Genetic Identification of Clones of *Armillaria mellea* in Coniferous Forests in Washington

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ABSTRACT

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Armillaria mellea establishes subterranean clones that traverse multiple hosts. The sizes of clones in coniferous forests in Washington were examined in this study. Incompatibility alleles were assayed for 13 fruiting bodies from three separate sites; these alleles were employed as markers to determine the clonal identity of each fruiting body. Only one clone was detected at each of the sites where multiple collections were made. Extensive clonal development of *A. mellea* on these sites is indicated, which contrasts with the much smaller sized clones in a maple (*Acer saccharum*) sugar bush in Vermont. The methods described are useful for identifying biological species and estimating clonal relationships of *A. mellea* present in a given region.

Additional key words: fungal genetics, incompatibility alleles, mating type distribution, shoestring root rot.

The potential for extended subterranean growth by *Armillaria mellea* (Vahl ex Fr.) Kummer creates the possibility for development of clones that traverse multiple hosts. A more accurate assessment of the nature of dispersal and infection, including the possibility of clones in local environs, is critical for understanding the nature of this organism and for controlling the disease it causes. The ability to distinguish the genetic individuality of isolates is essential to this endeavor. Adams (1) and Shaw and Roth (5) observed an interaction ("line of demarcation") when pairing certain isolates of *A. mellea*. Paired isolates that produced lines of demarcation were interpreted as belonging to different clones, whereas paired isolates that lacked demarcation were considered to be of the same clone. This interpretation differs from that of Ullrich and Anderson (7) in their genetic studies of *A. mellea* in maple (*Acer saccharum*) sugar bushes in New England (see biological species below). According to these interpretations, Adams (1) and Shaw and Roth (5) reported clones of extensive acreage in ponderosa pine forests in Oregon and Washington, whereas Ullrich and Anderson (7) reported much smaller clones in the maple forests. Interest in a definitive resolution to these differences has fostered the following collaboration.

A. mellea is a bifactorial heterothallic fungus (2–4,7) with multiple alleles (7). Compatibility between monosporous isolates is determined by macroscopic and microscopic criteria other than the formation of clamp connections and dikaryotic cells (the criteria commonly applied for most Homobasidiomycetes). Unpaired monosporous isolates and incompatible pairings of monosporous isolates produce colonies with fluffy aerial morphology. Compatible pairings yield colonies with depressed, crustose morphology. Isolates from fruiting body tissues, or vegetative material in nature, also produce crustose colonies resembling the mycelium resulting from compatible pairings.

A recent study of Anderson and Ullrich (2) indicated that *A. mellea* is a complex of at least ten reproductively isolated groups (biological species) in North America. Intersterility between the biological species is absolute, but within each biological species compatibility is governed by bifactorial heterothallism. Ullrich and Anderson (7) observed a "raised brown line of mycelium" at the juncture of colonies of different biological species in culture. This reaction is evident in confrontations of either monosporous isolates or isolates from fruiting body tissues, but is more distinct in the latter. The raised brown line is absent at colony junctures of members of the same biological species. Isolates from the same biological species, but possessing different sets of incompatibility alleles (and thus representing different clones) produce no raised line.

We believe that the raised line observed by Ullrich and Anderson (7) may be equivalent to the line of demarcation observed by Adams (1) and Shaw and Roth (5). Therefore, the absence of a line of demarcation would not be sufficient to determine that isolates belong to the same clone. In either case, bona fide mating interactions provide a more critical assay of clonal relationships.

In this study the incompatibility alleles of monosporous isolates

TABLE 1. Location, host, and clonal designation of fruiting bodies assayed

Fruiting body	Location	Host	Clonal designation
131	Site I, Meadow Butte, Klickitat Co., WA	*Pinus ponderosa*	A
132	Site I, 450 m from 131	*Pinus ponderosa*	A
133	Site II, near Mt. Adams, WA, (13 km from Meadow Butte)	*Pinus ponderosa*	B
134	Site II, 100 m from 133 and 135	*Pinus contorta*	B
135	Site II, 100 m from 133 and 134	*Abies grandis*	B[a]
113	Site II (113–119 collected	*Abies grandis*	B[a]
114	from within 1 m to	*Abies grandis*	B
115	400 m of one another)	*Abies grandis*	B
116		*Abies grandis*	B
117		*Abies grandis*	B
118		*Abies grandis*	B
119		*Abies grandis*	B
136	Site III East of Klickitat River (18 km from Site II, 22 km from Site I)	*Pinus ponderosa*	C

[a]Owing to small sample size and random assortment, not all incompatibility alleles were recovered from this fruiting body. Analysis is consistent with, but does not prove, clonal designation.

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derived from 13 fruiting bodies were employed as naturally occurring genetic markers for distinguishing clones. This technique already has been used to identify clones of *A. mellea* in a maple sugar bush in Vermont (7). In such an analysis, differing alleles indicate different clones, whereas identical alleles are assumed to indicate a single clone. Although the former interpretation derives directly from the definition of clone, the latter case could be due to spore dispersal and random mating rather than clonal development. The large number of alleles in *A. mellea* (7) and the distribution patterns of basidiospores in Homobasidiomycetes (6) favor the interpretation of identical alleles as multiple isolations of the same clone.

MATERIALS AND METHODS

Sporocarps were collected in mixed conifer forests near Glenwood, Washington. The location and host upon which each sporocarp was collected is recorded in Table 1. A sporefall of each was collected on paper. Monosporous cultures from each sporeprint were isolated and maintained on the enriched malt extract medium (SR) of Shaw and Roth (5). To assay mating interactions and determine mating types, up to ten monosporous isolates from a fruiting body were paired in all combinations. For pairings, a 1 mm^3 piece of inoculum was placed 5–10 mm from similar inoculum of the mate on SR. Pairings were incubated 4–6 wk at room temperature in darkness. Then each pairing was scored for compatibility or incompatibility on the basis of macroscopic appearance (ie, depressed crustose vs. fluffy aerial morphology, respectively). Mating-type designations were assigned on the basis of these reactions, and two to four tester strains (each of distinct mating type) were selected from each fruiting body for pairing with testers from the other fruiting bodies. Table 2 illustrates specimen mating interactions between testers from fruiting bodies possessing: identical sets of mating type alleles; completely different sets of mating type alleles; and one allele in common.

RESULTS AND DISCUSSION

Table 1 lists geographical and host origins of all collections used in this study. Clonal designation of each fruiting body, as determined by assay of incompatibility alleles, also is provided in

	136-8 (A1B1)	136-10 (A2B2)	136-5 (A1B2)	136-9 (A2B1)	113-5 (A3B3)	113-4 (A3B2)	114-7 (A4B3)	114-9 (A3B2)	115-4 (A3B3)	115-2 (A4B2)	116-1 (A4B3)	116-8 (A3B2)	117-5 (A4B3)	117-8 (A3B2)	118-4 (A3B3)	118-5 (A4B2)	119-1 (A4B2)	119-6 (A3B3)	131-1 (A4B4)	131-7 (A5B5)	132-4 (A5B4)	132-7 (A4B5)	133-4 (A3B2)	133-9 (A4B3)	133-5 (A3B3)	133-7 (A4B2)	134-1 (A4B3)	134-9 (A3B2)	134-2 (A3B3)	134-5 (A4B2)	135-1 (A3B2)	135-2 (A3B2)
136-8 (A1B1)	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+						
136-10 (A2B2)		-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	-	+			+	+	-	+	+	-						
136-5 (A1B2)			-	+	+	-	+	-	+	-	+	-	+	-	+	-	-	+			+	+	-	+	+	-						
136-9 (A2B1)				-	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+						
113-5 (A3B3)					-	-	-	-	-	+	-	-	-	-	-	+	+	-														
113-4 (A3B2)						-	+	-	-	-	+	-	+	-	-	-	-	*														
114-7 (A4B3)							-	+	-	-	-	+	-	+	-	-	-	-														
114-9 (A3B2)								-	-	-	+	-	+	-	-	-	-	-														
115-4 (A3B3)									-	+	-	-	-	-	-	+	+	-														
115-2 (A4B2)										-	-	-	-	-	+	-	-	+														
116-1 (A4B3)											-	+	-	+	-	-	-	-														
116-8 (A3B2)												-	+	-	-	-	-	-														
117-5 (A4B3)													-	+	-	-	-	-			+	-	+	-	-	-						
117-8 (A3B2)														-	-	-	-	-			+	+	-	+	-	-						
118-4 (A3B3)															-	+	+	-														
118-5 (A4B2)																-	-	+														
119-1 (A4B2)																	-	+			+	-	-	-	+	-						
119-6 (A3B3)																		-			+	+	-	-	-	+						
131-1 (A4B4)																			-	+	-	-	+	-	+	-	-	+	+	-	+	+
131-7 (A5B5)																				-	-	-	+	+	+	+	+	+	+	+	+	+
132-4 (A5B4)																					-	+	+	+	+	+	+	+	+	+	+	+
132-7 (A4B5)																						-	+	-	+	-	-	+	+	-	+	+
133-4 (A3B2)																							-	+	-	-	+	-	-	-	-	-
133-9 (A4B3)																								-	-	-	-	+	-	-	+	+
133-5 (A3B3)																									-	+	-	-	-	+	-	-
133-7 (A4B2)																										-	-	-	+	-	-	-
134-1 (A4B3)																											-	+	-	-	*	+
134-9 (A3B2)																												-	-	-	-	-
134-2 (A3B3)																													-	+	-	-
134-5 (A4B2)																														-	-	-
135-1 (A3B2)																															-	-
135-2 (A3B2)																																-

Fig. 1. Mating interactions between pairings of monosporous tester strains derived from sporeprints of each *Armillaria mellea* fruiting body included in the study. Number preceding the hyphen designates fruiting body of origin (Table 1). Number following the hyphen designates the tester derived from that fruiting body. Allelic designations of testers appear in parentheses. Interactions: +, compatible; −, incompatible; *, indeterminate in two replications; blank space indicates that pairing was not performed.

TABLE 2. Specimen mating interactions[a] between monosporous tester strains isolated from fruiting bodies of *Armillaria mellea* that posess various combinations of mating type alleles

	Identical mating type alleles				No mating type alleles in common				One mating type allele in common			
	A1 B1	A2 B2	A1 B2	A2 B1	A3 B3	A4 B4	A3 B4	A4 B3	A1 B3	A3 B4	A1 B4	A3 B3
A1 B1	−	+	−	−	+	+	+	+	−	+	−	+
A2 B2	+	−	−	−	+	+	+	+	+	+	+	+
A1 B2	−	−	−	+	+	+	+	+	−	+	−	+
A2 B1	−	−	+	−	+	+	+	+	+	+	+	+

[a]Interactions: +, compatible; and −, incompatible.

Table 1. The results of the mating interactions of testers by which the clonal orgainization was determined are presented in Fig. 1. For each locality in which several collections were made, only one clone was found. No line of demarcation or raised line was formed in any of the parings of monosporous isolates. These results (based on assay of incompatibility alleles) support the conclusions of Adams (1) and Shaw and Roth (5) that clonal development of *A. mellea* is common and that individual clones are extensive in certain coniferous forests of the Pacific Northwest.

The extent of clones found in this study of *A. mellea* in the western conifer forests contrasts markedly with the size of clones found in a maple sugar bush of Vermont (7). In the earlier eastern study, six clones were found in close proximity to one another and their dimensions were considerably smaller (maximum distance observed between isolates of a single clone was 50 m) than those of clones in the present study. In this study, the greatest distances between samples of each clone that were assayed more than once were: 450 m, site I; and 400 m, site II; however, the maximum size of each clone remains undetermined. The larger size of clones in the Pacific Northwest may be explainable by moisture conditions. In the Pacific Northwest (east of the Cascade Mountains) fruiting is infrequent because of sporadic rainfall, whereas in the eastern USA basidiocarps develop in most areas every fall. Alternatively, reduced rainfall may decrease the number of infections by providing moisture conditions unfavorable for germination. In either case, the large clones of the Pacific Northwest simply may reflect a reduced availability of spores capable of establishing new foci of infection. Therefore, large clones would develop from continued vegetative growth in the absence of new colonies from recently introduced propagules.

All isolates in this study (from three separate locations) belong to only one of the ten intersterile groups identified by Anderson and Ullrich (2). Adams (1) and Shaw and Roth (5) reported several groups of isolates that formed lines of demarcation with one another. If the formation of a line of demarcation indicates that isolates belong to different biological species, then the results of Adams (1) and Shaw and Roth (5) might imply that several biological species rather than clones were represented in those studies. This discrepency may reflect different sampling methods. Adams (1) and Shaw and Roth (5) obtained isolates from rhizomorphs, mycelial fans, and infected root tissues. In contrast, all isolates in this study originated from basidiospores. Therefore, any biological species that were not fruiting at the time of sampling remained undetected.

The assay of incompatibility alleles is a definitive means for determining the local distribution of *A. mellea* clones. This method requires monosporous *A. mellea* isolates which, because laboratory fruiting is rare, are obtainable only from natural fruiting. This method does not exclude the possibility of additional biological species existing vegetatively on the sites at the time of sampling. The distinction between biological species is discernible by the "raised brown line of mycelium." Therefore, a reasonably comprehensive understanding of the distribution of *A. mellea* within a region can be discerned by an examination of both sexual products and vegetative isolations.

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