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NUCLEAR MIGRATION IN DIPLOID–HAPLOID PAIRINGS OF ARMILLARIA OSTOYAE

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ABSTRACT

Diploid-haploid pairings to identify field isolates of Armillaria to species generally rely on morphological differences between diploid and haploid colonies (flat and fluffy colonies, respectively) and morphological changes in haploid tester colonies. Presumably, the change in morphology of the haploid tester is due to diploidization. Isozyme markers, aconitase and glucosephosphate isomerase, were used to follow nuclear migration from putatively diploid isolates into a haploid tester strain of A. ostoyae. After pairing diploid field isolates with a haploid tester for 3 or 4 weeks, subcultures were taken from the haploid tester at 2 and 10 mm from the confrontation zone. Subcultures from 2 mm were mostly flat; subcultures from 10 mm were fluffy, flat, or fluffy with flat sectors. Hyphal tip cultures from the subcultures were analyzed for the isozyme markers. All fluffy hyphal tip cultures had the isozyme phenotype of the haploid tester. Flat hyphal tip cultures either had the isozyme phenotype of the diploid parent or had a hybrid isozyme phenotype with markers of both the diploid parent and the haploid tester. The data suggest that in conspecific diploid-haploid pairings, the diploid nucleus usually migrates into the haploid mycelium and eventually displaces the haploid nucleus.

Key Words: Armillaria ostoyae, genetics, identification, isozymes

Identification of species of Armillaria (Agaricales: Tricholomataceae) in the field is difficult because characteristics of mycelial fans (Roll-Hansen, 1985) and rhizomorphs (Berubé and Dessureault, 1988, 1989; Guillaumin et al., 1989) vary among the species only to a limited extent, and because basidiomes (mushrooms) are seasonal and rare to uncommon in most regions. In vitro production of basidiomes is possible for some species (particularly A. ostoyae (Romagn.) Herink.), but the process is time-consuming (Guillaumin, 1986; Guillaumin et al., 1989; Harrington et al., 1992). This limitation and the limited variation among Armillaria species in cultural characteristics (Guillaumin et al., 1985, 1989; Rishbeth, 1986) make identification of field isolates difficult and have impeded progress on the elucidation of the biology of the various species. Electrophoresis of isozymes (Morrisson et al., 1985) and restriction fragments of DNA (Anderson et al., 1987; Jahnke et al., 1987; Smith and Anderson, 1989), DNA homology (Jahnke et al., 1987), DNA sequence analysis (Anderson and Stasovski, 1992) or antibodies (Burdsall et al., 1990) may prove feasible for species identifications in the future. For the present, critical identification of cultures relies on pairings with tester strains.

Although various pairing techniques have been utilized, most rely on the variation in culture morphology between single basidiospore strains (haploid) and isolates from decay, fans or rhizomorphs, which are putatively diploid (Hintikka, 1973). Most diploid isolates produce relatively little aerial mycelium, and the mycelium often has a reddish-brown crust. Haploid strains tend to produce more aerial mycelium and the colonies generally remain white. When sexually compatible haploid strains of the same species are placed near each other and allowed to grow together for 3 or more weeks, the fluffy, white mycelium may flatten and become darker or crustose. If the strains are of different species or are homoallelic at one or both of the two mating loci (the investigated species of Armillaria are tetrapolar, with two multiallelic loci determining sexual compatibility), the mycelia remain white or light tan, continue to produce abundant aerial
hyphae and do not become crustose (Korhonen, 1978). Evidence suggests that the colony type arising from conspecific pairings of haploids is diploid (Anderson and Ulrich, 1982; Korhonen, 1980; Korhonen and Hintikka, 1974; Larsen et al., 1992; Peabody et al., 1978).

Diploid isolates are not as easy to identify as haploid strains (Shaw and Loopstra, 1988; Siepmann, 1987; Wargo and Shaw, 1985). Diploid-haploid pairings can be made in a manner analogous to dikaryon-monokaryon pairings used in dikaryotic hymenomycetes (Korhonen, 1978). Although this technique has been used by many investigators (Anderson and Ulrich, 1982; Guillaumin and Berthelay, 1981; Harrington, 1987; Harrington et al., 1992; Kile, 1983; Mallet, 1990; Rishbeth, 1982), the genetic evidence for the conversion of the haploid to a diploid in diploid-haploid pairings has not been established (Ulrich and Anderson, 1988; Guillaumin et al., 1991).

The objective of our study was to determine the genetic basis for diploidization of diploid-haploid pairings of 

**MATERIALS AND METHODS**

Polymorphic isozymes were searched for in five single basidiospore strains and nine putatively diploid isolates of *A. ostoyae* from New Hampshire. One haploid strain (B286 from *Abies balsamea* (L.) Mill., Albany, New Hampshire) and two diploid isolates (B356 and B359 from *Tsuga canadensis* (L.) Carr. and *A. balsamea*, respectively, Bartlett, New Hampshire) were selected because of differences in electrophorograms for aconitase (ACO; EC number 4.2.1.3) and glucosephosphate isomerase (GPI; EC 5.3.1.9) (Figs. 1, 2).

Two series of diploid-haploid pairings (B356 × B286 and B359 × B286) were conducted on MEA (1% Difco malt extract, 1.5% Difco agar) in 50-mm-diam plastic petri dishes. Inocula were placed 5 mm apart and incubated at room temperature (20 to 25 C) with laboratory lighting. Subcultures were taken from the haploid tester side (B286) after 3 and 4 wk with five replicate plates for each sampling time. From each plate, one or two 3-mm-diam plugs were taken from a point 2 mm and 10 mm (generally the advancing margin) from the confrontation zone. Where the haploid tester had grown further than 10 mm, subcultures were also taken from the advancing margin. Subculture plugs were transferred to MEA and incubated under laboratory conditions for 7 to 10 days before evaluating for culture type (fluffy, flat, or fluffy with flat sectors).

Hyphal tips were excised using 40× magnification from subcultures taken at each sampling time and distance from the confrontation zone. Hyphal tips were taken without regard to subculture morphology but were selected on the basis of ease of excision, which resulted in a bias for fast-growing hyphal tips. A minimum of 40 hyphal tips were taken for each sampling time and sampling distance. Hyphal tips were transferred to MEA, incubated as above for 14 to 16 days, and scored for culture type.

Representative samples of hyphal tip cultures (fluffy and flat from each distance and time) were grown in liquid media for enzyme extraction. Plugs of mycelia from MEA plates were inoculated into 30 ml of liquid medium (20 mg malt extract and 2 mg yeast extract per ml) in 125 ml erlenmeyer flasks and incubated in still culture at 20 to 25 C for 3 to 4 wk. Mycelia (and rhizomorphs, if present) were vacuum-filtered, placed in prechilled mortars, frozen with liquid nitrogen, and ground to a fine powder. Enzymes were extracted by grinding with 0.5 to 0.75 ml of extraction buffer. Extraction buffer was prepared by mixing 0.2 M Na2PO4 (25 ml), glycerol (25 ml), deionized water (50 ml) and disodium EDTA (0.17 g), and adjusting the pH to 7.2 with 4 N NaOH. Lyophilized bovine serum albumin (1 g), sodium ascorbate (1 g), glutathione (1 g), NAD (1 mg), and NADP (1 mg) were added to the buffer and the pH readjusted to 7.2. Enzyme extracts were absorbed through miracloth onto 4 × 12 mm wicks of Whatman No. 3 MM chromatography paper and stored at −80 C.

Twelve percent starch gels were prepared 1 day in advance by the microwave method of Marty et al. (1984). Buffer systems D (pH 6.1) and E (pH 8.1) were continuous morpholine citrate systems run at 250 V constant voltage for 5 h (Conkle et al., 1982). Gels were loaded with up to 30 wicks, each representing a different isolate or strain. Wicks were removed after 10 min of electrophoresis at the specified voltage. Staining procedures were those of Marty et al. (1984).

Although genetic analysis of the segregation of isozyme electromorphs through meiosis was not conducted, the number of coding loci and the monomorphic or dimeric nature of the enzymes could be easily inferred (Micales et al., 1986). Electrophoresis of over 50 putatively diploid field isolates and haploid strains of *A. ostoyae* in this study and in other unpublished work (Rizzo and Harrington) indicated that ACO is monomorphic and has two loci. Although only one locus for ACO has been noted in some organisms (Pasteur et al., 1988), all haploid strains of *A. ostoyae* had two bands (Fig. 1). Putatively diploid field isolates of *A. ostoyae* had two, three, or four bands; four bands could be expected if the isolate was heterozygous at both loci. No heteromeric isozyme molecules were suggested by the electrophoretic mobility of the bands (Fig. 1), and ACO has been found to be monomorphic in other organisms (Pasteur et al., 1988).

The enzyme GPI has been shown to be dimeric with one locus in many organisms (Pasteur et al., 1988), including fungi (Linde et al., 1990), and appeared to be dimeric with a single locus in *A. ostoyae* (Fig. 2). All haploid strains tested had only one band for GPI, and a sampling of over 50 putatively diploid field isolates of *A. ostoyae* had either one band (putatively homozygous) or three bands (putatively heterozygous, with a strongly staining band migrating halfway between the
other two bands, consistent for a heteromeric isozyme) (Fig. 2).

RESULTS

After pairing for 3 or 4 wk with a putatively diploid field isolate, subcultures taken from the haploid tester mycelia were either fluffy, flat, or fluffy with flat sectors. In pairing field isolate B356 with haploid tester B286, all subcultures taken from B286 at 2 mm from the confrontation zone had a flat culture morphology (TABLE I), suggesting diploidization. Subcultures taken at 10 mm from the confrontation zone were either fluffy or fluffy with flat sectors (TABLE I). The few subcultures taken from beyond 10 mm were all fluffy (data not shown). All hyphal tips taken from 10 mm subcultures (even those taken from flat sectors) grew into fluffy colonies, but many of the hyphal tip cultures taken from 2 mm subcultures grew into flat colonies (TABLE I).

In pairing field isolate B359 with B286, 2 mm subcultures taken from the haploid tester after 3 wk yielded mostly sectored cultures, and those taken after 4 wk yielded mostly flat cultures (TABLE I). All subcultures from 10 mm were either flat or sectored (TABLE I). The few subcultures taken from further than 10 mm were all fluffy (data not shown). About one-third of the hyphal tip cultures taken from 2 mm subcultures were flat (TABLE I). From the 4 wk subcultures taken at 10 mm, only 3 of 40 hyphal tip cultures were flat (TABLE I).

Markers for two isozymes allowed for the determination of nuclear phenotype of hyphal tip cultures.

TABLE I

<table>
<thead>
<tr>
<th>Pairing</th>
<th>Time (wk)</th>
<th>Distance (mm)</th>
<th>Subcultures</th>
<th>Hyphal tip cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fluffy</td>
<td>Flat</td>
</tr>
<tr>
<td>B286 × B356</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>B286 × B359</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

* Distance is measured from the confrontation zone between the paired cultures.

* Numbers are the percentage of subcultures (n = 10) or hyphal tip cultures (n = 40) that were fluffy, flat, or fluffy with flat sectors.
cultures. A diagrammatic representation of the electrophoretic phenotypes observed in the parent strains and hyphal tip cultures is shown in Figs. 3 and 4. Haploid tester strain B286 had one band for GPI and two bands for ACO (Figs. 1–4). Because there was only one band resolved (band 1b), diploids B356 and B359 were considered to be homozygous for GPI (Figs. 3, 4). In contrast, both B356 and B359 were considered to be heterozygous for ACO1 (Figs. 3, 4). Isolate B356 was considered to be heterozygous for ACO2 but B359 was considered to be homozygous for the ACO2 locus.

Isozyme phenotypes of selected flat and fluffy hyphal tip cultures corresponded in almost all cases with culture morphology (TABLE II). All fluffy hyphal tip cultures matched the isozyme phenotype of the haploid tester (B286). Twenty-five flat, hyphal tip cultures from haploid tester mycelia had the isozyme phenotype of the respective diploid parent, four cultures had isozyme phenotypes of both the parent and tester (a hybrid), and three cultures had the isozyme phenotype of the haploid tester strain (TABLE II, Figs. 3, 4).

Consistent with the dimeric nature of GPI, the putative hybrid culture from pairing B356 × B286 had three bands: one band from the homozygous diploid, one band from the haploid tester, and a heteromeric band whose migration was intermediate between the other two bands (Figs. 2, 3). Three putative hybrids of B359 × B286 also had three bands for GPI, including a heteromeric band (Fig. 4). In the B359 × B286 hybrid, staining for ACO resulted in four bands: the three bands found in the diploid and band 2a of the haploid tester (Figs. 1, 4).

**DISCUSSION**

Isozyme markers from diploid parent isolates moved into the mycelium of a haploid tester strain of *A. ostoyae*, supporting the hypothesis that there is a genetic basis for the changes in culture morphology used as a criterion for identification of *Armillaria* species. In those cases where the flat, hyphal tip cultures showed only the isozyme phenotype of the diploid parent, it could be argued that the flat subcultures resulted from invasive growth of diploid hyphae rather than from diploidization of the haploid tester mycelium. This could easily be envisioned where the subcultures used for hyphal tipping originated from only 2 mm from the confrontation line. However, in four of the 32 flat, hyphal tip cultures tested, isozyme phenotypes indicative of hybrids were found, and two of these hybrids originated from subcultures taken 10 mm from the confrontation line. In both B356 × B286 and B359 × B286 hybrids, heteromers of the dimeric enzyme GPI were found, which suggests that alleles of both the haploid and diploid parents were in the same hyphal tip and that both were being expressed in the tested hyphal tip culture.
It has been suggested that in compatible, diploid-haploid pairings of Armillaria species, the diploid nucleus replaces the haploid nucleus in the tester mycelium (Guillaumin et al., 1991; Ullrich and Anderson, 1988) as has been reported for dikaryon-monokaryon (di-mon) pairings of Trametes versicolor (L.: Fr.) Pilát (Aylmore and Todd, 1984). Our data are consistent with this hypothesis and indicate that after hyphal fusion, the diploid nucleus slowly migrates into the haploid mycelium and co resides with the haploid nucleus in many of the hyphal tips. Although most subcultures taken from the haploid tester mycelium were flat in morphology, most hyphal tip cultures from those subcultures were fluffy and had only the isozyme markers of the haploid tester strain. Isozyme markers of only the diploid parent were found in most flat, hyphal tip cultures, which suggests that the diploid nucleus eventually displaces the haploid nucleus. The infrequent hybrid isozyme phenotype may represent a transitional phase. Although our study was limited in scope, culture morphology and isozyme patterns suggest that diploidized tester mycelia are, at least initially, mosaics of haploid, diploid, and hybrid hyphal tips of both the diploid and haploid parent nuclei.

An alternative hypothesis for diploidization is that the diploid nucleus undergoes reduction (haploidization) prior to hyphal fusion or in the haploid tester mycelium, and one of the resulting haploid nuclei fuses with the haploid nucleus of the tester to form a new, recombinant diploid. Apparent haploidizations of diploid nuclei have been observed in basidial primordia of Armillaria, which give rise to the dikaryotic hyphae in the gills, and transient dikaryotic stages in the life cycle are known (Korhonen and Hintikka, 1974; Korhonen, 1980, 1983; Larsen et al., 1992; Tommerup and Broadbent, 1975). Guillaumin (1986) found limited evidence (based on mating-type alleles) of recombinant dipl oids following diploid-haploid pairings of A. ostoyae. However, vegetative dipl oids of Armillaria are believed to be very stable (Anderson and Ullrich, 1982), and our data further suggest their stability. None of the 29 flat, hyphal tip cultures had isozyme patterns consistent with a recombination between the diploid parent and the haploid tester. However, the use of additional nuclear markers (isozyme or molecular) and the testing of more isolates could allow for detection of potential low frequency recombination events.

Korhonen (1983) observed nuclear migration through eroded septa in haploid-haploid pairings of Armillaria. He found that nuclear migration was very slow (approximately 1.1 mm per day) compared with migration rates in other hymeno mycetes of 3–4 mm per hour (Aylmore and Todd, 1984; Korhonen, 1983). Because of slow nuclear migration, a long period of time may be required for the entire mycelium of an Armillaria subculture to become converted to a diploid. It appears, however, that only a portion of a mycelium needs to be diploidized to induce a change in culture morphology from fluffy to flat.

The reliability of identifications using diploid-haploid pairings is greatly improved if only fresh tester isolates with exceptionally fluffy mycelia are used (Korhonen, 1978) and subcultures are taken from haploid tester mycelium after 4–6 wk (Harrington et al., 1992). Small plugs of mycelium are excised from the haploid tester at 2 mm behind the confrontation zone, from the ad-

<table>
<thead>
<tr>
<th>Pairing</th>
<th>Distance (mm)*</th>
<th>Culture morphology</th>
<th>Isozyme phenotypeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haploid</td>
</tr>
<tr>
<td>B286 × B356</td>
<td>2</td>
<td>Fluffy</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Flat</td>
<td>0</td>
</tr>
<tr>
<td>B286 × B359</td>
<td>2</td>
<td>Fluffy</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Flat</td>
<td>2</td>
</tr>
</tbody>
</table>

*Distance is measured from the confrontation zone between the paired cultures.

b Numbers represent the total number of hyphal tip cultures tested. Data from 3 and 4 wk were combined.
vancing margin of the tester at the site opposite the confrontation, and from a point between the other two samples. These plugs are transferred to MEA and incubated for 7 to 10 days. Subculures of the tester with a morphology different from the unpaired tester indicate that the tester has been diploidized and that the unidentified isolate and the tester are conspecific.

Siepmann (1987) found that in many diploid-haploid pairings between different species, the haploid tester would eventually (after 2 or 3 months) overgrow the diploid mycelium. If the haploid was of the same species, the haploid tester would change in morphology, but mycelia of the haploid tester would remain restricted to the line where the two mycelia met. This technique may prove useful for identification of field isolates of Armillaria species, but the genetic basis of the reaction is not known. Similarly, putatively diploid isolates may be identified to species by pairing with other diploid isolates on MEA. A black line usually develops between diploid isolates if they are different species, and this can be used to confirm results based on diploid-haploid pairings (Korhonen, 1978; Ullrich and Anderson, 1978). The black line is more easily seen if L-DOPA is added to the confrontation zone (Hopkin et al., 1989). Another alternative is the induction of haploidization of the diploid isolates using benomyl (at 25 ppm) and then using the induced haploid strains in haploid-haploid pairings (Anderson and Yacoob, 1984). We have found, however, that the diploid-haploid pairing technique with subculturing is the most reliable method for identification of diploid isolates of North American Armillaria species, and now a genetic basis for this test has been established for A. ostoyae.

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