

Community Analysis Reveals Close Affinities Between Endophytic and Endolichenic Fungi in Mosses and Lichens

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Abstract Endolichenic fungi live in close association with algal photobionts inside asymptomatic lichen thalli and resemble fungal endophytes of plants in terms of taxonomy, diversity, transmission mode, and evolutionary history. This similarity has led to uncertainty regarding the distinctiveness of endolichenic fungi compared with endophytes. Here, we evaluate whether these fungi represent distinct ecological guilds or a single guild of flexible symbiotrophs capable of colonizing plants or lichens indiscriminately. Culturable fungi were sampled exhaustively from replicate sets of phylogenetically diverse plants and lichens in three microsites in a montane forest in southeastern Arizona (USA). Intensive sampling combined with a small spatial scale permitted us to decouple spatial heterogeneity from host association and to sample communities from living leaves, dead leaves, and lichen thalli to statistical completion. Characterization using data from the nuclear ribosomal internal transcribed spacer and partial large subunit (ITS-LSU rDNA) provided a first estimation of host and substrate use for 960 isolates representing five classes and approximately 16 orders, 32 families, and 65 genera of Pezizomycotina. We found that fungal communities differ at a broad taxonomic level as a function of the phylogenetic placement of their plant or lichen

hosts. Endolichenic fungal assemblages differed as a function of lichen taxonomy, rather than substrate, growth form, or photobiont. In plants, fungal communities were structured more by plant lineage than by the living vs. senescent status of the leaf. We found no evidence that endolichenic fungi are saprotrophic fungi that have been “entrapped” by lichen thalli. Instead, our study reveals the distinctiveness of endolichenic communities relative to those in living and dead plant tissues, with one notable exception: we identify, for the first time, an ecologically flexible group of symbionts that occurs both as endolichenic fungi and as endophytes of mosses.

Introduction

Symbioses between fungi and photosynthetic organisms were associated with the early colonization of land by plants [7, 22, 42, 49] and the subsequent diversification of the most species-rich fungal lineages [9, 25, 29, 33]. The best-known fungal partners in such associations are mycorrhizal fungi, lichen-forming mycobionts, and plant pathogens (see [29]). Yet members of every major plant lineage also harbor fungal endophytes—highly diverse, horizontally transmitted fungi that live within asymptomatic above-ground tissues such as leaves ([2, 39, 46]; Class 3 endophytes, sensu [43]). Fungal endophytes are especially common among the Pezizomycotina (Ascomycota), representing at least five classes, dozens of families, and large numbers of previously unknown species [6, 19, 24].

Fungal symbionts resembling endophytes also live inside apparently healthy lichen thalli, forming persistent and symptomless infections ([6]; see also [21, 30, 32, 38, 47]). These “endolichenic fungi” represent lineages of Ascomycota that are distinct from lichen mycobionts (the primary fungal component of the lichen thallus), lichenicolous fungi (which

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fruit or are otherwise symptomatic on thalli), and incidental fungi on thallus surfaces ([6]; see also [31, 33]). They are known from every lichen species sampled to date at sites ranging from the Arctic to the tropics (see [6]) but have been characterized in only a few communities [6, 30, 32, 47]. In focal species, microdissection demonstrates that they live in close association with photobionts and are relatively rare in the mycobiont-dominated cortices and medulla [6]. They appear to be horizontally transmitted and comprise a high diversity of Pezizomycotina.

The observation that lichen thalli can harbor genera that are typically found as endophytes of plants, combined with ecological similarities between endophytes and endolichenic fungi (asymptomatic growth in living tissues, intimate association with a photosynthetic organism, high diversity, and horizontal transmission; [6, 21, 32, 47]), has led to uncertainty regarding the ecological distinctiveness of endolichenic fungi compared with endophytes. A previous study that addressed this issue compared fungi from corticolous lichens and foliar endophytes from trees on which those lichens grew, finding little evidence that dominant fungal species occurred in both leaves and lichens [47]. However, that study considered only vascular plants and only corticolous lichens, and did not sample endolichenic or endophytic communities to statistical completion. Subsequent studies have confirmed that endolichenic fungi are common in lichens with different substrates [30, 32] and that they share a close evolutionary relationship with endophytes [6]. However, no previous study has specifically addressed the distinctiveness of endolichenic fungi relative to other guilds through intensive sampling of phylogenetically diverse plants and lichens.

Here we evaluate whether endophytic and endolichenic fungi represent distinct ecological guilds or a single guild of flexible symbiotrophs capable of colonizing plants and lichens indiscriminately. In a biotically rich montane forest in southeastern Arizona, USA, we exhaustively sampled culturable fungi from fully replicated sets of phylogenetically diverse plants and lichens in three microsites. The small spatial scale of our study permitted us to sample to statistical completion and to decouple spatial heterogeneity from host associations. To provide a robust context for interpreting the apparent specificity of endophytic fungi (from living photosynthetic tissues of plants) and endolichenic fungi (from living thalli), we concurrently examined culturable fungi from dead leaves in tree canopies and leaf litter in the same microsites. Our resulting library of over 1,000 fungal isolates is drawn from the largest sample of co-occurring lichens and plants to date, spanning a diversity of lichen substrate uses, growth forms, photobionts, and mycobionts, as well as phylogenetically diverse plants ranging from mosses to angiosperms. Although preliminary in lieu of reciprocal infection experiments, this study provides a first perspective on host use by ascomycetous fungi in co-occurring lichens and plants, indicating that

endolichenic fungi represent a largely distinct guild—with the exception of a previously unexplored group of ecologically flexible symbionts that occur both as endophytes in mosses and as endolichenic fungi.

Materials and Methods

In June 2007, fresh tissues of 11 representative species of plants (Table 1) and lichens (Table 2) were collected in the eastern Chiricahua Mountains, southeastern Arizona (Cochise Co., Coronado National Forest; 31°53'00" N, 109°12'18" W). Part of the Madrean “sky island archipelago,” the Chiricahuas comprise a rainfall, temperature, and vegetation gradient ranging from the Sonoran and Chihuahuan deserts to oak woodlands (1,550–1,750 m above sea level (m.a.s.l.)), pine-oak woodlands and forests (1,750–2,200 m.a.s.l.), and conifer-dominated montane forests (2,200–2,400 m.a.s.l.) [8]. The climate of the region is arid to semiarid, with a pronounced dry season (March–June) between winter and summer wet seasons [8]. At the Southwestern Research Station in Portal, AZ (1,646 m.a.s.l.), mean annual rainfall is 548.6 mm and the mean annual temperature is 11.7°C (1909–2006; Western Regional Climate Center: <http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?az6716>).

The study site consisted of a semi-open natural drainage and rocky hillside ca. 9.5 km north-northwest of the Southwestern Research Station (2,100 m.a.s.l.). The area has an open canopy of *Pinus arizonica* var. *arizonica* interspersed with oaks (*Quercus hypoleucoides*, *Quercus gambelii*, *Quercus rugosa*), alligator juniper (*Juniperus deppeana*), Douglas fir (*Pseudotsuga menziesii*), and velvet ash (*Fraxinus velutina*), and an understory comprised of grasses, mosses, ferns (primarily *Woodsia plummerae*), small shrubs, and cacti (especially *Echinocereus triglochidiatus*). Epiphytic and saxicolous lichens are abundant.

Based on previous work in the mountains of southern Arizona [27, 28] hosts were sampled intensively at a local scale with the goal of achieving statistical completion in recovering culturable fungal diversity. In each of three microsites located ca. 30 m apart along a 100 m transect, replicate sets of phylogenetically diverse lichens and plants were sampled in very close physical proximity to one another, allowing spatial heterogeneity in fungal distributions to be decoupled from host affiliations. Whereas samples across multiple transects and sampling times would be useful for expanding the scope of inference of the present study, the high richness and abundance of fungi present challenges of scale that are being addressed in further work.

In this study, material from 33 individual plants (one individual of 11 representative species in each of the three microsites; Table 1) and 30 lichens (encompassing one or more thalli of ten lichen genera in each of three microsites;

Table 1 Endophytic fungi: plant host, lineage and family; number of plant tissue segments examined and isolates recovered; isolation frequency per microsite for three microsities; number of isolates sequenced, number of putative species, and diversity (Fisher's alpha) using OTU based on 95% ITS-LSU rDNA sequence similarity

Host	Host lineage	Host family	Leaf segments ^a	Isolates recovered	Isolation frequency/ microsite \pm SD	Isolates sequenced (%)	Putative species (95% CI)	Fisher's alpha (95% CI) ^c
<i>Leucobryum</i> sp. ^d	Moss	Bryaceae	141	21	0.15 \pm 0.02	19 (91%)	11	10.9 (4.5, 17.4)
<i>Ceratodon purpureus</i>	Moss	Ditrichaceae	143	30	0.21 \pm 0.16	30 (100%)	14	10.2 (4.9, 15.6)
<i>Woodsia plummerae</i>	Fern	Dryopteridaceae	144	7	0.05 \pm 0.09	5 (71%)	4	9.3 (0.2, 18.4)
<i>Juniperus deppeana</i>	Conifer	Cupressaceae	144	22	0.15 \pm 0.12	19 (86%)	10	8.5 (3.3, 13.9)
<i>Pinus arizonica</i> var. <i>arizonica</i>	Conifer	Pinaceae	144	9	0.06 \pm 0.02	9 (100%)	2	0.8 (-0.3, 1.9)
<i>Pseudotsuga menziesii</i> var. <i>glauca</i>	Conifer	Pinaceae	144	21	0.15 \pm 0.22	16 (76%)	3	1.1 (-0.1, 2.3)
<i>Eragrostis intermedia</i>	Angiosperm	Poaceae	144	2	0.01 \pm 0.02	2 (100%)	1	N/A
<i>Quercus rugosa</i>	Angiosperm	Fagaceae	130	0	0	N/A	N/A	N/A
<i>Quercus gambelii</i>	Angiosperm	Fagaceae	128	1	0.01 \pm 0.01	1 (100%)	1	N/A
<i>Quercus hypoleucoides</i>	Angiosperm	Fagaceae	144	4	0.03 \pm 0.05	3 (75%)	1	N/A
<i>Fraxinus velutina</i>	Angiosperm	Oleaceae	125	1	0.01 \pm 0.02	1 (100%)	1	N/A
Total			1,531	118	0.08 \pm 0.11 ^b	105 (89%)	36 (28, 44)	19.4 (13.0, 25.7)

^a All hosts started with 144 leaf segments (i.e., 48 per microsite for three microsities), but a small number were lost to contamination, overgrowth, and desiccation

^b Average infection frequency

^c Fisher's alpha was not calculated for species with <4 isolates sequenced, but all hosts from which endophytes were obtained were included in community-wide endophyte diversity analyses. Differences in diversity among species were not calculated due to small sample sizes

^d This plant was not identified to species because of limited taxonomic resources for the region. However, accessions of this species were morphologically consistent among all microsities

Table 2) was collected. For endophytes (defined functionally as fungi occurring within living, asymptomatic tissues of plants), we collected three branches containing healthy leaves (trees, shrubs), three shoots (grass, fern), or one small mat (mosses; 4–9 cm²) per species in each microsite. From four tree species (*J. deppeana*, *P. arizonica*, *P. menziesii*, and *Q. rugosa*), dead leaves attached to branches in tree canopies (hereafter, dead leaves) and leaves in below-crown leaf litter in an intermediate state of decomposition (hereafter, leaf litter) also were collected using the same individual plants sampled for endophytic fungi (Table 3). For endolichenic fungi (defined functionally as fungi occurring within living, asymptomatic lichens), portions of mature thalli sufficient for herbarium specimens and fungal isolations were collected. Specimens were deposited at the Robert L. Gilbertson Mycological Herbarium at the University of Arizona (ARIZ, accessions LI-2009-1-LI.2009-30) and the Cryptogamic Herbarium at Duke University (DUKE, accessions 48138-48166).

Isolation of Fungi

Plant and lichen material was transported in plastic and paper bags, respectively, to the laboratory and processed within 24 h.

Debris and plant material was removed from lichens with aid of a dissecting microscope. Each leaf and thallus was washed thoroughly in running tap water prior to surface sterilization. To maximize recovery of diverse fungi, 5–15 leaves per individual (or 2 cm² from each moss sample) were cut to yield ca. 150–200 tissue segments (each ca. 2 mm²), which were surface sterilized and selected haphazardly to yield 48 segments per species per microsite. A similar approach was used for lichen thalli, except that those that did not fragment naturally were cut into 2 cm² pieces prior to surface-sterilization, and into 2 mm² pieces under sterile conditions thereafter, for a total of 48 haphazardly selected segments per species per microsite. Cutting lichen thalli after surface sterilization does not limit the recovery of endolichenic fungi, and the slightly different methods used here for plant vs. lichen samples do not affect isolation frequency or apparent composition [6].

Segments were surface-sterilized by agitating sequentially in 95% ethanol for 30 s, 10% bleach (0.5% NaOCl) for 2 min, and 70% ethanol for 2 min [4], and then surface dried under sterile conditions before plating on 2% malt extract agar, which supports growth of diverse endophytes [15, 16] and endolichenic fungi [6, 32]. Plates were sealed with Parafilm and incubated under ambient light/dark condition at room temperature (ca. 21.5°C) for up to 1 year.

Table 2 Endolichenic fungi: host species, order, substrate, growth form and photobiont; number of lichen tissue segments examined and isolates recovered; isolation frequency; number of isolates sequenced, number of putative species, and diversity (Fisher's alpha) using OTU based on 95% ITS-LSU rDNA sequence similarity

Host	Host order	Substrate	Growth form	Photobiont ^a	Thallus segments ^b	Isolates recovered	Isolation frequency/ microsite \pm SD	Isolates sequenced (%)	Putative species (95% CI)	Fisher's alpha (95% CI) ^c
<i>Dermatocarpon</i> spp. ^d	Verrucariales	Saxicolous	Foliose	<i>Myrmecia</i> sp./other unicellular algae	143	23	0.16 \pm 0.15	20 (87%)	8	4.9 (1.5, 8.4)
<i>Flavopunctelia praesignis</i>	Lecanorales	Epiphytic	Foliose	<i>Trebouxioioid</i>	144	70	0.49 \pm 0.14	64 (91%)	18	8.3 (4.5, 12.2)
<i>Punctelia hypoleucites</i>	Lecanorales	Epiphytic	Foliose	<i>Trebouxioioid</i>	144	110	0.76 \pm 0.11	97 (88%)	19	7.1 (3.9, 10.2)
<i>Usnea hirta</i>	Lecanorales	Epiphytic	Fruiteuse	<i>Trebouxioioid</i>	144	47	0.33 \pm 0.03	45 (96%)	14	7.0 (3.3, 10.6)
<i>Pseudevernia intensa</i>	Lecanorales	Epiphytic	Fruiteuse	<i>Trebouxioioid</i>	144	71	0.49 \pm 0.17	61 (86%)	14	6.4 (3.1, 9.6)
<i>Xanthoparmelia viriduloumbrina</i>	Lecanorales	Saxicolous	Foliose	<i>Trebouxioioid</i>	138	57	0.41 \pm 0.00	56 (98%)	16	7.5 (3.8, 11.2)
<i>Lecidea tessellata</i>	Lecideacea	Saxicolous	Crustose	<i>Trebouxioioid</i> / <i>Chlorosarcinopsis</i>	142	37	0.26 \pm 0.06	36 (97%)	10	4.6 (1.7, 7.4)
<i>Physcia caesia</i>	Teloschistales	Saxicolous	Foliose	<i>Trebouxioioid</i>	144	80	0.56 \pm 0.33	70 (88%)	20	9.4 (5.3, 13.5)
<i>Peltigera</i> spp. ^d	Peltigerales	Terricolous/ Muscicolous	Foliose	<i>Nostoc</i> sp.	143	65	0.45 \pm 0.27	63 (97%)	19	9.2 (5.1, 13.4)
<i>Diploschistes muscorum</i> sensu Lumbsch	Ostropales	Saxicolous	Crustose	<i>Trebouxioioid</i> / <i>Trentepohlia</i>	144	39	0.27 \pm 0.05	35 (90%)	16	11.4 (5.8, 17.0)
Total					1430	599	0.42 \pm 0.22 ^e	547 (91%)	53 (46, 60)	14.5 (10.6, 18.4)

^a Photobiont information based on Brodo et al. (2001) and Elix et al. (1987) (for *Trentepohlia* record in *Diploschistes* s.l.s)

^b All hosts started with 144 tissue segments, but a small number were lost to contamination, overgrowth, and desiccation

^c Fisher's alpha did not differ among lichen species ($F_{19,29}=0.70$, $P=0.7042$)

^d Microsites 1 and 3 contained *P. rufescens*; microsite 2 contained *P. canina*. Microsite 1 contained *D. tamini*, microsite 2 contained *D. tenue* and *D. americanum*, and microsite 3 contained *D. tenue*. Each genus was treated as a single taxon

^e Average infection frequency

Table 3 Fungi from dead leaves in tree canopies (dead leaf fungi (DLF)) and leaf litter (LLF): plant host, lineage, and family; number of plant tissue segments examined and isolates recovered; isolation frequency; number of isolates sequenced, number of putative species, and diversity (Fisher's alpha) using OTU based on 95% ITS-LSU rDNA sequence similarity

Host	Host lineage	Host family	Leaf segments ^a	Isolates recovered	Isolation frequency/ microsite \pm SD	Isolates sequenced (%)	Putative species (95% CI)	Fisher's alpha (95% CI) ^b
Dead leaves from canopy								
<i>Quercus rugosa</i>	Angiosperm	Fagaceae	144	34	0.24 \pm 0.39	32 (97%)	8	3.4 (1.0, 5.7) a
<i>Juniperus deppeana</i>	Conifer	Cupressaceae	141	87	0.62 \pm 0.18	82 (94%)	23	10.6 (6.3, 15.0) b
<i>Pinus arizonica</i> var. <i>arizonica</i>	Conifer	Pinaceae	144	36	0.25 \pm 0.40	31 (86%)	3	0.8 (-0.1, 1.5) a
<i>Pseudotsuga menziesii</i> var. <i>glauca</i>	Conifer	Pinaceae	142	29	0.20 \pm 0.21	19 (66%)	3	1.0 (-0.1, 2.1) a
DLF total			571	186	0.33 \pm 0.32 ^c	164 (89%)	32 (25, 40)	11.8 (7.7, 15.9)
Leaf litter								
<i>Quercus rugosa</i>	Angiosperm	Fagaceae	143	29	0.20 \pm 0.18	27 (90%)	7	3.1 (0.8, 5.3) B, C
<i>Juniperus deppeana</i>	Conifer	Cupressaceae	144	43	0.30 \pm 0.09	37 (86%)	16	10.7 (5.5, 16.0) A
<i>Pinus arizonica</i> var. <i>arizonica</i>	Conifer	Pinaceae	143	57	0.40 \pm 0.35	55 (96%)	8	2.6 (0.8, 4.4) C
<i>Pseudotsuga menziesii</i> var. <i>glauca</i>	Conifer	Pinaceae	144	29	0.20 \pm 0.03	25 (86%)	10	6.2 (2.4, 10.0) A, B
LLF total			574	158	0.28 \pm 0.19 ^c	144 (91%)	33 (28, 38)	13.5 (8.9, 18.0)

^a All hosts started with 144 tissue segments, but a small number were lost to contamination, overgrowth, and desiccation

^b Different letters represent significant differences ($P < 0.05$) based on pairwise comparisons of diversity for DLF (lowercase) and LLF (uppercase) using bootstrap randomization in PAST (1,000 replicates)

^c Average infection frequency

Fungi growing from plant or lichen tissues were isolated into pure culture, vouchered in sterile water, and deposited at ARIZ (accession nos. AZ0001-1110). This study focuses only on isolates recovered in culture; uncultured fungi will be addressed in forthcoming work. Overall, 4,176 tissue segments were examined (Tables 1, 2, and 3).

DNA Extraction, PCR, Sequencing

Total genomic DNA was extracted directly from every isolate following [5]. The nuclear ribosomal internal transcribed spacers and 5.8 s gene (ITS rDNA; ca. 600 bp) and an adjacent portion of the nuclear ribosomal large subunit (LSU rDNA; ca. 500 bp) were amplified by the polymerase chain reaction (PCR) as a single fragment using primers ITS1F or ITS5 and LR3 [20, 51, 53]. Each 20- μ l reaction mixture included 10 μ l of Sigma Readymix REDTaq with MgCl₂ (St. Louis, MO), 0.16 μ l of each primer (50 mM), 0.5 μ l of DNA template, and 9.18 μ l of PCR-quality water. Cycling reactions (94°C for 3 min; 36 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min; and 72°C for 10 min) were run on an MJ Research PTC200 thermocycler (Waltham, MA). SYBR Green I stain (Molecular Probes, Invitrogen; Carlsbad, CA) was used to detect DNA bands on a 1% agarose gel. All products yielded single bands.

PCR products were cleaned, quantified, and normalized at the University of Arizona Genetics Core facility.

Bidirectional sequencing was performed on an Applied Biosystems 3730x/DNA Analyzer (Foster City, CA). The software applications *phred* and *phrap* [11, 12] were used to call bases and assemble contigs with automation provided by the ChromaSeq package in Mesquite v. 1.06 (<http://mesquiteproject.org>). Base calls were verified by inspection of chromatograms in Sequencher v. 4.5 (Gene Codes, Ann Arbor, MI). Sequences have been deposited in GenBank under accession numbers HM122760-HM123719.

Species Richness and Diversity

Because isolates rarely sporulated in culture, molecular data were used to delineate operational taxonomic units (OTU). Although the high degree of intraspecific variability within fungal ITS rDNA precludes delimiting all species using a fixed level of divergence (e.g., 3%; see [37]), recent examination of four genera in the Sordariomycetes and Dothideomycetes, including common inhabitants of lichens and both living and dead leaves [6, 41], demonstrated that genotype groups delimited by 5% ITS rDNA divergence (95% sequence similarity) conservatively estimate sister species boundaries when compared against published phylogenies [50]. Although most studies use only ITS data to delimit OTU, the addition of partial LSU data in this study provides greater phylogenetic information for the

same sequencing effort without resulting in significant changes to 95% and 100% OTU groups (U'Ren, J. M., unpublished).

In comparing tools for assembling ITS-LSU rDNA OTU (e.g., DOTUR, [44]; three FastGroupII algorithms: PSI, PSI with gaps and SeqMatch, [54]; and Sequencher, as in [4]), Sequencher with manual editing and a minimum of 40% overlap delimited groups that were most often congruent with clades found by Bayesian phylogenetic analyses (Electronic Supplementary Material Fig. S1), and was more amenable than algorithms designed for bacterial 16S sequences [26, 50]. Therefore, Sequencher was used to designate OTU corresponding to 95% sequence similarity as a basis for further analyses. Key conclusions do not differ when OTU based on 100% similarity are used (data not shown).

OTU accumulation curves, rarefaction curves, and bootstrap estimates of total richness (which are more precise than Chao and Jackknife estimates when data sets contain many rare taxa; [34, 40]) were inferred in EstimateS v. 8.0 (<http://vicero.eeb.uconn.edu/EstimateS>) using 50 randomizations of sample order without replacement. Total richness (number of putative species) was assessed with PAST v. 1.88 (<http://folk.uio.no/ohammer/past/>). Diversity was measured using Fisher's alpha [14], a parameter of the log series model that is robust to variation in sample size [34, 48]. Bootstrap randomizations of Fisher's alpha were conducted in PAST (1,000 replicates) to compare diversity among samples, with confidence intervals calculated in SPADE (<http://chao.stat.nthu.edu.tw/softwareCE.html>) when key assumptions for analysis of variance (ANOVA) were not met. All other statistical analyses were done in JMP v. 7.0.2 (SAS Institute, Cary, NC).

Taxonomic Composition and Similarity of Fungal Assemblages

Taxonomic composition was estimated at the class level by querying all sequences with BLASTn in the NCBI non-redundant nucleotide database [1] followed by comparison with a maximum likelihood phylogenetic analysis containing named taxa for confirmation (data not shown). A χ^2 test was used to test the null hypothesis that classes of Pezizomycotina were distributed equitably among different tissue types (living leaves, lichen thalli, dead leaves, leaf litter) and host lineages (living leaves: moss, fern, conifer, angiosperm; dead leaves and leaf litter: conifers, angiosperm; lichens: Verrucariales, Teloschistales, Peltigerales, Ostropales, Lecideaceae, and Lecanorales, and among species in the Lecanorales) using percent-normalized data. Because fungi-matching unnamed/environmental sequences could not be reliably assigned taxonomic affiliation using BLAST (see [50]), fungi with top hits that matched environmental samples or otherwise unidentified fungi were excluded (36

isolates representing 11 OTU). All taxonomic identification below the level of class was treated with caution due to the prevalence of misidentified sequences in GenBank and challenges associated with the limitations of the BLAST algorithm (see [4, 36, 50, 52]).

Similarity among partitions of the fungal community was assessed at the OTU level using Jaccard's index (based on presence/absence data only) and the Morisita–Horn index (based on isolation frequency) [3, 18]. Indices were calculated in EstimateS v. 8.0, were based only on non-singleton genotypes, and were compared statistically with a custom PERL script for bootstrap randomizations written by T. Wheeler (University of Arizona).

Results

Over 1 year of incubation, 1,061 isolates were recovered (Tables 1, 2, and 3). Isolation frequency, defined as the percent of tissue segments bearing culturable fungi, differed among tissue types (Wilcoxon rank-sum, $\chi^2_3=36.43$, $P<0.0001$): culturable fungi were recovered significantly less frequently from living leaves than from lichen thalli, dead leaves, and leaf litter (Table 4).

Endophytic fungi were recovered from all plant species examined except *Q. rugosa* (Table 1) and were isolated from up to 21% of tissue segments per species. Isolation frequency differed among plant lineages (Wilcoxon rank-sum, $\chi^2_3=16.46$, $P=0.0009$), with angiosperms having a significantly lower isolation frequency than mosses or conifers (Table 4). Isolation frequency did not differ significantly among microsites ($F_{2,30}=2.62$, $P=0.0896$).

Endolichenic fungi were recovered from every thallus, and were isolated from 16% to 76% of tissue segments examined per lichen species (Table 2). Isolation frequency differed as a function of the ordinal placement of lichen mycobionts (log-transformed with one outlier removed, $F_{5,23}=3.00$, $P=0.0314$) and was greatest in Teloschistales, Lecanorales, and Peltigerales (Table 4). There was weak evidence for a higher isolation frequency in epiphytic vs. saxicolous lichens; foliose and fruticose vs. crustose lichens; and lichens with exclusively trebouxoid photobionts vs. lichens with other photobionts (see Table 2). However, once the effect of lichen taxonomy was taken into account these factors were not significant (ANOVA on residuals following test for ordinal effect, $P=0.2326$ (substrate), $P=0.1984$ (growth form), $P=0.6352$ (photobiont); lack-of-fit F test, $F_{2,22}=0.34$, $P=0.7177$). Isolation frequency did not differ significantly among microsites ($F_{2,27}=0.53$, $P=0.5954$).

Table 4 Summary of host taxa sampled, isolates sequenced, species richness, 95% confidence intervals of species richness, bootstrap estimate of richness, and diversity (Fisher's alpha) for each community partition and host lineage

	Host species	Isolation frequency/ microsite \pm SD ^a	Sequences	Putative species	Richness 95% CI (lower)	Richness 95% CI (upper)	Bootstrap estimate richness	Fisher's alpha (95% CI) ^b
Endophyte	11	0.08 \pm 0.11 A	105	36	29.22	42.78	43.44	19.4 (13.0, 25.7) A
Mosses	2	0.18 \pm 0.11 b	49	19	13.65	24.35	23.21	11.4 (6.3, 16.5) a
Fern	1	0.05 \pm 0.09 a, b	5	4	1.3	6.7	5.06	9.3 (0.2, 18.4) a
Conifers	3	0.12 \pm 0.13 b	44	15	10.14	19.86	18.34	8.0 (4.0, 12.1) a
Angiosperms	5	0.01 \pm 0.02 a	7	4	1.85	6.15	4.79	3.9 (0.1, 7.7) a
Endolichenic fungi	10 ^a	0.42 \pm 0.22 B	547	53	46.95	59.05	60.68	14.5 (10.6, 18.4) B
Verrucariales	1	0.23 \pm 0.12 c	20	8	5.28	10.72	9.8	4.9 (1.5, 8.4)
Lecanorales	5	0.49 \pm 0.18 a	323	39	30.04	47.96	46.62	11.6 (8.0, 15.2)
Teloschistales	1	0.56 \pm 0.33 a, b	70	20	16.13	23.87	23.35	9.4 (5.3, 13.5)
Peltigerales	1	0.45 \pm 0.27 a, b, c	63	19	14.38	23.62	22.64	9.2 (5.1, 13.4)
Ostropales	1	0.27 \pm 0.05 b, c	35	16	12.25	19.75	19.28	11.4 (5.8, 17.0)
Lecideaceae	1	0.26 \pm 0.06 b, c	36	10	6.72	13.28	11.79	4.6 (1.7, 7.4)
Dead leaf fungi (DLF)^c	4	0.33 \pm 0.32 B	164	32	22.78	41.22	38.6	11.8 (7.7, 15.9) B
Conifers	3	0.36 \pm 0.31 a	132	26	18.4	33.6	30.79	9.7 (6.0, 13.4) a
Angiosperm	1	0.24 \pm 0.39 a	32	8	2.74	11.26	9.17	3.4 (1.0, 5.7) b
Leaf litter fungi (LLF)^c	4	0.28 \pm 0.19 B	144	33	28.26	37.74	37.9	13.5 (8.9, 18.0) B
Conifers	3	0.30 \pm 0.20 a	117	27	23.32	30.68	30.62	11.0 (6.9, 15.2) a
Angiosperm	1	0.20 \pm 0.18 a	27	7	3.79	10.21	8.29	3.1 (0.8, 5.3) b

^a Different letters represent significant differences ($P < 0.05$) based on ANOVA with post-hoc Tukey–Kramer HSD comparisons of infection frequency among substrates (uppercase), major plant lineages, or lichen orders (lower case).

^b Different letters represent significant differences ($P < 0.05$) based on pairwise comparisons of diversity among major plant lineage using bootstrap randomization in PAST (1,000 replicates) (lowercase letters), or significant differences among tissue types detected by ANOVA with post-hoc Tukey–Kramer HSD comparisons (uppercase letters). No significant differences were observed among lichen families (no superscripts shown).

^c Per-taxon comparisons of Fisher's alpha values for DLF and LLF are given in Table 3

Fungi from dead leaves in tree canopies (i.e., dead leaf fungi (DLF)) and leaf litter (leaf litter fungi (LLF)) were recovered from every individual and were isolated from 20–62% (DLF) and 20–40% (LLF) of tissue segments per species. DLF were isolated 2.1 times more frequently than LLF from *J. deppeana* ($t_4 = 2.76$, $P = 0.0510$), but in all other host species, DLF and LLF were isolated with similar frequencies (Table 3). Isolation frequency from dead leaves and leaf litter did not differ among major plant lineages (conifers vs. angiosperm; Table 4) or microsite (pooled DLF and LLF data, $F_{2,9} = 0.09$, $P = 0.9166$).

Richness and Diversity

High-quality sequence data from 960 isolates yielded 95 OTU based on 95% ITS-LSU rDNA similarity (Fisher's alpha = 26.2). These represented 354 unique genotypes (based on 100% ITS-LSU rDNA similarity; Fisher's alpha = 201.5). Overall, 33.7% of OTU and 66.3% of genotypes were singletons (i.e., found only once).

Our sampling effort was statistically sufficient to capture the total OTU richness of culturable DLF (Fig. 1). For endophytic, endolichenic, and LLF fungi, differences between bootstrap estimates and the upper 95% confidence intervals around observed richness ranged from only 0.16 to 1.63 OTU (Table 4), indicating that the majority of culturable fungi in these groups were recovered. Based on minute differences between observed and expected richness, these data were used to compare diversity of culturable fungi in each substrate.

Although diversity of endophytes in mosses and conifers exceeded that in angiosperms by more than twofold, diversity did not differ significantly among major plant lineages (Table 4). Similarly, diversity of endolichenic fungi did not differ significantly among lichen species (Table 2), lichen orders (Table 4), or as a function of lichen growth form, substrate, or photobiont (ANOVA on residuals following test of ordinal effect: $P = 0.7400$ (substrate), $P = 0.8937$ (growth form), $P = 0.8524$ (photobiont); lack-of-fit F test, $F_{2,22} = 0.07$, $P = 0.9369$).

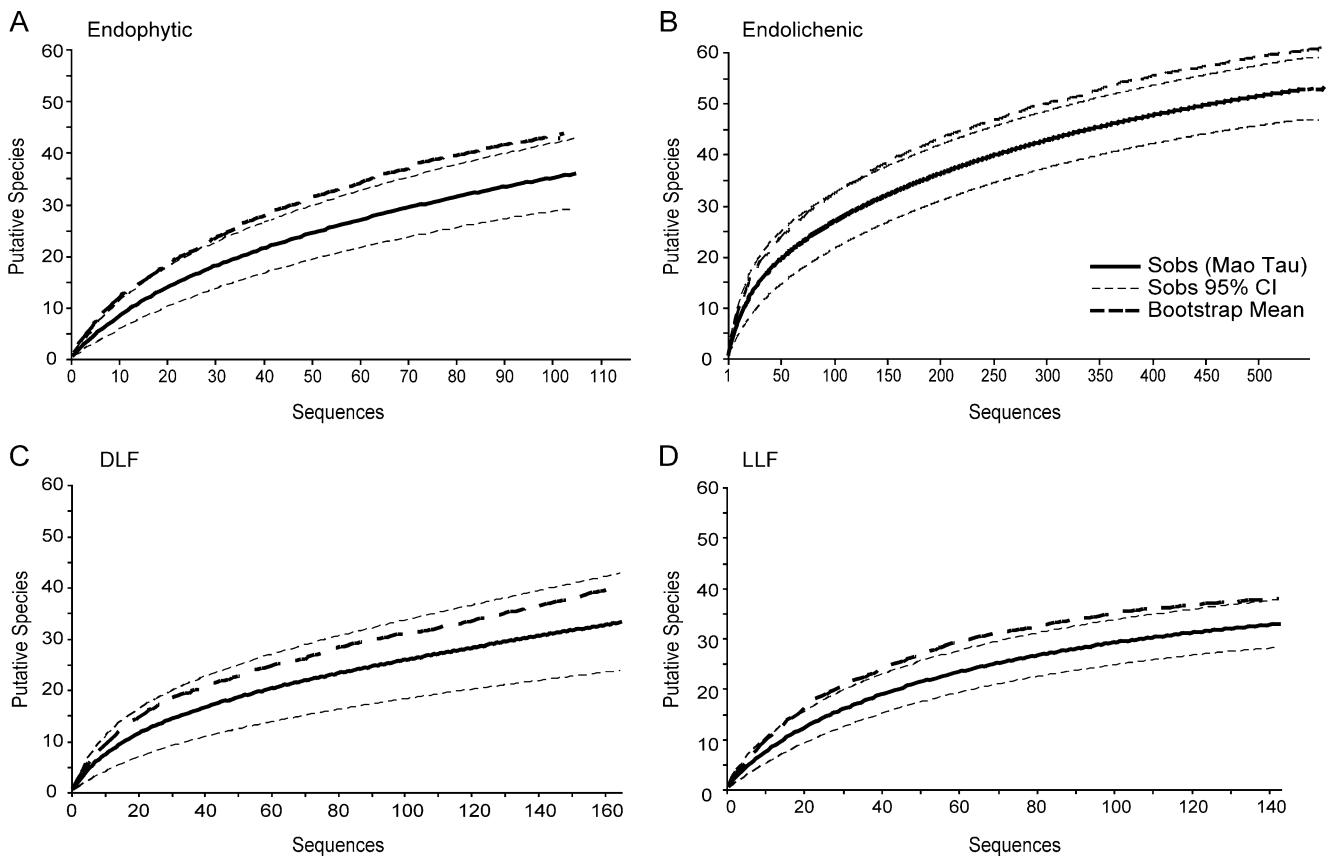


Figure 1 Species accumulation curves (S_{obs} Mao Tau), 95% confidence intervals, and bootstrap estimates of richness based on ITS-LSU rDNA OTU (95% sequence similarity) for **a** 105 isolates of endophytic fungi from living photosynthetic tissues of 11 species of

plants; **b** 547 endolichenic fungi from living thalli of ten lichen genera; **c** 165 isolates of fungi from dead leaves in canopies of four tree species (dead leaf fungi, DLF); and **d** 143 isolates of fungi from leaf litter beneath four tree species (leaf litter fungi, LLF)

The diversity of endophytes (Fisher's alpha) exceeded that of endolichenic fungi by a factor of 1.3 ($F_{1,4}=16.09$, $P=0.0160$) despite the higher isolation frequency and species richness of fungi found in lichens overall (Tables 1 and 2). When constrained to equal numbers of isolates ($N=105$, matching the number of sequenced endophytic isolates), endophytes (36.0 species; 95% CI=29.2, 42.8) were more species-rich than endolichenic fungi (27.5 species; 95% CI=22.1, 33.0; isolate-based rarefaction; Fig. 2a). Host-based rarefaction highlighted the higher species richness of endolichenic fungi in the overall sample (Fig. 2b; Electronic Supplementary Material Fig. S2), reflecting the fivefold greater numbers of endolichenic fungi vs. endophytic fungi recovered here.

Cumulative diversity of endophytes (Table 1; Fisher's alpha=19.4) was significantly greater than that of DLF and LLF (Table 3) ($F_{2,6}=13.37$, $P=0.0062$). However, these guilds differed in the number of host species and individuals surveyed. When analyses were constrained to species for which endophytes, DLF and LLF were collected, diversity of endophytes in *J. deppeana*, *P. arizonica*, and *P. menziesii*

(Fisher's alpha=8.0) was similar to that of DLF (Fisher's alpha=9.7) and LLF (Fisher's alpha=11.0) ($F_{2,6}=0.22$, $P=0.8097$). The diversity of DLF and LLF differed significantly among plant species (Table 3) and between conifers and *Q. rugosa* (Table 4). DLF and LLF communities contained similar numbers of OTU (Table 3); however, LLF from *P. arizonica* and *P. menziesii* were four- to sixfold more diverse than DLF from the same hosts ($P=0.027$ and 0.004) (Table 3).

Composition of Fungal Assemblages

All fungi sequenced here are members of the Pezizomycotina (Ascomycota; $n=939$ sequences with defined taxonomy; the remaining 21 isolates were classified as either "uncultured fungus" or "fungal endophyte"). Based on BLAST analyses coupled with phylogenetic analyses, five classes and approximately 16 orders, 32 families, and 65 genera of Pezizomycotina were represented. Pezizomycetes were most common (43.2% of isolates; ca. six families and 20 genera), followed by Sordariomycetes (28.9% of isolates; ca. eight families and 18 genera), Leotiomyces (17.2% of isolates; ca. four

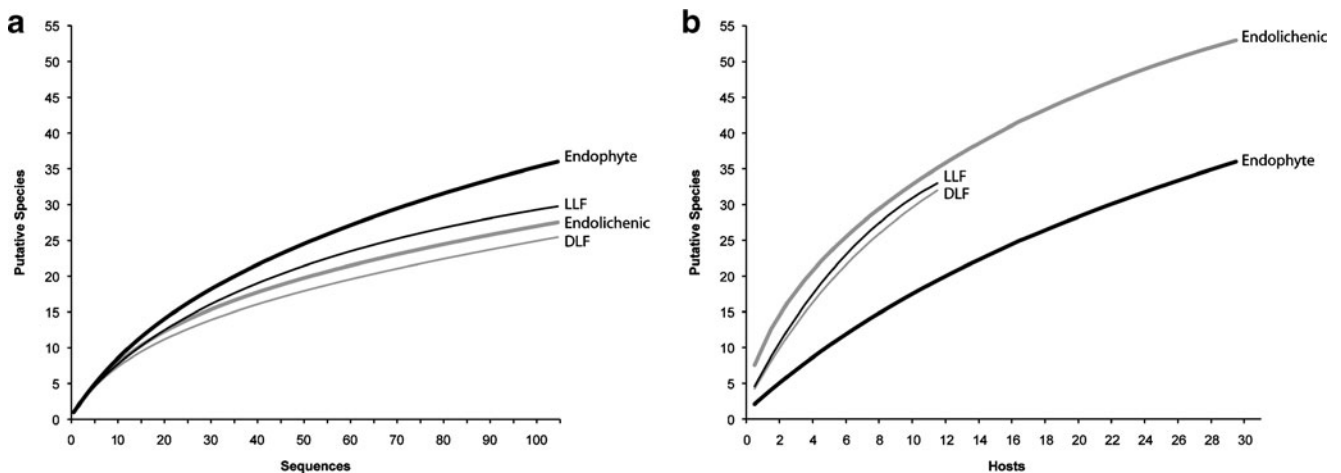


Figure 2 a Species-based rarefaction based on ITS-LSU rDNA OTU (95% sequence similarity) for endophytes, endolichenic fungi, dead leaf fungi (DLF), and fungi from leaf litter beneath trees (leaf litter fungi, LLF) constrained to equal numbers of isolates ($n=105$); b host-based rarefaction based on ITS-LSU rDNA OTU (95% sequence

similarity) for endophytic fungi from living photosynthetic tissues of 33 individual plants, endolichenic fungi from living thalli of 30 lichens, fungi from dead leaves in canopies of 16 trees (DLF), and fungi from leaf litter beneath 16 trees (LLF)

families and six genera), Dothideomycetes (8.7% of isolates; ca. 13 families and 19 genera) and Eurotiomycetes (0.4% of isolates; one family and two genera). After accounting for differences in sample sizes, the null hypothesis that these five classes of Pezizomycotina were equitably distributed among endophytes, endolichenic fungi, DLF and LLF was rejected ($\chi^2_9=61.99$, $P<0.0001$) (Fig. 3a).

Within living tissues, the relative abundance of the most common classes differed significantly among major plant lineages ($\chi^2_9=415.71$, $P<0.0001$; Fig. 3b) and lichen orders ($\chi^2_{10}=180.86$, $P<0.0001$; Fig. 3c). Endophyte assemblages in mosses were dominated by Pezizomycetes (>70% of isolates), the focal fern by Dothideomycetes, conifers by Leotiomyces, and angiosperms by Sordariomycetes and Dothideomycetes. Each plant lineage yielded at least three classes of Pezizomycotina except angiosperms, which yielded only two. The majority of endolichenic fungi were Pezizomycetes (>55% of isolates) (Fig. 3a). After lichen-ordinal effects were taken into account, no pattern was observed with regard to endolichenic taxonomy and lichen growth form, substrate, or photobiont (data not shown). Within the Lecanorales, the most thoroughly sampled order, Pezizomycetes made up more than half of all isolates in every host species (55.7–95.1% of isolates; Fig. 3d). Endolichenic fungi recovered here did not include any previously known lichenicolous fungi or mycobionts.

When analyses were restricted to three conifers for which endophytes, DLF and LLF were recovered, taxonomic composition at the class level was consistent ($\chi^2_6=10.46$, $P=0.1065$) and was dominated by Leotiomyces (Fig. 3e). Class-level taxonomy also was consistent between DLF and LLF from *Q. rugosa* (Fig. 3f). When

equal numbers of isolates were considered ($N=105$), endophytes, endolichenic fungi, DLF and LLF each harbored a similar phylogenetic diversity of fungi (defined by the number of putative orders recovered; range: 8.0–8.7 orders for five random draws of 105 isolates/tissue type).

At the OTU level, assemblages of endolichenic fungi were significantly more similar to endophytes than to DLF and LLF based on both presence/absence data and isolation frequency (Fig. 4a). This pattern reflects OTU that were recovered as endolichenic fungi and endophytes of mosses (15 OTU representing Dothideomycetes, Pezizomycetes, and Sordariomycetes). The resulting similarity of endolichenic fungi and endophytes from mosses significantly exceeded similarity between endolichenic fungi and endophytes of vascular plants (Fig. 4b).

When analyses were restricted only to those hosts for which endophytes, DLF, and LLF were recovered, endophytes were significantly more similar to DLF than to LLF when species abundance was taken into account (Fig. 4c). This pattern is illustrated by the composition and relative abundance of the most common fungi in leaves of *P. arizonica* and *P. menziesii*, although variation among host species was observed (Fig. 5).

Discussion

Because many genera of fungi commonly observed as endophytes also occur within asymptomatic lichen thalli [6, 21, 30, 32, 47], the ecological distinctiveness of endolichenic fungi has been uncertain. The goal of this study was to clearly define the degree of ecological similarity and

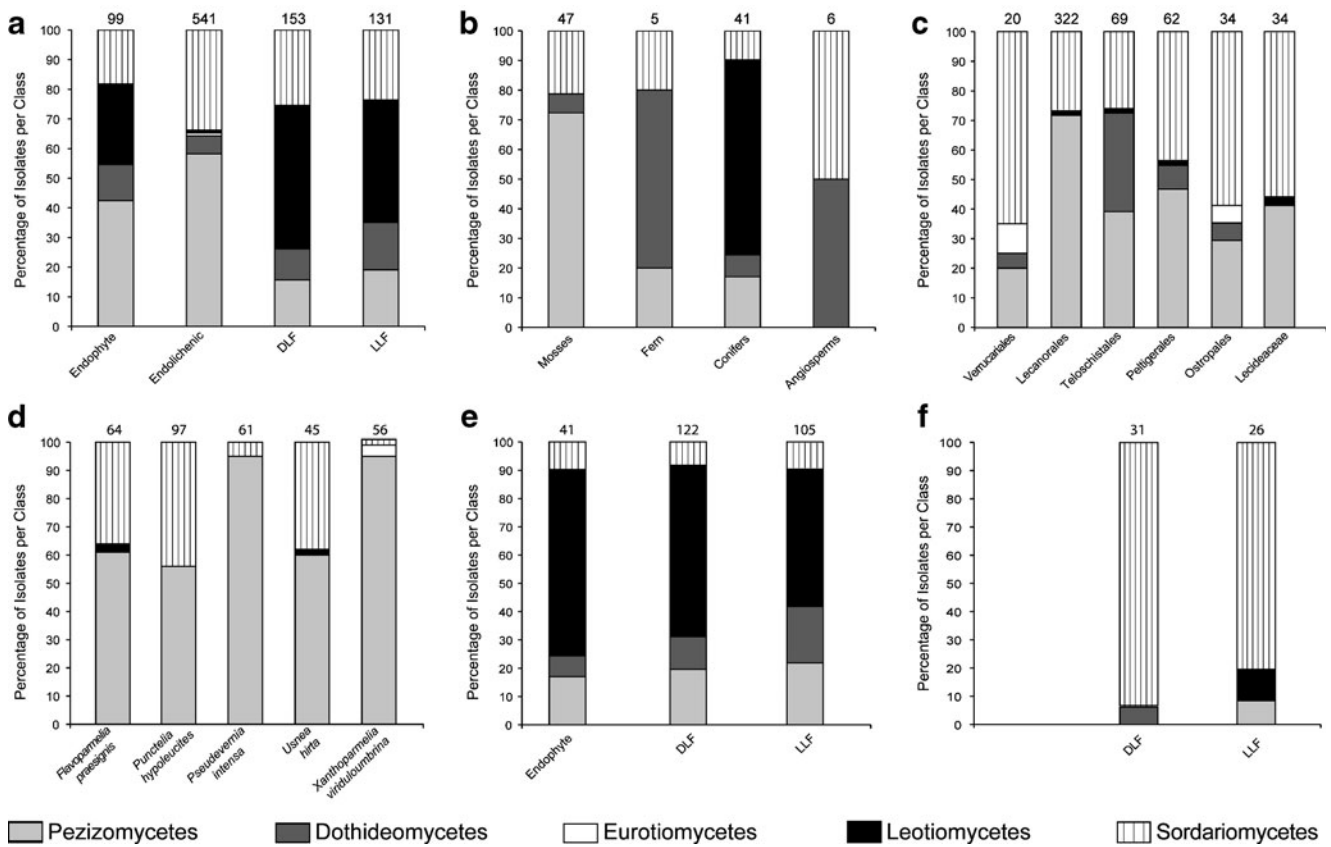


Figure 3 Percent of isolates representing each of five classes of Pezizomycotina (Pezizomycetes, Dothideomycetes, Eurotiomycetes, Leotiomyces, and Sordariomycetes) as a function of **a** host tissue type (endophytic fungi from living photosynthetic tissues of plants; endolichenic fungi from living lichen thalli; fungi from dead leaves in the canopy (DLF), and fungi from leaf litter (LLF); **b** major plant lineage (endophytes only); **c** lichen order/family (endolichenic fungi

only); **d** Lecanorales species (endolichenic fungi only); **e** host tissue type, considering only three host species for which endophytes, DLF, and LLF were isolated (*J. deppeana*, *P. arizonica*, and *P. menziesii*); and **f** host tissue type, considering *Q. rugosa* from which DLF and LLF were sampled. The total number of sequences assessed is listed at the top of each stacked bar

taxonomic overlap between endolichenic fungi and fungi from other substrates by intensively comparing isolation frequency, species richness, diversity, and taxonomic composition with endophytes and fungi inhabiting dead leaves in the tree canopy and leaf litter. Although the present study was limited to only three microsites, considered only the dominant plant and lichen species, comprised a limited number of representatives of each host lineage, and evaluated only culturable fungi (see below), the resulting library provides evidence for three primary conclusions regarding fungal symbionts from plants and lichens in this montane community.

Firstly, fungal communities appear to differ at a broad taxonomic level as a function of the phylogenetic placement of their plant or lichen hosts (Fig. 3b, c). At the class level, endolichenic fungal assemblages differed as a function of lichen taxonomy, rather than substrate, growth form, or photobiont. Because endolichenic fungi associate physically with photobionts in lichen thalli (see [6]), these data point to an unexpected role of mycobionts in shaping

the composition of endolichenic communities (Fig. 3c, d). Similarly, class-level composition of endophytes and fungi from senescent leaves differed as a function the phylogenetic placement of hosts (Fig. 3e, f), suggesting that fungal assemblages in these plants are structured, at a broad taxonomic level, more by plant lineage than by the living vs. senescent status of the leaf.

Secondly, the present study does not provide support for the suggestion that endolichenic fungi are simply incidental or saprotrophic fungi that have been ‘entrapped’ by lichen thalli (see discussion in [6, 32]). Although many classes of fungi were found in both living lichen thalli and non-living leaves, analyses at the OTU level indicate that relative to endolichenic fungi, DLF and LLF represent either different species or a very small number of shared species that differ markedly in abundance. This underscores the potential for the same fungal lineages to have diversified in parallel in lichen thalli (as symbiotrophs) or in dead plant tissue (as saprotrophs), but likely not as species that encompass both ecological

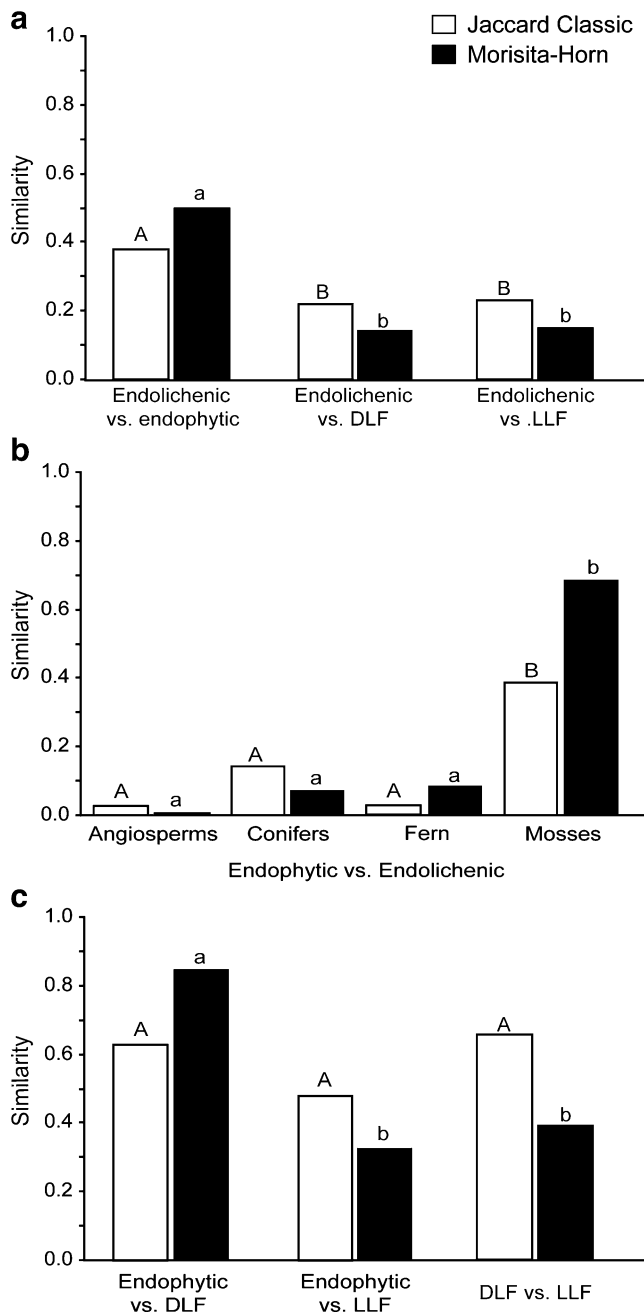


Figure 4 Pairwise similarity of fungal assemblages based on non-singleton OTU based on 95% ITS-LSU rDNA genotypes as a function of **a** host tissue type (corresponding to living or dead plant or lichen hosts for endophytes, endolichenic fungi, DLF, LLF); **b** endophytes from angiosperms, conifers, ferns, and mosses versus endolichenic fungi; and **c** host tissue type, considering only three host species for which endophytes, DLF and LLF were isolated (*J. deppeana*, *P. arizonica*, and *P. menziesii*). Different letters above each bar represent statistically significant differences ($P < 0.05$) after *t* tests with a randomized distribution of observed data (lowercase, Morisita-Horn; uppercase, Jaccard)

modes. In contrast, ca. 25% of the conifer-associated fungi studied here are found both in senescent leaves and as endophytes, supporting previous suggestions that some endophytes encompass lifestyles spanning both symbiosis

and decay (e.g., [41]). The degree of overlap between endophytic and putatively saprotrophic communities differed between Pinaceae and Cupressaceae, and should be evaluated in additional plant lineages.

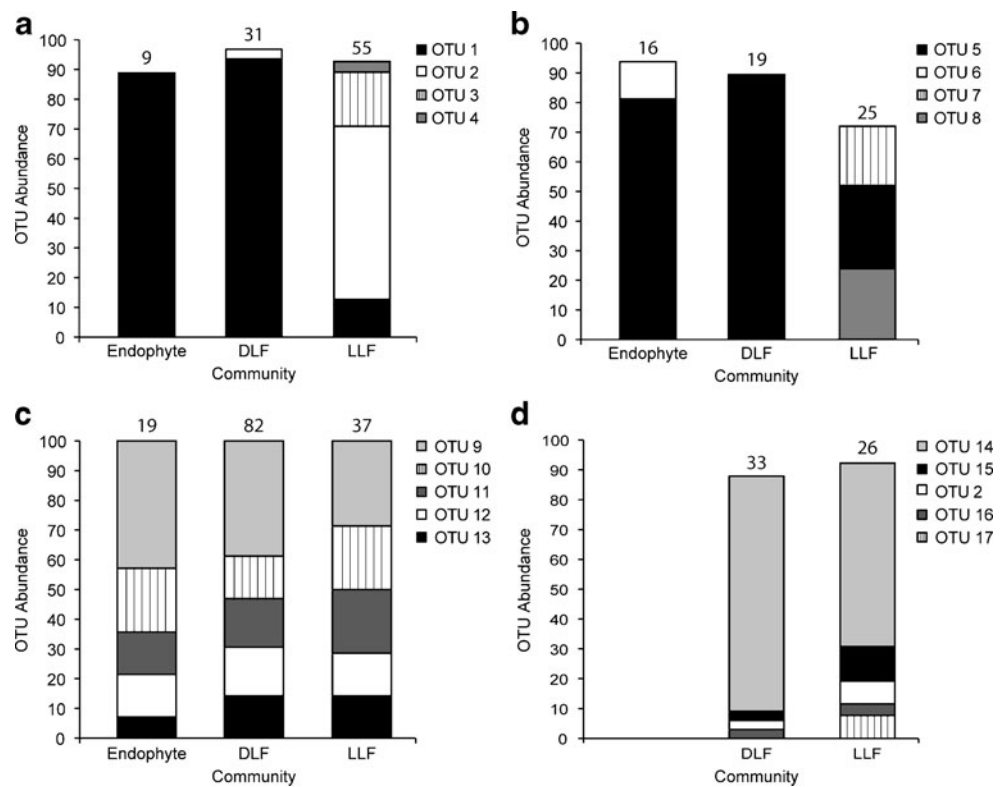
Thirdly, our study reveals that some fungi are capable of forming both endolichenic and endophytic symbioses. The high similarity of endophytes and endolichenic fungal communities observed here (Fig. 4a) is driven strongly by OTU that occur in both mosses and lichens (Fig. 4b). This similarity is consistent regardless of lichens' epiphytic or saxicolous habit, growth form, or taxonomy (data not shown) and may reflect numerous structural and ecological similarities between lichens and mosses, including non-vascular architecture, the ability to deal with desiccation, and the capacity of some species to associate with cyanobacteria (e.g., *Sphagnum* sp.; see [10]). Consistent with Suryanarayanan et al. [47] we found little evidence for congruence of endolichenic communities and endophytes of vascular plants, even in cases in which endophytes were isolated from the foliage of the trees on which endolichenic-containing lichens were collected. Strikingly, fungi capable of colonizing both mosses and lichens occur in three classes of Pezizomycotina.

Both mosses, here representing the Bryophyta, and lichens, here harboring primarily green algal photobionts in lineages closely related to land plants [35], contained fungal symbiont communities dominated by Pezizomycetes, one of the earliest-diverging lineage of Pezizomycotina [45]. In contrast, more recently evolved vascular plants were dominated by classes of Pezizomycotina that arose more recently (see phylogenetic estimation in [45]): the Dothideomycetes, Sordariomycetes, and Leotiomycetes. These observations suggest that the evolution of land plants may have structured the evolution of endophytic fungi from ancestral endolichenic fungi (see [6]). However, testing this hypothesis will require careful phylogenetic analyses informed by a greater breadth of sampling within plant lineages, as well as special attention to fungi from two later-diverging lineages (Dothideomycetes, Sordariomycetes) that also occurred, albeit more rarely than Pezizomycetes, as symbionts of mosses and lichens.

Although conclusions at the OTU level are based on groups defined by 95% ITS-LSUrDNA similarity, they do not differ when groups based on 100% similarity are used. Although unlikely, it is possible that strains with fully identical ITS-LSUrDNA genotypes represent different species: in some fungal groups these loci evolve too slowly to reliably diagnose species boundaries (e.g., *Aspergillus* spp.; see [23]), potentially leading to artificial "lumping" of disparate organisms into a single genotype group. Future inoculation experiments using culture libraries will be important for confirming these observations. In the meantime, 100% similarity over the >1,000 bp reads used here

Figure 5 Percent abundance of the most common OTU in each host plant species as endophytes, DLF, or LLF.

a *Pi. arizonica*; **b** *Ps. menziesii*; **c** *J. deppeana*; and **d** *Q. rugosa*. The taxonomic affiliation of each OTU was estimated using a BLASTn query of the NCBI database: OTU 1, 5, 9 (Helotiales, Leotiomycetes); OTU 2 (Rhytismatales, Leotiomycetes); OTU 3, 10 (Pleosporales, Dothideomycetes); OTU 4, 13 (Unidentified Ascomycota); OTU 6 (Hypocreales, Sordariomycetes); OTU 7, 8, 11, 12 (Pezizales, Pezizomycetes); OTU 14 (Phyllachorales, Sordariomycetes); OTU 15, 17 (mitosporic Sordariomycetes); OTU 16 (mitosporic Ascomycota). The total number of sequences assessed is listed at the top of each stacked bar



provides a strong signal for overlap between endolichenic and moss-endophytic communities.

Importantly, the above conclusions are based only on cultured isolates. Culture-free approaches consistently show that fungal diversity is higher than estimated by culturing alone, and that the two approaches are complementary for capturing a representative perspective on community composition. Arnold et al. [4] showed that culturing endophytes from *Pinus taeda* markedly underestimated diversity when compared with a clone-based method, suggesting that diversity of endophytic and endolichenic fungi is likely larger than the high values estimated here. That study found that the same classes of fungi were recovered by cloning and culturing, with one exception—the Sordariomycetes, which were common among cultures, but never were found by cloning. Although few studies are available that explicitly compare culturing and cloning results from the same plant material, previous work (e.g., [4, 17]) suggests that our conclusions regarding patterns at higher taxonomic levels are likely stable. Because a culture-free approach would expand current estimates of diversity at the OTU level, as well as address the surprising lack of cultured endophytes in leaves of *Q. rugosa* and rarity of isolates from *P. arizonica*, such methods are a target for future work. However, the low isolation frequency from these hosts may not reflect culture bias, but instead result from sampling in the hot dry season before the onset of seasonal rains, which are associated with an increase in endophyte isolation frequency [13].

Overall, our study provides insights regarding the taxonomic composition of endolichenic communities and their ecological connections to endophytes and saprotrophs. Our data suggest that endolichenic fungi are largely distinct from fungal communities in living and dead photosynthetic tissues of plants with the exception of moss endophytes. This finding suggests that endolichenic fungi may have a greater degree of ecological specificity compared with endophytes as a whole, which include some species capable of forming endolichenic symbioses and others that persist in non-living tissues (saprotrophic fungi). By distinguishing ecological specialists from those more flexible species that inhabit multiple tissue and host types, this study provides a framework for future work regarding the evolution of trophic modes in the diverse Pezizomycotina and empirical assessments of host- and substrate specificity and ecological function.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Arnold AE, Maynard Z, Gilbert GS (2001) Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycol Res* 105:1502–1507
- Arnold AE, Mejia LC, Kyllö D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci USA* 100:15649–15654
- Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R (2007) Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* 99:185–206
- Arnold AE, Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* 88:541–549
- Arnold AE, Miadlikowska J, Higgins KL, Sarvate SD, Gugger P, Way A, Hofstetter V, Kauff F, Lutzoni F (2009) A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? *Syst Biol* 58:283–297
- Barr DJS (2001) Chytridiomycota. In: McLaughlin DJ, McLaughlin EG, Lemke PA (eds) *The Mycota*, vol. 7A. Springer, New York
- Barton AM (1994) Gradient analysis of relationships among fire, environment, and vegetation in a southwestern USA mountain range. *Bulletin of the Torrey Botanical Club* 121:251–265
- Berbee ML (2001) The phylogeny of plant and animal pathogens in the Ascomycota. *Physiol Mol Plant Pathol* 59:165–187
- During HJ, Van Tooren BF (1990) Bryophyte interactions with other plants. *Bot J Linn Soc* 104:79–98
- Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8:186–194
- Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8:175–185
- Faeth SH, Hammon KE (1997) Fungal endophytes in oak trees: long-term patterns of abundance and associations with leafminers. *Ecology* 78:810–819
- Fisher RA, Corbet AS, Williams CB (1943) The relation between the number of species and the number of individuals in a random sample of an animal population. *J Anim Ecol* 12:42–58
- Fröhlich J, Hyde KD (1999) Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodivers Conserv* 8:977–1004
- Fröhlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with palms. *Mycol Res* 104:1202–1212
- Gallery RE, Dalling JW, Arnold AE (2007) Diversity, host affinity, and distribution of seed-infecting fungi: a case study with *Cecropia*. *Ecology* 88:582–588
- Gamboa MA, Bayman P (2001) Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). *Biotropica* 33:352–360
- Ganley RJ, Brunsfeld SJ, Newcombe G (2004) A community of unknown, endophytic fungi in western white pine. *Proc Natl Acad Sci USA* 101:10107–10112
- Gardes M, Bruns TD (1993) ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. *Mol Ecol* 2:113–118
- Girlanda M, Isocrono D, Bianco C, Luppi-Mosca AM (1997) Two foliose lichens as microfungus ecological niches. *Mycologia* 89:531–536
- Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. *Science* 293:1129–1133
- Henry T, Iwen PC, Hinrichs SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J Clin Microbiol* 38:1510–1515
- Herre EA, Mejia LC, Kyllö DA, Rojas EI, Maynard Z, Butler A, Van Bael SA (2007) Ecological implications of anti-pathogen effects of tropical endophytes and mycorrhizae. *Ecology* 88:550–558
- Hibbett DS, Gilbert L-B, Donoghue MJ (2000) Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* 407:506–508
- Higgins KLH, Coley P, Kursar T, Arnold AE (2010) Culturing and direct PCR reveal highly diverse and host generalist endophytes in tropical forest grasses. *Mycologia* (in press)
- Hoffman M, Arnold AE (2008) Geography and host identity interact to shape communities of endophytic fungi in cupressaceae trees. *Mycol Res* 112:331–344
- Hoffman M, Arnold AE (2010) Diverse bacteria inhabit hyphae of phylogenetically diverse fungal endophytes. *Appl Environ Microbiol* 76:4063–4075
- James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, Lumbsch HT, Rauhut A, Reeb V, Arnold AE, Amtoft A, Stajich JE, Hosaka K, Sung GH, Johnson D, O'Rourke B, Crockett M, Binder M, Curtis JM, Slot JC, Wang Z, Wilson AW, Schussler A, Longcore JE, O'Donnell K, Mozley-Standridge S, Porter D, Letcher PM, Powell MJ, Taylor JW, White MM, Griffith GW, Davies DR, Humber RA, Morton JB, Sugiyama J, Rossmann AY, Rogers JD, Pfister DH, Hewitt D, Hansen K, Hambleton S, Shoemaker RA, Kohlmeyer J, Volkmann-Kohlmeyer B, Spotts RA, Serdani M, Crous PW, Hughes KW, Matsuura K, Langer E, Langer G, Untereiner WA, Lucking R, Budel B, Geiser DM, Aptroot A, Diederich P, Schmitt I, Schultz M, Yahr R, Hibbett DS, Lutzoni F, McLaughlin DJ, Spatafora JW, Vilgalys R (2006) Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* 443:818–822
- Kannangara B, Rajapaksha RSCG, Paranagama PA (2009) Nature and bioactivities of endolichenic fungi in *Pseudocyphellaria* sp., *Parmotrema* sp. and *Usnea* sp. at Hakgala montane forest in Sri Lanka. *Lett Appl Microbiol* 48:203–209
- Lawrey JD, Diederich P (2003) Lichenicolous fungi: interactions, evolution, and biodiversity. *Bryologist* 106:80–120
- Li W-C, Zhou J, Guo S-Y, Guo L-D (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Divers* 25:69–80
- Lutzoni F, Pagel M, Reeb V (2001) Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 411:937–940
- Magurran AE (2004) *Measuring biological diversity*. Blackwell Publishing Ltd., Oxford
- Nickrent DL, Parkinson CL, Palmer JD, Duff RJ (2000) Multigene phylogeny of land plants with special reference to bryophytes and the earliest land plants. *Mol Biol Evol* 17:1885–1895
- Nilsson RH, Kristiansson E, Ryberg M, Larsson K-H (2005) Approaching the taxonomic affiliation of unidentified sequences in public databases—an example from the mycorrhizal fungi. *BMC Bioinform* 6:178
- Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson K-H (2008) Intraspecific ITS variability in the kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evol Bioinform* 4:193–201
- Petrini O, Hake U, Dreyfuss MM (1990) An analysis of fungal communities isolated from fruticose lichens. *Mycologia* 82:444–451
- Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS (eds) *Microbial ecology of leaves*. Springer, New York
- Poulin R (1998) Comparison of three estimators of species richness in parasite component communities. *J Parasitol* 84:485–490
- Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R (2007) A phylogenetic evaluation of whether

- endophytes become saprotrophs at host senescence. *Microb Ecol* 53:579–590
42. Redecker D, Kodner R, Graham LE (2000) Glomelean fungi from the Ordovician. *Science* 289:1920–1921
 43. Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. *New Phytol* 182:314–330
 44. Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* 71:1501–1506
 45. Spatafora JW, Sung GH, Johnson D, Hesse C, O'Rourke B, Serdani M, Spotts R, Lutzoni F, Hofstetter V, Miadlikowska J, Reeb V, Gueidan C, Fraker E, Lumbsch T, Lucking R, Schmitt I, Hosaka K, Aptroot A, Roux C, Miller AN, Geiser DM, Hafellner J, Hestmark G, Arnold AE, Budel B, Rauhut A, Hewitt D, Untereiner WA, Cole MS, Scheidegger C, Schultz M, Sipman H, Schoch CL (2006) A five-gene phylogeny of Pezizomycotina. *Mycologia* 98:1018–1028
 46. Stone JK, Bacon CW, White JFJ (2000) An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF (eds) *Microbial endophytes*. Marcel Dekker, New York
 47. Suryanarayanan TS, Thirunavukkarasu GN, Hariharan GN, Balaji P (2005) Occurrence of non-obligate microfungi inside lichen thalli. *Sydowia* 57:120–130
 48. Taylor LR (1978) Bates, Williams, Hutchinson—a variety of diversities. In: Mound LA, Warloff N (eds) *Diversity of insect faunas*. Blackwell Publishing Ltd., Oxford
 49. Taylor TN, Krings M (2005) Fossil microorganisms and land plants: associations and interactions. *Symbiosis* 40:119–135
 50. U'Ren JM, Dalling JW, Gallery RE, Maddison DR, Davis EC, Gibson CM, Arnold AE (2009) Diversity and evolutionary origins of fungi associated with seeds of a neotropical pioneer tree: a case study for analyzing fungal environmental samples. *Mycol Res* 113:432–449
 51. Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* 172:4238–4246
 52. Vilgalys R (2003) Taxonomic misidentification in public DNA databases. *New Phytol* 160:4–5
 53. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR protocols: a guide to methods and applications*. Academic Press, San Diego
 54. Yu Y, Breitbart M, McNairnie P, Rohwer F (2006) FastGroupII: a web-based bioinformatics platform for analyses of large 16 S rDNA libraries. *BMC Bioinform* 7:57