INCOMPATIBILITY BETWEEN Laetiporus CINNINATUS AND L. SULPHUREUS IN CULTURE

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ABSTRACT
Pairings were conducted between single basidiospore isolates obtained from 9 collections of Laetiporus belonging to three Laetiporus restriction groups (LRG). Seventy one of 81 pairings between LRGs I and VII, both of which have yellow pores and fruit on trunks or stumps of hardwoods, resulted in a change in culture morphology to a denser, more pigmented mycelium than that present in the single spore cultures. Allozyme analysis of 35 of these pairings indicated that nuclear exchange had occurred. All of the 162 pairings between LRGs I or VII and LRG IV, which has white pores and fruits on the ground near hardwoods, resulted in the formation of a dark pigmented line between the single spore isolates. No nuclear migration was indicated in any of these pairings based on allozyme analysis. These results confirm previous reports that suggest LRGs I and VII are probably the same species, L. sulphureus. LRG IV undoubtedly does not belong to this species but represents L. cincinnatus. Preliminary results of pairings between sibling single basidiospores using the culture morphology criteria suggest that both of the species have bipolar multi-allelic mating systems.

Keywords: allozyme, bipolar, isozyme, mating systems, Polyporaceae

INTRODUCTION
Laetiporus sulphureus (Fr.) Murr. in North America has referred to several forms that differ in their ecology and/or pore color. Restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction amplified ITS region of the nuclear ribosomal DNA also shows consistent differences between these forms (Banik et al., 1996, 1998). Using the RFLP data, Banik et al. (1998) identified seven restriction groups within L. sulphureus, which were termed Laetiporus restriction groups (LRG) I-VII. The RFLPs of each of the LRGs were distinct except LRG VII appeared to be a combination of the RFLPs observed in LRGs I and VI. The restriction patterns obtained for LRG VII appeared such that they could have resulted
from matings between individuals from LRG I and LRG VI. Basidiomes of all three of these groups possess yellow pore layers and fruit on stumps and trunks of hardwoods. These characteristics are generally attributable *L. sulphureus* sensu stricto.

LRG IV basidiomes possess white pore layers and occur on the ground near the base of trees, characters that coincide with the taxon described as *Polysorus cincinnatus* Morgan (Morgan 1885). Banik et al. (1998) proposed the new combination *L. cincinnatus* (Morgan) Burdsall, Banik & Volk. In their view, the lack of combined RFLP patterns indicative of genetic exchange between LRG IV and the other LRGs, along with the distinct differences in gross morphology and ecology, substantiated the assertion that *L. cincinnatus* and *L. sulphureus* were reproductively isolated and therefore different species.

In this study, the proposed reproductive isolation between LRGs I and VII (putatively *L. sulphureus*) and LRG IV (*L. cincinnatus*) is examined using pairings between single spore isolates in culture. Because *Laetiporus* lacks clamp connections allozyme markers will be used as an independent test of nuclear migration and sexual compatibility. The compatibility of LRGs I and VII is also tested.

**MATERIALS AND METHODS**

**Isolate collection and allozyme typing**

Single spore and tissue isolates were obtained from three basidiomes each of LRGs I, IV and VII (Table 1). Basidiospores were routinely obtained by suspending sections of the pore surface over oak wood extract medium (OWEM) until basidiospores were deposited on the medium surface. OWEM was prepared by boiling 50 gr red oak sawdust in 1 L water for 1 hour and straining through two layers of cheesecloth to obtain oak wood extract. After autoclaving, 500 mL of the extract was combined with 500 mL water containing 20 gr agar. After 2-4 days incubation at room temperature, germinated single basidiospores were transferred, along with a small piece of underlying medium, to 15 x 45 mm vials containing 2 mL of OWEM. After several months the resulting single spore isolates were transferred to 16 x 125 mm culture tubes containing 1.5 % malt extract and 2 % agar (MEA) for long term storage. Tissue isolates were obtained by excising small pieces of basidiome trama onto MEA.

Tissue isolates from each of the collections were subjected to allozyme analysis for the dimeric enzyme glucose 6-phosphate (GPI) in order to obtain markers for use as aids in interpreting pairing results. A small amount of mycelium was scraped from cultures growing on medium consisting of 4 % potato dextrose agar (Difco), 0.5% agar and 0.25 % gallic acid (PDAgal) and ground in a ground glass tissue homogenizer with the addition of 100 µL extraction buffer (0.05 M Tris-HCl, pH 7.1). The resulting homogenate was centrifuged at 13800 relative centrifugal force for
three minutes in a Eppendorf microcentrifuge, and the supernatant was used for allozyme analysis using cellulose acetate electrophoresis. The procedure followed the instructions provided by the manufacture of the cellulose acetate gel plate electrophoresis apparatus (Helena Laboratories, Beaumont, TX) (Hebert and Beaton, 1993). The running buffer was Tris-glycine (0.25 M Trizma-base, 1.9 M glycine, pH 8.5) and gels were run for 30 min at 200 volts. Gels were stained for GPI activity using the technique of Richardson et al. (1986), modified by the addition of 2 mL of 1.5 % agar and the use of 0.1 M Tris, pH 7.4 as the stain buffer. Gels were incubated for 10 - 60 min in the dark at room temperature before being photographed and scored for allozyme activity.

Table 1. Field data for nine collections of *Laetiporus* from three *Laetiporus* restriction goups (LRG), from which basidiospores were obtained for incompatibility studies.

<table>
<thead>
<tr>
<th>LRG</th>
<th>collection number</th>
<th>date collected</th>
<th>host</th>
<th>color</th>
<th>position</th>
<th>location</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>GR-12</td>
<td>10/11/95</td>
<td>hardwood</td>
<td>yellow</td>
<td>log</td>
<td>Grant Co, WI</td>
</tr>
<tr>
<td></td>
<td>NJ-2</td>
<td>10/8/95</td>
<td><em>Quercus</em> sp</td>
<td>yellow</td>
<td>trunk</td>
<td>Morris Co, NJ</td>
</tr>
<tr>
<td></td>
<td>TJV 95-84</td>
<td>10/8/95</td>
<td><em>Prunus serotina</em></td>
<td>yellow</td>
<td>trunk</td>
<td>Dane Co, WI</td>
</tr>
<tr>
<td>IV</td>
<td>DA-37</td>
<td>10/15/97</td>
<td><em>Q. velutina</em></td>
<td>white</td>
<td>soil</td>
<td>Dane Co, WI</td>
</tr>
<tr>
<td></td>
<td>WS-1</td>
<td>9/30/95</td>
<td>-</td>
<td>white</td>
<td>soil</td>
<td>Washington Co, WI</td>
</tr>
<tr>
<td></td>
<td>TJV 95-49</td>
<td>9/3/95</td>
<td><em>Acer saccharum</em></td>
<td>white</td>
<td>soil</td>
<td>Sauk Co, WI</td>
</tr>
<tr>
<td>VII</td>
<td>CT-I</td>
<td>10/5/95</td>
<td><em>Acer sp.</em></td>
<td>yellow</td>
<td>stump</td>
<td>Darien Co, CT</td>
</tr>
<tr>
<td></td>
<td>TJV 95-53</td>
<td>9/8/95</td>
<td><em>Acer sp.</em></td>
<td>yellow</td>
<td>trunk</td>
<td>Baraga Co, MI</td>
</tr>
<tr>
<td></td>
<td>TJV 95-83</td>
<td>10/7/95</td>
<td><em>Quercus sp.</em></td>
<td>yellow</td>
<td>log</td>
<td>Dane Co, WI</td>
</tr>
</tbody>
</table>

**Pairings between and within LRGs**

Three single spore isolates were selected at random from each of the three collections representing either LRG I, IV or VII, for a total of 27 single spores isolates. These isolates were paired in all possible combinations on PDAgal. Two replicates of each pairing were done, one in 150 x 25 mm and one in 90 x 15 mm plastic Petri plates. Pairings were conducted by placing one agar plug, approximately 5 mm$^2$ cut from cultures growing on PDAgal, of each of the two isolates to be paired in contact with each other in the center of the plate. A second plug of each isolate was placed 2 cm away from the first plug, such that the four plugs in the plate were in line, with the plugs from one isolate on one side of the plate and the plugs from the other on the opposite side. After incubating at 25 C for 7 to 10 days, the morphologies of the pairings were recorded. Two basic categories of morphologies were observed, one in which the two single spore isolates appeared to fuse and the other in which the two isolates remained distinct.
Allozyme analysis was performed on 59 unique pairings in which the two single spore isolates remained distinct. Two samples from each of these pairings, one from each side, were analyzed by removing a small amount of mycelium and treating it as described earlier. Allozyme analysis was performed on 54 pairings in which the two single spores appeared to fuse by removing a small amount of mycelium from the center fused area. In all pairings analyzed, the two single spore isolates possessed different allozymes so that the detection of heterodimeric activity could be used as an indicator for nuclear combination.

**Pairings within collections**

To determine the mating systems present in *L. cincinnatus* and *L. sulphureus*, all sibling single spore isolates from four collections were paired in all combinations using the protocol described above. Collections WS-1, DA-37 (LRG IV), TJV 95-53(LRG VII), and GR-12 (LRG-I), possessing 18, 23, 20, and 15 single spore isolates respectively, were used. The pairings were incubated and scored as described above.

**RESULTS**

**Basidiospore collection and initial allozyme analysis**

Basidiospores of all collections reliably germinated on OWEM with germination percentages ranging from 20 - 80%. Survival of the germinated basidiospore when transferred to OWEM was close to 100%.

Four allozyme types, designated A - D were identified in tissue isolates from the 9 collections (Fig. 1). Each of the allozyme patterns possessed only a single band, indicating that each collection was homozygous at the GPI locus. All three LRG IV collections and one LRG VII collection were type B. One collection each of LRG I and LRG VII was type A and one of each was type C. The remaining LRG I collection was type D.

![Fig. 1](image-url) Representative GPI allozyme patterns obtained from isolates of *Laetiporus* by electrophoresis on cellulose acetate gels. Lanes A-D) Homodimeric patterns from nine different tissue isolates belonging to three LRGs: type A found in LRGs I and VII, type B found in LRGs IV and VII, type C found in LRGs I and VII, type D found in LRG I. The remaining lanes are the heterodimeric patterns obtained from pairings of basidiospores of different allotypes.
Pairings within and between LRGs

Within each LRG, 45 pairings were made between the 9 single spore isolates from three different collections. In LRG I, 31 of these pairings exhibited fusion (Fig. 2a) in which the center area was denser and often more pigmented than that of the mycelium resulting from the single spore isolates growing from the outer seed plugs. This included all 27 pairings between isolates from different collections. The 9 self pairings exhibited fusion without a change in morphology (Fig. 2b). In the remaining 5 pairings, the single spore isolates remained separate, although the formation of a distinct line separating them did not occur (Fig. 2c). Pairings exhibiting this morphology always involved sibling basidiospores. Allozyme analysis of 7 pairings that exhibited fusion with a change to a denser, more pigmented morphology, also demonstrated heterodimeric GPI activity (Table 2, Fig. 1).

Twenty-five of the 45 pairings within LRG VII exhibited fusion with the center area becoming denser and more pigmented. Non-sibling pairings accounted for 21 of these. The 9 self pairings fused without a change in morphology as did 4 sibling pairs. Six non-sib pairs involving the same single spore isolate of collection TJV-95-53, as well as one sibling pairing with this isolate, exhibited non-fusion without a line morphology. Fourteen fusion pairings with a change in morphology were analyzed for GPI activity, and they all exhibited heterodimeric activity (Table 2, Fig. 1).

Twenty-one of 36 non-sibling pairings between the three LRG IV collections expressed fusion morphology with increased density and pigmentation as did 6 sibling pairings. In the remaining 6 non-sibling pairings the two single spore isolates remained separate without the formation of a line. All of these 6 pairings involved the same single spore isolate of collection WS-1. The 9 self pairings all exhibited fusion without a change in density or pigmentation as did 2 sibling pairs. In one sibling pair the isolates remained separate without line formation (Table 2). Allozyme analysis of these pairings could not be conducted because all isolates were the same GPI allotype.

Eighty-one unique basidiospore isolate pairings were made between each two LRGs. Between LRG I and LRG VII these resulted in 71 pairings in which fusion with increased density and pigmentation occurred. Eight of the ten remaining pairings involved the same single spore isolate, and these pairings mostly remained separate without line formation as did the last two pairings. Thirty five of the pairings between LRG I and VII were subjected to allozyme analysis and all of them exhibited heterodimeric pattern types (Table 2, Fig 1). Two of these pairings exhibited the separate type morphology and samples were taken from both sides of the pairing. Heterodimeric GPI activity was detected on only one side of each of these pairings.
Fig. 2. Culture morphologies observed in pairings between single basidiospore isolates of *Laetiporus*: a) fusion reaction with increased density and pigmentation of mycelium, b) fusion reaction without an increase in density or pigmentation, c) separation of single spore isolate(s) without the formation of a distinct line, d) separation of the single spore isolates with the formation of a distinct line.
Table 2. Results of pairings between basidiospore isolates of *Laetiporus* from nine collections belonging to three LRGs and frequency of recovery of heterodimeric allozyme patterns.

<table>
<thead>
<tr>
<th>Restriction groups paired</th>
<th>Number of pairs</th>
<th>Number of pairs rated with each reaction type</th>
<th>Number of pairings with heterodimeric GPI activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>fusion with increased density</td>
<td>dense line of separation</td>
</tr>
<tr>
<td>I-I</td>
<td>45</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>I-IV</td>
<td>81</td>
<td>0</td>
<td>81</td>
</tr>
<tr>
<td>I-VII</td>
<td>81</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>IV-IV</td>
<td>45</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>IV-VII</td>
<td>81</td>
<td>0</td>
<td>81</td>
</tr>
<tr>
<td>VII-VII</td>
<td>45</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

*Allozyme analysis was not done within LRG IV because all collections were the same allotype.*

Pairings between either LRG I or LRG VII and LRG IV all resulted in the formation of a dense, darkly pigmented line separating the two single spore isolates (Fig. 2d). Allozyme analysis of 26 pairings between LRG IV and LRG I and 31 pairings between LRG IV and LRG VII failed to detect heterodimeric GPI activity (Table 2).

The results were the same between both replicates for 270 of 278 pairings made. All but one of the 8 pairings that did not agree were between sibling single spore isolates. These pairings were all rated as fusions without increased density or as remaining separate without line formation.

**Pairings within collections**

Reaction types observed in pairings of single spore isolates from the same fruiting body were the same as those observed in within-LRG pairings. None of the intracollection pairings exhibited a dense dark line as seen between LRG IV and LRG I or VII. Fusion with increased density and pigmentation (Fig. 2a) occurred in 43 %, 48 %, 51 %, and 52 % of the non-self pairings between sibling single spores of DA-37, 95-53, WS-1, and GR-12 respectively. The pattern for this morphology among the pairings fit that expected for a bipolar mating system except for 1 of the 231 DA-37 pairings, 2 of the 136 WS-1 pairings, and 2 of the 120 GR-12 pairings. In addition, one single spore isolate of collection TJV-95-53 and one of WS-1 did not express the increased density morphology in any pairings with sibling single spore isolates, and these two isolates were removed from the study. These same single spore isolates rarely or never elicited the increased density morphology in within-LRG pairings. Self pairings exhibited fusion in all instances (Fig. 2b). The non-self pairings that did not fuse with increased density of the mycelium either fused without the density change or remained separate without dark line formation (Fig. 2c). No discernible pattern accompanied the occurrence of these latter two morphologies. Because of the homozygous condition of the GPI locus for each of the basidiomes from
which the basidiospores were isolated, allozyme analysis of the intracollection pairings was not possible.

**DISCUSSION**

The use of OWEM facilitated the collection of viable basidiospores of *Laetiporus*. Previous attempts to obtain single spore isolates using other media had been thwarted by the lack of basidiospore germination. With a reliable way to obtain basidiospores a critical assessment of the incompatibility within the genus is now possible.

The occurrence of the heterodimeric GPI activity in pairings between single spore isolates from different collections of either LRG I or VII substantiates the compatibility of these isolates. To confirm that heterodimer formation resulted from nuclear exchange and not mycelial mixing, the mycelia from ten pairs of separately grown single spore isolates were ground together and analyzed for GPI activity. The pairs of single spore isolates chosen had exhibited heterodimeric activity when they were paired during the compatibility tests. None of the ten pairs of separately grown single spore isolates exhibited heterodimeric activity when mixed during grinding. Also, samples taken from five pairings between LRG IV and LRGs I or VII exhibited the homodimeric GPI activity of each of the single spore isolates, but no heterodimeric activity was detected. In these cases, invasive growth of mycelia between the two single spore isolates occurred but nuclear exchange did not take place. Thus it appears that heterodimeric enzyme is formed only when the two isotypes are present in a biologically active thallus.

Heterodimeric GPI activity correlates consistently with the morphological change of the cultures to a denser, more pigmented state, thus substantiating that this morphology is indicative of compatibility. This type of dimorphism has been described for other fungi, such as *Armillaria mellea* (Vahl: Fr.) Kummer (Hintikka, 1973). In *Laetiporus*, the dimorphism is comparatively subtle, but by using the pairing protocol described it can be consistently and reliably used to assess compatibility.

The allozyme activity and morphological data confirm that LRG I and LRG VII are sexually compatible and represent *L. sulphureus* sensu stricto. The consistent formation of a dark line in all pairings between LRG IV and LRGs I or VII accompanied by the lack of heterodimeric GPI activity confirm that LRG IV is incompatible with *L. sulphureus*. Undoubtedly, LRG IV is a different species, and its designation as *L. cincinnatus* is appropriate. For both species, the almost complete compatibility between collections indicates the presence of multi-allelic mating system.

The pattern of compatible reactions in within-basidiome pairings fits almost exactly that expected for a bipolar mating system for both *L. sulphureus* and *L. cincinnatus*. Incompatible reactions in these crosses apparently appear either as fusions without an increase in density or a separation of the two single spores without the formation of a dark line. The
two single spore isolates that did not fit the mating system pattern also did not exhibit the expected fusion with increased density morphology in within-LRG pairings. The most likely explanation for this is that these putative single spore isolates were in fact heterokaryotic. In preliminary pairing results using known heterokaryons and haploids, we have observed reactions similar to those observed with these two isolates (unpublished). Also allosyme analysis of two pairings involving one of the questionable single spore isolates suggest that nuclear migration occurred in only one direction. This might be expected if a homokaryon were paired with a heterokaryon.

The presence of a bipolar mating system is not unexpected since many other brown rot fungi have this type of mating system (Ryvarden, 1991). Confirmation of the mating system type should be possible if single spores can be obtained from basidiomes that are heterozygous at the GPI locus, making allosyme analysis of sibling pairings possible.

The conspecificity of LRG VI with *L. sulphureus* still remains to be examined. No single spore isolates of LRG VI currently exist so it was not included in this study. Also, the status of LRG II, present in the southern United States, and LRG III, which occurs on conifers, still need to be addressed. Work is currently underway to examine the compatibility among these groups and with *L. sulphureus* and *L. cincinnatus* using the techniques presented here.

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LITERATURE CITED


