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# A comparison of the community diversity of foliar fungal endophytes between seedling and adult loblolly pines (*Pinus taeda*)

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## ABSTRACT

Fungal endophytes represent one of the most ubiquitous plant symbionts on Earth and are phylogenetically diverse. The structure and diversity of endophyte communities have been shown to depend on host taxa and climate, but there have been relatively few studies exploring endophyte communities throughout host maturity. We compared foliar fungal endophyte communities between seedlings and adult trees of loblolly pines (*Pinus taeda*) at the same seasons and locations by culturing and culture-independent methods. We sequenced the internal transcribed spacer region and adjacent partial large subunit nuclear ribosomal RNA gene (ITS–LSU amplicon) to delimit operational taxonomic units and phylogenetically characterize the communities. Despite the lower infection frequency in seedlings compared to adult trees, seedling needles were receptive to a more diverse community of fungal endophytes. Culture-free method confirmed the presence of commonly cultured OTUs from adult needles but revealed several new OTUs from seedling needles that were not found with culturing methods. The two most commonly cultured OTUs in adults were rarely cultured from seedlings, suggesting that host age is correlated with a selective enrichment for specific endophytes. This shift in endophyte species dominance may be indicative of a functional change between these fungi and their loblolly pine hosts.

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## Introduction

Plants harbor numerous and phylogenetically diverse species of endophytic fungi without symptoms of disease. Those that are horizontally transmitted among hosts with localized infections in above ground tissues (Class 3 endophytes, *sensu* Rodriguez *et al.* [2009]) are especially diverse and their ecological roles remain largely unknown. A number of studies have

suggested that some endophytic species have beneficial effects on their hosts, including pathogen defense (Minter 1981; Arnold *et al.* 2003), herbivore resistance (Diamandis 1981; Carroll 1988; Miller *et al.* 2008), as well as heat and drought tolerance (Bae *et al.* 2009). In return, it is assumed that endophytic fungi benefit from the interaction by acquiring protection and nutrition from their hosts and, in many cases, reproducing sexually on dead tissues of their host

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plant (Carroll & Carroll 1978; Saikonen et al. 1998). However, the nature of the plant-endophyte symbiosis likely falls along a mutualism-parasitism continuum (Saikonen et al. 1998; Sieber 2007) depending on the host-endophyte genotype–genotype interaction, environmental context, and the state of the host health (Carroll 1988; Redman et al. 2001), for example. Exploring endophyte species diversity and composition across environmental gradients and host contexts will help identify endophytic species with ecologically distinct roles as well as present a useful means to understand the environmental variables responsible for structuring fungal diversity.

Comparisons of endophyte communities across host age, i.e., seedling to adult stage, have been rarely conducted (although see Ferreira Rodrigues 1994), whereas comparisons over leaf age (young to old leaves) are more commonly explored (Espinosa-Garcia & Langenheim 1990; Ferreira Rodrigues 1994; Frohlich et al. 2000; Arnold & Herre 2003). Comparison of species abundance and richness across host tissue types is also limited in studies of foliar fungal endophytes with proper molecular data (although see Sandberg et al. 2014). Aspects of fungal endophyte communities that are unique to different life stages of a host are likely to give clues about host specificity, coevolution, host mechanisms for recruiting horizontally transmitted endophytic fungi as well as, potentially, any beneficial effects on host fitness, which are challenging to assess experimentally for endophytic fungi (Sieber 2007).

The goal of this study was to compare communities of foliar fungal endophytes in adult trees vs. seedlings of loblolly pines (*Pinus taeda*) in North Carolina's Duke Forest and to identify the most common and recurring endophyte species at these two different stages of host development. Foliar fungal communities were sampled during two seasons (summer and winter) for both adult trees and seedlings with culturing methods. Sampling was supplemented with culture-independent cloning to identify unculturable or slow-growing endophytic fungi. We used both taxonomy-dependent and operational taxonomic unit (OTU)-based approaches to characterize the fungal endophyte communities using the nuclear ribosomal internal transcribed spacer (ITS) and adjacent partial large subunit (nrLSU) RNA coding region. Exploring fungal endophytes in *P. taeda* of the Duke Forest using these molecular markers also allowed us to compare our results with an older endophyte diversity study of *P. taeda* by Arnold et al. (2007).

## Materials and methods

### Sampling pine needles and tissue processing

In 2010, the Free-Air CO<sub>2</sub> Enrichment (FACE) project in the Blackwood Division of Duke Forest (Orange County, NC) was completed (Hendrey et al. 1999) and half of each experimental plot was deforested. This led to the natural regeneration of pine seedlings in the newly opened half of each plot adjacent to adult *Pinus taeda* trees that were 20–30 y old. Three of the plots that had previously received ambient CO<sub>2</sub> treatments (1, 5, and 6) were targeted for sampling.

Field sampling was conducted in summer (June–August 2012) and winter (December 2012–January 2013), representing four sampling treatments; adult-summer, adult-winter, seedling-summer, seedling-winter, respectively. At each of the three plots, 11 to 12 of the oldest second-year needles from three adult trees (total of nine trees), were sampled using tree pruners or by climbing observation towers from multiple branches at various compass directions between 6 and 8 m above the ground. Second-year needles are distinguished from first-year needles by the color of their fascicle sheaths. For seedlings, we increased the sampling to six individuals per plot (total of 18 seedlings) due to lower isolation frequency compared to adult needles and sampled 11 to 20 needles per seedling. The seedling needles all consisted of the oldest primary juvenile needles growing near the lower part of the seedling, which are not fascicled and which are between 3 and 4 cm long (Bormann 1956). By sampling the oldest needle tissues from both adult trees and seedlings, we were able to compare the most established fungal endophyte communities from the respective age classes.

Needles were surface-sterilized by briefly immersing them in 95 % ethanol, followed by 2 min in 0.5 % hypochlorite and 2 min in 70 % ethanol. Two random 2 mm sections distributed across the needle length were cut and placed on 2 % malt extract agar (MEA) slants in 1.5 ml centrifuge tubes under sterile conditions. A total of 1284 and 408 needle segments from seedlings and adult trees (Table 1), respectively, were allowed to incubate on MEA, a growth media amenable to a broad group of fungal species (Arnold et al. 2007), for at least two months before cultures were sampled for genotyping. The culturing study was complemented with culture-free sampling where pine needles from adult trees and seedlings were collected in June of 2013, surface-sterilized, cut into sections in the same manner as for cultured samples and then bulked by host age. The culture-free method revealed whether the culture-dependent approach missed fungal species that are abundant in the communities of the two age classes.

### Genotyping fungal endophytes

For cultured fungal endophytes, at least fifty randomly selected isolates from each sampling treatment were initially processed for genotyping. Small pieces of mycelium (ca. 1 mm × 1 mm) were placed in 0.6 ml tubes with 50 μL of glass beads (400 μm VWR silica beads) and 50 μL of Tris–EDTA buffer. DNA was extracted by vortexing the tubes at maximum speed for 2 min. Products were diluted with 200 μL of TE buffer for direct use in PCR. We amplified the nuclear ribosomal internal transgenic spacers (ITS1, 5.8S nrRNA, ITS2), and partial 28S large subunit (nrLSU) RNA regions using the primers ITS1F (Gardes & Bruns 1993) and LR3 (Vilgalys & Hester 1990) following PCR protocol outlined in Arnold et al. (2007) with an initial denaturation step of 95 °C for 4 min, followed by 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 90 s, and a final incubation for 10 min at 72 °C. PCR products were cleaned with ExoSAP-IT (Affymetrix) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) followed by analysis with an Applied Biosystems 3730xl DNA Analyzer. The ITS1 or ITS2 region is typically

used for environmental DNA barcoding (Schoch et al. 2012; Bazzicalupo et al. 2013) and diversity estimation (Nilsson et al. 2008; U'Ren et al. 2009) for fungi, while the nrLSU region has been one of the most commonly used markers for phylogenetic inferences in Fungi (Moncalvo et al. 2000; Arnold et al. 2007). For adult samples, due to low OTU diversity that was detected in a preliminary survey, the number of genotyped cultures was increased by matching restriction fragment length polymorphism (RFLP) patterns of fifty extra random isolates from each season with those previously sequenced. The ITS–LSU region was amplified and subjected to restriction enzyme digestion by AluI and MspI. All ITS–LSU amplicons with unique RFLP bands were sequenced.

For cloning, samples from different plots were bulked by host age and ground under liquid nitrogen with mortar and pestle. DNA was extracted using Qiagen DNeasy kit (Qiagen) and the ITS–LSU region was amplified as previously described, but at two annealing temperatures of 50 °C and 52 °C, because different annealing temperatures are known to reveal different microbial community compositions (Schmidt et al. 2013). PCR products were ligated to a cloning vector with the TOPO PCR cloning kit (Life Technologies). Forty-eight fungal clones from both host ages at each annealing temperature were genotyped using RFLP markers. Clones with unique RFLP bands were sequenced. Three to seven clones or isolates of each RFLP group were sequenced and found to be >99 % identical within a group, validating the reliability of RFLP markers.

Sequencing for both cultured isolates and clones used a two-step process. Samples were first unidirectionally sequenced from the 5' direction to cover the most variable ITS region using ITS1F. Unique isolates (<99.6 % similarity) based on the ITS region were resequenced from the 3' direction with LR3. Some sequences were removed due to poor quality. Sequencher 5.0 was used to call bases and assemble reads into consensus sequences and determine sequences with unique ITS regions.

### Clustering and phylogenetic analyses

The ITS2 sequences were used to delimit operational taxonomic units (OTUs) using 90 %, 95 %, 97 %, and 99 % sequence similarity (Suppl. Table 1). A 95 % grouping criterion is commonly used to approximate fungal species boundaries (Arnold & Lutzoni 2007) but ITS variation is often clade-dependent (Nilsson et al. 2008). Hence, the conservative 99 % grouping was used in all following phylogenetic and multivariate community analyses (Pitkaranta et al. 2008). Results are also reported with and without singletons. Sequence clustering was performed with UCLUST, which is a *de novo* clustering approach without a reference database (Edgar 2010) in QIIME (Caporaso et al. 2010).

We chose one representative sequence from each ITS2 99 % OTU cluster and used its linked partial LSU sequence to build our phylogenetic tree. The LSU sequences were aligned with reference sequences collected from GenBank, which included 149 representatives from all major classes within the Ascomycota and Basidiomycota (Suppl. Table 2). References were chosen to help identify OTUs to the class level with high bootstrap support. We also included LSU sequences of 29 endophytic isolates and 17 endophytic environmental clones from

Arnold et al. (2007)'s study on adult loblolly pine needles sampled in 2004 (Suppl. Table 3). LSU sequences were manually aligned in MacClade 4.08a (Maddison & Maddison 2005) and ambiguous regions were identified using the LSU secondary structure model for *Saccharomyces cerevisiae* (Cannone et al. 2012).

We constructed LSU phylogenies using RAxML (Stamatakis 2006; Stamatakis et al. 2008) implemented within SNAP Mobyly workbench (Price & Carbone 2005) with three sequence data sets: i) unambiguously aligned sites only, ii) unambiguously aligned sites with five ambiguously aligned regions recoded with PICS-Ord (Luecking et al. 2011), and iii) unambiguously aligned sites and ambiguously aligned regions recoded with PICS-Ord with a conservative backbone constraint tree, which consisted of 38 reference taxa based on several published multigene phylogenies (Spatafora et al. 2006; Wang et al. 2006; Schoch et al. 2009; Prieto et al. 2013; Machouart et al. 2014; Suppl. Table 2 and Suppl. Fig 1). Alignments with and without delimited ambiguous regions are deposited in TreeBASE (accession 17939). The results of the nonparametric bootstrap analyses were based on 1000 pseudoreplicates.

We report the potential identities of some endophyte OTUs based on high similarities of ITS2 or ITS1 to known species using BLAST (criteria: query cover  $\geq$ 95 %, max identity  $\geq$ 95 %, published in peer-reviewed journals; Suppl. Table 4). However, inferring species identities with BLAST has many limitations and should only be considered as a temporary reference to understand the species diversity of a community.

### Multivariate community analysis

We compared the composition of fungal OTUs between needles from adult trees and seedlings by performing hierarchical clustering analyses in UniFrac (Lozupone et al. 2006) with the ML tree built using ambiguous regions coded by PICS-Ord and the conservative backbone constraint tree. We performed each analysis with or without singletons as well as weighted or unweighted with abundance data, assigning more weight on abundant or rare lineages, respectively. The communities were analyzed in groups of equal numbers of individuals (3) per age category, which correspond to whole plots for adult samples and half of each plot for seedling samples. We assessed the robustness of the clusters at the smallest sample size within a group using jackknife analysis with 1000 permutations in the UniFrac framework. UniFrac distances between pairs of groups were also used as input for principal coordinates analysis to illustrate the variation among the fungal endophytic communities in different host ages and seasons.

Rarefaction curves, extrapolations, and bootstrap estimates of total OTU richness were inferred between host age communities identified by culturing using EstimateS v9.1.0 (Colwell 2013). Rarefaction curves were extrapolated to 2 $\times$  the number of genotyped isolates, beyond which the variance increases greatly. Bootstrap estimates were inferred with 100 randomizations of sample order. Rarefaction curves using 99 %, 95 % and 90 % ITS2 similarity criteria are reported in the supplemental files (Suppl. Fig 2). Alpha diversity was calculated in the R package *vegan* with a parametric estimator, Fisher's alpha, and a non-parametric Simpson's diversity index. Diversity indices were calculated for the entire

community for each age as well as per group of three individuals within each age category. Community samples were also randomized and subsampled without replacement to yield 1000 random partitions of 48 sequences each. The distributions of diversity indices from the random partitions were compared between adult and seedling culture samples with t-tests as well as against the observed values from respective cloning samples with one-sample t-tests. We excluded Fisher's alpha values over 1000, characteristic of small sample sizes with high species richness, from statistical analyses. The Wilcoxon sign-rank test was used to test if certain fungal endophyte OTUs were more common in one host age than the other by comparing the isolation frequencies of the most commonly isolated OTUs from seedling and adult individuals from the same plots.

## Results

*Pinus taeda* seedlings yielded endophytic fungal growth from 180 out of 1284 needle segments (14.0 %), whereas leaves from adult trees yielded growth from 313 out of 408 needle segments (76.7 %). The isolation frequencies differed significantly between seedlings and adult trees ( $t = 9.23$ ,  $df = 6.68$ ,  $p < 0.01$ ). Random fungal isolates were genotyped from seedlings (57 winter and 50 summer; 107 total) and adult trees (95 winter and 97 summer; 192 total; Table 1). We sequenced 96 fungal clones from both adult trees and seedlings to identify any endophytic OTUs that may not be detected by our culturing method. A total of 491 isolates and clones were genotyped (Table 1) of which 182 were sequenced bidirectionally with ITS1F and LR3 primers (GenBank accession KM519195–KM519376; Suppl. Tables 4 and 5). RFLP matched 170 isolates and clones as one of eleven OTUs (Table 1, Suppl. Fig 3, Suppl. Table 4).

shared 16 OTUs (Fig 1). Seedlings had 51 OTUs not found in adults whereas adults had 23 OTUs not found in seedlings. In both seedlings and adults, the majority of non-overlapping OTUs were singletons, 82.4 % (42/51) and 73.9 % (17/23), respectively. Since the OTU communities between seasons were not structurally significant for either seedlings or adult trees (Fig 2, Suppl. Fig 4a, c), they were combined and analyzed together hereafter.

Based on 99 % ITS2 similarity, 192 clones clustered to 39 OTUs. Only one OTU was shared between seedlings and adult trees (Fig 1). About half of non-overlapping OTUs were singletons for the cloned communities, 51.5 % (17/33) for seedlings and 40.0 % (2/5) for adults.

Both culturing and cloning detected far more singletons in the seedlings than in adults (Fig 1). In total, culturing and cloning methods identified 118 ITS2 OTUs, of which 17 overlapped between seedling and adult endophyte communities, which correspond to 18.1 % (17/94) and 41.5 % (17/41) OTUs from the respective communities. Cloning revealed 28 (71.8 % of total cloned OTUs) more OTUs that were not found by culturing, whereas culturing revealed 79 (87.8 % of total cultured OTUs) more OTUs that were not found by cloning.

Out of 118 ITS2 OTUs, 83 (70.3 %) had high ITS similarity to known species, 27 (22.9 %) had high similarity to unnamed sequences, and 8 (6.8 %) had no high similarity in GenBank (Suppl. Table 4). The final dataset for the phylogenetic analysis consisted of 454 base pairs of the LSU from 149 reference sequences (Suppl. Table 2), 46 sequences from Arnold et al. (2007; Suppl. Table 3), and 117 OTUs from this study (Suppl. Table 4). Two OTUs (e50ss002 and e50ss004) that grouped in different OTUs based on ITS2 clustering had identical LSU sequences and were merged, thereby condensing the original 118 OTUs to 117. Out of 454 LSU nucleotides, 292 were unambiguously aligned and five ambiguous regions were identified

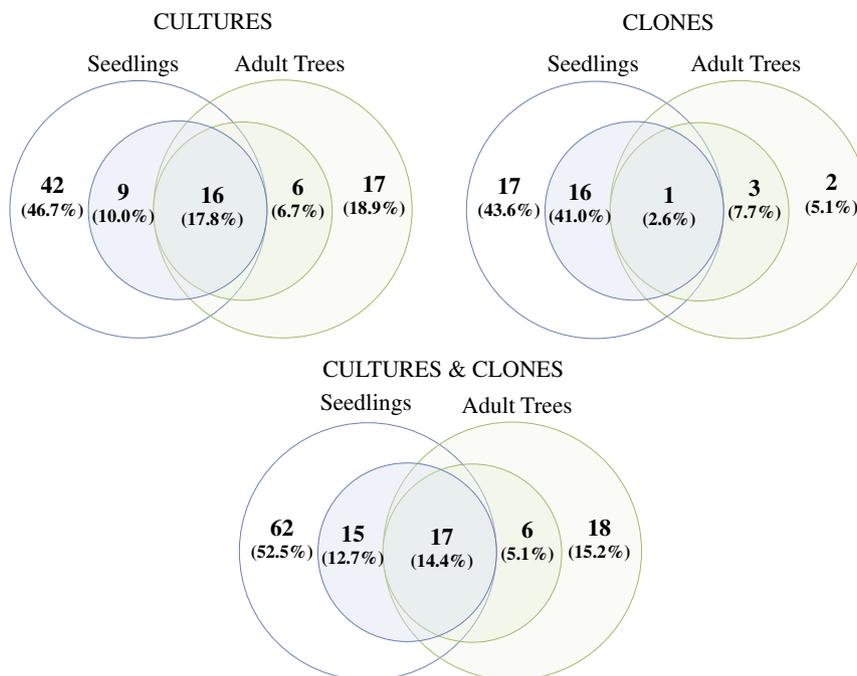
**Table 1 – Sampling summary and genotyping methods of endophytic fungi from needle tissues of seedling and adult *P. taeda* from summer and winter seasons. Isolation frequencies indicate fractions from which fungal cultures were isolated from the total of 2 mm needle segments on MEA media. Genotyped culture fractions indicate those genotyped out of the total number of cultured isolates. Cultures were either genotyped by bidirectional sequencing, unidirectional sequencing, or RFLP matching. Percent OTUs indicate OTUs out of a total of 118 OTUs delimited using the conservative 99 % similarity criterion.**

	Isolation frequency	Genotyped	Bidirectionally sequenced	Unidirectionally sequenced	Identified by RFLP match	99 % ITS2 OTUs
Summer seedling	98/564 (17.4 %)	50 (51 %)	36 (72 %)	14 (28 %)	0	35 (29.7 %)
Winter seedling	82/720 (11.4 %)	57 (69 %)	57 (100 %)	0	0	37 (31.4 %)
Summer seedling clone	N/A	96	28 (29 %)	25 (26 %)	43 (45 %)	34 (28.8 %)
Summer adult	142/204 (69.6 %)	97 (68 %)	32 (33 %)	45 (46 %)	20 (21 %)	18 (15.3 %)
Winter adult	171/204 (83.8 %)	95 (55 %)	27 (28 %)	49 (52 %)	19 (20 %)	25 (21.2 %)
Summer adult clone	N/A	96	2 (2 %)	6 (6 %)	88 (92 %)	6 (5.1 %)
Total	N/A	491	182 (56.7 %)	321 (65.4 %)	170 (34.6 %)	118

### Clustering and phylogenetic analysis

Based on 99 % ITS2 similarity, 299 cultured isolates clustered to 90 OTUs. Communities from seedlings and adult trees

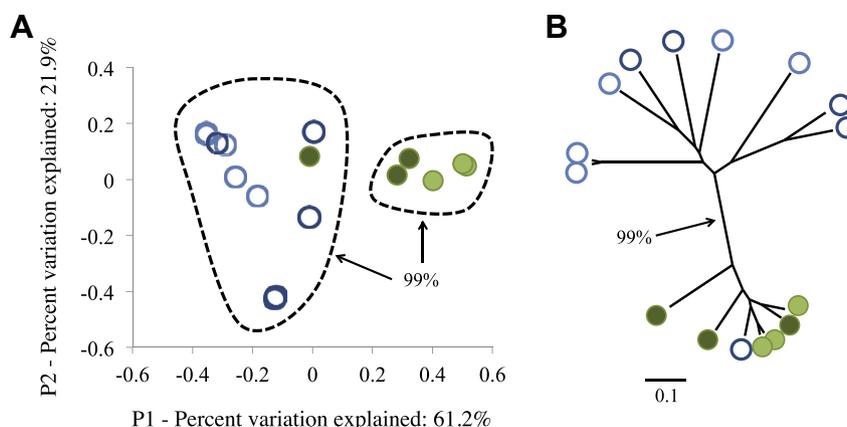
(positions 24–39, 63–92, 133–157, 341–411, 431–450), resulting in 162 ambiguous sites being coded by PICS-Ord. Major classes of Ascomycota and Basidiomycota were resolved as monophyletic and typically with high bootstrap support



**Fig 1 – Venn diagram of endophytic fungi (OTUs = 99 % ITS2 similarity groups) recovered from needle tissues of seedlings and adult trees of *P. taeda* using culturing and cloning methods. The number and percentage of overlapping, non-overlapping non-singleton, and non-overlapping singleton OTUs are represented in the circles. Outer circles represent singletons, OTUs that are found only once in the comparison. Inner circles represent non-singletons.**

( $\geq 70\%$ ), except the Dothideomycetes (Fig 3). Although the Dothideomycetes was not resolved as monophyletic (here forming a polytomy with Arthoniomycetes), all of our OTUs within this class grouped with known Dothideomycetes species with high support.

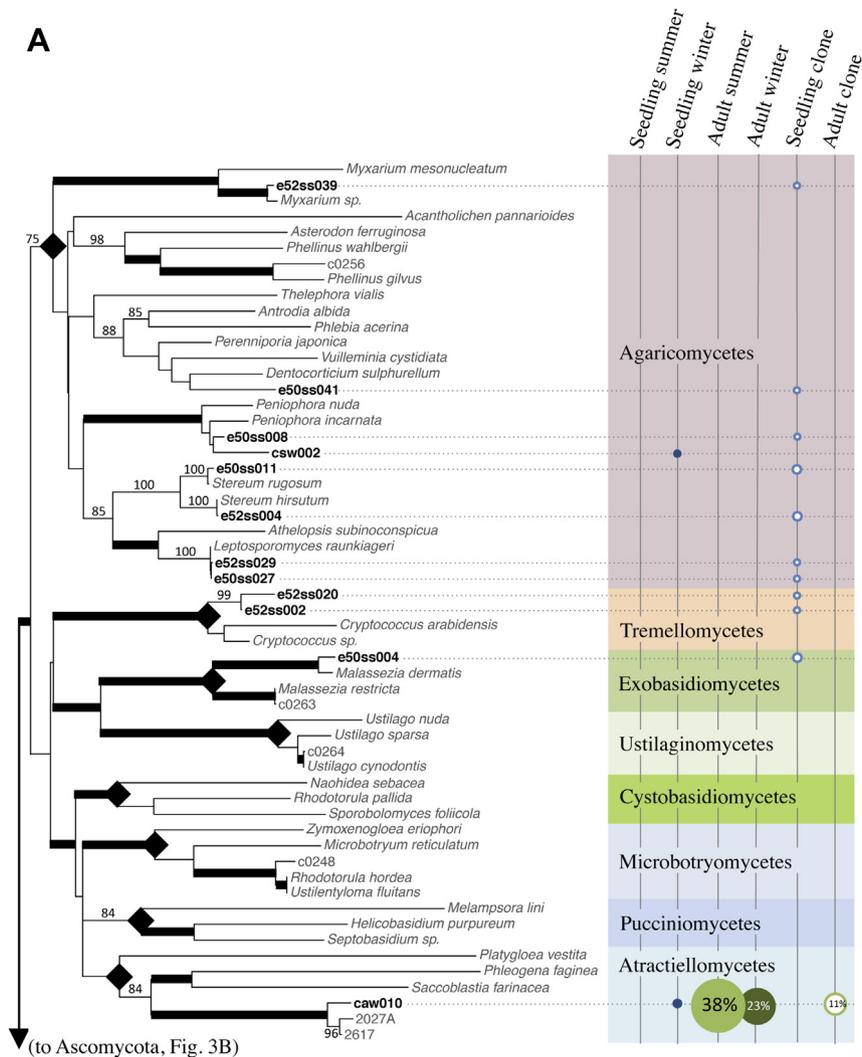
The OTUs detected by our study were distributed across four classes within the Basidiomycota and six classes within the Ascomycota (Fig 3). One OTU (e50ss030) could not be placed within a known fungal class, but a follow-up was not pursued since the OTU was a singleton identified by



**Fig 2 – Comparison of fungal endophyte communities cultured from needles of seedlings and adult trees of *P. taeda* sampled in the summer and winter. (A) Principal coordinates analysis of fungal endophyte communities with weighted abundance without singletons using the UniFrac metric. Dotted circles indicate  $>99\%$  jackknife support clusters after 1000 permutations. (B) Hierarchical clustering of communities with unweighted abundance without singletons using UPGMA based on tree represented in Fig 3. Jackknife support value (1000 permutations) is shown for the edge separating the two clusters. Scale bar shows UniFrac distance. Open blue circles represent fungal endophyte communities in seedling needles and closed green circles represent those in needles of adult trees. Light blue or green circles indicate communities from summer samples and the dark blue and green circles indicate communities from winter samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

cloning. The most commonly cultured OTU from adult trees, sampled during the summer and winter (caw010; 38.1 % and 23.2 %, respectively), was resolved within the Atractiellomycetes (Basidiomycota) and had no high similarity to BLAST searches. The second most commonly cultured OTU from adult trees, for both summer and winter (cas039; 35.1 % and 17.9 %, respectively), had 99 % ITS2 similarity to the Dothiomyces species *Septorioides pini-thunbergii* (Quaedvlieg

et al. 2013). In contrast, the most commonly isolated OTUs from seedlings were from the Sordariomycetes (e.g., csw046, css003, csw043, csw026; Table 2). However, no single Sordariomycetes OTU was found more than 9.3 % of the time at either host age. The Sordariomycetes had the greatest OTU richness from both adult tree and seedling communities, with 53 OTUs overall (44.9 %) and 35 of these (35/53; 66.0 %) being singletons.



**Fig 3 – Maximum likelihood (ML) phylogenetic tree based on 312 LSU rRNA sequences showing the phylogenetic placement of 117 fungal endophyte OTUs found in needles of *P. taeda* seedlings and adult trees as part of this study (shown in bold) within the broader context of the dikarya represented by 149 reference species and 46 genotypes from Arnold et al. (2007). Phylogenetic analyses were conducted in three ways: i) 292 unambiguously aligned sites only, ii) 292 unambiguously aligned sites with 162 ambiguous sites recoded with PICS-Ord, and iii) 292 unambiguously aligned sites with 162 ambiguous sites recoded with PICS-Ord and with conservative backbone constraint tree (Suppl. Fig 1). The tree shown here is derived from analysis type iii) The number of isolates or clones for each OTU (99 % ITS2) in each sample is represented by the size of the circles on the right. The percentage of isolates or clones for OTUs that made up more than 10 % of the sample is indicated within the circles. Clones are from pine needle tissue collected from summer. The scale bar indicates the number of substitutions per site for a unit branch length. Branches were bolded if ML bootstrap values were >70 % for all three types of analyses. When branches were only highly supported by analysis types ii and iii, the ML bootstrap values of the analysis type ii were indicated near branches. Nodes for major fungal clades (mostly classes) are indicated with a diamond. Sequences from Arnold et al. (2007) are represented by grey code names (e.g., 2235, c0280) and starts with ‘c’ if it was a clone.**

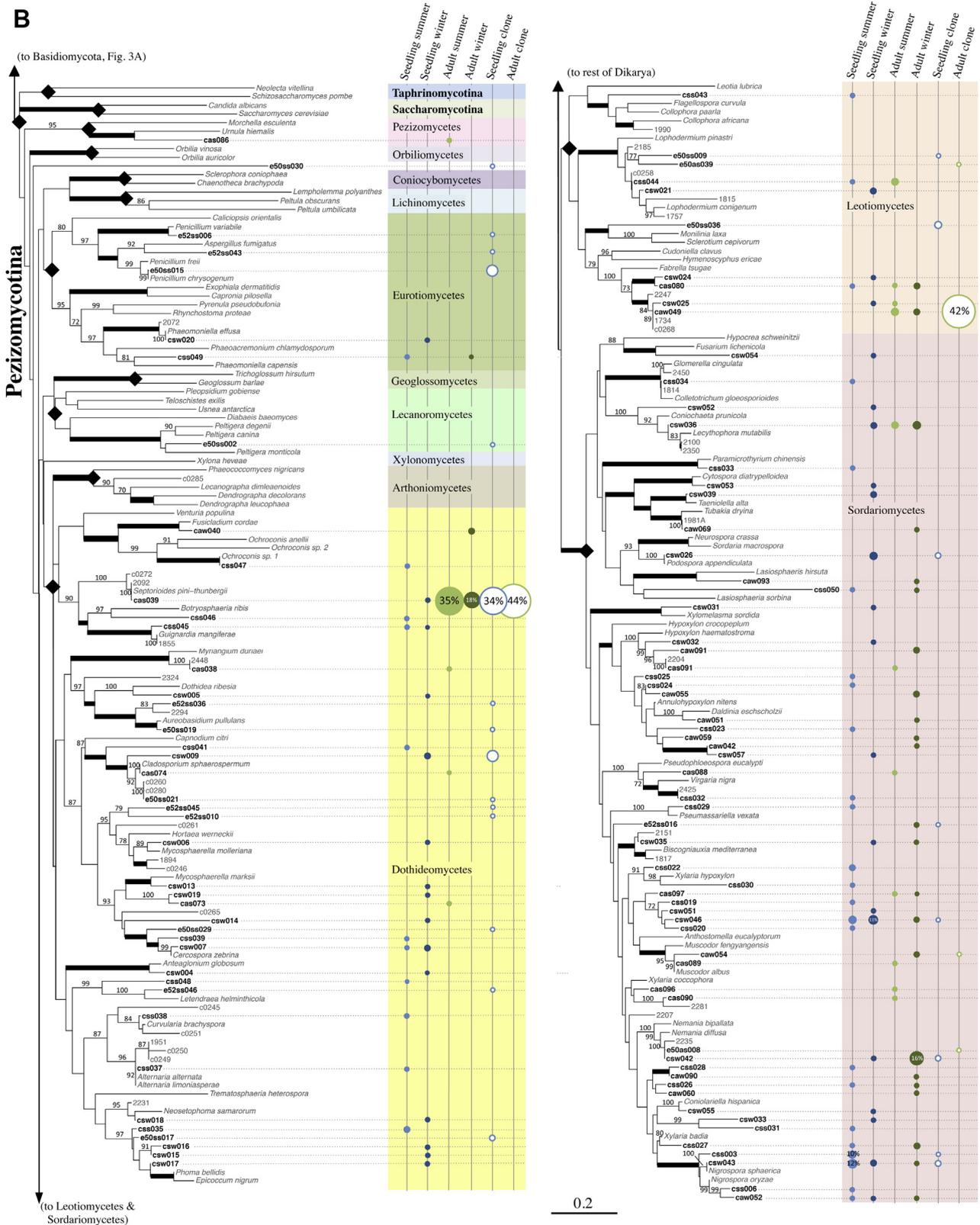


Fig 3 – (continued).

**Table 2 – Taxonomic distribution of fungal endophytes in needles of seedling and adult *P. taeda* detected with culturing and cloning methods. Summer and winter samples are pooled for culturing. The three most commonly found OTUs from each sampling type are indicated with superscripts 1, 2, and 3. The class with the most OTUs from each sampling type is bolded.**

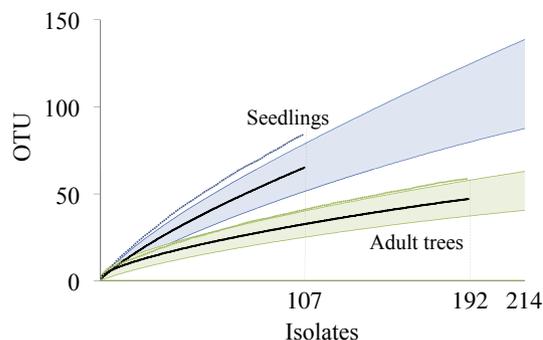
Taxonomic group/OTU	% Genotyped isolate		% Clone	
	Seedling	Adult	Seedling	Adult
Ascomycota	98.1	69.3	83.3	88.5
Sordariomycetes	<b>62.6</b>	<b>30.7</b>	12.5	2.1
csw046	9.3 <sup>1</sup>	0.01	0.01	0
csw043	7.5 <sup>2</sup>	0.01	5.2	0
csw026	4.5 <sup>3</sup>	0	2.1	0
css003	4.5 <sup>3</sup>	0	0.9	0
csw042	1.9	7.8 <sup>3</sup>	2.1	0
Leotiomycetes	7.5	8.3	4.2	41.7
caw049	0	3.6	0	41.7 <sup>2</sup>
Dothideomycetes	26.2	29.2	<b>53.1</b>	<b>44.8</b>
cas039	0.9	26.6 <sup>2</sup>	34.4 <sup>1</sup>	43.7 <sup>1</sup>
csw009	2.8	0	8.3 <sup>3</sup>	0
Lecanoromycetes	0	0	2.1	0
Eurotiomycetes	1.9	0.5	13.5	0
e50ss015	0	0	9.4 <sup>2</sup>	0
Pezizomycetes	0	0.5	0	0
Basidiomycota	1.9	30.7	15.6	11.5
Tremellomycetes	0	0	2.1	0
Agaricomycetes	0.9	0	11.5	0
Atractiellomycetes	0.9	30.7	0	11.5
caw010	0.9	30.7 <sup>1</sup>	0	11.5 <sup>3</sup>
Exobasidiomycetes	0	0	2.1	0
Unknown	0	0	1.0	0

Cloning identified OTUs in three more classes (Exobasidiomycetes, Tremellomycetes, and Lecanoromycetes) compared to what was found with culturing (Fig 3, Table 2). More Dothideomycetes were detected by cloning than any other class for both seedling and adult fungal communities, although this was mostly due to the overrepresentation by one OTU – cas039. For clones obtained from adult trees, three common OTUs (caw049, caw010, and cas039) made up the majority of the community and only three other OTUs were detected (Fig 3, Table 2).

### Multivariate community analyses

Endophyte communities in needles of adult trees were highly similar to each other compared to those of seedling needles. The jackknife analysis supported the separate clustering of fungal communities from adult trees vs. seedlings (>99%), except in the case of one adult sample or one seedling sample (Fig 2), which tended to cluster in the other group depending on whether the analysis weighted or unweighted abundance data, respectively. Results were similar with and without singletons (data not shown).

Based on 99% ITS2 similarity, OTU diversity was greater for seedlings than for adult trees (67 OTUs/107 isolates vs. 37 OTUs/192 isolates; Fisher's alpha = 76.7 vs. 13.6; Simpson's index = 3.9 vs. 2.4, respectively). Fisher's alpha was statistically significant between adult and seedling communities per three individuals ( $t = 3.4$ ,  $df = 8.5$ ,  $p < 0.01$ ) but not



**Fig 4 – Rarefaction curves of endophytic fungal communities from needle tissues of *P. taeda* seedlings and adult trees. Curves include black line representing observed 99% OTUs, shaded area around curve representing the 95% confidence intervals, and extrapolated curve up to 2× the number of isolates from the seedling community. Dotted lines represent bootstrap species richness. Communities only include cultured isolates. Rarefaction curves of clones and cultures using 90%, 95% and 99% ITS2 similarity are reported in Suppl. Fig 2.**

significant for Simpson's index ( $t = 1.9$ ,  $df = 6.1$ ,  $p = 0.11$ ). Randomization analyses for partitions of 48 cultured isolates indicated that the richness was significantly different for both Fisher's alpha ( $t = 89.5$ ,  $df = 1021.3$ ,  $p < 0.001$ ) and Simpson's index ( $t = 141.9$ ,  $df = 1075.9$ ,  $p < 0.001$ ; Suppl. Fig 5). OTU diversity was also greater with environmental cloning for seedlings than for adult trees (34 OTUs/96 clones vs. 6 OTUs/96 clones; Fisher's alpha = 18.8 vs. 1.4; Simpson's index = 0.9 vs. 0.6, respectively). Randomization analyses of cultured samples indicated that the Simpson's index and Fisher's alpha values for cloning were both significantly lower than expected from culturing alone (Suppl. Fig 5).

Rarefaction curves for the fungal OTUs detected in adult *Pinus taeda* approached a plateau but did not for seedlings (Fig 4). The 95% confidence interval for the endophyte community from adult trees remained lower than the observed richness of endophyte OTUs in the endophyte community from seedlings.

To test whether particular OTUs associated more with needles from adult trees or seedlings, we compared the frequency of the three most common OTUs from each host age by the Wilcoxon sign-rank test by pairing adult and seedling communities from the same plots. We found that two of the OTUs, caw010 and cas039, were significantly more represented in adult communities than in seedlings (Table 3).

## Discussion

This study compares the fungal endophyte communities between seedlings and adult trees of *Pinus taeda* using molecular data and identifies fungal OTUs that are common vs. unique to these two age categories to better understand fungal endophyte specialization. We found that the two most commonly isolated endophytic fungal OTUs from adult trees, an unknown

**Table 3 – Most commonly cultured OTUs from adult and seedling tissues (Table 2). Average frequency and standard deviation of each OTU per plot per season are indicated for adult trees and seedlings. Results of the Wilcoxon signed-rank test to determine if commonly cultured endophytes were found in needles of adult trees or seedlings more often than by chance.**

OTU	% In adult	% In seedling	Wilcoxon rank test
caw010	31.1 ± 15.4	0.6 ± 1.4	$p < 0.05$
cas039	25.3 ± 15.2	1.1 ± 2.7	$p < 0.05$
csw042	8.1 ± 10.2	1.2 ± 2.8	$p = 0.18$
csw046	3.3 ± 5.8	8.1 ± 6.0	$p = 0.28$
csw043	2.4 ± 5.8	6.9 ± 6.2	$p = 0.42$
css003	0.0 ± 0.0	5.3 ± 6.6	$p = 0.18$
csw026	0.0 ± 0.0	4.0 ± 6.2	$p = 0.37$

species from the class Atractiellomycetes (caw010) found 26.6 % of the time and a species tentatively identified as *Septorioides pini-thunbergii* (cas039) found 30.7 % of the time, which were also found by Arnold et al. (2007), were found rarely in needles of seedlings as cultures (Table 3). This suggested that these species are specialized to adult needles and the canopy environment compared to needles of seedlings and the environment close to the soil. The most abundantly found OTUs from seedlings (Table 2) were not isolated significantly more from seedlings than from adult trees (Table 3). Furthermore, most of the endophyte OTUs from seedlings were only found once (Fig 1). The high OTU richness in the seedlings may be due to seedlings having little to no specificity to most fungal endophytic species, thereby becoming vulnerable to a diverse group of fungal species, endophytic, pathogenic, and saprotrophic species alike, compared to adult needles. Alternatively, a more exhaustive sampling might reveal that the OTU richness from needles of seedlings and adult trees are not significantly different. However, differences in the relative abundances of certain OTUs are clear between the two host age categories, suggesting that some endophyte OTUs may specialize on adult tissues and have distinct ecological roles from other endophytes. Whether this is mainly due to differences in innate susceptibility between adult trees and seedlings or differences in selection by the correlating environmental factors, such as the inoculum community, proximity to the canopy or soil, microclimatic factors, or life spans of different leaf types, is still unknown.

Isolation frequency for foliar fungal endophytes increases with leaf age or exposure duration (e.g., Ferreira Rodrigues 1994; Kumaresan & Suryanarayanan 2002; Arnold & Herrera 2003) whereas an increase with host age has not been previously observed (Espinosa Garcia & Langenheim 1990; Ferreira Rodrigues 1994). In this study, we found that seedlings that were not under a canopy had considerably lower isolation frequency than leaves of adjacently growing adult *P. taeda*. Since leaf age was not controlled between the seedlings and adult trees, it is possible that seedling needles were younger than needles collected from adult trees even though we sampled the oldest healthy needles available from seedlings at the time. However, despite the possible younger leaf age of seedlings, we found greater OTU richness as well as alpha diversity

in the endophyte community from seedlings than from adult needles. Endophyte studies comparing species richness across leaf ages have mixed findings from no difference (Frohlich et al. 2000), an increase (Kumaresan & Suryanarayanan 2002), to an initial increase and then decrease in species richness (Espinosa Garcia & Langenheim 1990) with increasing leaf age. Espinosa Garcia & Langenheim (1990) found that endophyte diversity tended to increase from one to three-year-old leaves of coastal redwoods (*Sequoia sempervirens*) and then decrease in fully mature needles that were eight years or older. They also found that the young tissues of redwood sprouts from the base of trees tended to have higher diversity than the mature tissues from the canopies of trees, which had a more uneven community with several dominating fungal species. However, Ferreira Rodrigues (1994) compared fungal endophyte communities between saplings and mature trees of Amazonian palm (*Euterpe oleracea*) and found no difference in diversity. It should be noted that these previous studies comparing fungal endophyte communities across leaf or host ages were based on morphological data from cultures only and hence, have limited inference in estimating species richness (Arnold et al. 2007). However, Espinosa Garcia & Langenheim (1990)'s study finds similar patterns as ours, suggesting that a decrease in species diversity of fungal endophytes with plant maturity (i.e., seedlings or sprouts vs. adult leaves as opposed to leaf maturity) may be a common pattern among conifers like *S. sempervirens* or *P. taeda* and distinct from non-conifers, like *E. oleracea*. Furthermore, a decrease in diversity of symbiotic fungal species with plant maturity has also been found in mycorrhizal associations as well as rhizosphere communities (Houlden et al. 2008; Husband et al. 2002a; Husband et al. 2002b). Given our findings along with similar correlations in other symbiotic systems, symbiont specialization associated with plant maturity may be common.

#### Culturing vs. environmental PCR

Cloning did not identify more distinct 99 % ITS2 OTUs than culturing per sampling effort for both host ages (Table 1). In adult communities, cloning only identified two new OTUs, both of which were singletons (Fig 3), and did not suggest that we were missing any significant fungal lineages with culturing methods (Suppl. Fig 2). In contrast, cloning identified 26 new OTUs (three new classes) in seedling communities, of which seven were non-singletons (Figs 1 and 3, Table 2). Some non-singleton OTUs were also from lineages uniquely detected with cloning, such as *Stereum* spp. in the Agaricomycetes (e52ss004 and e50ss011), *Malassezia* spp. in the Exobasidiomycetes (e50ss004), and a Leotiomycetes OTU with high ITS similarity to *Monilina* sp. (e50ss036). Some OTUs unique to cloning, such as e50ss015 (*Penicillium* sp.), have been found as cultures in other studies (Vega et al. 2010; Sandberg et al. 2014), suggesting that factors other than the ability to grow on MEA, such as competing endophytic species, might affect an endophyte's ability to be cultured. Lastly, OTUs detected only with cloning may represent non-endophytic fungi from inefficient surface-sterilization. For example, *Malassezia* spp. are common fungi found on the human skin (Findley et al. 2013) and *Peltigera* spp. (e50ss002 and e50ss005) are lichens

with a widespread distribution (Martinez et al. 2003). Arnold et al. (2007) also found *Malassezia* spp. and unexpected lichen species from cloning.

OTU cas039 made up the majority of the clones, but not cultures, from seedling samples, despite being one of the most culturable OTUs from adult needles. We think the particular seedling samples we used for the bulk DNA extraction had an unusually high infection rate by this fungal species or there is a primer-binding bias. We found that different annealing temperatures did not significantly affect the proportion of cas039 clones (data not shown). In the future, different pairs of primers may be a better way to overcome primer-bias.

We found relatively few isolates belonging to the genus *Lophodermium* (Helotiales, Leotiomyces), which was the most commonly cultured fungus in Arnold et al. (2007)'s study. Even common endophyte OTUs may have interannual variations that were not detected in this study, although sampling was conducted over two different seasons and no significant differences were detected (Fig 2). Alternatively, *Lophodermium* endophytes may have been dominant in Arnold et al. (2007)'s study due to their comparatively fast growth rates, which increases their likelihood of culturing, especially from larger (>2 mm) leaf sections. This is further supported by Arnold et al. (2007) finding considerably lower frequency of *Lophodermium* endophytes with cloning than expected based on their culturing results.

#### A diffuse mutualism with key players?

Despite the increasing number of studies of endophytic fungi within multiple host species and environments (Carroll 1988; Ganley et al. 2004; Rodriguez et al. 2009; U'Ren et al. 2010; Sun et al. 2012; Zimmerman & Vitousek 2012), their ecological roles and factors underlying the community diversity remain mostly unknown (Rodriguez et al. 2009). Evidence supporting horizontally-transmitted endophytic fungi as mutualists are rare and circumstantial (Faeth & Fagan 2002; Sieber 2007 but see Carroll 1988; Arnold et al. 2003; Ganley et al. 2008) and have been under great speculation (Faeth 2002; Sieber 2007), mainly due to their low host specificity and their high phylogenetic diversity within hosts (Herre et al. 1999). Fungal endophyte communities within plants may be an example of a diffuse mutualistic network where the benefits are low for the individual host, but are high for the host population (Carroll 1988; Stanton 2003; Merckx & Bidartondo 2008). Furthermore, the mutualism may be 'unevenly diffuse', where one or a few fungal species are particularly important on a specific host individual despite the high diversity and high number of fungal partner species throughout the geographic range of the host species (Jordano 1987; Gove et al. 2007; Ridout & Newcombe 2015).

We found that some fungal endophytes are consistently more common than others at the adult stage, which suggests that these fungal endophytes are either better adapted to adult tissues, preferentially selected by adult tissues, their spores are disproportionately abundant in the canopy compared to the ground level, or a combination of these factors. Such species may have ecological roles that are distinct

from others and should be further investigated. In pine needles, seedlings and adults alike contain various secondary metabolites, including phenolics, flavonoids, condensed tannins, and monoterpenes (Kainulainen et al. 1996) and their relative abundances with age depend on the particular compound (Oleszek et al. 2002; Thoss et al. 2007), which may affect the community structure of endophytes. They also vary in their vulnerability to different stress factors (Boege & Marquis 2005) as well as exposure to different fungal species even when they occur in close spatial proximity, which may lead to differential selection of endophytic fungi with different beneficial functions. However, host-specific species may still remain rare in the community despite any beneficial roles due to other ecological factors (Wilson & Yoshimura 1994; Reveillaud et al. 2014), e.g., poor survival outside of host or competition with other endophytes. The future challenge will be to identify functional differences among common and rare endophytic fungal species as well as understanding what factors affect the diversity of endophytes within individual hosts.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funbio.2015.07.003>.

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