Genetic and Morphological Characterization of *Cladobotryum* Species Causing Cobweb Disease of Mushrooms

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Cobweb disease of mushrooms is characterized by the growth of coarse mycelium covering affected mushrooms (5). Fruiting bodies of *Agaricus bisporus* can be attacked at any stage in their development, although it is predominantly later flushes that are affected, especially if early infection is inadequately controlled. In Ireland and Great Britain, the causal agent of cobweb disease generally has been identified as *Cladobotryum dendroides* (= *Dactylium dendroides*; teleomorph, *Hypomyces rosellus*), a soil-inhabiting fungus that may be introduced into mushroom casing in the form of spores or mycelium. *Cladobotryum mycophilum* (teleomorph, *Hypomyces odoratus*) and *Cladobotryum varium* (teleomorph, *Hypomyces aurantius*) also have been described as inciting cobweb disease of mushrooms (3). In the early 1970s, the methylbenzimidazole carbamate (MBC) fungicides, benomyl and carbendazim, were used to control fungal diseases of mushrooms (4), but by the mid-1980s, the first sign of resistance to MBC fungicides occurred in Great Britain (9). In 1992, following extensive use of carbendazim in the Republic of Ireland, resistance in cobweb disease organisms to MBC fungicides was reported. An Ireland-wide epidemic ensued within 6 months; the disease spread through a mushroom crop in 24 to 48 h (14). The epidemic was characterized by a colony morphology distinct from that previously seen; a dense, granular mat was produced over the casing layer, which lacked much of the “pink” coloration that hitherto typified the disease.

Nucleotide sequence analysis of the β-tubulin gene of the MBC fungicide-resistant isolates revealed a point mutation at codon 50 (13). A PCR-based assay exploiting this mutation enables rapid differentiation of sensitive and resistant isolates of the pathogen. Since these fungicide-resistant isolates are responsible for the increased incidence of cobweb disease in the Irish and British mushroom industries and have the potential to spread to mushroom industries elsewhere, our objective was to determine the identity of the fungus. To achieve this we compared (i) the conidial morphology of the cobweb pathogen with those of *C. dendroides* and *C. mycophilum* and (ii) molecular data with those of *Cladobotryum* species reported to be associated with cobweb disease of *A. bisporus* (3).

### MATERIALS AND METHODS

**Fungal cultures.** Forty-three isolates of *Cladobotryum/Hypomyces* species, reported to be associated with cobweb disease of *A. bisporus*, were obtained from culture collections, fellow researchers, and cobweb-infested mushroom compost from several geographic locations (Table 1). The type culture of *Cladobotryum penicilliatus* was also included in this study because, although it is not reported to be associated with cobweb disease of mushrooms, the latter has conidial dimensions similar to those of *C. mycophilum* and *C. varium* (7). Isolates were grown at 20°C on malt extract agar (MEA; Oxoid, Basingstoke, England) in 90-mm-diameter petri plates. Most isolates were tested for benzimidazole fungicide sensitivity by a poison plate assay (13).

The isolates originally identified as *C. dendroides/H. rosellus* and *C. mycophilum/H. odoratus* were examined for conidium size, the number of septa per conidium, the presence or absence of phialide extensions/rachises, and a conspicuous basal hilum on the conidia. Chlamydospore and/or microsclerotium production was also noted (6, 17).

**DNA extraction.** Isolates were grown on sterile discs of cellophane over MEA at 20°C for 3 to 4 days. Mycelium was removed and DNA was extracted by a phenol-chloroform extraction method (15) but with a third phenol-chloroform extraction step. The genomic DNA pellet was resuspended in 250 µl of sterile distilled water and quantified following electrophoresis on a 1% (wt/vol) agarose (Pharmacia, Uppsala, Sweden) gel (18).

PCR and nucleotide sequencing of the rDNA. Initial amplifications of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene fragment were performed by using a Perkin-Elmer (PE) Applied Biosystems (Warrington, England) GeneAmp PCR System 2400 and primers ITS1 and ITS4 (Table 2) (19). Amplifications (50 µl) were performed by using 100 µg of...
C. varium 95 (MUCL-8223)
C. asterophorum 91
C. mycophilum
C. dendroides 19

by using a double-stranded DNA template (0.2 M MgCl₂, and visualized with a UV transilluminator (18). Each PCR product was separated by electrophoresis on 1.2% (wt/vol) agarose gels, stained with ethidium bromide, and 7 min at 72°C. Aliquots of each PCR product from these amplifications were 1 min at 94°C; 30 cycles of 15 s at 94°C, 15 s at 58°C, and 15 s at 72°C; and 1 cycle each primer. The following reaction program was used: initial denaturation cycle, 1.5 mM MgCl₂, 2U of Ampli

genomic DNA, 1.5 mM MgCl₂, 2 U of Ampli Taq polymerase (PE Applied Biosystems), 0.2 mM each deoxynucleoside triphosphate (dNTP), i.e., dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.2 μM each primer. The following reaction program was used: initial denaturation cycle, 1 min at 94°C; 30 cycles of 15 s at 94°C, 15 s at 58°C; and 15 s at 72°C; and 1 cycle of 7 min at 72°C. Aliquots of each PCR product from these amplifications were separated by electrophoresis on 1.2% (wt/vol) agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator (18). Each PCR product was purified by using the Wizard DNA Clean Up system (Promega, Southampton, England) according to the manufacturer’s instructions. Isolates were sequenced by using a double-stranded DNA template (0.2 μg μl⁻¹) and 1 μM of forward (ITS1 or ITS3) or reverse (ITS2 and ITS4) primers according to the protocol supplied with the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems). Sequencing was conducted with a PE Applied Biosystems Model 373A DNA Sequencer by the method recommended by the manufacturer. The sequence data of complementary strands were compared visually and aligned by using CLUSTAL V (11). A phylogenetic tree was constructed from genetic distance values calculated with the DNA sequence analysis software package MEGA (12).

RAPD-PCR and data analysis. Amplification reactions were performed in 50-μl volumes containing 20 to 30 ng of template DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 1 μM each dNTP, 1 μM primer, and 1 U of Ampli Taq polymerase. PCR was performed with a PE Applied Biosystems 480 thermocycler as follows: 45 cycles of 30 s at 95°C, 1 min at 36°C, and 2 min at 72°C, and a final cycle of 7 min at 72°C. The following primers (Operon, Alameda, California) were used: A5, A8, C20, E19, G17, and Y15 (Table 2). Randomly amplified polymorphic DNA (RAPD) banding patterns were scored on the basis of the presence or absence of bands of equal size, and a neighbor-joining phylogenetic tree was constructed based on pairwise distances calculated by using the Jaccard coefficient and the RAPDdistance 1.04 statistical package (1).
TABLE 2. Primer sequences used for PCR, DNA sequencing, RAPD and microsatellite analysis of isolates of Cladobotryum and Hypomyces species

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>ITS1</td>
<td>TCCTGTAAGTTGAACCGGCA</td>
</tr>
<tr>
<td>ITS2</td>
<td>GCTGCGTTCCTCATGATCC</td>
</tr>
<tr>
<td>ITS3</td>
<td>GCATCGATGAAAGACGACG</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCCTCGGTATTGATGTC</td>
</tr>
<tr>
<td>OP-A5</td>
<td>AGGGTTCTTGG</td>
</tr>
<tr>
<td>OP-A8</td>
<td>GTCAGTGGAG</td>
</tr>
<tr>
<td>OP-C20</td>
<td>ACCTCCGCA</td>
</tr>
<tr>
<td>OP-E19</td>
<td>ACCGGATAGT</td>
</tr>
<tr>
<td>OP-G17</td>
<td>ACGCAGCGCA</td>
</tr>
<tr>
<td>OP-Y15</td>
<td>AGTCGCCCCTT</td>
</tr>
<tr>
<td>DAGP1</td>
<td>GGTAGGGTCGTCGGCCAC</td>
</tr>
<tr>
<td>DAGP2</td>
<td>ACGGGAAAACGATGTTGATCG</td>
</tr>
</tbody>
</table>

Cloning and sequencing of a RAPD fragment containing a microsatellite. An 800- to 900-bp polymorphic band, identified in the resistant isolates, was generated by RAPD-PCR using primer E19 and excised from a 1.5% (w/vol) low-melting-point agarose gel (Gibco BRL, Paisley, Scotland). The band was purified by using the Wizard PCR Preps DNA Purification System (Promega), cloned into the pGEM T Vector System (Promega), and transformed into Escherichia coli JM109. Successful constructs were sequenced on an automated PE Applied Biosystems Model 373A DNA Sequencer.

Amplification of the GA repeat microsatellite. Amplification reactions were performed with primers designed from the flanking regions of the microsatellite nucleotide sequence of the cloned RAPD fragment. Reaction volumes were as described for RAPD-PCR except that 0.4 μM concentrations (each) of the forward (DAGP1) and reverse (DAGP2) primers were used. PCR conditions were as follows: initial denaturation cycle of 2 min at 95°C, 35 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C, and a final cycle of 7 min at 72°C.

Nucleotide sequence accession numbers. The sequences for the microsatellites and ITS regions of Cladobotryum/Hypomyces species have been deposited in the EMBL database under accession no. Y17087 through Y17092 and Y17094 through Y17104 (Table 1).

RESULTS

Morphological characteristics. Isolate 109, received from the United States as a Dactylum sp., fits the descriptions of C. dendroides (6, 16); notably, conidia were 7 to 12 μm wide and had one to three septa, with a conspicuous basal hilum and a secondary extension (rachis) evident on the phialides. Chlamydochepes were produced, and some microsclerotia were present. Isolates 98, 99, and 100, also originally identified as C. dendroides, failed to produce conidia, probably as a result of prolonged storage; hence no morphological data were available.

MBC-sensitive isolates 88, 90, 91, 98, 104, 105, 106, 107, 108, 110, and 111, originating outside Ireland, had conidia similar in size (7 to 12 μm) and septation to isolate 109, but the conspicuous basal hilum and phialide extensions typical of C. dendroides were absent. Chlamydospores and microsclerotia were produced by these isolates. No morphological differences between these isolates and the Irish MBC-resistant and -sensitive isolates were observed, except that some of the resistant isolates did not produce microsclerotia.

Nucleotide sequence analysis of ITS regions. ITS sequences divided the Cladobotryum/Hypomyces isolates associated with cobweb disease into four groups, representing four distinct species (Fig. 1). The type culture of C. penicillatum formed a fifth distinct species, showing 42 variable sites within the ITS 1 region (EMBL accession no. AJ012830) compared to C. mycophyllum. C. penicillatum, therefore, shows greater variation within this region compared to C. mycophyllum than do the other three Cladobotryum species studied. The sequences of the three C. dendroides isolates (99, 100, and 109), which grouped together, differed by 2 bp in the ITS 1 region. Two isolates (98 and 107) received as C. dendroides grouped instead with the majority of isolates studied and apparently had been misidentified. The sequences of these isolates and of those obtained from the mushroom industry, were 99.6 to 100% similar to those of isolates 108 and 111, identified by the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands) as C. mycophyllum, and to those of isolates 88, 90, and 91, also identified as C. mycophyllum (Table 1). These isolates subdivided into three intraspecific ITS groups (Fig. 1); the nucleotide variability between these groups was 0.2 to 0.4%, and they differed from each other by a single nucleotide in the ITS 2 region. The nucleotide sequences within each subgroup were identical. All of the isolates in subgroups 1 and 3 (Fig. 1) were MBC sensitive; most were obtained from the mushroom industry in Ireland, Great Britain, Australia, Canada, or the United States. All the isolates in subgroup 2, except isolate 24, which was deposited with the International Mycological Institute (Egham, England) in 1982, were MBC resistant; most were obtained from the Irish mushroom industry, and two were obtained from Great Britain (Fig. 1; Table 1).

DNA polymorphism identified by RAPD-PCR. Analysis of the RAPD patterns produced by the six primers differentiated the Cladobotryum/Hypomyces isolates into interspecific groups in a similar manner to the ITS sequencing (data not shown). C. dendroides isolates 99, 100, and 109 grouped together and were distinct from Cladobotryum asterophorum and C. varium. The remainder of the isolates analyzed, which were distinct from these three species and were now believed to be C. mycophyllum, divided into two distinct groups: MBC sensitive and MBC resistant. The MBC-resistant isolates from Ireland clustered strongly together, with the two British isolates (87 and 89) showing slight divergence. The MBC-sensitive isolates were
much more divergent. A polymorphic band identified by using primer OP-E19 distinguished MBC-resistant isolates.

Microsatellite GA repeats in *C. mycophilum*. The band identified by RAPD-PCR to distinguish MBC-resistant isolates was purified and cloned. Fragments amplified by using primers designed from regions flanking the microsatellite nucleotide sequence revealed length polymorphism among the *C. mycophilum* isolates (Fig. 2). These microsatellites contained different numbers of GA repeats, giving rise to the length variation. The MBC-resistant isolates all contained the same number of GA repeats and formed a single clade. MBC-sensitive isolates from Europe (including Ireland and Great Britain), Australia, and North America had different numbers of GA repeats, which tended to group them according to geographic origin. Clade formation based on identical microsatellite DNA sequence data was as follows: isolates 5, 7, 8, 10, 18, 87, and 89 (MBC-resistant isolates from Ireland and Great Britain); isolates 13 and 19 (MBC-sensitive isolates from Australia); isolates 104, 105, and 110 (MBC-sensitive isolates from North America); isolates 106 and 107 (MBC-sensitive isolates from North America); isolates 11, 25, 50, 83, 88, 90, 91, and 108 (MBC-sensitive isolates from Europe). Isolates 24, 66, and 111 each gave a unique length polymorphism for this region.

**DISCUSSION**

Variation in the morphology of *Cladobotryum* spp. associated with cobweb disease of mushrooms has led to their uncertain identification. The pathogen in Great Britain and Ireland has generally been referred to as *D. dendroides* (= *C. dendroides*) (9, 13). Although the observations on the conidium and phialide characteristics of the *Cladobotryum* isolates we obtained from the Irish and United Kingdom mushroom industries did not appear to fall strictly within the descriptions of either *C. dendroides* or *C. mycophilum* (6, 17), we suggest that the majority are more likely to be *C. mycophilum* than *C. dendroides*. Gams (8) concurred with this view and identified these isolates as *C. mycophilum* despite the lack of the characteristic odor. This result contradicts previous reports (17) that none of the agaricicolous species of *Hypomyces*, including *C. mycophilum*, are associated with *A. bisporus*.

One of the isolates, originally identified as a *Dactylum* sp. (isolate 109) from the United States, very closely matched the morphological description of *C. dendroides* and was identified as such by Gams (8). This isolate, therefore, was important when isolates were compared at the molecular level. Molecular analyses of this isolate and two isolates (99 and 100) obtained from culture collections and identified as *C. dendroides* showed them to be distinct from the majority of cobweb pathogen isolates studied.

Most of the cobweb pathogen isolates obtained from different geographic regions, including both MBC-sensitive and MBC-resistant isolates from Ireland and Great Britain, were 99.6 to 100% similar in their ITS nucleotide sequences and were regarded as one species, with minor nucleotide variability splitting them into three subgroups. MBC-sensitive isolates in subgroup 1 included isolates originally identified by the Centraalbureau voor Schimmelcultures and the International Mycological Institute as *C. mycophilum*. The one MBC-sensitive isolate, 24, which was grouped with the MBC-resistant isolates was deposited with the International Mycological Institute in 1982, before MBC resistance was detected in the cobweb pathogen. We conclude that the isolates in all three subgroups are *C. mycophilum* and are distinct from the other three *Cladobotryum/Hypomyces* species which also have been reported to cause cobweb disease (3). Despite the fact that previous reports have indicated a considerable overlap in conidial dimensions among *C. mycophilum*, *C. varium*, and *C. penicillatum* (7), nucleotide sequence analysis indicates that none of the isolates from infected mushroom compost in the present study is *C. penicillatum*.

RAPD analysis of the isolates renamed *C. mycophilum* showed that the MBC-resistant isolates clustered strongly together compared with the much more divergent MBC-sensitive isolates, suggesting that the former may be clonal. It is most likely that the Irish MBC-resistant *C. mycophilum* isolates, first detected after the cobweb epidemic in 1992, originated from a single site in Ireland. MBC resistance in Great Britain was reported before 1992; hence, although they clustered with the Irish MBC-resistant isolates, the British MBC-resistant isolates showed some variation from them, suggesting that they may have arisen independently. No MBC resistance was found in isolates obtained from outside Ireland or Great Britain.

The number of GA repeats in the microsatellites from these *C. mycophilum* isolates, in general, correlated with their geographical origin and appeared to confirm the clonal nature of the MBC-resistant isolates. While most of the European MBC-sensitive isolates analyzed had considerably fewer GA repeats than the MBC-resistant isolates, MBC-sensitive isolate 66 from Ireland possessed only one GA repeat fewer than the MBC-resistant isolates, suggesting that it may be related to the parental form of the resistant isolates. Microsatellites, which are reported to be widespread in fungi (10), could be used as molecular markers in population studies of *C. mycophilum*.

This study provides some understanding of the causal agents of cobweb disease in the cultivated mushroom *A. bisporus*. Cobweb disease has been associated with several *Cladobotryum* species (3), and although we do not dispute this fact, we suggest that *C. mycophilum* may now be the most common causal agent. The taxonomy of the genus *Cladobotryum* (*Hypomyces*)...
is well documented (6, 16, 17); however, the lack of type cultures and the apparent effects of prolonged storage on sporulation in available cultures present difficulties in their identification. Accurate identification of the cobweb pathogen, which can be achieved by using molecular markers, is necessary for those working with cultivated mushrooms, especially in view of the potential for \textit{C. mycophilum} to develop fungicide resistance. In relation to MBC resistance in particular, we wish to correct the identification of the cobweb pathogen in our earlier publication (13) to \textit{C. mycophilum}.

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**REFERENCES**


