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HOST AND GEOGRAPHIC STRUCTURE OF ENDOPHYTIC AND ENDOLICHENIC FUNGI AT A CONTINENTAL SCALE[1]

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- *Premise of the study:* Endophytic and endolichenic fungi occur in healthy tissues of plants and lichens, respectively, playing potentially important roles in the ecology and evolution of their hosts. However, previous sampling has not comprehensively evaluated the biotic, biogeographic, and abiotic factors that structure their communities.
- *Methods:* Using molecular data we examined the diversity, composition, and distributions of 4154 endophytic and endolichenic Ascomycota cultured from replicate surveys of ca. 20 plant and lichen species in each of five North American sites (Madrean coniferous forest, Arizona; montane semideciduous forest, North Carolina; scrub forest, Florida; Beringian tundra and forest, western Alaska; subalpine tundra, eastern central Alaska).
- *Key results:* Endolichenic fungi were more abundant and diverse per host species than endophytes, but communities of endophytes were more diverse overall, reflecting high diversity in mosses and lycophytes. Endophytes of vascular plants were largely distinct from fungal communities that inhabit mosses and lichens. Fungi from closely related hosts from different regions were similar in higher taxonomy, but differed at shallow taxonomic levels. These differences reflected climate factors more strongly than geographic distance alone.
- *Conclusions:* Our study provides a first evaluation of endophytic and endolichenic fungal associations with their hosts at a continental scale. Both plants and lichens harbor abundant and diverse fungal communities whose incidence, diversity, and composition reflect the interplay of climatic patterns, geographic separation, host type, and host lineage. Although culture-free methods will inform future work, our study sets the stage for empirical assessments of ecological specificity, metabolic capability, and comparative genomics.

Key words: Ascomycota; diversity; endolichenic fungi; endophytic fungi; lichens; plant–fungal symbioses; symbiotrophs.

In both natural and agricultural ecosystems, plants form symbiotic relationships with diverse and ecologically important fungi (Petrini, 1996; Agrios, 2005). Although the most widely recognized fungal symbionts are mycorrhizal fungi and plant pathogens, members of every major lineage of land plants (including bryophytes, ferns and their allies, conifers, and angiosperms) also harbor fungal endophytes—highly diverse, horizontally transmitted fungi that live within overtly healthy aboveground tissues such as leaves and stems (i.e., class 3 endophytes, sensu Rodriguez et al., 2009; e.g., Carroll, 1986; Clay, 1990; Rodrigues, 1994; Dobranic et al., 1995; Fisher, 1996; Faeth and Hammon, 1997; Davis et al., 2003; Arnold and Lutzoni, 2007; Higgins et al., 2007; Davis and Shaw, 2008; Hoffman and Arnold, 2008; U'Ren et al., 2010). In contrast to the systemic and vertically transmitted endophytes associated with many cool-season grasses (class 1 endophytes, Rodriguez et al., 2009), endophytes of most plants form highly localized infections, with hyphae usually growing intercellulary (but occurring within single epidermal cells in some host species; Petrini, 1991; Stone et al., 2000). Plant tissues accumulate fungal infections as a function of exposure to inoculum, which often is positively associated with tissue age; thus, a single mature leaf can frequently harbor numerous endophyte species (Carroll, 1995; Lodge et al., 1996; Gamboa and Bayman, 2001; Arnold et al., 2003). Although most endophytes are thought to be commensal or mildly parasitic, many provide benefits to their hosts, mediating defense against pathogens and herbivores and influencing plant responses to abiotic stressors such as drought (Costa Pinto et al., 2000; Arnold et al., 2003; Arnold and Engelbrecht, 2007; Márquez et al., 2007; Mejía et al., 2008). Endophytes are common in photosynthetic tissues of plants in biomes ranging from hot deserts to tropical rainforests and tundra (Arnold and

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Lutzoni, 2007), but the scale of their diversity and broad patterns of their host associations are not well understood.

Fungal symbionts resembling endophytes (i.e., endolichenic fungi) appear similarly ubiquitous in asymptomatic thalli of lichens (Suryanarayanan et al., 2005; Arnold et al., 2009; Ding et al., 2009; U'Ren et al., 2010), wherein they associate primarily with photobionts (Arnold et al., 2009). Although they have been characterized from a limited number of lichen species and geographic areas, current evidence suggests that like endophytes, endolichenic fungi are horizontally transmitted, form highly localized infections, and represent at least five classes of nonlichenized Pezizomycotina, dozens of families, and large numbers of previously unknown species (Arnold et al., 2009; U'Ren et al., 2010). Phylogenetic analyses and ancestral state reconstructions of trophic modes indicate that endophytic and endolichenic fungi are closely related and that endolichenic fungi may have provided the evolutionary source for transitions to endophytism multiple times across the Ascomycota (Arnold et al., 2009).

The distribution and biodiversity of these symbiotrophic fungi has been examined at scales ranging from a single leaf or host individual to landscape and continental distributions (e.g., Carroll, 1995; Arnold and Lutzoni, 2007; Davis and Shaw, 2008; Hoffman and Arnold, 2008; Arnold et al., 2009). At a global scale, species diversity of endophytes appears to peak in the seasonally wet tropics and decreases with increasing latitude (Arnold and Lutzoni, 2007, but see Suryanarayanan et al., 2011), and relatively few endophyte species or genotypes appear to be shared among closely related hosts in different geographic locations (Fisher et al., 1994, 1995; Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; Arnold et al., 2009). At smaller spatial scales, symbiotrophic fungal communities often differ among co-occurring plant species (Petrini, 1996; Arnold et al., 2000; Suryanarayanan and Kumaresan, 2000) and between physically proximate plants and lichens (Suryanarayanan et al., 2005; U'Ren et al., 2010), with the exception of a recently identified and ecologically flexible group of symbiotrophic fungi that inhabits both lichens and mosses (U'Ren et al., 2010). However, despite the increasing number of studies evaluating endophyte biodiversity and distributions (e.g., Pan and May, 2009; Saunders et al., 2010; Vega et al., 2010; Higgins et al., 2011), no study to date has (1) simultaneously examined endophytic and endolichenic fungi in multiple lineages of phylogenetically diverse hosts using consistent methods, a replicated study design, and molecular analyses to differentiate morphologically similar strains, nor (2) placed these surveys into a larger context by evaluating similar pools of host species in multiple biogeographic provinces and bioclimatic zones.

Here, we present the results of a large-scale survey of endophytic and endolichenic fungal communities associated with locally replicated, phylogenetically diverse sets of 20–23 plant and lichen host taxa in each of five sites across North America. This culture-based effort resulted in 4791 isolates, which is the largest and most geographically extensive collection of endophytic and endolichenic fungi sampled to date. We use molecular data (ITS rDNA-partial LSU rDNA) from 4154 of these isolates to examine (1) how isolation frequency and diversity of fungi differ as a function of host type (plant vs. lichen), host lineage (mycobiont order or plant division), abiotic factors, and geographic location; (2) the geographic heterogeneity of endophytic and endolichenic communities at local and continental scales; and (3) the influence of host type and identity on the composition of endophytic and endolichenic communities at levels ranging from fungal classes to genotypes.

MATERIALS AND METHODS

Five sites representing five distinct environmental, biological, and biogeographic regions were chosen for this study (Table 1): the Madrean Sky Island Archipelago of southeastern Arizona (AZC); the Appalachian Mountains of western North Carolina (NCH); subtropical scrub forest in Florida (FLA); Beringian tundra and boreal forest in the Seward Peninsula ecoregion of western Alaska (AKN); and inland, subalpine tundra in the Interior Highlands of eastern central Alaska (AKE). Within each site, we selected species of plants and lichens that were phylogenetically diverse and representative of the region (Hultén, 1965; Radford et al., 1968; Barton, 1994; Brodo et al., 2001; http://www.archbold-station.org/station/html/datapub/species/lists/plantlist.html). Sites were chosen to represent biologically and geographically distinct locations, as well as areas where endophytic and endolichenic fungi have not been previously studied in detail.

For this study, we define a community as the assemblage of culturable fungi inhabiting apparently healthy tissues of the dominant plants and lichens in each site. For analyses, we subdivide these communities by host type (plant or lichen) and host lineage (mycobiont class or plant division). Our sampling was designed to capture a statistically complete sample of endophytic and endolichenic richness within each site (i.e., such that estimated richness falls within the 95% confidence intervals of observed richness) to permit meaningful comparisons of diversity and composition among communities (see Carroll, 1986; Gotelli, 2008).

In each site, we collected fresh tissues of 10 or 11 species of plants and thalli of 7–12 genera of lichens representing 12–18 species (Tables 2, 3) from each of

TABLE 1. Geographic and abiotic characteristics of North American sites sampled for endolichenic and endophytic fungi from 2007 to 2008.

Site	Vegetation type	Collection date	Latitude	Longitude	Elevation (m.a.s.l.)	Mean annual precipitation (mm)	Mean annual temperature (C)	Mean growing season (d)
Chiricahua Mountains, Arizona (AZC)	Temperate coniferous forest	Jun-07	31°55′47″N	109°22′56″W	2300	512.3	12.3	202
Highlands Biological Station, North Carolina (NCH)	Granite outcrop in temperate semideciduous forest	Jul-07	35°01′41″N	83°160′72″W	1100	2091.7	11.3	252
Archbold Biological Station, Florida (FLA)	Subtropical scrub forest	Mar-08	27°11′19″N	81°20′15″W	35–65	1315.7	22.2	365
Eagle Summit, Alaska (AKE)	Subalpine low shrub tundra	Jun-08	65°29′04″N	145°24′13″W	1113	267.5	–2.8	142
Nome, Alaska (AKN)	Beringian tundra and coniferous forest	Jun-08	64°30′04″N	165°24′23″W	6	405.1	–3.1	122

Notes: Additional site information provided in U'Ren et al. (2010). Alaska vegetation types are based on information from http://www.hort.purdue.edu/newcrop/cropmap/alaska/maps/AKeco3.html. Climate data were obtained from the Western Regional Climate Center (http://www.wrcc.dri.edu/CLIMATEDATA.html) or the Southeastern Regional Climate Center (http://www.sercc.com/climateinfo/historical/historical.html).

three replicate microsites. Microsites were located ca. 30 m apart along a 100 m transect. Individual plants and lichens were typically within close proximity to one another within each microsite (<1 to 10 m apart). The only exception was in the survey of endophytic and endolichenic fungi associated with *Picea*, *Bryoria*, and *Hypogymnia* in AKN; see Tables 2, 3.

From each microsite, three small branches containing healthy leaves (trees, shrubs), three shoots (grasses, ferns), or one small mat (mosses; 4–9 cm^2) per species were collected for endophytic fungi. Mature lichen thalli sufficient for herbarium specimens and fungal isolations were collected for endolichenic fungi. Overall, the isolation frequency of endophytic or endolichenic fungi, defined as the percentage of tissue segments containing cultivable fungi, was quantified for 105 host species: 52 plant species/site combinations, including representative Bryophyta, Pteridophyta, Lycopodiophyta, Pinophyta, and Angiospermae (Table 2), and 53 lichen species/site combinations, encompassing mycobionts from 10 orders and a diversity of growth forms, substrates, and photobionts (Table 3). Isolation frequency is used as a proxy for degree of host tissue colonization (see Johnson and Whitney, 1989; Carroll, 1995; Lodge et al., 1996) reflecting the positive relationship observed between hyphal density within leaves and isolation frequency of endophytes (Arnold, 2002).

Specimens of host material were deposited at the University of Arizona Herbarium (ARIZ) and the Cryptogamic Herbarium at Duke University (DUKE) (AZC accessions 48138–48166; NCH accessions 190848–190850, 190858–190885; FLA accessions 190809, 190886–190908, 190910–190915; AKE accessions 190916–190945; AKN accessions 190946–190981).

***Isolation of fungi*—**Plant and lichen material was transported in plastic and paper bags, respectively, to the laboratory and processed within 24 h (except for AKN: 48 h) following U'Ren et al. (2010). Each sample was washed thoroughly in running tap water for 30 s. Plant tissue was cut into 2 mm^2 segments prior to surface-sterilization. Lichen thalli were cut into ca. 2 cm^2 pieces, surface-sterilized, and then cut under sterile conditions into 2 mm^2 segments. This difference in handling does not influence fungal growth or estimates of diversity (U'Ren et al., 2010). 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 (Arnold et al., 2007), and surface-dried under sterile conditions. Forty-eight segments per host species per microsite were placed on 2% malt extract agar (MEA) in Petri dishes (16 tissue segments/dish) or 1.5 mL microcentrifuge tubes (1 segment/tube). Plates or tubes were sealed with Parafilm and incubated under ambient light/dark condition at room temperature (ca. 21.5°C) for up to 1 year. Emergent fungi were isolated into pure culture, vouchered in sterile water, and deposited at the Robert L. Gilbertson Mycological Herbarium at the University of Arizona (Appendix S1; see Supplemental Data with the online version of this article). Overall, 14 989 tissue segments were examined (Table 4).

***DNA extraction, PCR, sequencing*—**Total genomic DNA was extracted directly from every isolate following Arnold and Lutzoni (2007). The nuclear ribosomal internal transcribed spacers and 5.8S gene (ITS rDNA; ca. 600 bp) and an adjacent portion of the nuclear ribosomal large subunit (LSU rDNA; ca. 500 bp) were PCR-amplified as a single fragment using primers ITS1F or ITS5 and LR3 (White et al., 1990; Gardes and Bruns, 1993; Vilgalys and Hester, 1990; ITS rDNA-partial LSU rDNA) following U'Ren et al. (2010). ITS rDNA was amplified using primers ITS1 and ITS4 (White et al., 1990) for 56 isolates that failed to amplify using the primers listed above. PCR conditions are described in U'Ren et al. (2010).

PCR products were evaluated by staining with SYBR Green I (Molecular Probes, Invitrogen, Carlsbad, California, USA) after electrophoresis on a 1% agarose gel. All positive amplicons yielded single bands. PCR products were cleaned, quantified, normalized, and sequenced directly with the Applied Biosystems BigDye Terminator v3.1 cycle sequencing kit and the original PCR primers at the University of Arizona Genetics Core. Bidirectional sequencing was performed on an Applied Biosystems 3730*xl* DNA Analyzer (Foster City, California, USA).

The software applications *phred* and *phrap* (Ewing and Green, 1998; Ewing et al., 1998) were used to call bases and assemble contigs with automation provided by the ChromaSeq package in the program Mesquite v. 1.06 (http://mesquiteproject.org). Base calls were verified by inspection of chromatograms in the program Sequencher v. 4.5 (Gene Codes, Ann Arbor, Michigan, USA). Sequences have been deposited in GenBank under accession numbers JQ758514–JQ762015 (also see online Appendix S2 for AZC accession numbers).

***Species richness and diversity*—**Operational taxonomic units (OTU) were defined using molecular data because isolates seldom sporulated in culture. A distance matrix based on pairwise Needleman-Wunsch alignments was generated with the needledist module in the program ESPRIT (Sun et al., 2009) under the default parameters. Gaps of any length were treated as a single evolutionary event and terminal gaps were not penalized (Sun et al., 2009). To increase computational speed for large data sets, ESPRIT removes sequences with pairs of *k*-mer distances larger than 0.5 (Sun et al., 2009).

The program mothur (Schloss et al., 2009) was used to cluster sequences into OTU using the complete-linkage method (i.e., furthest neighbor) and to calculate richness and OTU overlap among communities. Sequences were assembled into groups at the genotype level (i.e., 100% sequence similarity), as well as at 99%, 97%, and 95% sequence similarity (online Appendix S3). Previous assessment of four endophyte-rich genera in the Sordariomycetes and Dothideomycetes demonstrated that 5% ITS rDNA divergence (95% sequence similarity) conservatively estimates sister species boundaries when compared against published phylogenies (U'Ren et al., 2009), such that OTU clustered at the 95% sequence similarity level were used as a proxy for species. Our main conclusions did not differ when OTU were defined using greater stringency (data not shown).

OTU accumulation curves, rarefaction analyses, and bootstrap estimates of total richness were inferred in the program EstimateS v. 8.0 (http://viceroy.eeb.uconn.edu/EstimateS) using 50 randomizations of sample order without replacement. Fisher's alpha, a parameter of the log series model that is robust to variation in sample size (Fisher et al., 1943; Taylor, 1978; Magurran, 2004), was calculated in the program PAST v. 1.88 (Hammer et al., 2001) for 39 endophytic and 47 endolichenic host-species/site combinations. Hosts for which fewer than eight isolates were genotyped were included in whole-community diversity measures, but not in diversity assessments for individual host species. All other statistical analyses comparing isolation frequency, richness, and diversity were done in the program JMP v. 9.0.0 (SAS Institute, Cary, North Carolina, USA).

Mean annual temperature, diurnal temperature range, annual temperature, length of the growing season (number of days per year with mean nighttime temperature above 0°C), mean annual precipitation, and latitude (Western Regional Climate Center and Southeastern Regional Climate Center) were investigated as explanatory variables for regression analyses of isolation frequency and diversity. Mean annual temperature was excluded from further analyses because it was strongly correlated with latitude and growing season (latitude: $r = 0.9639$, $P = 0.0082$; growing season: $r = 0.9496$, $P = 0.0135$). Mean annual precipitation and growing season were selected as explanatory variables because they were not significantly correlated with one another ($r = 0.6760$, $P = 0.2104$). For each statistical test, the fit of the model was assessed using a lack-of-fit F test (online Appendix S4).

***Taxonomic composition and similarity of fungal communities*—**Class-level taxonomy for each isolate was estimated using the top hit from BLASTn queries (Altschul et al., 1990; ITS rDNA-partial LSU rDNA) of the curated ITS sequence database at the Alaska Fungal Metagenomics Project (http://www.borealfungi.uaf.edu/) and the AFTOL database (http://aftol1.biology.duke.edu/pub/blast/blastUpload; LSU rDNA only). Only hits with >90% sequence identity and >50% query and subject coverage were used. With these criteria, six isolates (representing three OTU) could not be reliably assigned taxonomic affiliation using BLAST. Basidiomycota comprised <3% of the overall data set. They were excluded from all analyses and will be addressed in a future study.

A χ^2 test was used to test the null hypothesis that classes of Pezizomycotina were distributed equally among taxonomic lineages of plants and lichens following U'Ren et al. (2010). The distribution of fungal classes across lichens with different substrates, photobionts, and growth forms was not analyzed because in many cases these traits were confounded with site and/or mycobiont lineage (Table 3).

Similarity among communities was assessed for all nonsingleton OTU with Jaccard's index (JI, based on presence/absence data only), and two indices that take isolation frequency into account: the Morisita–Horn index (MH) and the new-normalized estimate of shared species, NNESS (Trueblood et al., 1994). NNESS is based on the expected number of species shared between two random samples of size *m* drawn from a population. JI, MH, and NNESS range from 0 (no similarity) to 1 (identical communities). Because NNESS is based on random subsamples of size *m*, it is more robust than MH to differences in sample size. JI was calculated in the program EstimateS v. 8.0, MH in mothur (Schloss et al., 2009), and NNESS in BiodivR (Hardy, 2010).

Analysis of similarity (ANOSIM; Clarke, 1993) and non-metric multidimensional scaling (NMDS), an ordination method that uses rank-order information

in a dissimilarity matrix (Gauch, 1982), were conducted in the program PAST v. 1.88 (Hammer et al., 2001) using all nonsingleton OTU (i.e., those OTU occurring more than once in the entire data set). ANOSIM uses distance measures to test the null hypothesis that there are no differences in species composition between two or more groups (Warwick et al., 1990; Clarke, 1993). Distances (defined as 1-Morisita's index) were converted to ranks and the test statistic R calculated as the difference of mean ranks between vs. within groups. Significance was computed by 10 000 permutations of group membership.

Partial Mantel tests (Smouse et al., 1986; Legendre and Troussellier, 1988) based on the distribution of Pearson correlation coefficient (r) with 10 000 random permutations (see Manly, 1997; Legendre, 2000) were used to examine the influence of climatic similarity and geographic distance on endophytic and endolichenic community composition for each site, while holding effects of either climate or intersite distance constant. Climatic similarity was based on Euclidean distances of the factor scores from the principal component analysis (PCA) of two abiotic variables (length of growing season and mean annual precipitation, Table 1) at each site following Rissler and Apodaca (2007). Community dissimilarity matrices were calculated using 1-NNESS.

RESULTS

Overall, 4791 fungal isolates were obtained in culture, corresponding to emergence of fungi from 31.8% ± 27.3% of surface-sterilized segments of photosynthetic tissue from lichen and plant species collected from five sites across North America. Fungi were isolated from all host species except *Quercus rugosa* at AZC, *Salix pulchra* at AKN, and *Betula nana* at AKN (Tables 2, 3).

***Differences in isolation frequency as a function of host type*—**Fungi were isolated nearly twice as frequently from lichen tissue segments (41.0% ± 2.5 of segments; mean per species ± SE; 3124 isolates overall) than from plant tissue segments (23.0% ± 2.2; 1667 isolates overall; Wilcoxon rank sum $\chi^2_1 = 32.499$, $P < 0.0001$; Table 4). For both plants and lichens, isolation frequency differed significantly among sites (Table 4; online Appendix S4), although patterns were not consistent between host types (e.g., AKN had the lowest isolation frequency for endolichenic fungi, whereas AZC had the lowest isolation frequency for endophytic fungi). For endophytes, intersite differences in isolation frequency reflected differences among angiosperms, but not Pinophyta or mosses (which had consistently high isolation frequencies in all sites; Appendix S4). Isolation frequency also differed more than 3-fold as a function of host lineage (i.e., mycobiont order and plant division) after taking into account the effect of site (Table 5; online Appendix S4).

***Isolation frequency and abiotic factors*—**The isolation frequency of endolichenic fungi from 52 species of lichens increased linearly as a function of growing season, but was not sensitive to mean annual precipitation (Fig. 1). The isolation frequency of endophytic fungi from 48 species of plants increased linearly as a function of both growing season and annual precipitation (Fig. 1). This trend is strong for endophytes from vascular plants only, but not for endophytes of mosses (online Appendix S4). Mean isolation frequencies for endophytic and endolichenic fungi were significantly associated with latitude ($P < 0.05$; online Appendix S4).

***Completeness of sampling*—**Sequence data from 4154 endophytic and endolichenic Ascomycota yielded 715 unique genotypes (313 of which were unique to lichens, 247 were unique to plants, and 155 were shared between both host types,

TABLE 2. Isolation frequency, putative species richness, and diversity of endophytic fungi from 52 plant hosts from five sites across North America.

Site *	Host division	Host family	Host species	Leaf segments †	Isolates recovered	Isolation frequency/microsite ± SD	Isolates sequenced (%)	Ascomycota sequences	Ascomycota putative species	Fisher's alpha
AZC	Bryophyta	Bryaceae	*Leucobryum* sp.	141	21	0.15 ± 0.02	19 (91%)	19	13	18.14
AZC	Bryophyta	Ditrichaceae	*Ceratodon purpureus*	143	30	0.21 ± 0.16	30 (100%)	30	15	11.93
AZC	Pteridophyta	Dryopteridaceae	*Woodsia plummerae*	144	7	0.05 ± 0.09	5 (71%)	5	4	9.28
AZC	Pinophyta	Cupressaceae	*Juniperus deppeana*	144	22	0.15 ± 0.12	19 (86%)	19	10	8.54
AZC	Pinophyta	Pinaceae	*Pinus arizonica* var. *arizonica*	144	9	0.06 ± 0.02	9 (100%)	9	2	0.79
AZC	Pinophyta	Pinaceae	*Pseudotsuga menziesii* var. *glauca*	144	21	0.15 ± 0.22	16 (76%)	16	3	1.08
AZC	Angiospermae	Poaceae	*Eragrostis intermedia*	144	2	0.01 ± 0.02	2 (100%)	2	1	0.79
AZC	Angiospermae	Fagaceae	*Quercus rugosa*	130	0	0	N/A	N/A	N/A	N/A
AZC	Angiospermae	Fagaceae	*Quercus gambelii*	128	1	0.01 ± 0.01	1 (100%)	1	1	N/A
AZC	Angiospermae	Fagaceae	*Quercus hypoleucoides*	144	4	0.03 ± 0.05	3 (75%)	3	1	N/A
AZC	Angiospermae	Oleaceae	*Fraxinus velutina*	125	1	0.01 ± 0.02	1 (100%)	1	1	N/A
NCH	Bryophyta	N/A	Unknown moss	144	66	0.46 ± 0.35	60 (91%)	60	14	5.74
NCH	Bryophyta	Hypnaceae	*Hypnum* sp.	144	68	0.47 ± 0.23	65 (96%)	65	24	13.75
NCH	Lycopodiophyta	Selaginellaceae	*Selaginella tortipila*	144	17	0.12 ± 0.17	14 (82%)	14	8	7.75
NCH	Pteridophyta	Dryopteridaceae	*Dryopteris marginalis*	131	5	0.04	3 (60%)	3	3	N/A
NCH	Pinophyta	Pinaceae	*Pinus strobus*	114	14	0.14 ± 0.11	13 (93%)	13	6	4.32
NCH	Pinophyta	Pinaceae	*Tsuga canadensis*	144	54	0.38 ± 0.26	49 (91%)	49	13	5.78
NCH	Angiospermae	Sapindaceae	*Acer rubrum*	119	48	0.36 ± 0.38	48 (10%)	48	10	3.84
NCH	Angiospermae	Poaceae	*Danthonia* sp.	144	133	0.92 ± 0.07	124 (93%)	124	9	2.22
NCH	Angiospermae	Ericaceae	*Kalmia latifolia* L.	144	29	0.20 ± 0.31	29 (100%)	29	6	2.29
NCH	Angiospermae	Fagaceae	*Quercus montana*	144	60	0.42 ± 0.33	57 (95%)	57	6	1.69
FLA	Bryophyta	Bryaceae	*Leucobryum* sp.	144	55	0.38 ± 0.25	54 (98%)	53	14	6.20
FLA	Lycopodiophyta	Selaginellaceae	*Selaginella arenicola*	144	38	0.26 ± 0.26	30 (79%)	30	23	45.11

TABLE 2. Continued.

Site *	Host division	Host family	Host species	Leaf segments †	Isolates recovered	Isolation frequency/ microsite ± SD	Isolates sequenced (%)	Ascomycota sequences	Ascomycota putative species	Fisher's alpha
FLA	Pinophyta	Pinaceae	*Pinus elliottii*	144	138	0.96 ± 0.04	130 (94%)	73	32	21.73
FLA	Pinophyta	Pinaceae	*Pinus clausa*	144	52	0.36 ± 0.07	47 (90%)	18	11	12.01
FLA	Angiospermae	Poaceae	*Aristida stricta*	144	39	0.27 ± 0.22	35 (90%)	34	14	8.90
FLA	Angiospermae	Arecaceae	*Serenoa repens*	144	65	0.45 ± 0.40	58 (89%)	41	10	4.21
FLA	Angiospermae	Fagaceae	*Quercus inopina*	144	80	0.56 ± 0.25	51 (64%)	50	19	11.17
FLA	Angiospermae	Fagaceae	*Quercus chapmanii*	144	18	0.13 ± 0.08	15 (83%)	13	5	2.97
FLA	Angiospermae	Bromeliaceae	*Tillandsia usneoides*	144	25	0.17 ± 0.02	25 (100%)	25	11	7.50
FLA	Angiospermae	Ericaceae	*Vaccinium vitis-idaea*	144	38	0.26 ± 0.34	30 (79%)	30	10	5.25
AKE	Bryophyta	Hylocomiaceae	*Pleurozium schreberi*	144	126	0.88 ± 0.02	108 (86%)	107	19	6.71
AKE	Bryophyta	Polytrichaceae	*Polytrichum commune*	144	26	0.18 ± 0.11	24 (92%)	19	16	47.84
AKE	Lycopodiophyta	Lycopodiaceae	*Huperzia selago*	144	18	0.13 ± 0.08	17 (94%)	14	10	15.64
AKE	Pinophyta	Pinaceae	*Picea glauca*	144	8	0.06 ± 0.03	7 (88%)	6	6	N/A
AKE	Angiospermae	Betulaceae	*Betula nana*	144	2	0.01 ± 0.01	2 (100%)	2	2	N/A
AKE	Angiospermae	Cyperaceae	*Carex* sp.	144	3	0.02 ± 0.04	1 (33%)	1	1	N/A
AKE	Angiospermae	Ericaceae	*Cassiope tetragona*	144	75	0.52 ± 0.12	69 (92%)	69	21	10.28
AKE	Angiospermae	Ericaceae	*Empetrum nigrum*	144	13	0.09 ± 0.05	12 (92%)	12	10	28.23
AKE	Angiospermae	Ericaceae	*Ledum groenlandicum*	144	21	0.15 ± 0.17	19 (90%)	19	7	4.00
AKE	Angiospermae	Salicaceae	*Salix pulchra*	144	3	0.02 ± 0.02	2 (67%)	2	2	N/A
AKN	Bryophyta	Hylocomiaceae	*Pleurozium schreberi*	144	32	0.22 ± 0.04	32 (100%)	32	18	17.00
AKN	Bryophyta	Polytrichaceae	*Polytrichum commune*	144	18	0.13 ± 0.10	8 (44%)	7	4	3.87
AKN	Pteridophyta	Equisetaceae	*Equisetum arvense*	144	28	0.19 ± 0.06	27 (96%)	26	12	8.64
AKN	Pinophyta	Pinaceae	*Picea glauca* ‡	144	68	0.47 ± 0.37	65 (96%)	65	8	2.39
AKN	Angiospermae	Salicaceae	*Salix pulchra*	144	0	0	N/A	N/A	N/A	N/A
AKN	Angiospermae	Betulaceae	*Betula nana*	144	0	0	N/A	N/A	N/A	N/A
AKN	Angiospermae	Cyperaceae	*Carex* sp.	144	2	0.01 ± 0.01	1 (50%)	1	1	N/A
AKN	Angiospermae	Ericaceae	*Cassiope tetragona*	144	9	0.06 ± 0.00	9 (100%)	9	8	34.61
AKN	Angiospermae	Rosaceae	*Dryas octopetala*	144	22	0.15 ± 0.25	20 (91%)	20	3	0.97
AKN	Angiospermae	Ericaceae	*Empetrum nigrum*	144	1	0.01 ± 0.01	1 (100%)	1	1	N/A
AKN	Angiospermae	Ericaceae	*Ledum groenlandicum*	144	32	0.22 ± 0.13	31 (97%)	31	9	4.25

* AZC = Chiricahua Mountains, Arizona; NCH = Highlands Biological Station, North Carolina; FLA = Archbold Biological Station, Florida; AKE = Eagle Summit, Alaska; and AKN = Nome, Alaska.

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

‡ Hosts were collected ca. 60 km east of Nome, in Council, AK, the nearest site with trees.

TABLE 3. Isolation frequency, putative species richness, and diversity of endolichenic fungi from 53 lichen hosts from five sites across North America.

Host order	Host species	Substrate [a]	Growth form [a]	Photobiont [a]	Thallus segments†	Isolates recovered	Isolation frequency/ microsite ± SD	No. isolates sequenced (%)	Ascomycota		
									Sequences	Putative species	Fisher's alpha
Site AZC: Chiricahua Mountains, Arizona											
Verrucariales	*Dermatocarpon* spp.‡	Saxicolous	Foliose	*Myrmecia* sp./other unicellular algae	143	23	0.16 ± 0.15	20 (87%)	20	9	6.29
Lecanorales	*Flavopunctelia praesignis*	Epiphytic	Foliose	Trebouxioid	144	70	0.49 ± 0.14	64 (91%)	64	23	12.86
Lecanorales	*Punctelia hypoleucites*	Epiphytic	Foliose	Trebouxioid	144	110	0.76 ± 0.11	97 (88%)	97	27	12.40
Lecanorales	*Usnea hirta*	Epiphytic	Fruticose	Trebouxioid	144	47	0.33 ± 0.03	45 (96%)	45	15	7.87
Lecanorales	*Pseudevernia intensa*	Epiphytic	Fruticose	Trebouxioid	144	71	0.49 ± 0.17	61 (86%)	61	17	7.81
Lecanorales	*Xanthoparmelia viriduloumbrina*	Saxicolous	Foliose	Trebouxioid	138	57	0.41 ± 0.00	56 (98%)	56	18	9.18
Lecideaceae	*Lecidea tessellata*	Saxicolous	Crustose	Trebouxioid/ *Chlorosarcinopsis*	142	37	0.26 ± 0.06	36 (97%)	36	15	9.65
Teloschistales	*Physcia caesia*	Saxicolous	Foliose	Trebouxioid	144	80	0.56 ± 0.33	70 (88%)	70	25	13.91
Peltigerales	*Peltigera* spp.‡	Terricolous/ Muscicolous	Foliose	*Nostoc* sp.	143	65	0.45 ± 0.27	63 (97%)	63	20	10.00
Ostropales	*Diploschistes muscorum* sensu *Lumbsch*	Saxicolous	Crustose	Trebouxioid/ *Trentepholia*	144	39	0.27 ± 0.05	35 (90%)	35	21	22.16
Site NCH: Highlands Biological Station, North Carolina											
Lecanorales	*Lecanora orenoides*	Saxicolous	Crustose	Trebouxioid	144	44	0.31 ± 0.10	40 (91%)	40	21	17.87
Lecanorales	*Cladonia subtenuis*	Terricolous	Fruticose	Trebouxioid/ *Asterochloris* sp.	144	45	0.31 ± 0.20	39 (87%)	39	14	7.82
Lecanorales	*Flavoparmelia caperata*	Epiphytic	Foliose	Trebouxioid	144	79	0.55 ± 0.13	65 (82%)	65	26	16.06
Lecanorales	*Pseudevernia consocians*	Epiphytic	Fruticose/ Foliose	Trebouxioid	144	43	0.30 ± 0.19	39 (91%)	39	19	14.62
Lecanorales	*Parmotrema reticulatum*	Epiphytic	Foliose	Trebouxioid	144	95	0.66 ± 0.19	82 (86%)	82	25	12.25
Lecanorales	*Usnea* spp.‡	Epiphytic	Fruticose	Trebouxioid	144	8	0.06 ± 0.02	8 (100%)	8	3	1.74
Lecanorales	*Xanthoparmelia conspersa*	Saxicolous	Foliose	Trebouxioid	144	67	0.47 ± 0.10	63 (94%)	63	20	10.10
Umbilicariales	*Lasallia* spp.‡	Saxicolous	Foliose	Trebouxioid/ *Pseudotrebouxia*	144	28	0.19 ± 0.16	26 (93%)	26	15	14.77
Peltigerales	*Peltigera praetextata*	Terricolous/ Muscicolous	Foliose	*Nostoc* sp.	144	17	0.12 ± 0.20	17 (100%)	17	7	4.45
Peltigerales	*Sticta beauvoisii*	Muscicolous/ Epiphytic	Foliose	*Nostoc* sp.	144	70	0.49 ± 0.37	56 (80%)	56	23	14.58
Ostropales	*Diploschistes scruposus*	Saxicolous	Crustose	Trebouxioid	144	69	0.48 ± 0.16	62 (90%)	62	25	15.56
Site FLA: Archbold Biological Station, Florida											
Lecanorales	*Cladonia evansii*	Terricolous	Fruticose	Trebouxioid/ *Asterochloris* sp.	144	138	0.96 ± 0.04	133 (96%)	133	48	26.95
Lecanorales	*Cladonia leporina*	Terricolous	Fruticose	Trebouxioid/ *Asterochloris* sp.	144	105	0.73 ± 0.27	102 (97%)	102	45	30.78
Lecanorales	*Cladonia* spp.‡	Terricolous	Fruticose	Trebouxioid/ *Asterochloris* sp.	144	112	0.78 ± 0.21	102 (91%)	102	40	24.23
Lecanorales	*Cladonia subtenuis*	Terricolous	Fruticose	Trebouxioid/ *Asterochloris* sp.	144	96	0.67 ± 0.28	86 (90%)	86	34	20.76
Lecanorales	*Parmotrema perforatum*	Epiphytic	Foliose	Trebouxioid	144	44	0.31 ± 0.07	41 (93%)	41	15	8.52
Lecanorales	*Parmotrema rampoddense*	Epiphytic	Foliose	Trebouxioid	144	63	0.44 ± 0.44	58 (92%)	58	26	18.10
Lecanorales	*Parmotrema tinctorum*	Epiphytic	Foliose	Trebouxioid	144	87	0.60 ± 0.34	85 (94%)	82	34	21.77
Lecanorales	*Usnea* spp.‡	Epiphytic	Fruticose	Trebouxioid	144	80	0.56 ± 0.21	80 (100%)	80	29	16.34
Arthoniales	*Herpothallon rubrocinctum*	Epiphytic	Crustose	*Trentepholia*	144	59	0.41 ± 0.30	50 (85%)	50	27	23.94
Teloschistales	*Pyxine eschweileri*	Epiphytic	Foliose	Green unicellular (chlorococcoid)	144	90	0.63 ± 0.08	79 (87%)	78	26	13.65

TABLE 3. Continued.

Host order	Host species	Substrate [a]	Growth form [a]	Photobiont [a]	Thallus segments†	Isolates recovered	Isolation frequency/ microsite ± SD	No. isolates sequenced (%)	Ascomycota		
									Sequences	Putative species	Fisher's alpha
Site AKE: Eagle Summit, Alaska											
Lecanorales	*Alectoria ochroleuca* + *A. nigricans*	Terricolous	Fruticose	Trebouxioid	144	23	0.16 ± 0.19	20 (87%)	20	16	37.13
Lecanorales	*Flavocetraria cucullata*	Terricolous	Fruticose	Trebouxioid	144	42	0.29 ± 0.36	34 (81%)	34	17	13.53
Lecanorales	*Cladonia mitis*	Terricolous	Fruticose	Trebouxioid/ *Asterochloris sp.*	144	108	0.75 ± 0.25	95 (88%)	95	25	11.05
Lecanorales	*Dactylina arctica*	Terricolous	Fruticose	Trebouxioid	144	16	0.11 ± 0.02	11 (69%)	11	8	13.19
Lecanorales	*Masonhalea richardsonii*	Terricolous	Fruticose/ Foliose	Trebouxioid	144	5	0.03 ± 0.02	4 (80%)	4	4	N/A
Lecanorales	*Arctoparmelia separata*	Saxicolous	Foliose	Trebouxioid	144	121	0.84 ± 0.21	98 (81%)	98	27	12.31
Peltigerales	*Peltgera* spp.‡	Terricolous/ Muscicolous	Foliose	*Coccomyxa*/ *Nostoc* sp.	144	143	0.99 ± 0.01	122 (85%)	122	19	6.30
Rhizocarpaceae	*Rhizocarpon* cf. *geographicum*	Saxicolous	Crustose	Green unicellular (chlorococcoid)	144	98	0.68 ± 0.37	87 (89%)	87	24	10.95
Ophioparmaceae	*Ophioparma ventosa*	Saxicolous	Crustose	Trebouxioid	144	142	0.99 ± 0.02	128 (90%)	128	16	4.82
Umbilicariales	*Umbilicaria* spp.‡	Saxicolous	Foliose	Trebouxioid	144	101	0.70 ± 0.21	87 (86%)	87	28	14.30
Site AKN: Nome, Alaska											
Lecanorales	*Alectoria ochroleuca* + *A. nigricans*	Terricolous	Fruticose	Trebouxioid	144	2	0.01 ± 0.02	0	0	0	N/A
Lecanorales	*Bryoria* spp.*‡	Epiphytic	Fruticose	Trebouxioid	144	3	0.02 ± 0.02	2 (67%)	2	2	N/A
Lecanorales	*Flavocetraria cucullata*	Terricolous	Fruticose	Trebouxioid	144	4	0.03 ± 0.03	2 (50%)	2	2	N/A
Lecanorales	*Cetraria* spp.‡	Terricolous	Fruticose	Trebouxioid	144	3	0.02 ± 0.02	3 (100%)	3	3	N/A
Lecanorales	*Cladonia stellaris*	Terricolous	Fruticose	Trebouxioid/ *Asterochloris* sp.	144	21	0.15 ± 0.06	7 (33%)	7	7	N/A
Lecanorales	*Hypogymnia physodes**	Epiphytic	Foliose	Trebouxioid	144	11	0.08 ± 0.07	10 (91%)	10	8	18.50
Lecanorales	*Parmelia omphalodes*	Saxicolous	Foliose	Trebouxioid	144	27	0.19 ± 0.15	22 (81%)	22	10	7.07
Lecanorales	*Stereocaulon paschale*	Terricolous	Fruticose	Green alga/ Cyanobacteria	144	9	0.06 ± 0.06	6 (67%)	6	5	14.11
Lecideaceae	*Amygdalaria panaeola*	Saxicolous	Crustose	Green unicellular/ Cyanobacteria	144	98	0.68 ± 0.17	87 (89%)	87	24	10.95
Peltigerales	*Peltigera* spp.‡	Terricolous/ Muscicolous	Foliose	*Coccomyxa*/ *Nostoc* sp.	144	19	0.13 ± 0.06	17 (89%)	17	13	25.26
Ophioparmaceae	*Ophioparma ventosa*	Saxicolous	Crustose	Trebouxioid	144	79	0.55 ± 0.31	73 (92%)	73	19	8.34
Umbilicariales	*Umbilicaria proboscidea* + *U. phaea*	Saxicolous	Foliose	Trebouxioid	144	11	0.08 ± 0.03	6 (55%)	6	4	5.24

* Hosts were collected ca. 60 km east of Nome in Council, AK, the nearest site with trees.

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

‡ For some hosts, the same species could not be collected in each microsite. For statistical analyses, each genus was treated as a single taxon. In AZC, microsites 1 and 3 contained *Peltigera rufescens*; microsite 2 contained *P. praetextata*. Microsite 1 contained *Dermatocarpon taminium*; microsite 2 contained *D. tenue* and *D. americanum*; microsite 3 contained *D. tenue*. In NCH, microsite 1 contained *Usnea subscabrosa* and *U. cornuta* s.l.; microsite 2 contained *Usnea ceratina*, *U. subgracilis*, *U. subfloridana*; microsite 3 contained *Usnea cornuta* s.l., *U. subscabrosa*, *U. entoviolata*, *Usnea* sp. Microsite 1 contained *Lasallia papulosa*; microsites 2 and 3 contained *L. pensylvanica*. For FLA, microsite 1 contained *Cladonia didyma*; microsites 2 and 3 contained *C. subradiata*. Microsites 1 and 2 contained *Usnea mutabilis*; microsite 3 contained *U. subscabrosa*. In AKE, microsites 1 and 2 contained *Peltigera malacea*; microsite 3 contained *P. aphthosa*. Microsites 1 and 3 contained *Umbilicaria hyperborea*; microsite 2 contained *U. proboscidea*. In AKN, microsite 1 contained *Bryoria lanestris*; microsites 2 and 3 contained *Bryoria* sp. Microsites 1 and 2 contained *Cetraria laevigata*; microsite 3 contained *C. islandica*. Microsites 1 and 3 contained *Peltigera aphthosa*; microsite 2 contained *P. malacea*.

[a] Substrate, growth form, and photobiont information based on Brodo et al. (2001) and Elix et al. (1987) (for *Trentepohlia* record in *Diploschistes* s.l.).

TABLE 4. Isolation frequency, putative species richness, and diversity of endolichenic and endophytic fungi per site using OTU based on 95% ITS rDNA-partial LSU rDNA sequence similarity.

Host	Site	Tissue segments	Isolates recovered	Isolation frequency/ microsite ±SD *	Isolates sequenced (%)	Ascomycota sequences	Putative species (95% CI)	Bootstrap estimate richness	Fisher's alpha (FA) †	Mean FA/host species ±SD ‡
Lichen	AZC	1430	599	0.42 ± 0.17 A	547 (91%)	547	69 (60.0, 78.0)	77.47	21.39 (21.1, 21.68)	11.22 ± 4.55
	NCH	1584	565	0.36 ± 0.19 A	497 (88%)	497	77 (68.3, 85.7)	86.99	26.56 (26.21, 26.91)	11.80 ± 5.18
	FLA	1440	874	0.61 ± 0.19 A	816 (93%)	812	102 (93.1, 111.0)	113.78	34.62 (34.16, 35.08)	20.50 ± 6.57
	AKE	1440	799	0.55 ± 0.37 A	686 (86%)	686	45 (36.9, 53.2)	51.23	15.43 (15.22, 15.64)	13.73 ± 9,35
	AKN	1728	287	0.17 ± 0.22 B	235 (82%)	235	43 (37.0, 49.0)	48.47	10.05 (9.91, 10.19)	12.78 ± 7.10
	Total	7622	3124	0.41 ± 0.28	2781 (89%)	2777	225 (212.8, 237.2)	248.75	57.80 (57.72, 57.88)	14.05 ± 7.24
Plant	AZC	1531	118	0.08 ± 0.08 c	105 (89%)	105	39 (31.4, 46.6)	47.62	22.47 (21.8, 23.14)	7.22 ± 6.68
	NCH	1372	494	0.35 ± 0.25 ab	462 (94%)	462	70 (63.3, 76.7)	79.15	24.63 (23.89, 25.37)	5.26 ± 3.75
	FLA	1440	548	0.38 ± 0.24 a	475 (87%)	367	98 (87.6, 104.4)	115.54	43.5 (42.11, 44.89)	12.51 ± 12.65
	AKE	1440	295	0.21 ± 0.28 bc	261 (88%)	251	57 (49.7, 64.3)	64.41	26.33 (25.53, 27.13)	18.78 ± 16.61
	AKN	1584	212	0.13 ± 0.14 abc	194 (92%)	192	54 (45.1, 62.9)	63.91	25.48 (24.71, 26.25)	10.25 ± 12.02
	Total	7367	1667	0.23 ± 0.27	1497 (90%)	1377	252 (237.8, 266.2)	286.75	90.43 (90.22, 90.64)	10.45 ± 11.29

* Different letters represent significant differences ($P < 0.05$) based on ANOVA on residuals following test for host phylogeny effect with post hoc Tukey-Kramer HSD comparison of isolation frequency among lichens (uppercase) and plants (lowercase) from five sites.

† For each site, diversity and 95% confidence intervals were calculated using rarefaction at $N = 105$ for endophytes and $N = 235$ for endolichenic fungi, which is equal to the smallest sample size for endophytic and endolichenic fungi, respectively.

‡ Mean diversity per host species was significantly greater for endolichenic fungi than endophytes (*t* test, log-transformed Fisher's alpha, $t_{84} = 3.66$, $P = 0.0004$). Mean species richness of endolichenic fungi per host species (18.74 ± 10.85) significantly exceeded that of endophytes (8.79 ± 7.20) (*t* test, log-transformed OTU $t_{99} = -4.94$, $P < 0.0001$).

based on 100% ITS rDNA-partial LSU rDNA similarity; Fisher's alpha = 248.9) and 359 OTU (107 of which were found only in lichens, 134 were found only in plants, and 118 occurred in both host types, based on 95% ITS rDNA-partial LSU rDNA similarity; Fisher's alpha = 94.3). Despite sampling nearly 15 000 tissue pieces from 105 host species, our sampling effort was statistically insufficient to capture the total OTU richness of culturable endophytic and endolichenic fungi over the entire study (Fig. 2, Table 4). However, sampling sufficiently captured local richness of culturable endolichenic fungi within three sites (AZC, AKE, and AKN), and the bootstrap estimates exceeded the upper bound of the 95% confidence intervals around observed richness by only 1.29 to 2.78 OTU for NCH and FLA, respectively (Table 4). For endophytic fungi, sampling nearly captured local richness of culturable endophytic fungi within four sites (AKE, AKN, AZC, and NCH) with bootstrap estimates exceeding the upper bound of the 95% confidence intervals by only 0.11 to 2.45 OTU; however, the bootstrap estimate for FLA exceeded the observed by 11.14 OTU (Table 4). Therefore, we recovered the majority of culturable

TABLE 5. Taxonomic distribution of the number of lichen or plant host species sampled, host species per site, tissue pieces examined and isolates recovered; isolation frequency; number of isolates sequenced, number of putative species and bootstrap estimate, and diversity (Fisher's alpha [FA]) using OTU based on 95% ITS rDNA-partial LSU rDNA sequence similarity.

Host	Host classification	Host species	Sites	Mean isolation frequency ±SD *	Sequences	Ascomycota sequences	Ascomycota putative species (95% CI)	Bootstrap estimate richness	Mean FA/host species ± SD †
Lichen	Arthoniales	1	1	0.41 ± 0.30	50	50	27 (18.0, 36.0)	34.58	23.94
	Lecanorales	34	5	0.38 ± 0.31 B	1658	1657	187 (171.4, 202.6)	212.29	15.18 ± 7.74
	Lecideaceae	2	2	0.47 ± 0.26 AB	123	123	34 (28.5, 39.5)	39.50	10.30 ± 0.92
	Ophioparmaceae	2	2	0.77 ± 0.31 A	201	201	25 (20.7, 29.3)	27.73	6.58 ± 2.49
	Ostropales	2	2	0.38 ± 0.16 AB	97	97	42 (34.3, 49.7)	51.13	18.86 ± 4.67
	Peltigerales	5	4	0.44 ± 0.38 AB	275	275	67 (60.1, 73.9)	77.71	12.14 ± 8.30
	Rhizocarpaceae	1	1	0.68 ± 0.37	87	87	24 (17.5, 30.6)	29.30	10.95
	Teloschistales	2	2	0.59 ± 0.22 AB	151	148	50 (42.1, 57.9)	60.12	13.78 ± 0.18
	Umbilicariales	3	3	0.32 ± 0.32 AB	119	119	41 (34.3, 47.7)	49.53	11.44 ± 5.37
	Verrucariales	1	1	0.16 ± 0.15	20	20	9 (5.8, 12.2)	10.76	6.29
Plant	Bryophyta	9	5	0.34 ± 0.24 a	400	392	86 (76.7, 95.3)	97.82	14.58 ± 13.40 a
	Lycopodiophyta	3	3	0.17 ± 0.08 ab	61	58	40 (29.4, 50.6)	51.68	22.83 ± 19.69 a
	Pteridophyta	3	3	0.09 ± 0.08 ab	35	34	19 (14.3, 23.7)	23.45	8.96 ± 0.45 ab
	Pinophyta	9	5	0.30 ± 0.29 a	355	268	82 (74.4, 89.7)	94.65	7.08 ± 7.05 ab
	Angiospermae	28	5	0.18 ± 0.22 b	646	626	118 (107.2, 128.8)	134.24	7.83 ± 9.47 b

* Different letters after values represent significant differences ($P < 0.05$) based on ANOVA on residuals following test for site effect with post hoc Tukey-Kramer HSD comparison of isolation frequency among lichen mycobionts (uppercase) and plant divisions (lowercase). Arthoniales, Rhizocarpaceae, and Verrucariales only had one representative so were excluded from the analysis.

† Different letters represent significant differences ($P < 0.05$) based on ANOVA on residuals following test for site effects. There was no significant difference in diversity among mycobiont orders (online Appendix S4).

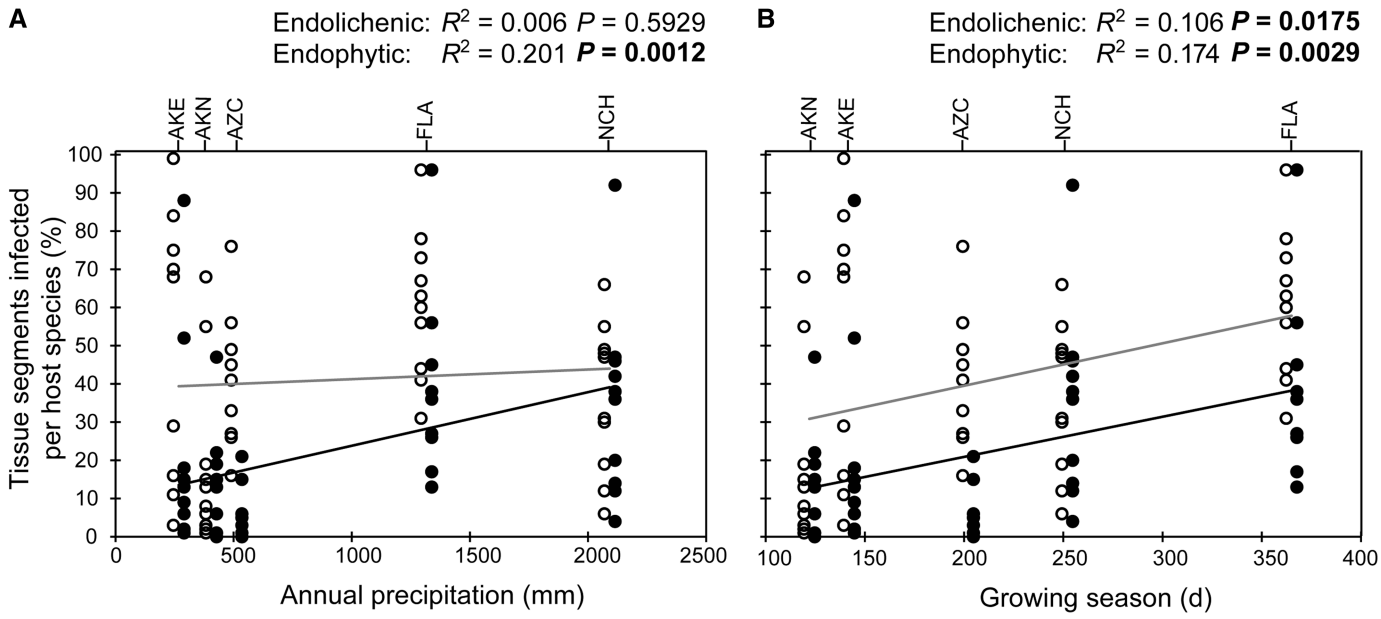

Fig. 1. Endophytic and endolichenic isolation frequency as a function of (A) mean annual precipitation and (B) mean length of growing season for 52 lichen and 48 plant species/site combinations in five sites across North America (Chiricahua Mountains, Arizona [AZC], Highlands Biological Station, North Carolina [NCH], Archbold Biological Station, Florida [FLA], Eagle Summit, Alaska [AKE], and Nome, Alaska [AKN]). Solid circles and black trendline indicate endophytic hosts; open circles and gray trendline indicate endolichenic hosts. For each explanatory variable, the coefficient of determination (R^2) and the P-value for ANOVA is presented for endolichenic and endophytic fungi. Points are jittered for readability.

fungi in these host–site combinations. Based on the small differences between observed and estimated richness, these data were used to compare diversity among substrates, sites, and host lineages.

***Differences in diversity as a function of host type*—**At the genotype and OTU levels, cumulative diversity of endophytic fungi exceeded that of endolichenic fungi by a factor of 1.2 and 1.6, respectively ($P < 0.0001$, determined by bootstrap randomization) (Table 4; also see online Appendix S3). However, on a per host species basis, the mean diversity of OTU from lichens significantly exceeded that from plants (Table 4). Mean diversity of endolichenic fungi per host species was similar among sites (Table 4); however, there was slight evidence that endophyte diversity per host species differed among sites (i.e., $P = 0.0689$; online Appendix S4).

Endolichenic diversity did not differ significantly as a function of mycobiont order, but diversity of endophytic fungi differed significantly among plant divisions (Table 5). Endophytic fungi from Lycopodiophyta and Bryophyta were significantly more diverse per host species than those from Angiospermae (Table 5).

***Diversity and abiotic factors*—**Endolichenic diversity increased linearly with increasing length of the growing season, but there was no effect of annual precipitation (Fig. 3, online Appendix S4; $N = 47$ lichen species/site combinations). Overall, endophyte diversity was not strongly related to annual precipitation or duration of the growing season (Fig. 3; online Appendix S4; $N = 39$ plant species/site combinations). However, the diversity of Pinophyta-associated endophytes per host species was positively correlated with mean length of growing season ($R^2 = 0.53$, $F_{1,6} = 6.70$, $P = 0.0413$). Latitude alone was not significantly associated with mean diversity of either endolichenic or endophytic fungi in these sites (online Appendix S4).

***Geographic heterogeneity in fungal communities*—**The composition of fungal communities differed significantly among sites (Fig. 4). Post hoc pairwise comparisons indicate significant differences in all cases ($P < 0.05$ using a Bonferroni correction) except for endophytic fungi from AZC and AKN, where isolation frequencies were relatively low (Table 4) and for which the difference is nearly significant (uncorrected $P = 0.0528$). These results could reflect our sampling of different species between sites, but we observe the same pattern when analyses are restricted to fungal communities from congeneric hosts (e.g., *Cladonia* spp. from NCH, FLA, and AKE: ANOSIM on Morisita distances, $R = 0.8155$, $P < 0.0001$). Overall, only one and two OTU from endophytic and endolichenic fungi, respectively, occurred across all five sites (<1% of OTU; tentatively identified as *Lecythophora* spp.; data not shown). Thirty-one percent of nonsingleton OTU occurred in only one site. When AKN and AKF are treated as a single site (broadly defined as tundra/boreal), 61% of nonsingleton OTU occurred in only one site.

Cluster analyses also reveal a strong effect of site on fungal communities (Fig. 4). Fungal communities from proximate sites (e.g., NCH and FLA) cluster more closely than communities from more geographically distant sites (Fig. 4). Sites in eastern and western North America are clearly demarcated by both NMDS and cluster analyses (Fig. 4). These analyses also show a secondary effect of host type: fungal communities isolated from plants and lichens within the same geographic location are more similar than fungal communities from the same host type in other sites (Fig. 4).

When compared across sites, similarity of fungal communities reflects environmental similarity more strongly than geographic proximity. Partial Mantel tests show that when the effect of

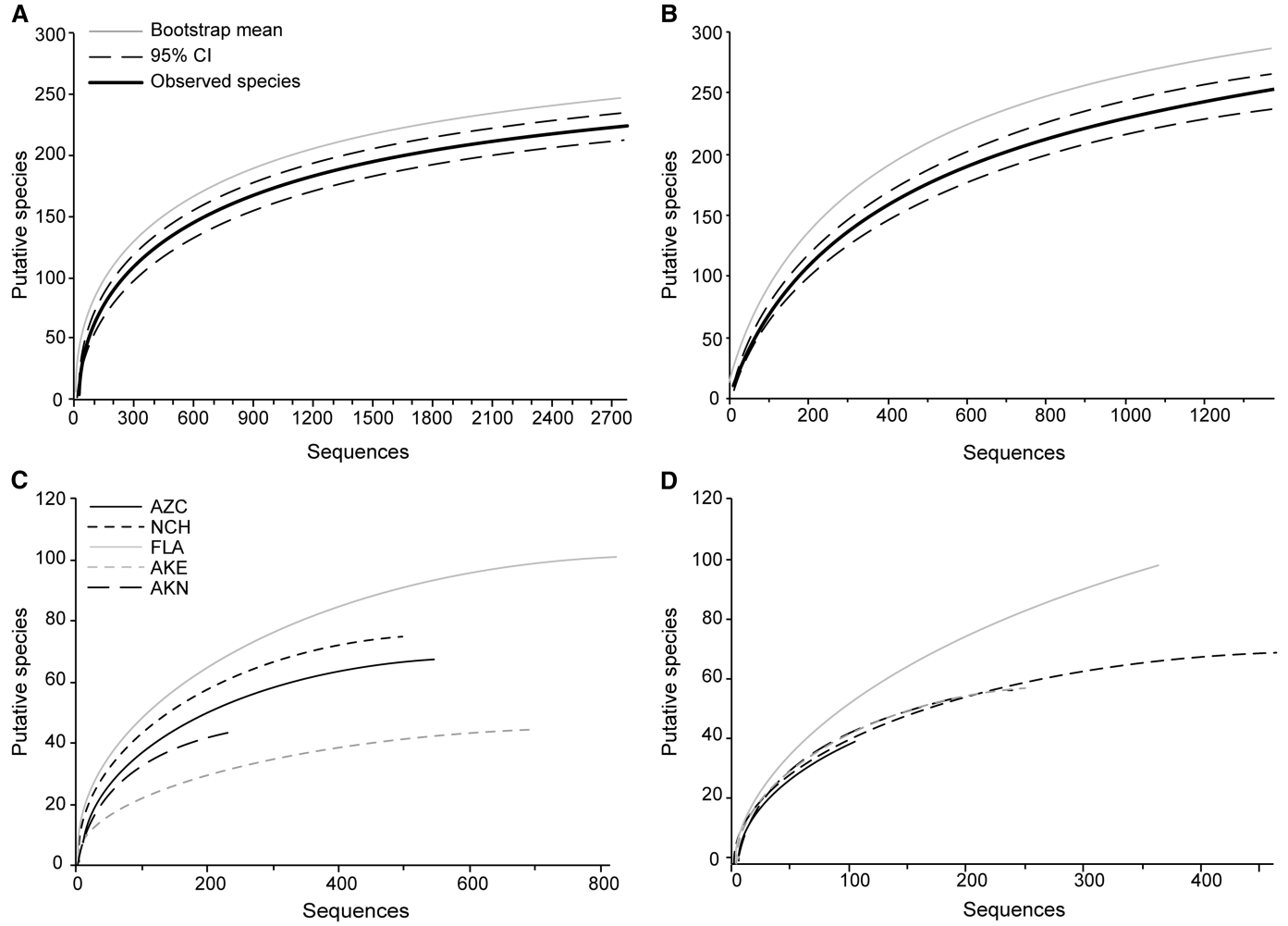

Fig. 2. Species accumulation curves (species observed: Mao Tau), 95% confidence intervals, and bootstrap estimate of richness based on ITS rDNA-partial LSU rDNA OTU (95% sequence similarity) for (A) 2777 isolates of endolichenic fungi from living thalli of 53 lichen species from five sites, (B) 1377 isolates of endophytic fungi from living photosynthetic tissues of 49 species of plants from five sites, (C) endolichenic fungi by site, and (D) endophytic fungi by site. Site abbreviations are as follows: AZC (Chiricahua Mountains, Arizona); NCH (Highlands Biological Station, North Carolina); FLA (Archbold Biological Station, Florida); AKE (Eagle Summit, Alaska); and AKN (Nome, Alaska).

geographic distance is removed, community similarity is positively associated with climatic similarity (endolichenic $r = -0.784$, $P = 0.0055$; endophytic $r = -0.757$, $P = 0.0073$). Yet, when the effect of climatic similarity is removed, community similarity is not positively associated with geographic proximity (endolichenic $r = -0.397$, $P = 0.2439$; endophytic $r = -0.333$, $P = 0.3483$).

***Distinctiveness of endophytic vs. endolichenic communities*—**In each site, endophytic and endolichenic communities differ significantly from one another, except in AKE where they nearly differ significantly (Fig. 5). This reflects a disparity between endophytes of vascular plants and endolichenic fungi: fungal communities in lichens were significantly more similar to those in mosses than to those in vascular plants (Wilcoxon pairwise comparison, $Z = -8.38$, $P < 0.0001$) (Fig. 5). Although strict-sense host specificity was rare, within-site comparisons showed that communities of fungi within lichens were more similar to one another than were communities in plants (Fig. 5), corresponding to higher host-specificity among endophytes than among endolichenic fungi (Fig. 6).

***Taxonomic composition of fungal communities*—**BLASTn queries of the Alaska Fungal Metagenomics Project ITS rDNA database and AFToL LSU rDNA database revealed that the majority of fungi sequenced here (97.2%) are Ascomycota (Pezizomycotina, $N = 4152$; Saccharomycotina, $N = 2$). Five classes and approximately 21 orders, 62 families, and 150 genera of Pezizomycotina were represented. Two identical isolates from *Serenoa repens* in FLA were the only members of Saccharomycotina found in the study (tentatively identified as *Pichia* sp.; data not shown). We did not find any previously known lichenicolous fungi or mycobionts.

Classes of Pezizomycotina were not evenly represented in our sample (ANOVA on logit-transformed percentages, $F_{4,42} = 15.67$, $P < 0.0001$). The majority were Sordariomycetes (67.5% of isolates), followed by Pezizomycetes (15.8% of isolates), Dothideomycetes (10.4% of isolates), Leotiomycetes (4.9% of isolates), and Eurotiomycetes (1.3% of isolates).

These classes of Pezizomycotina differed significantly in relative abundance among sites and in lichens vs. plants (Fig. 7).

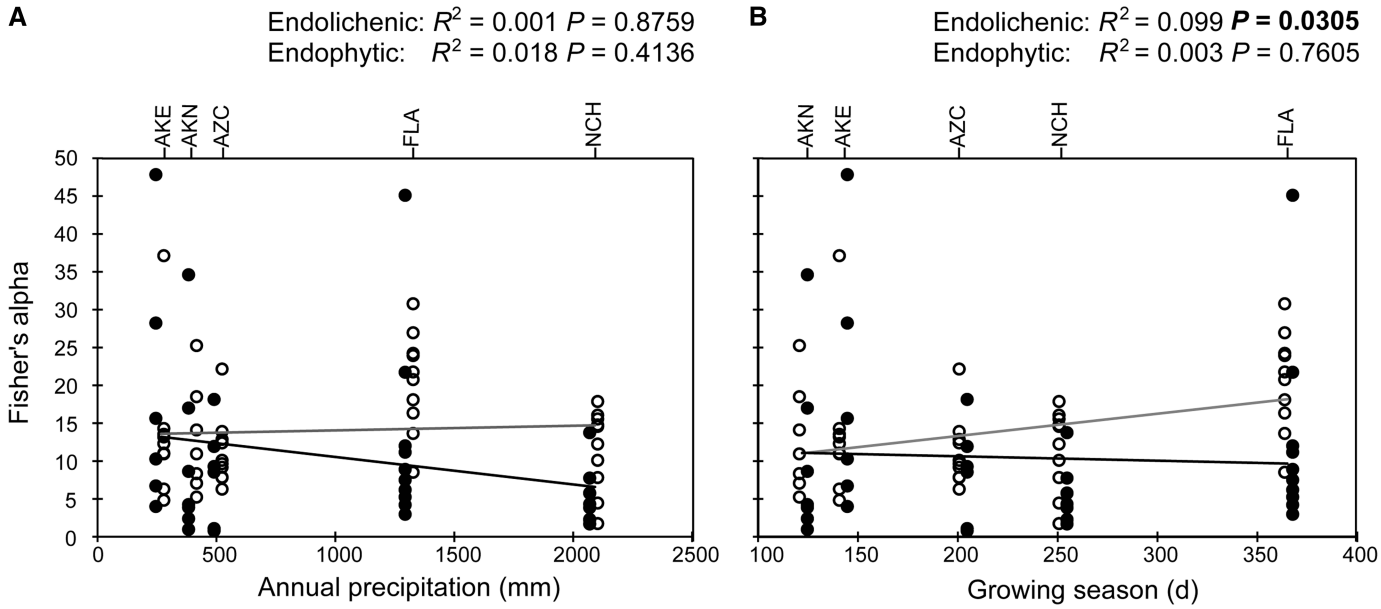

Fig. 3. Endophytic and endolichenic diversity as a function of (A) mean annual precipitation and (B) mean length of growing season for 47 lichen and 39 plant species/site combinations representing five sites across North America (Chiricahua Mountains, Arizona (AZC), Highlands Biological Station, North Carolina (NCH), Archbold Biological Station, Florida (FLA), Eagle Summit, Alaska (AKE), and Nome, Alaska (AKN)). Solid circles and black trendline indicate endophytic hosts; open circles and gray trendline indicate endolichenic hosts. For each explanatory variable, the coefficient of determination (R^2) and the P-value for ANOVA is presented for endolichenic and endophytic fungi. Precipitation × growing season was not significant for either endophytic or endolichenic fungal diversity. Points are jittered for readability.

DISCUSSION

Our study of plants and lichens in five sites across North America provides a first comparison of the associations of endophytic and endolichenic fungi with their hosts at a continental scale. Together our data indicate that the incidence, diversity, and composition of symbiotrophic fungi reflect the interplay of climatic patterns, geographic separation, host type, and host lineage. These data are important for understanding community assembly at local scale, as well as broader ecological and evolutionary associations between fungi and photosynthetic organisms. Perhaps most striking is our finding that congeneric and

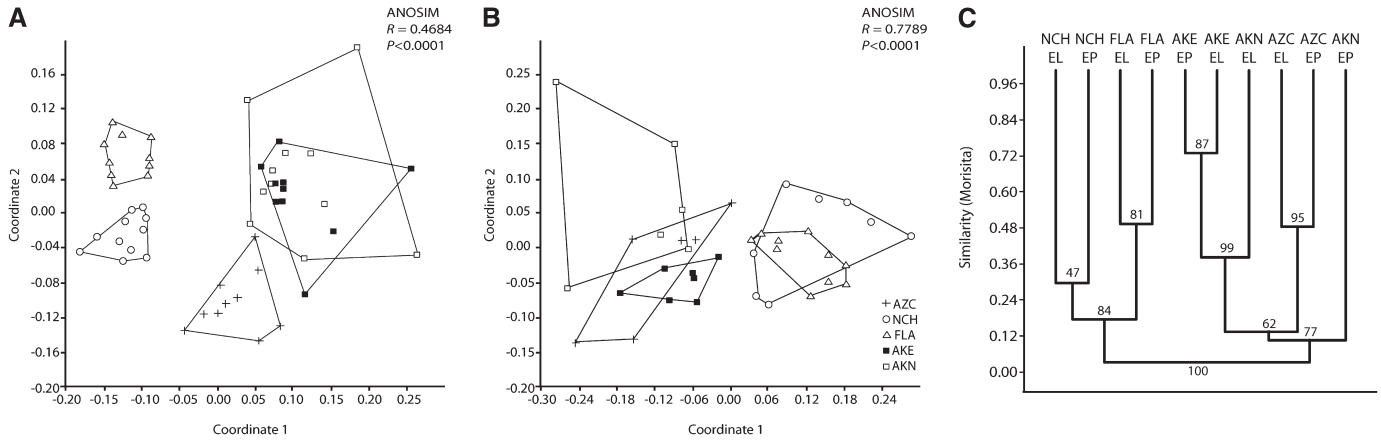

Fig. 4. Non-metric multidimensional scaling (NMDS) plots of (A) endolichenic and (B) endophytic fungal communities, showing the differences in community composition among sites (Chiricahua Mountains, Arizona (AZC), Highlands Biological Station, North Carolina (NCH), Archbold Biological Station, Florida (FLA), Eagle Summit, Alaska (AKE), and Nome, Alaska (AKN)). Each point represents the fungal community from a single lichen or plant host, and convex hulls (i.e., the smallest convex polygon containing all points) show the area occupied by points from each site. One-way analysis of similarity (ANOSIM) indicates significant differences in community composition among sites for both endophytic and endolichenic fungi. Post hoc pairwise comparisons indicate significantly different species composition between all sites ($P < 0.05$ after Bonferroni correction) except for endophytic fungi from AZC and AKN, which nearly differed significantly ($P = 0.0528$ uncorrected). (C) Unweighted pair-group average (UPGMA) dendrogram showing hierarchical clustering of endophytic and endolichenic communities from each site. Bootstrap values are based on 1000 bootstrap replicates. Hosts with <3 sequences and singleton OTU were excluded from analyses. The Morisita index was used to quantify community similarity for NMDS, ANOSIM, and UPGMA cluster analysis. Hosts from each microsite were pooled because communities of fungi from different microsites are not significantly different (ANOSIM $P > 0.05$), with the exception of microsites two and three for endolichenic fungi in AKE.

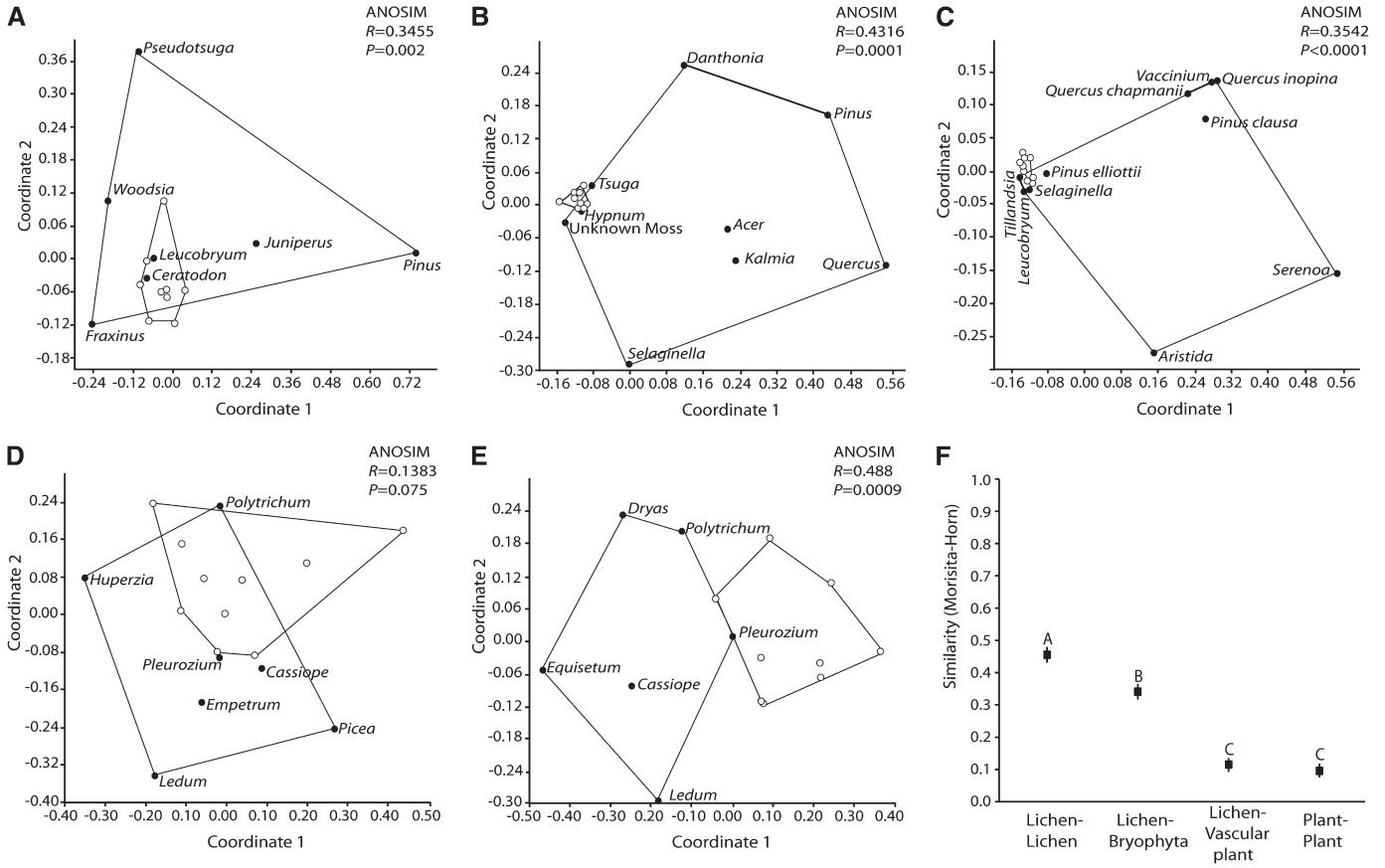

Fig. 5. Non-metric multidimensional scaling (NMDS) plots of endophytic and endolichenic fungal communities, showing the differences in community composition between lichen (open circles) and plant (closed circles) hosts from each site: (A) Chiricahua Mountains, Arizona (AZC); (B) Highlands Biological Station, North Carolina (NCH); (C) Archbold Biological Station, Florida (FLA); (D) Eagle Summit, Alaska (AKE); and (E) Nome, Alaska (AKN). Convex hulls show the area occupied by points from each host type. One-way analysis of similarity (ANOSIM) indicates significant differences ($P < 0.05$ after Bonferroni correction) in community composition between endophytic and endolichenic fungi in all sites but AKE, where endophytes and endolichenic communities nearly differ significantly. (E) Mean ± SEM pairwise similarity (Morisita-Horn) of endophytic and endolichenic fungal communities within the same site. Different letters represent significant differences ($P < 0.05$) based on post hoc Wilcoxon rank-sum comparisons; $\chi^2_3 = 290.19$, $P < 0.0001$. Hosts with <3 sequences and singleton OTU were excluded from analyses. The Morisita index was used to quantify community similarity for NMDS and ANOSIM.

confamilial lichens and plants living in different regions are infected by distinctly local symbionts. These local fungi may be similar in terms of higher taxonomy to those in other sites, but differ at shallow taxonomic levels.

Because of this turnover in species composition among sites, our collection of over 4700 isolates was insufficient to capture the estimated richness of these symbiotrophic fungi. Bootstrap estimates suggest at least 400 OTU and 849 genotypes of cultivable fungi in our sampled hosts. Overall, one-third of all OTU were found only once, precluding any inference regarding their host or geographic specificity. This number of singletons is lower than in many studies (see Arnold and Lutzoni, 2007; Arnold et al., 2007; Higgins et al., 2007; Davis and Shaw, 2008), reflecting our within-site sampling depth and replication. In each site, we captured or very nearly captured the total richness of cultivable endophytic and endolichenic fungi (Table 4), providing an opportunity to explore the factors shaping their ecological distributions.

Overall, fungi emerged from up to 61% of lichen thallus segments and 38% of photosynthetic tissue segments from plants per site. In each site, the incidence of cultivable endolichenic fungi ranged from ~1 to 5.25 times that of endophytic fungi. On a per host species basis, endolichenic fungi were more diverse than endophytic fungi in all sites except the two tundra locations (AKE and AKN). In the temperate zone, the higher abundance and diversity of endolichenic fungi likely reflects the longer exposure of lichen thalli to spore rain relative to the shorter lifetimes of leaves (some thalli can live up to 100 years; see Arnold et al., 2009). In AKE and AKN, isolation frequencies for endophytes generally were low, but those endophytes that were recovered had very high species richness, yielding high diversity values.

In general, endophytic fungi were particularly diverse in earlier-arising plant lineages such as mosses and lycophytes, especially compared to angiosperms (Tables 2, 4). In contrast, endolichenic diversity did not differ as a function of mycobiont order. Genetic distance among lichen mycobionts was smaller than among plants: a large fraction of focal lichens belong to one order (Lecanorales) and one class (Lecanoromycetes). The latter is estimated to have diversified 269–380 million years ago (Gueidan et al., 2011). In contrast, we sampled plant hosts spanning four major clades of land plants, which arose approximately

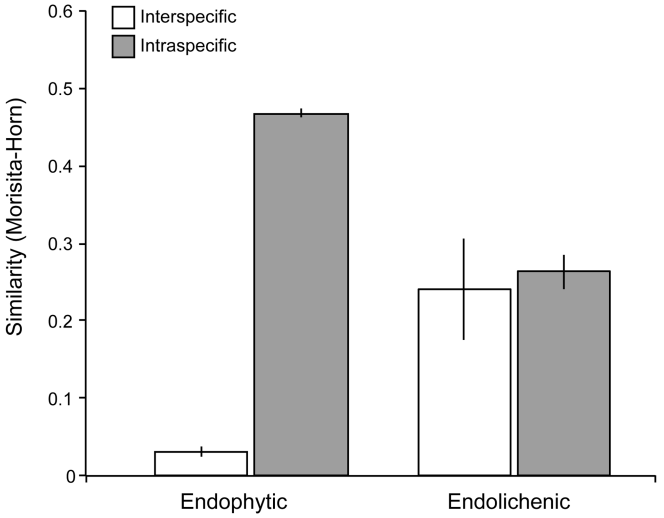

Fig. 6. Mean ± SEM pairwise similarity (Morisita–Horn) among individuals of the same host species (intraspecific) vs. similarity among individuals of different species (interspecific) for endophytic and endolichenic fungal communities within the same site. Similarity was significantly greater for conspecific hosts compared to heterospecific hosts for communities of fungi in plants (two-sided t test on logit-transformed Morisita–Horn; $t_{46} = 6.98$, $P < 0.0001$) and lichens ($t_{1624} = 2.67$, $P = 0.0076$). Hosts with <3 sequences and singleton OTU were excluded from analyses.

475 million years ago (Wellman et al., 2003; Steemans et al., 2009). This disparity in genetic distance may underlie the consistent levels of endolichenic diversity among lichens (Table 5) and greater host-generalism we observed among endolichenic fungi compared to endophytic fungi (Figs. 5F, 6).

Influence of abiotic factors on symbiotroph communities—Earlier studies have noted that endophytic communities from taxonomically related hosts differ in incidence, diversity, and composition among biogeographic regions (e.g., Fisher et al., 1995; Arnold et al., 2003; Arnold, 2007; Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008). Whether endolichenic fungi display similar patterns had not been studied previously, although a latitudinal gradient of isolation frequency and diversity was reported by Arnold et al. (2009). Dispersal limitation, limits to host range, local adaptive evolution, and environmental selection have been invoked to explain geographic heterogeneity of fungal communities (see Lumbsch et al., 2008; Peay et al., 2010), but the interplay of these factors has not been studied simultaneously in previous work.

We found that latitude alone was not a strong indicator of diversity when analyzed on a per host species basis. However, fungi in lichens were more diverse overall in more southerly sites, yielding a significant association between community-level diversity and latitude for endolichenic fungi (Table 4). No such pattern was observed for endophytes, but latitude became significant when our data were compared with tropical endophytes (data from Arnold and Lutzoni, 2007; Arnold et al., 2009; online Appendix S4).

Previous studies evaluating distributions of endophytic and endolichenic fungi have not decoupled latitude or geographic proximity from abiotic factors related to climate. We found that symbiotroph communities in sites that were closer together were more similar than those in more distant sites, but that differences among sites reflect climate more strongly than geographic distance alone. However, endolichenic and endophytic fungi differ somewhat in their associations with climate variables.

Mean length of the growing season, which is correlated with latitude but is more informative statistically, was positively associated with the isolation frequency and diversity of endolichenic fungi. This may reflect increased growth and photosynthetic rates of lichen thalli under warmer conditions (Coxson and Kershaw, 1983), through which more photosynthate might be available to these secondary mycobionts. Photosynthetic rates of photobionts also are influenced by factors such as thallus moisture content (Benedict, 1990), with many becoming physiologically active in the presence of fog or dew. This may explain the decoupling we observed between annual rainfall and endolichenic fungal diversity and abundance (Figs. 1, 3).

Overall, isolation frequency of endophytic fungi increased with increasing precipitation and growing season length, consistent with a strong relationship of rainfall and productivity on colonization and persistence by endophytic fungi in vascular plants (see Carroll, 1995; Arnold and Herre, 2003; Reiher, 2011). However, nonvascular plants displayed a different pattern: when analyzed alone, isolation frequency of moss-associated endophytes was not strongly influenced by annual rainfall or growing season length (online Appendix S4). In contrast with several previous studies (e.g., Ahlholm et al., 2002; Helander et al., 1993; but see Suryanarayanan et al., 2003), neither rainfall nor growing season was a strong predictor of endophyte diversity overall. However, growing season length was significantly correlated with endophyte diversity in conifers. This may reflect the fact that leaves of evergreens such as conifers accumulate endophytes as they age through multiple growing seasons (Bernstein and Carroll, 1977; Petrini and Carroll, 1981), whereas our estimates of isolation frequency and diversity for deciduous leaves represent colonization from a single growing season.

Here, we used mean length of growing season and annual precipitation to define climatic similarity, but other abiotic factors, as well as biotic factors such as local land use history, fire history, or plant diversity, density, and composition, also may be important. Future studies will sample hosts specifically to elucidate the biotic and abiotic factors influencing their distributions, as well as the geographic scale at which communities of endophytic and endolichenic fungi begin to turn over (see http://www.EnDoBiodiversity.org).

Taxonomic composition and host affiliation—The majority of endophytic and endolichenic fungi belonged to the same five classes of Pezizomycotina (Ascomycota), but a small number of Saccharomycotina (Ascomycota) and Basidiomycota also were found, especially in FLA (Table 4). Cultivable basidiomycetes occur frequently as endophytes of woody tissues, but also have been observed in foliar tissue, albeit rarely, using both culturing and direct PCR (Arnold et al., 2007; Pan et al., 2008; Rodriguez et al., 2009).

The taxonomic composition of fungal communities differed at both the class and OTU levels between lichens and plants. Lichens rarely contained Leotiomycetes ($N = 23$ isolates; 0.8%), which are common as endophytes of many conifers (Ganley et al., 2004; Ganley and Newcombe, 2006; Wang et al., 2006; Arnold et al., 2007), or Dothideomycetes ($N = 128$ isolates; 9.3%), which occur frequently as endophytes (Arnold et al., 2007; Arnold and Lutzoni, 2007; Higgins et al., 2007).

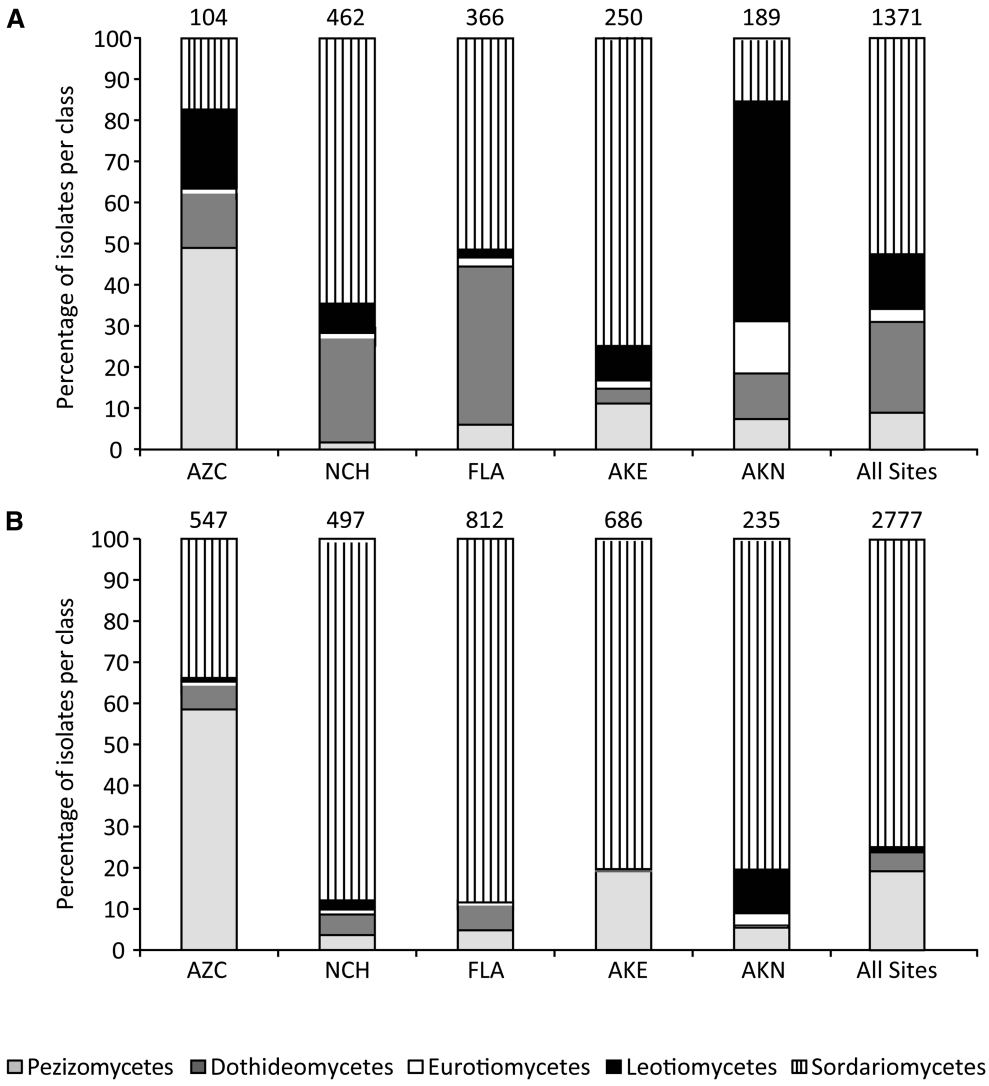

Fig. 7. Percentage of isolates representing each of five classes of Pezizomycotina (Pezizomycetes, Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes) as a function of site (Chiricahua Mountains, Arizona (AZC), Highlands Biological Station, North Carolina (NCH), Archbold Biological Station, Florida (FLA), Eagle Summit, Alaska (AKE), and Nome, Alaska (AKN)) for (A) endophytic fungi and (B) endolichenic fungi. The total number of sequences assessed is listed at the top of each stacked bar. These classes of Pezizomycotina were not equally distributed among sites (endolichenic $\chi^2_{16} = 141.76$, $P < 0.0001$, endophytic $\chi^2_{16} = 299.32$, $P < 0.0001$), nor among lichens vs. plants ($\chi^2_{16} = 185.21$, $P < 0.0001$).

At the OTU level, our results confirm earlier findings that communities of endolichenic fungi are largely distinct from endophytic fungi, with the exception of endophytes inhabiting mosses (see above, and U'Ren et al., 2010) and some cosmopolitan genotypes of Xylariaceae with broad host ranges (see Davis et al., 2003; Suryanarayanan et al., 2005). Endophytes of mosses were equally similar in composition to endolichenic communities from saxicolous, terricolous, epiphytic, and musicolous lichens (data not shown), such that their similarity is not solely due to overlapping niches (During and Van Tooren, 1990). Moreover, the OTU that colonize both mosses and lichens represent multiple classes (Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes, and Sordariomycetes) rather than a single evolutionary lineage. Thus, the similarity of endolichenic and moss-endophytic fungi may be explained by similar host architecture (i.e., nonvascular architecture), chemistry (associations with N_2-fixing cyanobacteria such as *Nostoc* sp.; Basilier et al., 1978; DeLuca et al., 2002), and/or ecology (water use and resistance to desiccation; Dilks and Proctor, 1974, 1976, 1979; Glime, 2007).

***Perspectives*—**Because our conclusions are based solely on cultured isolates and likely exclude species that require more specialized nutrients or are obligate symbionts, we likely underestimated fungal species richness and diversity. Similarly, missing species have the potential to alter our estimates of host and geographic affiliations. Culture-free approaches such as direct PCR and pyrosequencing can recover comparable or greater fungal diversity, as well as different taxonomic groups compared to culturing alone (Arnold et al., 2007; Gallery et al., 2007; Jumpponen and Jones, 2009); however, these methods also are subject to bias (see Kunin et al., 2010). For example, Tedersoo et al. (2010) found that ITS tag pyrosequencing errors led to an artificial increase in insertions and singletons compared to Sanger sequencing

and that estimates of ectomycorrhizal fungal richness and taxonomy were sensitive to primer selection, DNA extraction, and PCR methods. In addition, Arnold et al. (2007) and Higgins et al. (2011) found that certain clades of endophytic fungi recovered by culturing were not found from environmental PCR-based surveys of the same plant material. Undoubtedly the addition of culture-free methods would enhance the results presented here. In turn, our culture library sets the stage for empirical assessments of ecological specificity, multigene phylogenetics, metabolic capability, and comparative genomics. Such studies will shed light on functional trait distributions associated with geography, climate, and host use; inform host-symbiotroph coevolution at large and small spatial scales; and improve taxonomic tools for fungal diversity studies based on culture-independent methods.

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