York.
17. Hood, I. A., Redfern, D. B., and Kile, G. A. 1991. *Armillaria* in planted hosts. Pages 122-149 in: Armillaria Root Disease. C. G. Shaw III and G. A. Kile, eds. U.S. Dep. Agric. For. Serv. Agric. Handb. 691.
18. Jacobson, K. M., Miller, O. K., and Turner, B. J. 1993. Randomly amplified polymorphic DNA markers are superior to somatic incompatibility tests for discriminating genotypes in natural populations

of the ectomycorrhizal fungus *Suillus granulatus*. Proc. Natl. Acad. Sci. USA 90:9159-9163.
19. Kays, S., and Harper, J. L. 1974. The regulation of plant and tiller density in a grass sward. J. Ecol. 62:97-105.
20. Kile, G. A. 1983. Identification of genotypes and the clonal development of *Armillaria luteobubalina* Watling and Kile in Eucalypt forests. Aust. J. Bot. 31:657-671.
21. Kile, G. A., McDonald, G. I., and Byler, J. W. 1991. Ecology and disease in natural forests. Pages 102-121 in: Armillaria Root Disease. C. G. Shaw III and G. A. Kile, eds. U.S. Dep. Agric. For. Serv. Agric. Handb. 691.
22. Kohli, Y., Morrall, R. A. A., Anderson, J. B., and Kohn, L. M. 1992. Local and trans-Canadian clonal distribution of *Sclerotinia sclerotiorum* on canola. Phytopathology 82:875-880.
23. Kohn, L. M., Stasovski, E., Carbone, I., Royer, J., and Anderson, J. B. 1991. Mycelial incompatibility and molecular markers identify genetic variability in field populations of *Sclerotinia sclerotiorum*. Phytopathology 81:480-485.
24. Korhonen, K. 1978. Interfertility and clonal size in the *Armillariella mellea* complex. Karstenia 18:31-42.
25. Levy, M., Romao, J., Marchetti, M. A., and Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95-102.
26. Livingston, W. H. 1990. *Armillaria ostoyae* in young spruce plantations. Can. J. For. Res. 20:1773-1778.
27. McDonald, B. A., and Martinez, J. P. 1991. DNA fingerprinting of the plant pathogenic fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*). Exp. Mycol. 15:146-158.
28. Milgroom, M. G., Lipari, S. E., and Powell, W. A. 1992. DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. Genetics 131:297-306.
29. Raper, J. R. 1966. Genetics and Sexuality in Higher Fungi. Ronald Press, New York.
30. Rayner, A. D. M. 1991. The phytopathological significance of mycelial individualism. Annu. Rev. Phytopathol. 29:305-323.
31. Rayner, A. D. M., and Todd, N. K. 1982. Population structure in wood-decomposing basidiomycetes. Pages 109-128 in: Decomposer Basidiomycetes: Their Biology and Ecology. J. C. Frankland, J. N. Hedger, and M. J. Swift, eds. Cambridge University Press, Cambridge.
32. Rishbeth, J. 1978. Infection foci of *Armillaria mellea* in first-rotation hardwoods. Ann. Bot. (London) 42:1131-1139.
33. Rishbeth, J. 1985. Infection cycle of *Armillaria* and host response. Eur. J. For. Pathol. 15:332-341.
34. Rizzo, D. M., and Harrington, T. C. 1993. Delineation and biology of clones of *Armillaria ostoyae, A. gemina* and *A. calvescens.* Mycologia 85:164-174.
35. Shaw, C. G., III, and Roth, L. F., 1976. Persistence and distribution of a clone of *Armillaria mellea* in a ponderosa pine forest. Phytopathology 66:1210-1213.
36. Shaw, C. G., III, Stage, A. R., and McNamee, P. 1991. Modeling the dynamics, behavior, and impact of Armillaria root disease. Pages 150-156 in: Armillaria Root Disease. C. G. Shaw III and G. A. Kile, eds. U.S. Dep. Agric. For. Serv. Agric. Handb. 691.
37. Smith, M. L., and Anderson, J. B. 1989. Restriction fragment length polymorphisms in mitochondrial DNAs of *Armillaria:* Identification of North American biological species. Mycol. Res. 93:247-256.
38. Smith, M. L., and Anderson, J. B. 1994. Mitochondrial DNAs of the fungus *Armillaria ostoyae:* Restriction map and length variation. Curr. Genet. 25:545-553.
39. 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.
40. Smith, M. L., Duchesne, L. C., Bruhn, J. N., and Anderson, J. B. 1990. Mitochondrial genetics in a natural population of the plant pathogen *Armillaria.* Genetics 126:575-582.
41. Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy. W. H. Freeman, San Francisco.
42. Ullrich, R. C., and Anderson, J. B. 1978. Sex and diploidy in *Armillaria mellea.* Exp. Mycol. 2:119-129.
43. Whitney, R. D. 1989. Root rot damage in naturally regenerated stands of spruce and balsam fir in Ontario. Can. J. For. Res. 19:295-308.
44. Wilkinson, L. 1989. SYSTAT: The System for Statistics. SYSTAT, Inc., Evanston, IL.
45. Williams, E. N. D., Todd, N. K., and Rayner, A. D. M. 1984. Characterization of the spore rain of *Coriolus versicolor* and its ecological significance. Trans. Br. Mycol. Soc. 82:323-326.
46. Zolan, M., and Pukkila, P. 1986. Inheritance of DNA methylation in *Coprinus cinereus.* Mol. Cell Biol. 6:195-200.