Isolation and identification of the basidiomycete fungi associated with fairy rings in golf course putting greens

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INTRODUCTION

Nearly 60 species of basidiomycete fungi in the families Agaricaceae, Lycoperdaceae, Marasmiaceae, and Tricholomataceae have been associated with fairy rings on turf (2). The most severe symptom (Type I) leaves necrotic bands from 2 - 4 inches wide and up to 30 feet in diameter (**Image 1 & 4**). These necrotic bands are most likely a result of soil hydrophobicity caused by byproducts of fungal growth or the fungal mycelium itself. However, fungal secretion of toxic compounds and direct penetration of host tissue have also been implicated in Type I symptom formation (4,8).

Basidiomycetes are difficult to isolate from soil because they characteristically grow more slowly in culture than ascomycetes, zygomycetes, and deuteromycetes. Even when successfully isolated, basidiomycetes rarely produce sexual structures in culture upon which identification can be based. Therefore, identification of fairy ring fungi has relied solely on the formation of above-ground basidiocarps. This phenomenon does not always occur, and daily mowing practices on putting greens oftentimes don't allow basidiocarps to get to developmental stages that are morphologically relevant for identification. Above-ground basidiocarps may also not be truly indicative of the soil borne fungus responsible for fairy ring development. Fairy ring diagnosis without basidiocarps depends on observation of hyphae with clamp connections, which some basidiomycetes do not form, or on symptom expression, which can be confused with other soil borne turf diseases or abiotic factors.

Reports by golf course superintendents of inadequate fairy ring control are common, and it is not known which fairy ring species are most prevalent in golf putting greens. The different species involved are likely to differ in their temperature thresholds, sensitivity to fungicides, and other important factors that influence the effectiveness of management programs. Accurate isolation and identification methods are necessary to allow for distribution studies, and may lead to more directed management recommendations for superintendents.

OBJECTIVES

- Develop procedures to isolate basidiomycete fungi from fairy ring-infested soils.
- Identify basidiomycete isolates associated with fairy ring symptoms on creeping bentgrass and bermudagrass putting greens.

METHODS

Cup cutter-sized samples (10.8 cm diameter/12.7 cm depth) were collected from golf putting greens exhibiting fairy ring symptoms at twenty golf courses and two research farms in NC, SC, FL, and HI. Greens were established with either creeping bentgrass (cv. ten 'A-1', two 'Penncross, and one 'Crenshaw') or bermudagrass (cv. seven 'Tifeagle' and one 'Champion'). Puffballs later associated with symptoms were also collected from four bermudagrass greens ('Tifeagle') and three creeping bentgrass ('A-1' and 'Penncross') greens.

Isolation

Traditional soil block and soil sprinkle methods were evaluated. The soil block method involved exposing the soil profile to a humid, room temperature (21˚C) environment and isolating from mycelial cords or primordia as they became apparent. The sprinkle plate method is similar to the soil block, except soil particles were sprinkled on selective media for isolation. PDA was amended with antibiotics (50 µg/L of streptomycin, chloramphenicol, and tetracyclin), and benomyl (2 µg/L) for suppression of contaminant fungi. A method developed by Thorn et al. (11) was also evaluated. The method involved washing organic matter particles and plating them on a selective medium containing lignin, benomyl, and guaiacol. Lignin served as the major carbon source, and guaiacol acted as an indicator of laccase or peroxidase production, which is characteristic of many basidiomycetes.

Wood substrates have been used with success for isolation and capture of some basidiomycetes from soils (3,7). White birch toothpicks and pot stakes were placed at regular intervals throughout each soil block and used as baits (**Image 2**). In successful isolations, mycelium was scraped from the wood and cultured, or the wood was cut into small pieces and placed on selective media (**Image 3**).

For isolation from puffballs, a small amount of context tissue from the gleba was placed on Leonian media (1.25 g of KH_2PO_4, 0.625g of $MgSO_4$, 0.625g of peptone, 6.25g of maltose, 6.25g of malt extract, and 15g agar per 1L of distilled water) amended with antibiotics and 2 µg/L of benomyl (**Image 5 & 6**). As concluded by Swartz, this medium grew puffball mycelium well (6,9), and was used to isolate mycelial cords and primordia from the soil blocks of four samples **(Table 2)**.

Identification

Isolates were examined for presence of clamp connections or identifiable asexual or sexual structures. Genomic DNA from 64 cultures was extracted using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA). PCR amplification of rDNA regions ITS1, 5.8S rRNA, and ITS2 was performed using the universal fungal primers ITS5 and ITS4 and the basidiomycete-specific primers ITS1f and ITS4b (5). Thermal cycling conditions involved an initial denaturation step at 94°C for 1 min; followed by 32 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 2 min. Amplification products were purified with a Qiaquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sent to the Duke University DNA Sequencing Facility for cleanup, electrophoresis and fluorometric analysis. The consensus sequence for each isolate was determined from 1-2 reactions each with primers ITS1f and ITS4b. A BLAST search of GenBank was performed with each sequence, and four to six of the most similar sequences were downloaded for comparison. All sequences were aligned using the ClustalW method in MegAlign (7.1; DNASTAR Inc., Madison, WI) and adjusted by visual examination. Phylogenetic trees were constructed in MEGA 2.1 using the neighbor-joining algorithm from genetic distances calculated using the Kimura 2-parameter model. Due to the similarity in sequences among the puffball isolates, *M. oreades* EF187911.1 was used as an outgroup. Bootstrap values were calculated in MEGA 2.1 based on 1,000 random samples of the data set.

Image 1: Type I fairy ring symptom on 'Tifeagle' bermudagrass putting green.

Isolation from soil

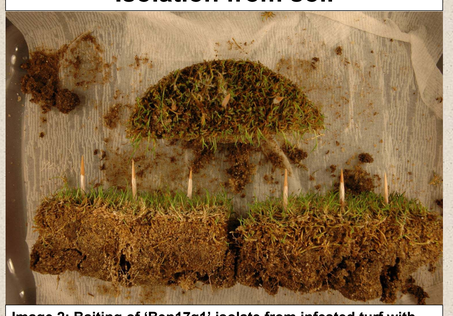

Image 2: Baiting of 'Ben17g1' isolate from infested turf with white birch toothpicks

Image 3: Guaiacol staining indicating laccase production by 'Ben17g1'

Image 4: Type I fairy ring symptom on 'A-1' creeping bentgrass putting green.

Isolation from puffball

Image 5: Puffballs from 'A-1' creeping bentgrass putting green.

Image 6: Hyphal growth of puffball isolate on Leonian medium.

Table 1: Isolates recovered from the 'Ben' site.

Isolate	Green	Collection Date	Isolation Method
Ben17g1	17g	8/27/06	Wood baiting
Benleaf17g2	17g	11/6/06	Wood baiting
Ben2gSB1	2g	11/6/06	Wood baiting

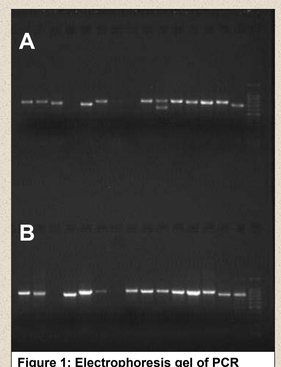

Figure 1: Electrophoresis gel of PCR products from universal fungal primers ITS4 and ITS5 (A), and basidiomycete specific primers ITS1f and ITS4b (B).

Table 2: Isolates recovered from fairy rings associated with puffballs

Isolate	Site	Turf Type	Location	Isolation Method
BHSBpuff1	BH	'Tifeagle' bermudagrass	FL	Soil Block/Primordia
EC3gSB EC16gSB	EC	'Penncross' bentgrass	NC	Soil Block/Cord
KapSoilMycel1	Kap	'Tifeagle' bermudagrass	HI	Soil Block/Cord
LCpuff1	LC	'Tifeagle' bermudagrass	SC	Puffball
LWA1puff2	LW	'A-1' bentgrass	NC	Puffball
LW19g5014	LW	'Penncross' bentgrass	NC	Puffball
LWTifpuff1 LWTifpuff2	LW	'Tifeagle' bermudagrass	NC	Puffball
MHSBpuffmyc1	MH	'Tifeagle' bermudagrass	NC	Puffball
OCNG706 b-d	OC	'A-1' bentgrass	NC	Puffball
Shillpuf 1a-2b	SH	'Penncross' bentgrass	NC	Puffball

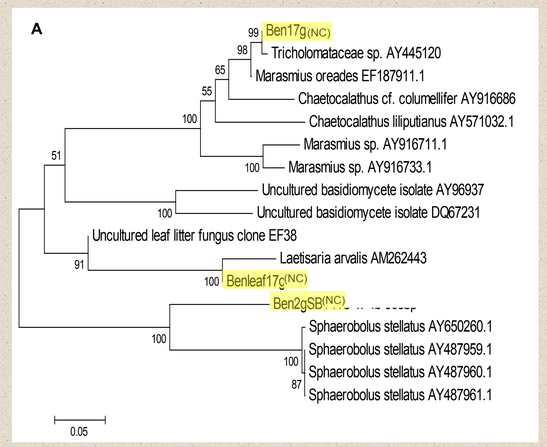

Figure 2: Neighbor joining phylograms produced from the sequences of ribosomal DNA regions ITS1, 5.8S, and ITS2 as amplified by the ITS1f and ITS4b primer set. Phylograms include sequences from three isolates recovered from one site with ring infested soils associated with mushrooms (A) and nineteen isolates recovered from puffballs or ring infested soils associated with puffballs (B), along with 4-6 of the most similar sequences obtained from a BLAST search of Genbank. Scale bar indicates the horizontal distance corresponding to genetic distance as calculated by the Kimura 2-parameter model. Bootstrap values are indicated adjacent to the nodes and are based on 1,000 resamplings of the data set.

RESULTS

- **Isolates from infested soil at the 'Ben' site possessed clamp connections, and were easily recovered on all of the selective media used (Table 1). Recovery of these isolates via wood baiting was particularly successful.**

- **Four cultures of puffball-forming fungi have been isolated and characterized from infested soils thus far. These cultures were recovered from mycelial cords or primordia formed on incubated soil blocks and subsequently isolated on modified Leonian media.**

- **Due to contamination by *Aspergillus*, *Curvularia*, and several other ascomycete and deuteromycete fungi, no basidiomycete fungi were isolated or characterized from soils of 18 of the 22 sampling sites with the methods described.**

- **Primers ITS1f and ITS4b exclusively amplified the ITS regions of isolates with clamp connections and isolates from puffball tissue (Figure 1). This PCR amplification also yielded an ~ 900 bp fragment that could be sequenced and used for phylogenetic analysis.**

- **Isolates from the 'Ben' site did not have similar ITS sequences. Ben17g1, which was collected in the summer of 2006, was clustered in a clade with a Tricholomataceae sp. and *Marasmius oreades*, while isolates collected in January of 2007 grouped in more distant clades (Figure 2A).**

- **Isolates from puffballs and puffball infested soils had more similar ITS sequences than those from the 'Ben' site. ITS sequences of isolates from the 13 isolates from NC and HI were similar to sequences of *Vascellum pratense*, while sequences of 6 isolates from FL, SC, and NC were similar to *Lycoperdon pusillum* (Figure 2B).**

DISCUSSION

The soil profile of golf putting greens is biologically diverse, and fungi that are fast growing and have many propagules will be routine contaminants in attempts to isolate slower growing basidiomycetes. Due to this, many of the methods outlined were unsuccessful in isolating basidiomycetes, particularly puffball fungi, from fairy ring infested soils. The use of different selective media may hold promise, however. Isolation of puffball species was not successful using the lignin-benomyl-guaiacol medium. Yet, a modified Leonian medium provided a more suitable semi-selective medium for hyphal growth, and will be utilized exclusively in further isolation attempts of puffballs from infested soils.

Although all techniques were successful in recovering basidiomycetes from the 'Ben' samples, baiting with birch wood was particularly conspicuous and simple. In recently received soil samples, basidiocarps have been observed fruiting from these wooden stakes, supplying morphological evidence for identification (**Image 7**).

Isolates successfully obtained from the 'Ben' site yielded interesting results. Isolate Ben 17g1 was recovered from a sample expressing fairy ring symptoms in the summer of 2007. This isolate showed sequence similarity to *Marasmius* sp. which have been commonly named as fairy ring pathogens, and have also been associated with the sterile white basidiomycete complex that can cause rots on snap bean, corn, rye and other crops in the Southeast U.S. (1,12). Isolates were obtained later the next winter from "white patch" symptoms on the same green (Benleaf17g2) and on a different green (Ben2gSB1). Isolates had similar culture morphology, with noticeable clamp connections, but hyphae were 2-3µm thinner. ITS sequences from the isolates did not align with the earlier collected Ben 17g1. They also grouped in distinctly separate clades, with Benleaf17g2 clustering closely with *Laetisaria arvalis*, (a reported biocontrol agent), and Ben2gSB1 clustering with several Genbank entries of *Sphaerobolus stellatus*, (the cannonball fungus). This demonstrates the seasonal diversity of basidiomycete fungi that are present in golf putting greens.

ITS sequences of the puffball isolates also yielded interesting results. On the basis of ITS sequence similarity with Genbank accessions, the 19 puffball isolates sampled are more closely related to either *Vascellum pratense* or *Lycoperdon pusillum* than to *Lycoperdon perlatum*, the most commonly named species associated with fairy rings on greens in the Southeastern U.S. The geographical distribution is also of interest, as an isolate from Hawaii groups with isolates from North Carolina in a clade with *Vascellum pratense*, while isolates from the Carolinas and a Florida isolate groups in a clade with *Lycoperdon pusillum*. Both *Vascellum pratense* and *Lycoperdon pusillum* have been reported as known causes of fairy rings in Japan. Terashima et al. has designed specific primers for both of these species (10), which will be utilized in future experiments aimed at detecting these fungi in fairy ring infested soils and in plant parts.

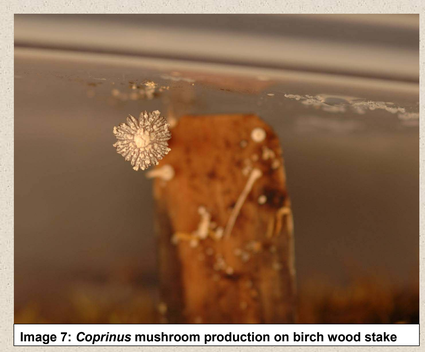

Image 7: *Coprinus* mushroom production on birch wood stake

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