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Host- and tissue-specificity of moss-associated *Galerina* and *Mycena* determined from amplicon pyrosequencing data

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ABSTRACT

The genetic diversity of two agaricoid saprotroph genera, *Galerina* and *Mycena*, and their distribution across bryophyte host species, and within bryophytes' photosynthetic and senescent tissues, was assessed using data from two pyrosequencing biodiversity inventories of bryophilous fungi. A total of 9 498 *Galerina* and 5 731 *Mycena* reads were mapped to branches broadly distributed throughout backbone trees, reflecting no obvious evolutionary specialization of particular fungal lineages to moss hosts/substrata. Although a few OTUs occurred with equal frequency across the hosts, most exhibited some degree of specialization to one or more bryophytes, indicating that the quality of different mosses as substratum varies between species. With one exception, all *Galerina* and *Mycena* OTUs were more frequent and abundant in senescent than photosynthetic tissues, likely reflecting saprotrophic nutritional modes in the fungi. A single *Mycena* OTU showed considerable colonization of both tissues, which may reflect an opportunistic parasitic or endophytic lifestyle.

Introduction

Members of the basidiomycete genera *Galerina* and *Mycena* are common throughout north temperate regions and most are thought to be generalist decomposers of plant material (Smith & Singer 1964; Smith 1971; Emmett *et al.* 2008; Gulden 2008; Boberg *et al.* 2011). Both produce a variety of extracellular laccase, peroxidase and cellulolytic enzymes that are effective at decomposing both the cellulosic and polyphenolic (e.g. lignin-like) components of plant cell walls (Steffen *et al.* 2000; Kellner *et al.* 2007; Steffen *et al.* 2007; Tortella *et al.* 2008; Baldrian 2009; Nagendran *et al.* 2009; Ibrahim *et al.* 2011; Kähkönen & Hakulinen 2011; Wolfe *et al.* 2012). While they are primarily saprotrophic, other life history strategies have been reported in both genera. In the case of *Galerina*, Redhead (1981) demonstrated that *Galerina paludosa* is, in fact, parasitic on *Sphagnum* mosses, while recent surveys have detected *Mycena* species as asymptomatic endophytic associates of a variety of healthy plant roots (Kernaghan & Patriquin 2011). Additionally, species of *Mycena* have been reported to form

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mycorrhizal associations with orchids (Martos et al. 2009; Ogura-Tsujita et al. 2009). In the boreal forest, both genera are regularly reported in association with mosses, or associated with various types of litter in 'mossy habitats' (Emmett et al. 2008; Gulden 2008), suggesting possible relationships with these plants either as saprotrophs or symbionts.

Next generation sequencing techniques have led to a revolution in microbial ecology by providing opportunities to generate unprecedented numbers of sequences and to detect both organisms present with extremely low abundance and those that cannot be cultured *in vitro* (Begerow et al. 2010; Ekblom & Galindo 2011). These techniques are currently used in studying broad level questions in fungal community diversity and ecology (e.g. Buée et al. 2009; Blaalid et al. 2012; Davey et al. 2012) but are less commonly used to address questions pertaining to specific fungal taxa. Using sequence data mined from two ITS2 pyrosequencing-based molecular surveys of fungal diversity associated with common mosses in the boreal forest, we investigate host and tissue preferences within *Mycena* and *Galerina* species associated with these mosses, as well as the genetic diversity that was detected within the genera.

Materials & methods

Data mining and bioinformatics

Galerina and *Mycena* sequences were mined from two pyrosequencing datasets investigating fungal diversity associated with mosses: (1) a study investigating moss-associated fungal diversity along an elevational gradient (Davey et al. 2013; MG-RAST Project Name: Bryophilous Fungi Across Elevation Gradient, MG-RAST ID: 4498705.3–4498719.3); (2) a study investigating responses of moss-associated fungal diversity to simulated nitrogen deposition (Davey et al. unpub.; MG-RAST Project Name: Bryophilous Fungi and Nitrogen Deposition, MG-RAST ID: 4510746.3–4510753.3). In Study 1, shoots of *Dicranum scoparium*, *Hylocomium splendens*, *Pleurozium schreberi* and *Polytrichum commune* were randomly sampled, during the summer of 2009, from 10 m × 10 m plots located in four different vegetation zones along each of two transects. The transects were 1 km long and traversed elevation gradients of approximately 200 vertical metres on the northwest-facing slopes of Synnfjell mountain in southern Norway (N 61°6′25.7 976″ E 9°48′7.34″). Five moss shoots of each species were collected, if present, in each of the eight plots, yielding a total of 150 shoots for fungal diversity analyses using pyrosequencing techniques (Davey et al. 2013). In Study 2, shoots of *Dicranum*, *Hylocomiu*, and *Pleurozium* were randomly sampled during the summer of 2010 in a Norway spruce forest near Kittilbu in Gausdal Vestfjell, Norway (61°10′N, 09°90′E). For each moss species, a representative shoot was collected from eight individual moss colonies within three 15 × 15 m control plots and three 15 × 15 m plots that had been fertilized at a rate of 150 kg N $ha^{-1}a^{-1}$ for the previous 7 yr. The resulting 144 moss shoots were analysed for fungal biodiversity using pyrosequencing techniques (see Davey et al. unpub.). In both studies, moss shoots were cleaned of coarse debris, washed, and separated into green, photosynthetic and brown, senescent sections as described in Davey et al. (2012). Freeze-dried, crushed shoot fragments were stored in 2× cetyltrimethylammonium bromide (CTAB) extraction buffer at −80 °C until DNA extraction. Genomic DNA was extracted from photosynthetic and senescent shoot fragments and the fungal ITS 2 region was pyrosequenced as described in Davey et al. (2012) using the primer sets ITS1F/ITS4 (White et al. 1990; Gardes & Bruns 1993) and ITS3/ITS4 (White et al. 1990) in a nested PCR, with each shoot fragment being individually tagged at both ends for downstream recognition during data analysis. Specificity of the general ITS1F/ITS4 and ITS3/ITS4 primer sets to the targeted genera has not been previously investigated, to the best of the author's knowledge, but multiple representatives of both genera were recovered in these studies and primer mismatches between the ITS1F, ITS, and ITS4 primers, as identified by BLAST comparison to the NCBI-nr database, were detected only in the final 5′ base of ITS3 for the genus *Mycena*, and as such are expected to have minimal impact on amplification.

A total of 972 403 ITS2 sequences were examined: 451 850 from Study 1 and 520 553 from Study 2. Sequences were quality-filtered, denoised, and clustered using Qiime v. 1.3.0 (Caporaso et al. 2010). Reads with length less than 250 bp, an average Phred quality score of less than 25, or errors in the tags were discarded. Those sequences with homopolymers of length >10 bp, ambiguous base calls (N), and more than one error in the primer sequence were also filtered from the dataset. In addition, a sliding window of 50 bp in length was used to identify regions of low sequence quality (average quality score <25) and truncate the sequence at the beginning of the low quality window. Truncated sequences still meeting the minimum length requirement (250 bp) were retained in the dataset. Denoiser v. 0.91 (Reeder & Knight 2010) as implemented in Qiime v 1.3.0 (Caporaso et al. 2010) was used to denoise the resulting reads, which were subsequently clustered into OTUs using a 97 % similarity threshold and the uclust algorithm as implemented in Qiime v. 1.3.0 (Caporaso et al. 2010; Edgar 2010). To account for unequal sequencing depth across the samples, the dataset was rarified to a depth of 800 sequences per moss shoot fragment, and those samples for which <800 sequences were obtained were discarded from the analyses. OTUs identified as *Mycena* or *Galerina* based on best BLAST match to the NCBI-nr database with the minimum criterion of 90 % identity and 70 % coverage were extracted from the dataset. Due to the unreliability of identifying short sequence fragments to the species level (Ovaskainen et al. 2010) based on BLAST matches to existing databases, OTUs were identified to genus and subsequently numbered. To mitigate the possible effects of sequencing errors (Tedersoo et al. 2010) and tag switching during PCR (Carlsen et al. 2012), 'presence' in a shoot fragment was defined as a minimum of five sequences, resulting in the effective exclusion of global singleton OTUs and low abundance incidences that may represent tag-switching events.

Statistic analyses and genetic diversity

Backbone trees for *Galerina* and *Mycena* were generated in RaxML (Stamatakis 2006) from full length ITS reference sequences downloaded from GenBank using a GTR gamma

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rate heterogeneity model. Support values for both trees were calculated from 1 000 bootstrap replicates. The extracted pyrosequencing reads were aligned with the ITS2 region of these reference sequences using MAFFT version 6.925 (Katoh 2008) and subsequently mapped to the backbone trees using the Evolutionary Placement Algorithm (EPA) (Berger *et al.* 2011) as implemented on the RaxML web server (available: http://sco.h-its.org/exelixis/software.html). Frequency and abundance of every OTU occurring in >10 shoot fragments was examined in photosynthetic versus senescent tissues of the mosses, and in each of the four hosts. Pearson's chi-squared test was used to assess whether differences in frequencies were significant, and whether the total number of reads recovered for each host and tissue type differed significantly from what would be expected at random, given their frequency of occurrence.

Results

Data characteristics and genetic diversity

A total of 38 OTUs representing 4.1 % of the dataset's total sequence diversity were identified as belonging to the genera *Galerina* and *Mycena*. Twenty *Galerina* OTUs (9 498 reads) were recovered and they occurred in 149 shoot fragments, while 18 *Mycena* OTUs (5 731 reads) were recovered from 84 shoot fragments. Of the 38 OTUs detected, only 11 occurred in >10 shoot fragments. The taxonomic affinity of these OTUs based on best BLAST match to the NCBI-nr database is indicated in Table 1. Co-occurrence of the two genera was low (23 %), and within each genus, multiple OTUs generally did not co-occur in a single shoot fragment. Seventy-five percent of shoots in which *Galerina* occurred contained only a single OTU of the genus, and similarly, 85 % of shoots containing *Mycena* were host to only a single OTU of the genus.

Both the *Galerina* and *Mycena* sequences were mapped by EPA to a variety of nodes throughout the backbone trees, and were not restricted to single lineages (Figs 1 and 2). In the case of *Galerina*, the majority of sequences mapped to *Galerina sphagnicola* (32 %) and *Galerina pseudocamerina* (34 %), with substantial proportions of the sequences also mapping to *Galerina pumila* (18 %), *Galerina fallax* (11 %), *Galerina luteolosperma* (9 %), and *Galerina calyptrata* (8 %) (Fig 1). Sequences identified as belonging to *Mycena* mapped primarily to *Mycena galopus* (36 %) with significant proportions of the sequences also mapping to *Mycena metata* (27 %) and *Mycena simia* (14 %) (Fig 2).

Host preference

A number of OTUs (*Galerina* spp. 1,2,4,5 and *Mycena* spp. 2,3) were detected in all hosts (Fig 3A). However, *Galerina* sp. 1 and *Mycena* sp. 2 were still detected both more frequently and in greater abundance in *Dicranum* and *Hylocomium*, respectively, than would be expected at random (Fig 3A and B). Although *Galerina* spp. 4 and 5, and *Mycena* spp. 3 were not detected significantly more frequently in any of the hosts, the total number of reads detected was significantly higher in *Pleurozium* and *Polytrichum*, respectively, than expected at random (Fig 3B). *Mycena* sp. 1 was found in all hosts except *Dicranum* but was both significantly more frequent and more abundant in *Pleurozium* and *Hylocomium*. *Galerina* spp. 6 and 7 were detected in only *Hylocomium* and *Pleurozium*, but were significantly more abundant in *Pleurozium*. Conversely, while *Mycena* sp. 4 was found in the same species, it was most abundant in *Hylocomium* (Fig 3A and B). *Galerina* spp. 3 occurred exclusively in *Dicranum* (Fig 3A).

Tissue preference

Although all OTUs occurring in >10 shoot fragments were detected in both photosynthetic and senescent tissues, most OTUs occurred at significantly higher frequency in brown tissues than in green tissues (Fig 3C). This difference was not statistically significant in *Mycena* sp. 2, and in contrast, *Mycena* sp. 4 occurred at higher frequency in green tissues, although the difference was non-significant. The total number of sequences recovered was also greater in brown tissues than in green tissues, with the exception of *Mycena* sp. 4. For most OTUs, significantly more reads were detected in the brown tissues than expected at random, although in *Galerina* sp. 2 and *Galerina* sp. 7, more reads were detected in the green tissues than expected at random (Fig 3D). In *Galerina* sp. 1 and

Table 1 – BLAST-based taxonomic affinity of the most abundant and frequent *Galerina* and *Mycena* OTUs detected in bryophyte tissues based on the most abundant sequence in each OTU

Name	Abundance	Frequency	Best BLAST match to NCBI-nr database			
			GenBank accession no.	Coverage	% Identity	Species
Galerina sp. 1	993	45	AJ585451	88	100	*Galerina fallax*
Galerina sp. 2	2 812	40	AJ585467	87	100	*Galerina hypnorum*
Galerina sp. 3	803	23	AJ585466	87	99	*Galerina calyptrata*
Galerina sp. 4	786	17	HM240525	97	99	*Galerina atkinsoniana*
Galerina sp. 5	897	17	AJ585453	87	99	*Galerina luteolosperma*
Galerina sp. 6	1 289	16	AJ585462	87	99	*Galerina cephalotrica*
Galerina sp. 7	1 597	13	AJ585477	84	99	*Galerina pumila*
Mycena sp. 1	2 820	31	JF908412	97	99	*Mycena metata*
Mycena sp. 2	1 255	23	GU234138	97	100	*Mycena simia*
Mycena sp. 3	380	15	EU846251	97	95	*Mycena tenax*
Mycena sp. 4	304	10	JF908467	97	99	*Mycena clavicularis*

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Fig 1 – RaxML generated backbone tree of *Galerina* species showing the EPA-based placements of all *Galerina* pyrosequencing reads. Bootstrap support values are indicated over branches. Branches on which *Galerina* reads were placed are indicated in green, and their width reflects the total number of reads that were placed on that branch.

Mycena spp. 1 and 4, the number of reads recovered from each of the tissue types did not differ significantly from what would be expected at random.

Discussion

Genetic diversity

Fully one-third (35 %) of the moss shoot fragments studied were colonized by one or more OTUs of *Galerina* or *Mycena*, and these groups represented just over 4 % of the total sequences recovered in the two studies that were data-mined. The sequences recovered represent considerable genetic diversity within both genera, as evidenced by their mapping to nodes that are broadly distributed across the backbone tree of each genus. Although in both genera the majority of reads mapped to a relatively small number of nodes, smaller numbers of reads (<500 each) were mapped to a wide variety of nodes. This suggests that many species of *Galerina* and *Mycena* have the capacity to colonize bryophyte substrates to some degree, but a relatively small number do so with considerable success. Indeed, most of the species to which a large number of sequences were mapped and to which the OTUs showed taxonomic affinity through BLAST searches are known to be bryophilous or sometimes occur with bryophytes (*G. sphagnicola*, *G. pumila*, *G. fallax*, *G. luteolosperma*, *M. galopus*; Emmet *et al.* 2008; Gulden 2008). However, considerable numbers of reads also mapped to *G. pseudocamerina* and *M. metata* which, based on fruit-body inventories, are best known from conifer litter, indicating that although bryophyte substrates may be unsuitable for the production of fruit bodies in these species, the fungi may still play an important role in bryophyte litter decomposition. Although the unique composition of bryophyte cell walls (Popper & Fry 2003) suggests decomposing moss materials may represent a unique niche, the mapping of

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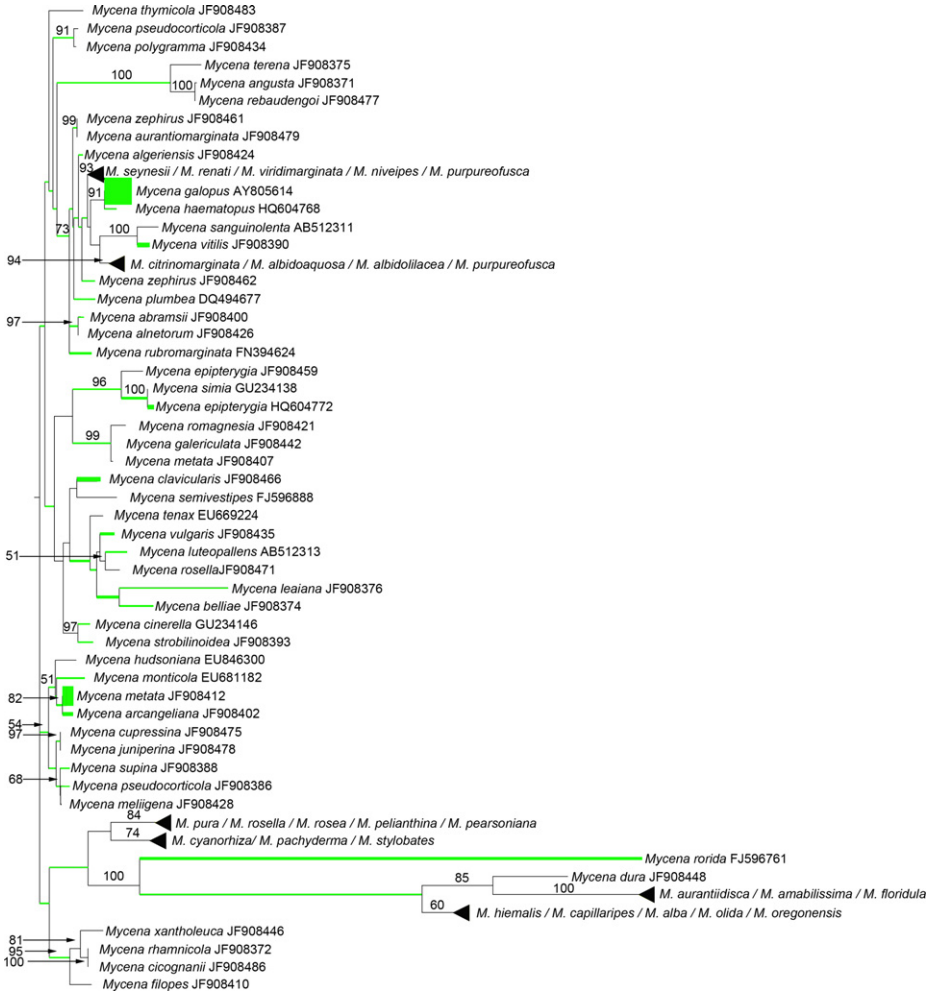

Fig 2 – RaxML generated backbone tree of *Mycena* species showing the EPA-based placements of all *Mycena* pyrosequencing reads. Bootstrap support values are indicated over branches. Branches on which *Mycena* reads were placed are indicated in green, and their width reflects the total number of reads that were placed on that branch.

sequences to many branches of the backbone tree would suggest that there has not been a distinct evolutionary specialization in the ecological strategy of *Galerina* and *Mycena*, or a subset of each genus, towards the colonization of mosses. However, conclusions about evolution must be drawn with caution because (a) a limited number of bryophyte species are represented here, (b) abundance of fungal rDNA is not a consistent estimate of fungal biomass between species (Baldrian *et al.* 2012) and (c) both *Galerina* (Gulden *et al.* 2005) and *Mycena* (Moncalvo *et al.* 2002) are polyphyletic genera, and cannot be truly considered evolutionary units in this context.

Host preferences

Although *Galerina* and *Mycena* species are often reported in association with a particular class of substrate or litter (e.g. *Sphagnum* mosses, decaying conifer wood, needle litter) or as fruiting among 'mosses' in a very general sense (Smith and Singer 1964, Smith 1971; Emmett *et al.* 2008; Gulden 2008), they are generally not considered to occupy extremely limited ecological niches. Indeed, the majority of frequently occurring OTUs of both *Galerina* and *Mycena* were detected in all or most of the hosts investigated. Although it is not possible to determine whether the DNA detected represents spores or actively growing mycelium, it is assumed that washing with Triton-X detergent and subsequent repeated rinsing steps have largely removed any spores adherent to the surface of the mosses, and as such 'presences' are thought to represent active mycelial growth. Accordingly, those OTUs present in all hosts seem likely to be producing a suite of extracellular enzymes that is well suited to the broad spectrum decomposition of plant cell walls, a hypothesis that is supported by the detection of such enzyme apparatus in the genome of *Galerina* species (Nagendran *et al.* 2009; Wolfe *et al.* 2012). However, a number of those OTUs occurring in all hosts occurred more abundantly in a single host than would be expected at random. Furthermore, other OTUs exhibited distinct preferences for a single host, occurring with it both at higher frequency and abundance than would be expected at random. It would seem that while the broad spectrum of degradative enzymes produced by *Galerina* and *Mycena* species (Steffen *et al.* 2000; Kellner *et al.* 2007; Steffen *et al.* 2007; Tortella *et al.* 2008; Baldrian 2009; Nagendran *et al.* 2009; Ibrahim *et al.* 2011; Kähkönen & Hakulinen 2011; Wolfe *et al.*

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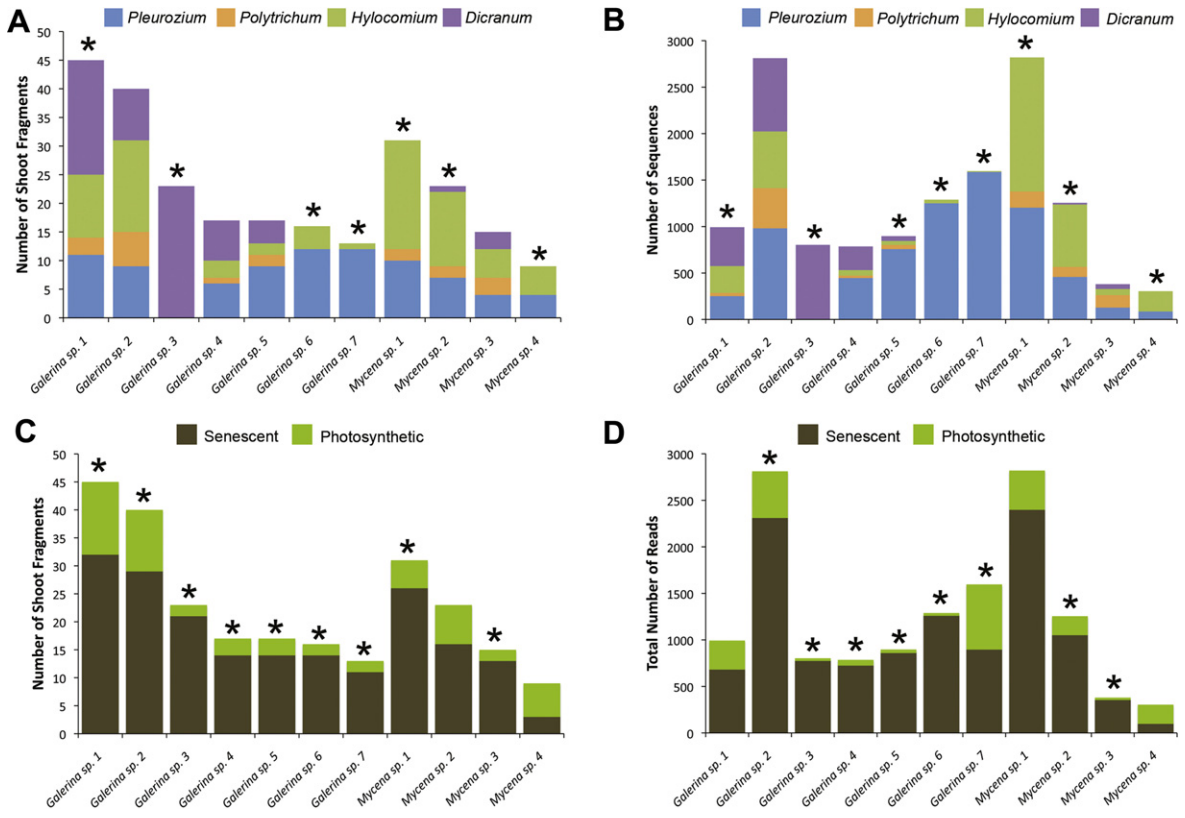

Fig 3 – The frequency of occurrence (A,C) and total abundance (B,D) of the 11 most common *Mycena* and *Galerina* OTUs in the four host species (A,B) and photosynthetic versus senescent tissues (C,D). OTUs in which the frequency or abundance in particular hosts is significantly different than would be expected at random are marked with an asterisk (*).

2012) likely allows them to colonize a variety of moss hosts, they still exhibit predilections for particular moss hosts that may represent more nutritionally rich or more readily colonized substrates. In particular, *Pleurozium* was colonized more heavily than other hosts, indicating that it may represent a particularly suitable substrate for the fungi, as was postulated by Davey *et al.* (2009, unpublished), who found *Pleurozium* and *Hylocomium* supported significantly more fungal biomass than other moss hosts. Although multiple OTUs showed preferences for the same hosts, it should be noted that there was a low degree of co-occurrence between the two genera, and even between OTUs of the same genus. This suggests either that the distribution of these OTUs is highly patchy, or that competition may be occurring between them, and individual OTUs are effective at excluding one another at the scale of a single moss shoot. Given that anthropogenic global change (e.g. climate warming, nitrogen deposition) can have significant and lasting effects on the species composition of bryophyte communities (e.g. Gignac 2001; Strengbom *et al.* 2001; Elmendorf *et al.* 2012), the detection of host preferences within two common saprotrophic basidiomycete genera often occurring with mosses may indicate that they will be similarly affected by global change.

Tissue preferences

Galerina and *Mycena* are both thought to be saprotrophic genera, although there are reports of some instances of biotrophic parasitism of mosses (Redhead 1981; Gulden 2010) by *Galerina*, and of both root endophytism (Kernaghan & Patriquin 2011) and orchid mycorrhizal symbiosis (Martos *et al.* 2009; Ogura-Tsujita *et al.* 2009) occurring in *Mycena*. Assuming again that 'presences' in our study represent active mycelial growth, the detection of the majority of *Galerina* and *Mycena* OTUs predominantly in the senescent tissues of the mosses is supportive of an ecological role for these taxa as saprotrophs colonizing moribund and senescent portions of the lower moss stems. Despite both *Galerina* and *Mycena* producing a variety of extracellular enzymes (Steffen *et al.* 2000; Kellner *et al.* 2007; Steffen *et al.* 2007; Tortella *et al.* 2008; Baldrian 2009; Nagendran *et al.* 2009; Ibrahim *et al.* 2011; Kähkönen and Hakulinen 2011; Wolfe *et al.* 2012) that could potentially facilitate infection and colonization of living plant tissues, there was little evidence of extensive colonization of green tissues by most OTUs. Recent research has demonstrated that mosses have a well developed set of defences against microbial attacks comparable to those found in vascular plants (Akita & Valkonen 2002; Andersson *et al.* 2005; Mekuria *et al.* 2005; Wang *et al.* 2005; de Leon *et al.* 2007), lending support to the idea that *Mycena* and *Galerina* are largely unsuccessful against the moss host's defences and only occasionally manage to opportunistically colonize living, photosynthetic cells in the upper green parts of the shoots. However, in a single instance, *Mycena* sp. 4, an OTU was detected both more frequently and more abundantly in green tissues. Although this OTU did not demonstrate a significant preference for

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photosynthetic tissues, it would appear that it is at least capable of circumventing host defences in order to specifically colonize the photosynthetic tissues. Our detection of a *Mycena* species colonizing the green tissues of mosses suggests the interactions between bryophytes and *Mycena* species may not be limited to saprotrophism, supporting recent research demonstrating alternative ecological functions within this genus (Martos *et al*. 2009; Ogura-Tsujita *et al*. 2009; Kernaghan & Patriquin 2011) and providing impetus for further investigation of potential endophytic or parasitic interactions between them.

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