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A Phylogenetic Estimation of Trophic Transition Networks for Ascomycetous Fungi: Are Lichens Cradles of Symbiotrophic Fungal Diversification?

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Abstract.—Fungi associated with photosynthetic organisms are major determinants of terrestrial biomass, nutrient cycling, and ecosystem productivity from the poles to the equator. Whereas most fungi are known because of their fruit bodies (e.g., saprotrophs), symptoms (e.g., pathogens), or emergent properties as symbionts (e.g., lichens), the majority of fungal diversity is thought to occur among species that rarely manifest their presence with visual cues on their substrate (e.g., the apparently hyperdiverse fungal endophytes associated with foliage of plants). Fungal endophytes are ubiquitous among all lineages of land plants and live within overtly healthy tissues without causing disease, but the evolutionary origins of these highly diverse symbionts have not been explored. Here, we show that a key to understanding both the evolution of endophytism and the diversification of the most species-rich phylum of Fungi (Ascomycota) lies in endophyte-like fungi that can be isolated from the interior of apparently healthy lichens. These "endolichenic" fungi are distinct from lichen mycobionts or any other previously recognized fungal associates of lichens, represent the same major lineages of Ascomycota as do endophytes, largely parallel the high diversity of endophytes from the arctic to the tropics, and preferentially associate with green algal photobionts in lichen thalli. Using phylogenetic analyses that incorporate these newly recovered fungi and ancestral state reconstructions that take into account phylogenetic uncertainty, we show that endolichenism is an incubator for the evolution of endophytism. In turn, endophytism is evolutionarily transient, with endophytic lineages frequently transitioning to and from pathogenicity. Although symbiotrophic lineages frequently give rise to free-living saprotrophs, reversions to symbiosis are rare. Together, these results provide the basis for estimating trophic transition networks in the Ascomycota and provide a first set of hypotheses regarding the evolution of symbiotrophy and saprotrophy in the most species-rich fungal phylum. [Ancestral state reconstruction; Ascomycota; Bayesian analysis; endolichenic fungi; fungal endophytes; lichens; pathogens; phylogeny; saprotrophy; symbiotrophy; trophic transition network.]

Fungal symbionts have been proposed to be key to plants' ancient colonization of land (Selosse and LeTacon 1998; Heckman et al. 2001) and have long been recognized as nearly ubiquitous in the rhizosphere of extant plants (i.e., mycorrhizae; Malloch et al. 1980; Taylor et al. 2004). Equally omnipresent are endophytic fungi, which occur within apparently healthy above-ground plant tissues such as leaves and stems (Clay and Holah 1999; Stone et al. 2000; Clay and Schardl 2002; Saikkonen et al. 2004; Schulz and Boyle 2005). These primarily ascomycetous fungi are known from biomes ranging from dry deserts to Arctic tundra and tropical rainforests, from every major lineage of land plants, and from every plant species examined to date (Stone et al. 2000; Davis et al. 2003; Arnold 2007).

Like their better-studied counterparts in the rhizosphere, endophytes in above-ground tissues such as leaves (i.e., foliar endophytes) can influence plant defense against disease, deter herbivores, augment plant growth, enhance or impair photosynthetic efficiency and drought tolerance, and improve or inhibit the ability of plants to exploit extreme environments (Clay and Holah 1999; Clay and Schardl 2002; Redman et al. 2002; Arnold et al. 2003; Saikkonen et al. 2004; Schulz and Boyle 2005; Arnold and Engelbrecht 2007). Clavicipitaceous endophytes (i.e., members of the Clavicipitaceae, a family of filamentous Ascomycota) associated with some cool-season grasses have garnered the most attention from ecologists and evolutionary biologists (Clay and Schardl 2002), but these symbionts represent a special case: they are vertically transmitted, grow systemically, and represent only a single family. In contrast, the overwhelming majority of land plants harbor diverse endophytes that are horizontally transmitted, form numerous localized infections in host tissues, and encompass at least 5 classes of Ascomycota (as well as a much more limited number of Basidiomycota and other fungi; Fröhlich and Hyde 1999; Arnold et al. 2000, 2003; Stone et al. 2000; Davis et al. 2003; Ganley et al. 2004; Saikkonen et al. 2004; Schulz and Boyle 2005; Arnold and Lutzoni 2007; for an overview of recent fungal classification, Hibbett et al. 2007). Colonization of tissues such as leaves typically occurs via penetration of the cuticle, although in some cases stomata provide an entryway for fungi that germinate on exterior surfaces (Mejía et al. 2008). Following colonization, many endophytes grow intercellularly, although some endophytes of conifers grow within epidermal cells (Stone et al. 2000).

Endophytes increase in biomass as leaves age due to the accumulation of new infections and the slow growth of established infections, reaching a density of one endophytic isolate per every 2 mm of leaf tissue in some tropical angiosperms and temperate conifers (e.g., Arnold et al. 2003).

At present, reliable estimates regarding the number of fungal species capable of forming endophytic associations are not available. Relatively few host plant species have been sampled for endophytes, sampling methods and species concepts differ markedly among studies, and endophytes are often absent from culture collections because they frequently remain sterile (do not produce fruiting bodies) in culture (Arnold 2007). Even so, it is clear that endophytes represent a significant portion of the "missing fungi"—those comprising the disparity between the currently recognized number of fungi (64 657 species; see Kirk et al. 2001) and ≥ 1.5 million fungal species thought to exist (Hawksworth and Rossman 1997; Hawksworth 2001). Individual plants frequently host multiple endophyte species, and individual plant species associate with dozens to hundreds of endophytic symbionts across their geographic ranges (Arnold and Lutzoni 2007; Hoffman and Arnold 2008). Moreover, relatively few endophytic fungi are shared among related plants in different biogeographic provinces (Arnold and Lutzoni 2007), and different fungi dominate the communities of endophytes in sympatric plant species (Arnold 2007). Species accumulation curves for endophytes are frequently nonasymptotic even in boreal and arctic ecosystems and arid lands (Higgins et al. 2007; Hoffman and Arnold 2008). In some tropical forest trees, and in relatively mesic sites in the temperate zone, an apparently healthy, mature leaf may harbor dozens of endophyte species at the same time (e.g., Lodge et al. 1996; Arnold et al. 2003).

At present, the evolutionary origins of these highly diverse endophytes are not known. Like many apparently avirulent microbial symbionts, endophytes generally are thought to have descended from pathogenic ancestors (see Clay and Schardl 2002; also see Spatafora et al. 2007). Similarly, it has been proposed that endophytes are simply saprotrophs awaiting leaf senescence or that they represent a latent phase in the life cycle of virulent pathogens (Stanosz et al. 1997; Carroll 1999; Schulz and Boyle 2005), such that they may have close evolutionary affinities with saprotrophic and pathogenic lineages. Because lichens are thought to have preceded plants in colonizing land (Heckman et al. 2001), it is also plausible that lichens harbored the fungi ancestral to endophytes. Previous studies have shown that in addition to the lichen-forming mycobiont fungi, lichen thalli harbor diverse Ascomycota (primarily Pezizomycotina or euascomycetes; Petrini et al. 1990; Girlanda et al. 1997; Suryanarayanan et al. 2005; Li et al. 2007). However, most of these studies have not distinguished fungi from lichen surfaces from those occurring within thalli, and the evolutionary relationships of lichen-associated fungi to endophytes have not been evaluated.

With the exception of the special case of grass-associated endophytes (Spatafora et al. 2007), none of these hypotheses for the evolutionary origins of endophytic fungi has been addressed explicitly. Here, we explore the evolutionary origins of endophytism using the broadest sample to date of endophytic fungi from bryophytes, ferns and lycophytes, conifers, angiosperms, and newly recovered fungi from the interior of lichen thalli (endolichenic fungi) to inform our understanding of the major evolutionary transitions across the Ascomycota. We focus specifically on the Pezizomycotina, the ecologically diverse and economically important subphylum that accounts for approximately 98% of the 33 000 recognized species of Ascomycota (Spatafora et al. 2006). Our results indicate the evolutionary dynamism of the endophytic habit, highlight the importance of endolichenism and endophytism in the diversification of the Pezizomycotina, and suggest a remarkable role of lichens as cradles of symbiotroph diversification in the largest fungal phylum.

Materials and Methods

Fungal Isolations

Endophytic fungi were isolated in culture from the interior of surface-sterilized, apparently healthy photosynthetic tissues of 34 species of bryophytes, seed-free vascular plants, conifers, and angiosperms from arctic, boreal, temperate, and tropical sites (Table 1). Endolichenic fungi were isolated from the interior of surface-sterilized, apparently healthy lichen thalli from 8 species of foliose lichens from arctic, boreal, temperate, and tropical sites, including lichens that grow on tree trunks (*Lobaria scrobiculata*), in association with bryophytes (*Peltigera* spp. and *Nephroma arcticum*), and on exposed rocks (*Umbilicaria mammulata*) (Table 1).

Isolation of endophytic and endolichenic fungi commenced within 72 h of sample collection. After washing in tap water (30 s), plant samples were cut into 2 mm^2 pieces and surface sterilized by sequential immersion in 96% ethanol (10 s), 0.5% NaOCl (2 min), and 70% ethanol (2 min) (Arnold et al. 2000, 2003). Because of potential damage to lichen thalli by sterilants, we used a gradient of surface sterilization to recover endolichenic fungi. From 2 lichen thalli per species per site, two 2 cm^2 pieces per thallus were subjected to (a) washing in water only or washing in water followed by immersion in 96% ethanol for 10 s, followed by immersion in 0.5% NaOCl and 70% ethanol for (b) 10 s each, (c) 30 s each, (d) 2 min each, or (e) 2 and 4 min, respectively. A total of 120–720 plant tissue segments were examined for endophytes per plant species in each site, representing 3–9 individuals per host species per site and 3–9 leaves or photosynthetic stems per individual. A total of 128 surface-sterilized thallus fragments were examined per lichen species per site for endolichenic fungi.

After treatment, leaf and lichen pieces were pressed lightly for 10 s against 2% malt extract agar (MEA), which encourages growth by diverse fungi (Fröhlich

TABLE 1. (A) Study sites, hosts, and frequency of fungal endophyte infection among 2 mm^2 segments of photosynthetic tissues for 34 plant host species-site combinations, and diversity (Fisher's alpha) of endophytes for 25 representative plant hosts. (B) Study sites, hosts, and frequency of endolichenic fungal infections among 2 mm^2 segments of asymptomatic thalli for 16 lichen host species-site combinations, and diversity (Fisher's alpha) of endolichenic fungi for 15 representative lichen hosts. Frequencies of infection reflect the proportion of tissue segments from which endophytes or endolichenic fungi were isolated in culture. Fisher's alpha calculations are based on operational taxonomic units defined by 95% ITS rDNA sequence similarity.

Site description	Site ID	Latitude (°N)	Host species	Host family	Percent infection	Fisher's alpha
A. Plant hosts						
Tropical moist forest (TROP)	BCI	9.9	*Gustavia superba*	Lecythidaceae	99.5	11.4
	BCI	9.9	*Heisteria concinna*	Olacaceae	97.9	18.1
	BCI	9.9	*Laetia thamnia*	Flacourtiaceae	95.6	13.9
	BCI	9.9	*Ouratea lucens*	Ochnaceae	99.1	9.3
	BCI	9.9	*Theobroma cacao*	Malvaceae	99.0	11.6
	BCI	9.9	*Trichilia tuberculata*	Meliaceae	97.9	23.2
Upland Sonoran desert	TAZ	32.2	*Platycladus orientalis*	Cupressaceae	25.5	6.0
	TAZ	32.2	*Cupressus arizonica*	Cupressaceae	6.0	13.0
Coniferous forest	SCA	32.3	*Pinus ponderosa*	Pinaceae	78.0	ND
Temperate mixed forest (TEMP)	DNC	35.6	*Acer rubrum*	Aceraceae	29.0	12.8
	DNC	35.6	*Arundinaria gigantea*	Poaceae	33.4	8.8
	DNC	35.6	*Huperzia* sp.	Lycopodiaceae	18.3	7.7
	DNC	35.6	*Juniperus virginiana*	Cupressaceae	33.0	16.3
	DNC	35.6	*Magnolia grandiflora*	Magnoliaceae	19.0	14.6
	DNC	35.6	*Pinus taeda*	Pinaceae	96.0	15.9
	DNC	35.6	*Platycladus orientalis*	Cupressaceae	80.2	9.4
	DNC	35.6	*Polystichum acrostichoides*	Dryopteridaceae	26.0	4.2
Southern boreal forest (SBOR)	MAQ	50.3	*Bazzania* sp.	Lepidoziaceae	2.2	ND
	MAQ	50.3	*Dryas integrifolia*	Rosaceae	6.7	4.9
	MAQ	50.3	*Empetrum nigrum*	Ericaceae	3.0	2.6
	MAQ	50.3	*Juniperus horizontalis*	Cupressaceae	18.0	15.5
	MAQ	50.3	*Picea mariana*	Pinaceae	22.6	17.5
	MRQ	50.3	*Pinus banksiana*	Pinaceae	29.0	5.7
	MAQ	50.3	*Vaccinium vitis-idaea*	Ericaceae	14.0	3.8
Northern boreal forest (NBOR)	SHQ	54.8	*Betula nana*	Betulaceae	1.0	ND
	SHQ	54.8	*Empetrum nigrum*	Ericaceae	1.0	ND
	SHQ	54.8	*Huperzia selago*	Lycopodiaceae	6.0	8.4
	SHQ	54.8	*Kalmia angustifolia*	Ericaceae	1.0	ND
	SHQ	54.8	*Picea mariana*	Pinaceae	41.3	6.7
	SHQ	54.8	*Sphagnum* sp.	Sphagnaceae	3.9	6.4
	SHQ	54.8	*Vaccinium vitis-idaea*	Ericaceae	5.1	ND
Arctic tundra (ARC)	IQN	63.8	*Cassiope tetragona*	Ericaceae	1.0	ND
	IQN	63.8	*Dryas integrifolia*	Rosaceae	1.0	ND
	IQN	63.8	*Sphagnum* sp.	Sphagnaceae	1.0	ND
B. Lichen hosts						
High-elevation bog (TROP)	HCR	9.4	*Peltigera neopolydactyla* s.l.	Peltigeraceae	45.8	13.1
Temperate pine forest (TEMP)	RNC	35.3	*Peltigera neopolydactyla* s.l.	Peltigeraceae	30.5	10.6
Montane deciduous forest (TEMP)	BNC	36.1	*Umbilicaria mammulata*	Incertae sedis	39.5	11.4
North-temperate coniferous forest	HME	45.2	*Lobaria scrobiculata*	Lobariaceae	9.7	10.2
Southern boreal forest (SBOR)	MAQ	50.3	*Peltigera aphthosa*	Peltigeraceae	17.0	11.4
	MAQ	50.3	*Peltigera leucophlebia*	Peltigeraceae	18.8	6.3
	MAQ	50.3	*Peltigera neopolydactyla* s.l.	Peltigeraceae	13.2	3.1
	MRQ	50.3	*Lobaria scrobiculata*	Lobariaceae	14.6	6.4
Northern boreal forest (NBOR)	SHQ	54.8	*Peltigera aphthosa*	Peltigeraceae	11.8	4.8
	SHQ	54.8	*Peltigera malacea*	Peltigeraceae	15.3	2.5
	SHQ	54.8	*Peltigera neopolydactyla* s.l.	Peltigeraceae	13.2	5.1
	SHQ	54.8	*Peltigera scabrosa*	Peltigeraceae	13.2	4.2
	SHQ	54.8	*Nephroma arcticum*	Nephromataceae	9.0	5.9
Arctic tundra (ARC)	IQN	63.8	*Peltigera aphthosa*	Peltigeraceae	18.0	2.1
	IQN	63.8	*Peltigera malacea*	Peltigeraceae	13.9	3.5
	IQN	63.8	*Peltigera scabrosa*	Peltigeraceae	1.4	ND

Site abbreviations: BCI, Barro Colorado Island, Panama; TAZ, Tucson, AZ, USA; SCA, Santa Catalina Mountains, near Tucson, AZ, USA; DNC, Duke Forest, North Carolina, USA; MAQ, Mingan Archipelago, Québec, Canada; MRQ, Moisie River, Moisie, Québec, Canada; SHQ, Schefferville, Québec, Canada; IQN, Iqaluit, Nunavut, Canada; RNC, Raven Rock State Park, North Carolina, USA; BNC, Beech Mountain, North Carolina, USA; HME, near Howland, Maine, USA; HCR, Heredia, Costa Rica. Abbreviations in parentheses correspond to site information given in Figure 2a (TROP, TEMP, SBOR, NBOR, ARC). ND indicates that diversity was not determined.

and Hyde 1999; Arnold and Lutzoni 2007) to harvest any viable fungi that remained on external surfaces. Lichen pieces then were cut into 2 mm^2 pieces under sterile conditions. The 2 mm^2 pieces of lichen thalli and leaves were transferred to new 2% MEA plates to harvest cultivable fungi from the interior of tissues. Plates were sealed with Parafilm and incubated up to 1 year at room temperature under ambient light. Emergent fungi were subcultured on 2% MEA, photographed, and vouchered as living cultures in sterile water at the Robert L. Gilbertson Mycological Herbarium at the University of Arizona and the Duke University Herbarium.

Localization of Endolichenic Fungi within Lichen Thalli

To more precisely identify the location of endolichenic fungi within the lichen thallus, 4 fresh *U. mammulata* thalli were stringently surface sterilized (treatment e) and then dissected under sterile conditions to separate the cortices, medulla, and algal layer. Cultures were prepared as described above using each tissue type and randomly selected portions of intact, surface-sterilized thalli. The proportion of isolates with genotypes identical to known endolichenic fungi (100% ITS rDNA sequence similarity; see below) was assessed for each tissue type.

Primary Identification of Isolates

Because endophytic and lichen-associated fungi frequently lacked reproductive structures in culture, they could not be identified beyond the level of phylum on the basis of morphology alone. Following Arnold et al. (2007), total genomic DNA was extracted directly from 2058 fungal isolates recovered in this study, including 630 endolichenic fungi, 1368 endophytic fungi, and 60 representative fungi from lichen surfaces (i.e., fungi recovered from surface impressions of lichen thalli). The nuclear ribosomal internal transcribed spacer region (ITS rDNA) was amplified and sequenced for each isolate using standard fungal primers ITS1F or ITS5 and ITS4 (for detailed methods, see Arnold and Lutzoni 2007). Sequences were assembled manually using Sequencher 4.2 (Gene Codes Corporation, USA) or BioPython scripts (Kauff et al. 2007) that facilitated automatic base-calling, assignment of quality scores for base calls, and contig assembly by phred and phrap (Green 1996; Ewing et al. 1998). Automatic base calls and assemblies were manually verified in Sequencher.

All ITS rDNA consensus sequences were subjected to BLAST comparisons with GenBank to estimate the taxonomic placement of each isolate and to compare sequences against previously published data. Consensus sequences then were grouped into genotype groups on the basis of 90% ITS rDNA sequence similarity to select representative taxa for multilocus sequencing (Arnold et al. 2007). The nuclear ribosomal large subunit (LSU rDNA; ca. 1.4 kb; primers LR0R–LR7) and small subunit (SSU rDNA; ca. 1.0 kb; primers NS1–NS24) were amplified and sequenced for 118 representative isolates following Higgins et al. (2007). In sum, this study yielded 2058 novel ITS rDNA sequences and 118 novel LSU rDNA and SSU rDNA sequences, all of which have been submitted to GenBank (Appendix 1).

Genotype groups based on 95% ITS rDNA sequence similarity were used to delimit functional taxonomic units for ecological analyses based on concordance with phylotypes presented in Figure 2a and previous studies by the authors (Arnold and Lutzoni 2007; Arnold et al. 2007). Total species richness and taxon accumulation curves were estimated using EstimateS (Colwell 2004), a freeware program that provides rarefied taxon accumulation curves, calculates diversity indices, and implements bootstrap estimates (among others) of total richness (see Arnold et al. 2007). Diversity was measured using Fisher's alpha, which is robust for comparisons among samples of different sizes (Leigh 1999; Arnold et al. 2007).

Sequence Alignment and Phylogenetic Analyses

LSU rDNA and SSU rDNA sequences were incorporated into alignments of 241 representative Ascomycota (Appendix 2), which included all Pezizomycotina and relevant outgroup taxa for which both loci were available when this study was conducted. The geographic origins and hosts of 118 isolates obtained in this study and sequenced for LSU rDNA and SSU rDNA are shown in Appendix 3.

Data for the resulting 359 operational taxonomic units (OTU) were aligned manually with MacClade 4.07 (Maddison and Maddison 2003) based on the secondary structure model (Kjer 1995) of *Saccharomyces cerevisiae* (Cannone et al. 2002). The LSU rDNA alignment comprised 4894 sites, of which 3907 were excluded (Group I introns, spliceosomal introns, and ambiguously aligned regions; sensu Lutzoni et al. 2000) and 987 sites were included. The SSU rDNA alignment comprised 7904 sites, of which 1425 were included. Alignments for each locus have been submitted to TreeBase (November 2008).

Following Lutzoni et al. (2004) and Reeb et al. (2004), we used neighbor-joining (NJ) bootstrap proportions to detect topological conflicts between data partitions. Conflicts were considered significant if 2 different relationships (one monophyletic and the other non-monophyletic) for the same set of taxa were both supported with bootstrap values $\geq$70% (Mason-Gamer and Kellogg 1996). NJ bootstrap analyses were performed separately on the LSU rDNA and SSU rDNA data sets (1000 replicates with maximum likelihood [ML] distance implemented using PAUP*4.0b.10) (Swofford 2002). Models for nucleotide substitution were estimated using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.6 (Posada and Crandall 1998). For LSU rDNA: (GTR + I + G) 6-parameter model (A-C = 1.1091, A-G = 3.1485, A-T = 1.5285, C-G = 0.8015, C-T = 9.0842, G-T = 1.0000) with unequal base frequencies (A = 0.2412, C = 0.2252, G = 0.3405, T = 0.1932), gamma shape distribution (0.5868), and a proportion of invariable sites (I = 0.3586). For SSU rDNA: (GTR + I + G) 6-parameter model (A-C = 1.1981, A-G = 2.7972, A-T = 1.0975, C-G = 0.7272, C-T = 5.0709, G-T = 1.0000) with unequal base frequencies (A = 0.2406, C = 0.2188, G = 0.2946, T = 0.2460), gamma shape distribution (0.5031), and a proportion of invariable sites (I = 0.2839). Using this approach, no conflicts were detected between the LSU rDNA and SSU rDNA data sets, such that these data were combined to yield a total of 2412 characters for phylogenetic analyses.

Bayesian metropolis-coupled Markov chain Monte Carlo (B-MCMCMC) analyses were conducted with MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001) on the combined data using 4 chains, a 6-parameter model for

nucleotide substitution (GTR; Rodriguez et al. 1990), a gamma distribution, and a proportion of sites assumed to be invariable as determined with AIC. In the combined analysis, LSU rDNA and SSU rDNA were considered as 2 separate data partitions. Ten independent analyses of 5 000 000 generations each were initiated with random trees and sampled every 100th tree. Because these runs did not converge on the same average likelihood, we initiated 20 independent analyses for 5 000 000 generations each, starting each with a random tree taken from the postburn-in sample of a previous run with the highest average likelihood score and sampling every 100th generation. A 50% majority-rule consensus tree was calculated using PAUP* for a total of 100 000 post-burnin trees derived from 5 analyses (20 000 trees per run) that resulted in a higher average likelihood level than the remaining 15 analyses.

In addition to posterior probabilities derived from MrBayes analyses, phylogenetic confidence was estimated using BayesPhylogenies, which allows simultaneous implementation of multiple models of nucleotide substitution without partitioning the data a priori (Pagel and Meade 2004). We initiated 10 BayesPhylogenies runs of 1 000 000 generations each, starting with a random tree and sampling every 100th generation. In each subsequent run, the number of partitions was raised by 1 with gamma rate heterogeneity included or not (2 partitions with GTR, 2 partitions with GTR + G, 3 partitions with GTR, 3 partitions with GTR + G, etc.) until a maximum of 6 partitions with gamma rate heterogeneity was reached. Based on the average of likelihood scores for the last 2000 post-burnin trees from each of the 10 BayesPhylogenies runs, the likelihood score obtained from 4 partitions + gamma rate heterogeneity (−48431.532) was markedly better (using the rule of thumb of a minimum improvement of 32.23 log units as suggested by Pagel and Meade 2005) than the next best run (5 partitions without gamma rate heterogeneity; −48739.107), with a striking difference of 307.575 log units. Using these optimal settings, we initiated 20 independent BayesPhylogenies runs for 1 000 000 generations, sampling every 100th tree. A random starting tree for each of the 20 runs was obtained from the 8000 post-burnin trees resulting from the previous run (using 4 partitions + gamma rate heterogeneity). A 50% majority-rule consensus tree was calculated using PAUP* for a total of 20 000 post-burnin trees derived from 10 runs (2000 trees per run) that resulted in a higher average likelihood level than the remaining 10 runs.

For both Bayesian analyses (MrBayes and BayesPhylogenies), posterior probabilities were calculated based on post-burnin trees sampled from multiple separate MCMC runs to avoid the artifact of obtaining high posterior probabilities for wrong relationships, which is associated with very short internodes (Alfaro et al. 2003). This artifact would have been carried over to the reconstruction of ancestral states, upon which our main conclusions are based. Because this artifact results in part from the inherent stochasticity of Bayesian analyses strong support for wrong relationships associated with very short internodes is less likely if post-burnin trees from independent runs are pooled. Douady et al. (2003) proposed Bayesian analyses on nonparametric bootstrap pseudoreplicates as a solution to this problem, but this approach was not applicable for such a large data set and given the problem of convergence experienced here. Similarly, conducting Bayesian analyses that would allow polytomies during the MCMC process to solve this problem (Lewis et al. 2005) was not possible due to the lack of available software to implement this method on large data sets.

Ancestral State Reconstructions

Nodes for ancestral state reconstruction were selected based on their posterior probability support values. Only those nodes significantly supported by both MrBayes posterior probability (MB-PP $\geq$ 95) and BayesPhylogenies posterior probability (BP-PP $\geq$ 95), by MB-PP only (MB-PP $\geq$ 95), or in 2 cases by BP-PP only (BP-PP $\geq$ 95) were included. Ancestral states were reconstructed (using a Mesquite module, see below) on 1000 trees drawn randomly using the program rt.py (F. Kauff; available at http://www.lutzonilab.net/downloads) from the post-burnin 100 000-tree pool derived from 5 MrBayes analyses (20 000 post-burnin trees/analysis), which were used for the majority-rule consensus tree. For 2 nodes that were supported significantly only by BP-PP, ancestral states were reconstructed on 1000 trees drawn randomly from the 20 000 post-burnin trees derived from the 10 BayesPhylogenies analyses.

Terminal taxa were coded according to their lifestyles based on a literature review (Kirk et al. 2001) and discussions with experts or on the origin of cultures obtained in this study (endophytic, endolichenic, and lichen surface fungi). Because this study focused on photosynthetic tissues, mycorrhizal euascomycetes were not coded.

Ancestral state reconstructions used ML with the marginal global optimality criterion (Pagel 1999; Jackson 2004; corresponds to the fossil likelihood global reconstruction available through Discrete) as implemented in the LASRDisc module in Mesquite 1.05 (Jackson 2004; Maddison and Maddison 2004) with the "trace character over trees" option and root mode = (0.5, 0.5). An asymmetrical 2-parameter Markov *k*-state model allowing different rates of gains and losses was selected based on a likelihood ratio test showing this model to be significantly better than a Markov *k*-state 1-parameter model. A given ancestral state was assigned to a node if its raw likelihood was >2 log units higher than the likelihood value of the other ancestral state (default in Mesquite 1.05).

In Reconstruction 1, we examined the evolution of endophytism. Terminal taxa were coded as endophytes (State 1) if they represented 1) named taxa known to be endophytic (e.g., *Balansia henningsiana*); 2) fungi isolated from the interior of surface-sterilized plant tissues in this study; 3) named taxa that showed $\geq$95%

ITS rDNA similarity to fungi recovered from the interior of surface-sterilized plant tissues in this study; or 4) fungi recovered from other substrates in this study with ≥ 95% ITS rDNA similarity to endophytes as defined under Criteria 1 and 2. All other terminal taxa were coded as 0.

In Reconstruction 2, we examined the evolution of pathogenicity. Two coding systems were used to compensate for uncertainty with regard to the potential for fungi with unknown ecological roles to act as pathogens. In coding A, we coded as pathogenic (i.e., State 1) all terminal taxa known to be pathogens or parasites of living organisms and all terminal taxa whose lifestyles were unknown; other taxa were coded as nonpathogens (State 0). In coding B, we coded as pathogenic only those terminal taxa known unequivocally to act as pathogens of living organisms. To be conservative in assigning ancestral states, we considered only those reconstructions that were congruent between the 2 coding approaches (i.e., those that were not sensitive to coding A vs. coding B).

In Reconstruction 3, we examined the evolution of saprotrophy. Two coding systems were used to compensate for uncertainty regarding the potential for fungi with unknown lifestyles to act as saprotrophs. In coding A, we coded as saprotrophic all terminal taxa known to actively decay tissue and all taxa for which the lifestyle was not known (State 1); other taxa were coded as nonsaprotrophs (State 0). In coding B, we coded as saprotrophic only those terminal taxa known unequivocally to be saprotrophs. In assigning ancestral states, we considered only those reconstructions that were congruent between the 2 coding approaches.

In Reconstruction 4, we examined the evolution of lichenization. Terminal taxa were coded as lichen-forming mycobionts (1) or non–lichen-forming (0). Because there was no uncertainty regarding the ability of fungi to serve as mycobionts, only one coding system was used.

In Reconstruction 5, we examined the evolution of endolichenism. We considered terminal taxa to be endolichenic (State 1) if they represented 1) fungi isolated from the interior of lichen thalli under stringent surface sterilization (treatment d or e above; "true endolichenic fungi"); 2) lichen-associated fungi with ≥ 95% ITS rDNA sequence similarity to true endolichenic fungi; or 3) lichen-associated fungi with ≥ 95% ITS rDNA similarity to plant endophytes (considered endolichenic because they were recovered from a lichen). All other terminal taxa were coded as 0.

Rates of change (gains and losses) of the endobiotic lifestyle (endophytic + endolichenic) and lichen-forming mode were calculated for 100 random trees from the 1000 used to reconstruct ancestral states (above, using the same parameters) and compared using Wilcoxon signed-rank tests due to a significant deviation of the data from normality. One data point was removed from the analysis of gain/loss ratios (gain/loss ratio of 1 for endophytism, which exceeded the mean by more than 3 standard deviations).

Relative Time and Diversification Estimation

The phylogenetic chronogram (Fig. 2a) was obtained through penalized likelihood analyses (truncated Newton algorithm) of the 100 000-tree MrBayes consensus phylogram (sumt option) using r8s 1.7 (Sanderson 2004). The smoothing value (= 10) was determined using cross-validation, with the root fixed at the age 100. The chronogram was divided into 32 time periods of equal duration. In each period, we assessed the total number of lineages of euascomycetes present in the tree and the number of lineages leading to unequivocally lichenized or endophytic/endolichenic terminal taxa. Results are shown in Figure 2b.

Estimation of Evolutionary Transitions among Ecological Modes

The number of unambiguous transitions along branches between ecological modes was counted and summed across the tree. Transitions were considered unambiguous if coding for 2 states was contiguous (i.e., reconstructions shown with color in Fig. 2a were not separated by branches without coding). Results are shown in Figure 2c. An alternative result is shown in Supplementary Figure 1, where the coding of extant taxa was biased toward saprotrophy.

Estimation of Relative Abundance of Ecological Modes among Major Lineages

The total number of known species of lichen-forming mycobionts and lichenicolous fungi—an ecologically delimited group of fungi that fruit from living thalli or form symptomatic infections in lichens (Lawrey and Diederich 2003)—was estimated from the literature (Kirk et al. 2001) and in consultation with experts (see acknowledgments) and were scaled relative to the total number of species currently recognized in each ecological group (Kirk et al. 2001). Proportional representation of endophytic and endolichenic isolates in each group reflects our survey results and is based on ITS rDNA sequence data for 1998 endolichenic and endophytic fungi. Raw data are shown in Appendix 4.

RESULTS

Recovery and Characterization of Endolichenic and Endophytic Fungi

All plant and lichen species sampled in this study harbored endophytic or endolichenic fungi, respectively, regardless of sampling site or taxonomic placement (Table 1). Endophytic fungi were recovered in 1–99.5% of plant tissue segments examined per plant species (mean = 35.0 ± 6.4%); endolichenic fungi were recovered from 1.4% to 45.8% of tissue segments examined (mean = 17.8 ± 2.9%). We recovered no fungi from the exterior surfaces of the most stringently surface-sterilized host tissues (treatments d and e for lichens; standard treatment, equivalent to treatment d, for plants).

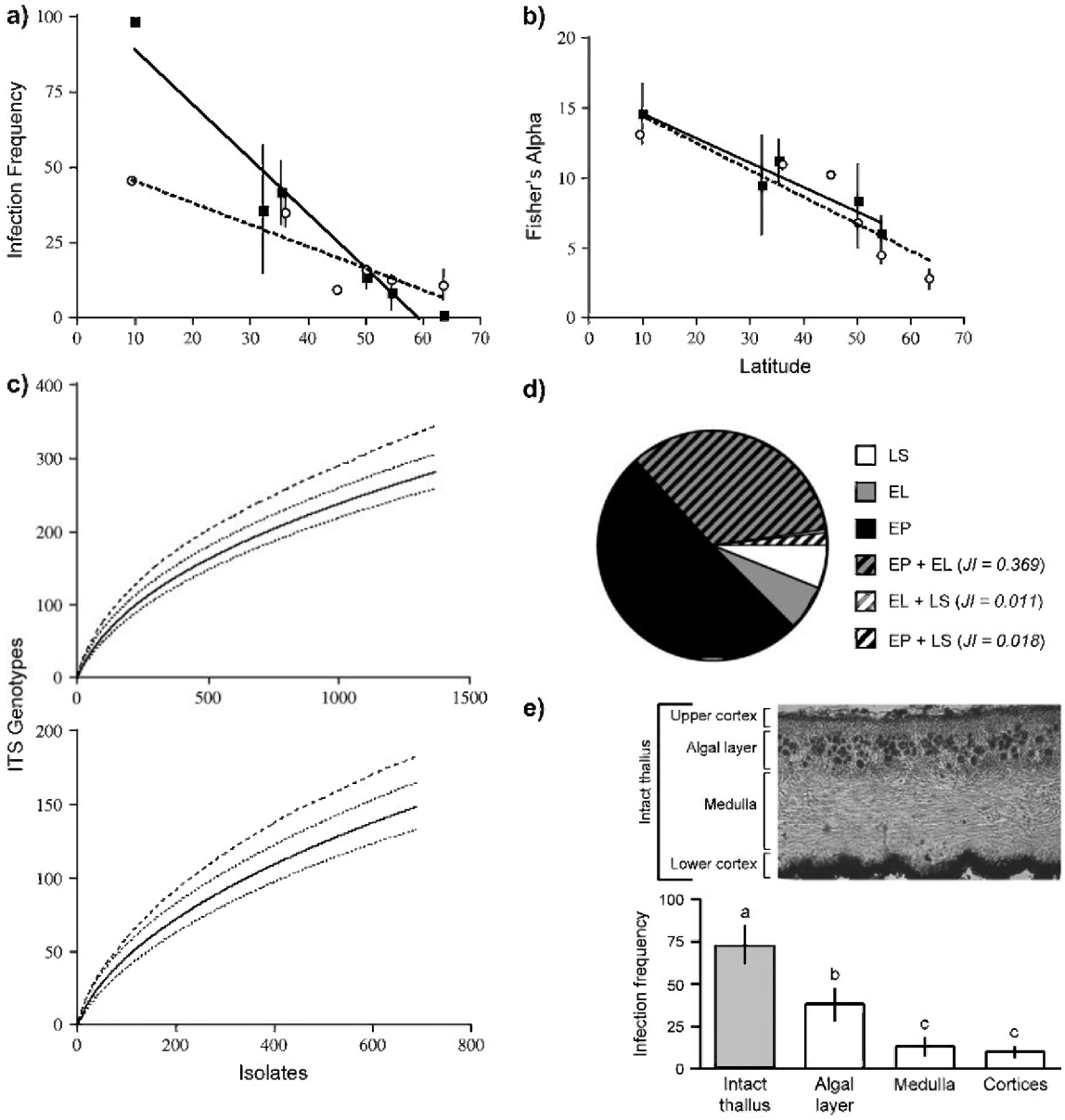


FIGURE 1. a) Infection frequency (mean ± standard error [SE]) of endophytic fungi among 2 mm^2 segments of surface-sterilized leaves and microphylls (black squares, solid line; 34 species-site combinations, including bryophytes, lycophytes, ferns, conifers, and angiosperms; $r^2 = 0.74$, $F_{1,32} = 93.20$, $P < 0.0001$) and endolichenic fungi from surface-sterilized fragments of asymptomatic lichens (open circles, dashed line; 16 species-site combinations, including Lobariaceae, Nephromataceae, Peltigeraceae, and *Umbilicaria mammulata*; $r^2 = 0.72$, $F_{1,14} = 38.39$, $P < 0.0001$) along a latitudinal gradient from tropical forests to the arctic (Table 1). b) Diversity (Fisher's alpha, mean ± SE) of endophytic fungi (black squares, solid line; 25 species-site combinations; $r^2 = 0.25$, $F_{1,22} = 7.35$, $P = 0.0128$) and endolichenic fungi (open circles, dashed line; 15 species-site combinations; $r^2 = 0.65$, $F_{1,13} = 24.57$, $P = 0.0023$), along a latitudinal gradient from tropical forests to the arctic (Table 1). c) Genotype accumulation curves (black solid lines) for representative endophytic fungi (upper panel) and endolichenic fungi (lower panel) from arctic, boreal, temperate, and tropical sites, sequenced for the nuclear ribosomal internal transcribed spacer (ITS rDNA). ITS rDNA genotypes reflect 95% sequence similarity, used here as a conservative proxy for species boundaries. Light dashed lines indicate 95% confidence interval based on 50 randomizations of sample order; heavy dashed lines indicate bootstrap estimate of total richness. d) Prevalence and taxonomic distribution of ITS rDNA genotypes among endophytic (EP) and lichen-associated fungi (endolichenic fungi, EL; fungi from lichen surfaces, LS) for all nonsingleton genotypes ($n = 177$, representing 2058 sequenced isolates from arctic, boreal, temperate, and tropical sites). ITS rDNA genotype groups are designated on the basis of 95% sequence similarity. Jaccard's index (JI) indicates shared genotypes among EP, EL, and LS fungi (0 = no shared genotypes; 1 = all genotypes shared). e) Preferential association of endolichenic fungi with the algal layer in the foliose lichen *U. mammulata*. Data indicate infection frequency (mean ± SE) for endolichenic fungi in an intact thallus (grey) and in discrete portions of dissected, surface-sterilized thalli (photo inset indicates different portions of thallus: cortices, algal layer, and medulla). Significant differences in infection frequency ($\alpha = 0.05$) are denoted by letters a, b, and c. Image courtesy of I. Brodo.

FIGURE 2. a) Phylogenetic relationships and relative divergence time among euascomycetes (=Pezizomycotina) and evolution of their ecological modes. Tree represents majority-rule consensus of 100 000 trees inferred using B-MCMCMC analysis of 1.4 kb of LSU rDNA + 1.0 kb of SSU rDNA data. Branch lengths represent relative time based on penalized likelihood (r8s). Named taxa represent all euascomycete species for which data for both loci were available (241 OTU; Appendix 2). Colors of taxon names and culture numbers indicate ecological mode: pale blue, endophytic; dark blue, endolichenic; red, pathogenic or parasitic; brown, saprotrophic; green, lichen forming; no color, ecological mode unknown or, in the case of cultures recovered here, present on the surface of lichen thalli; more than one color, known to have more than one ecological mode. Brown boxes after taxon names indicate unknown or ambiguous ecological roles that were coded as saprotrophic in a second analysis for a more in-depth examination of evolutionary transitions to and from saprotrophy. Abbreviations after culture numbers indicate host plant lineages (Angio, angiosperm; Conif, conifer; Lyco, lycophyte) or lichen genera (Pelt, *Peltigera*; Umb, *Umbilicaria*) and bioclimatic zone of origin (ARC, Arctic; NBOR, northern boreal forest; SBOR, southern boreal forest; TEMP, temperate forest; TROP, tropical forest; Appendix 3). Endophytes were isolated from fully surface-sterilized leaves or microphylls. Degree of surface sterilization for lichens is indicated for each isolate: Endo, isolated from the most stringently sterilized thallus pieces (treatments d and e; see Materials and Methods); all other treatments (a–c) are listed as water, 10 s, or 30 s corresponding to immersion times in 0.5% NaOCl and 70% ethanol. Thickened branches indicate $\geq 95\%$ posterior probability inferred from both MrBayes and BayesPhylogenies. If only one method provided significant support, values are shown below (MrBayes) or above branches (BayesPhylogenies). Pie charts indicate results of conservative ancestral state reconstruction for significantly supported nodes on 1000 Bayesian trees using maximum likelihood and show the proportion of reconstructions in which a given ancestral state was significantly supported. Pie charts are color coded in the inset with the following abbreviations: A = node not present (lightest), 0 = significantly reconstructed as not being the specified ecological mode (darker), 1 = significantly reconstructed as being the specified ecological mode (darkest), and E = reconstruction of ancestral state was equivocal (intermediate shade between 0 and 1). b) Relative diversification of fungal lineages as a function of the tree in A, indicating the number of lineages of euascomycetes (= Pezizomycotina; upper panel) and of endolichenic and endophytic versus lichen-forming euascomycetes (lower panel) over relative time. c) Trophic transition network showing directionality and frequency of conservatively inferred evolutionary transitions among extant and significantly reconstructed ancestral ecological modes in euascomycetes, as a function of the tree in A, indicating the transitions to and from symbiotrophic (lichen forming [L], pathogenic/parasitic [P], endolichenic [EL], endophytic [EP]) and saprotrophic (S) modes. Ratios indicate number of transitions observed from a given state:number of transitions to that state. Arrows are thickened in proportion to the number of transitions represented. See Supplementary Figure 1 for an alternative trophic transition network derived from a coding biased toward saprotrophism, that is, when all taxa marked by a brown box and the letter "S" (a) were coded as saprotrophic instead of nonsaprotrophic. Ratios of transitions, with coding biased toward saprotrophism, are shown in parentheses. d) Relative frequency of endophytes, endolichenic fungi, lichenicolous fungi, and lichen-forming mycobionts among major lineages of euascomycetes based on a review of available literature and consultations with experts (see Acknowledgments) for lichenicolous and lichen-forming fungi, and the distribution of functional taxonomic units inferred from ITS rDNA data for 1998 isolates of endophytic and endolichenic fungi recovered in this study. Proportions reflect the number of species with a given lifestyle within each lineage divided by the total number of species known to have that lifestyle (see Appendix 4). Hatched bars indicate taxonomic uncertainty with regard to the placement of orders *incertae sedis*.

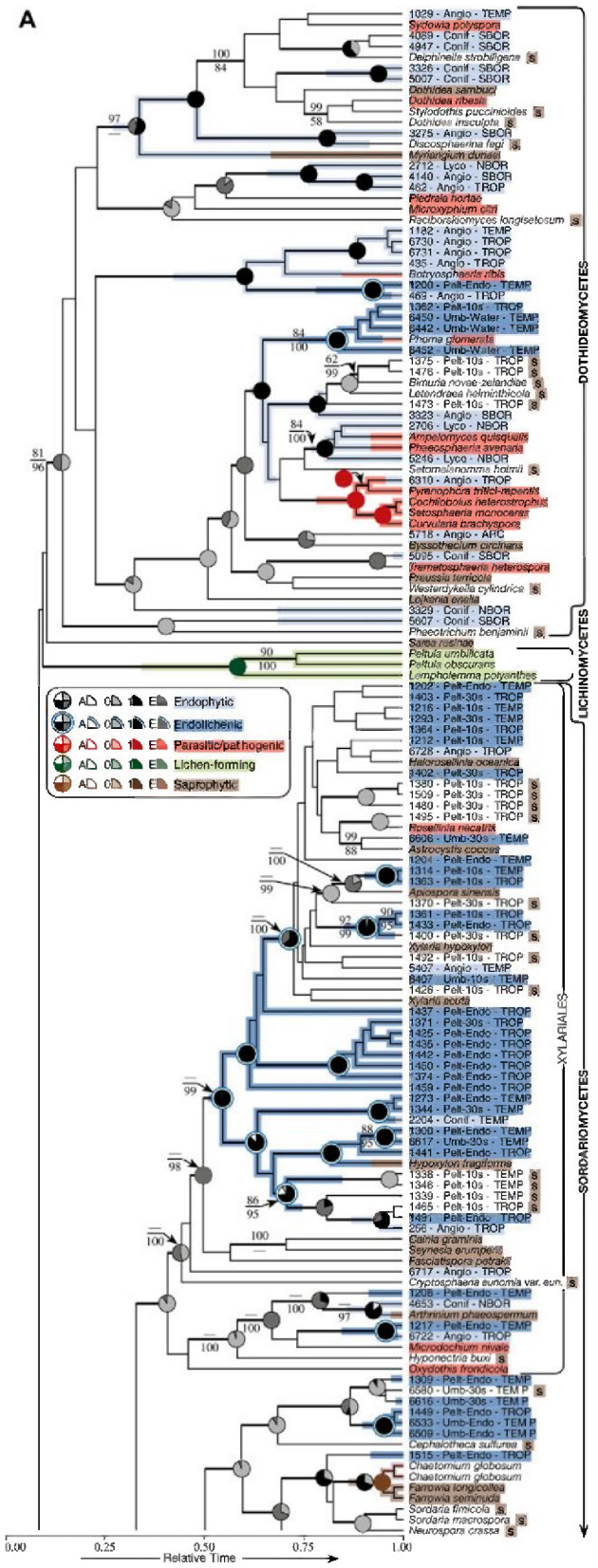
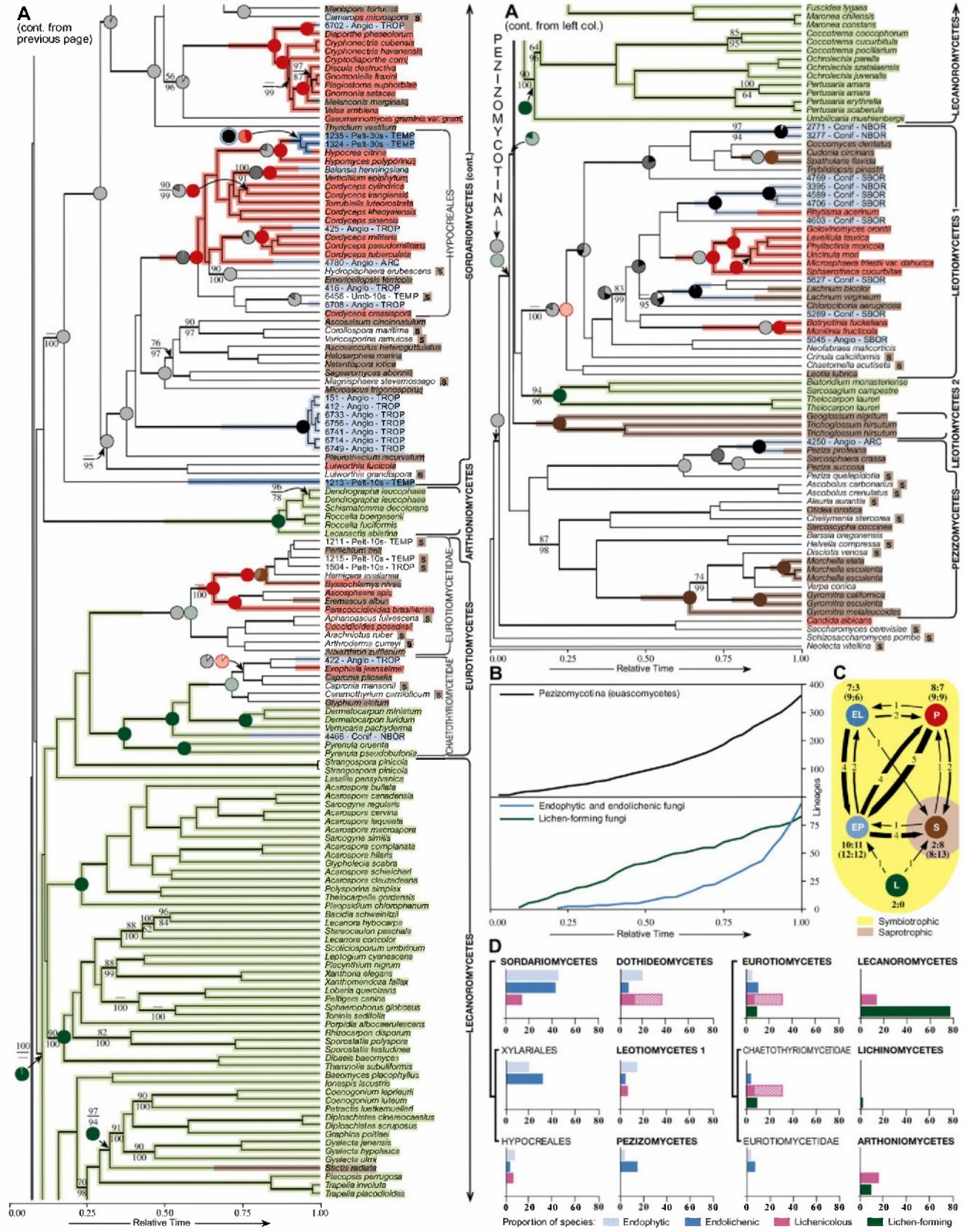


FIGURE 2. (Continued)


A
(cont. from previous page)
SORDARIOMYCETES (cont.)
HYPOCREALES
ARTHONIOMYCETES
EUROTIOMYCETES
EUROTIOMYCETIDAE
CHAETOTHYRIOMYCETIDAE
LECANOROMYCETES
Relative Time
A
(cont. from left col.)
PEZIZOMYCOTINA
LECANOROMYCETES
LEOTIOMYCETES 1
LEOTIOMYCETES 2
PEZIZOMYCETES
Relative Time
B
Pezizomycotina (euascomycetes)
Endophytic and endolichenic fungi
Lichen-forming fungi
Lineages
Relative Time
C
7:3 (9:6)
8:7 (9:9)
EL
P
EP
S
10:11 (12:12)
2:8 (8:13)
L
2:0
Symbiotrophic
Saprotrophic
D
SORDARIOMYCETES
DOTHIDEOMYCETES
EUROTIOMYCETES
LECANOROMYCETES
XYLARIALES
LEOTIOMYCETES 1
CHAETOTHYRIOMYCETIDAE
LICHINOMYCETES
HYPOCREALES
PEZIZOMYCETES
EUROTIOMYCETIDAE
ARTHONIOMYCETES
Proportion of species:
Endophytic
Endolichenic
Lichenicolous
Lichen-forming

Much like endophytes of plants, remarkably diverse fungi can be isolated readily from the interior of surface-sterilized lichen thalli (Fig. 1). Like endophytic fungi (Fig. 1a), these endolichenic fungi decrease linearly and rapidly in incidence and diversity from the tropics to the arctic (Fig. 1b, Table 1). Despite extensive sampling, taxon accumulation curves for endolichenic fungi remain nonasymptotic, resembling those for hyperdiverse endophytes (Fig. 1c). Fungi that shared $\geq 95\%$ ITS rDNA similarity with endophytic fungi were recovered frequently from the interior of lichen thalli but rarely from exposed thallus surfaces (Fig. 1d).

Localization of Endolichenic Fungi

Our surface sterilization methods suggested that endolichenic fungi occurred within, rather than upon, lichen thalli. Examination of thin cross-sections of 6 representative lichen thalli (*U. mammulata, P. neopolydactyla*) using scanning electron microscopy showed no evidence of entrapped spores within lichen thalli (data not shown). After dissection under sterile conditions, endolichenic fungi were isolated rarely from the mycobiont layers of the lichen thallus (i.e., medulla, cortices). Instead, culturable endolichenic fungi preferentially associated with the green algal photobiont, underscoring their apparent evolutionary and ecological similarity with endophytic fungi of green plants (Fig. 1e).

Phylogenetic Reconstructions

Using our broad sample of endophytic and lichen-associated fungi, we reconstructed the phylogenetic relationships and evolutionary history of ecological modes in the Ascomycota (Fig. 2a). Our analyses incorporated all data for the nuclear ribosomal small and large subunits (SSU rDNA, LSU rDNA) available for euascomycetes (= Pezizomycotina) and included 118 new isolates of endophytic, endolichenic, and lichen surface fungi associated with arctic, boreal, temperate, and tropical hosts.

We found that the capacity to form endophytic and endolichenic symbioses is widely distributed across the nonlichenized euascomycetes (Fig. 2a). There is little evidence for cocladogenesis of endophytic lineages with major lineages of land plants, in that fungi from lycophytes, conifers, and angiosperms are often intermixed with one another within and among well-supported clades.

Relative Age of Major Photobiont Symbioses in the Ascomycota

Relative to the lichen-forming Ascomycota (mycobionts), endophytic and endolichenic fungi originated later (minimum origin; Fig. 2b). Moreover, the rate of gain (39.45 ± 5.06) and loss (176.22 ± 17.86) of endophytic and endolichenic states significantly exceeds the rate of gain and loss for the lichen-forming state (gain, 0.93 ± 0.44; loss, 3.01 ± 1.15; $P < 0.0001$), revealing the greater evolutionary stability and persistence of the lichen symbiosis. Accordingly, endophytic and endolichenic Pezizomycotina are characterized by a more explosive rate of diversification than the lichen-forming lineages. Diversification of endophytic and endolichenic fungi parallels that of the euascomycetes as a whole and likely reflects the diversification of plants and lichens as substrates (Fig. 2b).

Together, these analyses suggest 2 fundamentally different patterns of diversification among photobiont-symbiotrophic euascomycetes: an evolutionarily ancient and canalized strategy (concentrated diversification) characterized by the lichen symbiosis and a recent and evolutionarily flexible strategy (phylogenetically diffuse diversification) characterized by endolichenic and endophytic fungi. The surprisingly linear and slightly convex diversification rate of lichen-forming fungi likely reflects 4 factors: 1) a relatively low extinction rate of lichen-forming fungi, suggested by the presence of many extant taxa representing ancient lichen-forming lineages (Fig. 2a); 2) land plant diversification, which provided new habitats for lichen diversification (e.g., muscicolous, epiphytic, foliicolous, and forest-associated lichens); 3) rare, ancient losses of the lichen symbiosis in the Stictidaceae, Eurotiomycetidae, and Chaetothyriales (Lutzoni et al. 2001), coupled with multiple recent losses of the lichen symbiosis through the colonization of other lichens (lichenicolous fungi) (Fig. 2d; Lawrey and Diederich 2003); and 4) taxon sampling, which was biased toward the inclusion of major lineages of lichen-forming fungi rather than capturing the species-level diversity of extant lichens.

Evolutionary Origins of Endophytism and Estimation of Major Trophic Transitions in the Ascomycota

Taking into account phylogenetic uncertainty (Pagel and Lutzoni 2002) and considering only those evolutionary transitions that were not sensitive to different coding criteria (see Materials and Methods), we conservatively estimated the number and direction of evolutionary transitions in major ecological modes among the euascomycetes (endophytism, endolichenism, lichen formation, parasitism/pathogenicity, and saprotrophy) (Fig. 2c). The resulting evolutionary trophic transition network provides first hypotheses regarding the evolution of symbiotrophic and saprotrophic lifestyles in the Pezizomycotina.

In contrast to previous expectations (see Clay and Schardl 2002; also see Spatafora et al. 2007), endophytic fungi were not consistently reconstructed as descendants of pathogenic ancestors. Transitions between endophytic and pathogenic states were most common, representing 9 of 29 conservatively estimated changes (31%); however, transitions in both directions occurred with similar frequency (Fig. 2c). Pathogen-to-endophyte transitions were especially common in the endophyte-rich Dothideomycetes and Sordariomycetes (Fig. 2a), which together contain the majority of ascomycetous plant pathogens (Berbee 2001). In several

clades, endophytism was reconstructed as ancestral to pathogenic species of ecological and economic significance (e.g., *Botryosphaeria ribis*, Dothideomycetes; *Rhytisma acerinum*, Leotiomycetes 1; Fig. 2a).

Similarly, endolichenic fungi were not consistently reconstructed as descendants of pathogenic ancestors (Fig. 2a,c). Instead, endolichenism appears to have served as an evolutionary source for transitions to parasitic/pathogenic, saprotrophic, and especially endophytic states, consistent with the hypothesis that lichens were readily available as hosts before land plants. Transitions from endolichenism were 1.5–2.3 times more common than transitions from other modes to endolichenism (Fig. 2c). Endolichenic-to-endophytic transitions occurred twice as frequently as transitions from endophytism to endolichenism (Fig. 2c).

We also found little evidence that endophytic and endolichenic fungi represent descendants of saprotrophic ancestors. Transitions from endophytic to saprotrophic states occurred 4 times more frequently than did saprotroph-to-endophyte transitions, and no saprotroph-to-endolichenic transitions were observed (Fig. 2c). In only one case was an endophyte reconstructed as descended from a saprotrophic ancestor: *Chaetomium globosum*, a ubiquitous bioactive fungus known as a pathogen, saprotroph, and endophyte (Fig. 2a; see Bharat et al. 2005).

When all transitions observed among the euascomycetes are considered, transitions to saprotrophy occurred 4.0 times as frequently as did transitions from saprotrophy to symbiotrophic states (Fig. 2a,c), suggesting that it is easier for euascomycetes infecting living tissue to become decomposers than vice versa. To ensure that this result was not an artifact of conservative coding for ecological modes, we coded all taxa with unknown ecological states as saprotrophic and then repeated the analysis (Supplementary Fig. 1). Transitions from symbiotrophy to saprotrophy remained 1.6 times more common than the reverse (Fig. 2c).

The evolutionary dynamism of endophytic and endolichenic fungi is underscored by numerous evolutionary host shifts in lineages containing these symbionts. For example, the mycoparasite *Hypocrea citrina* is part of a clade descended from an endolichenic ancestor (Fig. 2a). Similarly, the well-studied grass endophyte *B. henningsiana* (Hypocreales) appears to have descended from an insect-pathogenic or insect-parasitic ancestor (Fig. 2a), consistent with the observation that some entomopathogenic euascomycetes occur as endophytes in vascular plants (Arnold and Lewis 2005) and with the conclusions of Spatafora et al. (2007), who showed an evolutionary relationship among animal pathogens and endophytes in this clade.

Distinctiveness of Endolichenic Fungi Relative to Lichen-Forming Mycobionts and Lichenicolous Fungi

Despite the long-standing capacity of lichen-forming fungi to act as symbionts of photosynthetic organisms and the observation that more than 40% of the known Ascomycota species are lichen forming (Kirk et al. 2001), only one transition was observed from a lichen-forming (mycobiont) ancestor to a plant endophyte (endophyte 4466, Chaetothyriomycetidae; Fig. 2a,c). However, because the Eurotiomycetes include an unusual mixture of clades that are either 1) mostly nonlichenized or 2) mostly lichenized, additional discovery of nonlichenized Eurotiomycetes could shift the reconstruction of ancestral states from lichenized to nonlichenized for the deeper nodes within both this class and the Pezizomycotina in general. Therefore, further sampling could show that this transition is from a nonlichenized to an endophytic state rather than from a lichenized to an endophytic state, as suggested here.

We observed no transitions from lichenized ancestors to endolichenic species, and both endolichenic and endophytic fungi were rare to absent in the lichen-forming and secondarily nonlichenized clades (Fig. 2a). This disparity is confirmed by a larger sample encompassing all endophytic and endolichenic fungi recovered in our field surveys for which ITS rDNA sequence data were collected ($N = 1998$ isolates from 4 bioclimatic zones; Fig. 2d). Endophytic and endolichenic fungi are common among all major primary nonlichenized lineages of euascomycetes (Sordariomycetes, Dothideomycetes, Leotiomycetes, and Pezizomycetes) but are absent among the lichen-dominated clades (Lecanoromycetes, Arthoniomycetes, Lichinomycetes) and rare among the secondarily nonlichenized Eurotiomycetidae and Chaetothyriales (see Hibbett et al. [2007] for a detailed description of the classification of the fungi used here). In contrast, the majority of lichenicolous fungi are classified within lichen-dominated groups (e.g., Lecanoromycetes). Endolichenic fungi share with endophytes the habit of cryptic growth within an asymptomatic "host" (lichen thallus or plant) and therefore are distinct both taxonomically and ecologically from the approximately 1200 species previously recognized as lichenicolous fungi (Lawrey and Diederich 2003), as well as the approximately 13 500 species of mycobiont fungi that form lichen thalli (Kirk et al. 2001; Lutzoni et al. 2001).

Discussion

In this study, we isolated endophytes from the interior of asymptomatic photosynthetic tissues of bryophytes, lycophytes, ferns, conifers, and angiosperms sampled from 4 bioclimatic zones. This effort yielded the largest, most geographically extensive, and most taxonomically broad sample of previously unknown endophytic fungi to date. We recovered endophyte-like fungi (endolichenic fungi) associated with ecologically and phylogenetically distinct lichens along the same latitudinal gradient and found that these endolichenic fungi associate closely with the algal photobiont in lichen thalli. We characterized the diversity of these symbiotic communities using a fast-evolving molecular marker (ITS rDNA) and then used multiple loci to integrate these newly recovered fungi into the Ascomycota tree

of life. The result provides a first opportunity to examine the evolutionary origins of endophytism and other major ecological modes in the Ascomycota and to estimate the number and direction of major trophic transitions across the most diverse fungal phylum. Our study complements a recent effort examining the diversity of endophytic fungi from the tropics to the arctic (Arnold and Lutzoni 2007) by providing an explicit comparison with endolichenic fungi in a subset of sites sampled for both groups of symbionts, and for the first time offers a phylogenetic characterization of these little-known associates of lichens and plants.

Our results indicate that the euascomycetes symbiotic with photosynthetic organisms such as plants and lichen photobionts demonstrate a remarkable degree of evolutionary dynamism. Our analyses illustrate the importance of including novel fungi in studies examining phylogenetic relationships and ancestral states and underscore the need for further sampling (1) of novel fungi to test the conclusions suggested here and (2) of additional loci that will provide greater certainty regarding the phylogenetic relationships and ancestral states at deeper nodes. Our study also illuminates the challenge inherent in classifying the ecological modes of fungi, many of which (e.g., *C. globosum*) may have more than one ecological role. In inferring ancestral states and associated evolutionary transitions, we took this "ecological uncertainty" into account by performing multiple reconstructions with different codings for the ecological modes of terminal taxa and presented only those results that were consistent regardless of variable coding.

Although our data represent only the "tip of the iceberg" of diversity encompassed by endophytic fungi, endolichenic fungi, and the Ascomycota as a whole (see Fig. 1, Appendix 4), our preliminary results provide the basis for a series of testable hypotheses regarding the evolution of major ecological modes in the Pezizomycotina. In particular, our data highlight for the first time the previously unknown but important role of lichens in the diversification of the biotrophic Pezizomycotina. In addition to highly diverse lichen mycobionts (Kirk et al. 2001; Lutzoni et al. 2001) and lichenicolous fungi (Lawrey and Diederich 2003), lichen thalli harbor highly diverse endolichenic fungi that represent an evolutionary incubator for transitions to endophytic associations in plants. In turn, our analyses suggest that endophytism represents an evolutionarily dynamic state, with frequent transitions to and from pathogenicity.

Although transitions to saprotrophy also occur frequently across the Ascomycota, reversions from saprotrophy to symbiotrophic lifestyles (i.e., endophytism, endolichenism, pathogenicity, and lichenization) are rare, even when coding of terminal taxa is strongly biased to overrepresent saprotrophic states. This result is surprising in light of a recent study reporting that the ancestor of the Pezizomycotina was saprotrophic (James et al. 2006). However, that study did not take into account cryptic endophytic and endolichenic fungi. Moreover, our results suggest a key distinction between the euascomycetes associated with aboveground tissues and the ectomycorrhizal Basidiomycota, which may have undergone frequent transitions to and from saprotrophism over evolutionary time (Hibbett et al. 2000; but see also Bruns & Shefferson 2004). More frequent saprotroph-to-symbiotroph transitions are likely more common among the mycorrhizal Pezizomycotina, which—like the ectomycorrhizal Basidiomycota—would have access to alternative substrates such as organic debris in the rhizosphere (Hibbett et al. 2000).

Because endophytic and endolichenic fungi do not produce reproductive structures on living hosts, their generation times are tied directly to the life span of the substrate they inhabit. Long-lived leaves and lichens would constrain endophytic and endolichenic symbionts to longer generation times relative to saprotrophs, possibly providing a strong selective pressure against transitions or reversions to symbiosis. In turn, the apparent contradiction between long generation times and hyperdiversity of endophytic and endolichenic fungi can be resolved by the evolutionary flexibility of these symbiotrophic states. Transitions from endolichenism to endophytism would be favored given the shorter generation times of leaves (< 1–15 years) relative to long-lived lichen thalli (up to > 100 years). Endophytism could remain a viable strategy if endophytes act as ecological opportunists that form pathogenic (actively reproductive) infections in susceptible hosts while persisting as nonvirulent symbionts in other hosts. Despite extensive sampling of endophyte diversity (1368 endophyte strains), however, our BLAST comparisons never encountered an isolate with an ITS rDNA genotype identical to a known pathogen (see also Ganley et al. 2004). An exciting direction for future work lies in understanding the degree to which individual fungal genotypes are ecologically labile and in identifying the evolutionary drivers—including gene regulation, endohyphal bacterial endosymbionts, or host-specific responses—that underlie transitions in ecological modes.

Regardless of the evolutionary stability or age of associations, large-scale diversification in Fungi is consistently associated with the establishment of symbioses (Hibbett et al. 2000; Berbee 2001; Lutzoni et al. 2001, 2004; Lawrey and Diederich 2003; Taylor et al. 2004; James et al. 2006). Diversification of the Glomeromycota reflects the arbuscular mycorrhizal symbiosis (Taylor et al. 2004), and the diverse Agaricomycotina (hymenomycetes, Basidiomycota) reflect in large part the evolutionarily dynamic ectomycorrhizal symbiosis (Hibbett et al. 2000). Diversification in the Ascomycota, the most species-rich lineage of Fungi, reflects the dual symbiotic strategies of lichenization and lichenicolous associations (mostly Lecanoromycetes, Eurotiomycetes, Lichinomycetes, and Arthoniomycetes; Lutzoni et al. 2001) as well as the evolutionarily flexible symbioses embodied by endophytism and endolichenism (Dothideomycetes, Sordariomycetes, Leotiomycetes, and likely the Pezizomycetes [Arnold A.E., unpublished data]). Together,

these strategies correspond to 2 distinctive potential pathways leading to the evolution of pathogenicity in the euascomycetes: 1) endolichenic to endophytic to pathogenic and 2) lichen forming to pathogenic on hosts other than lichens, via lichenicolous lichens and lichenicolous fungi (Lutzoni et al. 2001). Both pathways involve the lichen symbiosis and define functional groups of euascomycete pathogens with distinct evolutionary histories.

Understanding the structure of the Ascomycota tree of life requires inclusion of the cryptic symbionts from leaves and lichens. In turn, understanding the evolution of virulence and mutualism, the diversity and evolution of important secondary metabolites (e.g., Dreyfuss and Chapela 1994; Bharat et al. 2005), and the scale of biodiversity in this economically and ecologically important phylum cannot be achieved without attention to the extremely diverse and largely unexplored endolichenic and endophytic fungi.

Supplementary Material

Appendices 1–4 and Supplementary Figure 1 can be found at http://www.sysbio.oxfordjournals.org/.

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References

Alfaro M.E., Zoller S., Lutzoni F. 2003. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Mol. Biol. Evol. 20:255–266.

Arnold A.E. 2007. Understanding the diversity of foliar fungal endophytes: progress, challenges, and frontiers. Fungal Biology Reviews 21:51–66.

Arnold A.E., Engelbrecht B.M.J. 2007. Fungal endophytes nearly double minimum leaf conductance in seedlings of a neotropical tree species. J. Trop. Ecol. 23:369–372.

Arnold A.E., Henk D.A., Eells R.L., 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 A.E., Lewis L.C. 2005. Ecology and evolution of fungal endophytes and their roles against insects. In: Vega F., Blackwell M., editors. Insect-fungal associations: ecology and evolution. Oxford: Oxford University Press. p. 74–96.

Arnold A.E., Lutzoni F. 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology. 88:541–549.

Arnold A.E., Maynard Z., Gilbert G.S., Coley P.D., Kursar T.A. 2000. Are tropical fungal endophytes hyperdiverse? Ecol. Lett. 3:267–274.

Arnold A.E., Mejia L., Kyllo D., Rojas E., Maynard Z., Robbins N., Herre E.A. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc. Natl. Acad. Sci. USA. 100:15649–15654.

Berbee M.L. 2001. The phylogeny of plant and animal pathogens in the Ascomycota. Physiol. Mol. Plant Pathol. 59:165–187.

Bharat P.B., Wijeratne M.K., Faeth S.H., Gunatilaka A.A.L. 2005. Globosumones A-C, cytotoxic orsellinic acid esters from the Sonoran Desert endophytic fungus *Chaetomium globosum*. J. Nat. Prod. 68:724–728.

Bruns T., Shefferson R.P. 2004. Evolutionary studies of ectomycorrhizal fungi: milestones and future directions. Can. J. Bot. 82:1122–1132.

Cannone K.M., Subramanian S., Schnare M., Collett J.R., D'Souza L.M., Du Y., Feng B., Lin N., Madabusi L.V., Müller K.M., Pande N., Shang Z.D., Yu N., Guttell R.R. 2002. The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinformatics. 3:2.

Carroll G.C. 1999. The foraging ascomycete [abstract]. In: 16th International Botanical Congress. St Louis (MO). p. 309.

Clay K., Holah J. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. Science 285:1742–1744.

Clay K., Schardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat. 160: S99–S127.

Colwell R.K. 2004. EstimateS: statistical estimation of species richness and shared species from samples, version 7 [Internet]. Available from http://viceroy.eeb.uconn.edu/EstimateS.

Davis E.C., Franklin J.B., Shaw A.J., Vilgalys R. 2003. Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: phylogenetics, distribution, and symbiosis. Am. J. Bot. 90:1661–1667.

Douady C.J., Delsuc F., Boucher Y., Doolittle W.F., Douzery E.J.P. 2003. Comparison of the Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol. Biol. Evol. 20: 248–254.

Dreyfuss M.M., Chapela I. 1994. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In: Gullo V.P., editor. The discovery of natural products with therapeutic potential. Boston (MA): Butterworth-Heinman. p. 49–80.

Ewing B., Wendl L.C., Green P. 1998. Base-calling of automated sequencer traces using phred I: Accuracy assessment. Genome Res. 8:186–194.

Fröhlich J., Hyde K.D. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? Biodiv. Conserv. 8:977–1004.

Ganley R.J., Brunsfeld S.J., Newcombe G. 2004. A community of unknown, endophytic fungi in western white pine. Proc. Natl. Acad. Sci. USA. 101:10107–10112.

Girlanda M., Isocrono D., Bianco C., Luppi-Mosca A.M. 1997. Two foliose lichens as microfungal ecological niches. Mycologia. 89: 531–536.

Green P. 1996. phrap [Internet]. Available from http://www.genome.washington.edu/.

Hawksworth D.L. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol. Res. 105:1422–1432.

Hawksworth D.L., Rossman A.Y. 1997. Where are all the undescribed fungi? Phytopath. 87:888–891.

Heckman D.S., Geiser D.M., Eidell B.R., Stauffer R.L., Kardos N.L., Hedges S.B. 2001. Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129–1133.

Hibbett D.S., Binder M., Bischoff J.F., Blackwell M., Cannon P.F., Eriksson O., Huhndorf S., James T., Kirk P.M., Lücking R., Lumbsch T., Lutzoni F., Matheny P.B., McLaughlin D.J., Powell M.J., Redhead S., Schoch C.L., Spatafora J.W., Stalpers J.A., Vilgalys R.,

Aime M.C., Aptroot A., Bauer R., Begerow D., Benny G.L., Castlebury L.A., Crous P.W., Dai Y.-C., Gams W., Geiser D.M., Griffith G.W., Gueidan C., Hawksworth D.L., Hestmark G., Hosaka K., Humber R.A., Hyde K., Koljalg U., Kurtzman C.P., Larsson K.H., Lichtward R., Longcore J., Miadlikowska J., Miller A., Monclavo J.-M., Mozley-Standridge S., Oberwinkler F., Parmasto E., Reeb V., Rogers J.D., Roux C., Ryvarden L., Sampaio J.P., Schuessler A., Sugiyama J., Thorn R.G., Tibell L., Untereiner W.A., Walker C., Wang Z., Weir A., Weiss M., White M., Winka K., Yao Y.-J., Zhang N. 2007. A higher-level phylogenetic classification of the Fungi. Mycol. Res. 111:509–547.

Hibbett D.S., Gilbert L.-B., Donoghue M.J. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. Nature. 407:506–508.

Higgins K.L., Arnold A.E., Miadlikowska J.M., Sarvate S.D., Lutzoni F. 2007. Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. Mol. Phylogenet. Evol. 42:543–555.

Hoffman M., Arnold A.E. 2008. Geography and host identity interact to shape communities of endophytic fungi in cupressaceous trees. Mycol. Res. 112:331–344.

Huelsenbeck J.P., Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 17:754–755.

Jackson V. 2004. LASRDisc: likelihood ancestral state reconstruction for discrete characters, v. 1.01 [Internet]. Available from http://ceb.csit.fsu.edu/lasrdisc.

James T.Y., Kauff F., Schoch C.L., Matheny P.B., Hofstetter V., Cox C.J., Celio G., Gueidan C., Fraker E., Miadlikowska J., Lumbsch H.T., Rauhut A., Reeb V., Arnold A.E., Amtoft A., Stajich J.E., Hosaka K., Sung G.-H., Johnson D., O'Rouke B., Crockett M., Binder M., Curtis J.M., Slot J.C., Wang Z., Wilson A.W., Schüßler A., Longcore J.E., O'Donnell K., Mozley-Standridge S., Porter D., Letcher P.M., Powell M.J., Taylor J.W., White M.M., Griffith G.W., Davies D.R., Humber R.A., Morton J.B., Sugiyama J., Rossman A.Y., Rogers J.D., Pfister D.H., Hewitt D., Hansen K., Hambleton S., Shoemaker R.A., Kohlmeyer J., Volkmann-Kohlmeyer B., Spotts R.A., Serdani M., Crous P.W., Hughes K.W., Matsuura K., Langer E., Langer G., Untereiner W.A., Lücking R., Büdel B., Geiser D.M., Aptroot A., Diederich P., SchmittI., Schultz M., Yahr R., Hibbett D.S., Lutzoni F., McLaughlin D.J., Spatafora J.W., Vilgalys R. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature. 443:818–822.

Kauff F., Cox C.J., Lutzoni F. 2007. WASABI: an automated sequence processing system for multi-gene phylogenies. Syst. Biol. 56: 523–531.

Kirk P.M., Cannon P.F., David J.C., Stalpers J.A. 2001. Dictionary of the Fungi. 9th ed. Wallingford, UK: CABI.

Kjer K.M. 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. Mol. Phylogenet. Evol. 4: 314–330.

Lawrey J.D., Diederich P. 2003. Lichenicolous fungi: interactions, evolution, and biodiversity. Bryologist. 106:80–120.

Leigh E.G. Jr. 1999. Tropical forest ecology. Oxford: Oxford University Press.

Lewis P.O., Holder M.T., Holsinger K.E. 2005. Polytomies and Bayesian phylogenetic inference. Syst. Biol. 54:241–253.

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.

Lodge D.J., Fisher P.J., Sutton B.C. 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. Mycologia. 88:733–738.

Lutzoni F., Kauff F., Cox C.J., McLaughlin D., Celio G., Dentinger B., Padamsee M., Hibbett D., James T.Y., Baloch E., Grube M., Reeb V., Hofstetter V., Schoch C., Arnold A.E., Miadlikowska J., Spatafora J., Johnson D., Hambleton S., Crockett M., Shoemaker R., Sung G.-H., Lucking R., Lumbsch T., O'Donnell K., Binder M., Diederich P., Ertz D., Gueidan C., Hansen K., Harris R.C., Hosaka K., Lim Y.-W., Matheny B., Nishida H., Pfister D., Rogers J., Rossman A., Schmitt I., Sipman H., Stone J., Sugiyama J., Yahr R., Vilgalys R. 2004. Assembling the fungal tree of life: progress, classification, and the evolution of subcellular traits. Am. J. Bot. 91: 1446–1480.

Lutzoni F., Pagel M., Reeb V. 2001. Major fungal lineages are derived from lichen symbiotic ancestors. Nature. 411:937–940.

Lutzoni F., Wagner P., Reeb V., Zoller S. 2000. Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. Syst. Biol. 49:628–651.

Maddison D.R., Maddison W.P. 2003. MacClade: analysis of phylogeny and character evolution, v. 4.07. Sunderland (MA): Sinauer.

Maddison W.P., Maddison D.R. 2004. Mesquite: a modular system for evolutionary analysis, v. 1.05 [Internet]. Available from http://mesquiteproject.org.

Malloch D.W., Pirozynski K.A., Raven P.H. 1980. Ecological and evolutionary significance of mycorrhizal symbioses in vascular plants (a review). Proc. Natl. Acad. Sci. USA. 77: 2113–2118.

Mason-Gamer K.M., Kellogg E.A. 1996. Testing for phylogenetic conflict among molecular data sets in the tribe Triticeae (Gramineae). Syst. Biol. 45:524–545.

Mejía L.C., Rojas E.I., Maynard Z., Arnold A.E., Van Bael S., Samuels G.J., Robbins N., Herre E.A. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol. Cont. 46:4–14.

Pagel M. 1999. The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. Syst. Biol. 48:612–622.

Pagel M., Lutzoni F. 2002. Accounting for phylogenetic uncertainty in comparative studies of evolution and adaptation. In: Lässig M., Valleriani A., editors. Biological evolution and statistical physics. New York: Springer. p. 148–161.

Pagel M., Meade A. 2005. Mixture models in phylogenetic inference. In: Gascuel O., editor. Mathematics of evolution and phylogeny. Oxford: Oxford University Press. p. 121–142.

Pagel M.A., Meade A.A. 2004. Bayesian estimation of ancestral character states on phylogenies. Syst. Biol. 53:571–581.

Petrini O., Hake U., Dreyfuss M.M. 1990. An analysis of fungal communities isolated from fruticose lichens. Mycologia. 82:444–451.

Posada D., Crandall K.A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics. 14:817–818.

Redman R.S., Sheehan K.B., Stout R.G., Rodriguez R.J., Henson J.M. 2002. Thermotolerance generated by plant/fungal symbiosis. Science. 298:1581.

Reeb V., Lutzoni F., Roux C. 2004. Contribution of *RPB*2 to multilocus phylogenetic studies of the Pezizomycotina (euascomycetes, Fungi) with special emphasis on the lichen-forming Acarosporaceae and evolution of polyspory. Mol. Phylogenet. Evol. 32: 1036–1060.

Rodriguez F., Oliver J.L., Marin A., Medina J.R. 1990. The general stochastic model of nucleotide substitution. J. Theoret. Biol. 142:485–501.

Saikkonen K., Wäli P., Helander M., Faeth S.H. 2004. Evolution of endophyte-plant symbioses. Trends Plant Sci. 9:275–280.

Sanderson M.J. 2004. r8s, v. 1.70 [Internet]. Available from http://ginger.ucdavis.edu/r8s.

Schulz B., Boyle C. 2005. The endophytic continuum. Mycol. Res. 109:661–686.

Selosse M.-A., LeTacon F. 1998. The land flora: a phototroph-fungus partnership? Trends Ecol. Evol. 13:15–20.

Spatafora J.W., Sung G.-H., Johnson D., Hesse C., O'Rourke B., Serdani M., Spotts R., Lutzoni F., Hofstetter V., Miadlikwoska J., Reeb V., Gueidan C., Fraker E., Lumbsch T., Lücking R., Schmitt I., Hosaka K., Aptroot A., Roux C., Miller A.N., Geiser D.M., Hafellner J., Hestmark G., Arnold A.E., Büdel B., Rauhut A., Hewitt D., Untereiner W.A., Cole M.S., Scheidegger C., Schultz M., Sipman H., Schoch C.L. 2006. A five-gene phylogeny of the Pezizomycotina. Mycologia. 98:1018–1028.

Spatafora J.W., Sung G.-H., Sung J.-M., Hywel-Jones N.L., White J.F. Jr. 2007. Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. Mol. Ecol. 16:1701–1711.

Stanosz G.R., Smith D.R., Guthmiller M.A., Stanosz J.C. 1997. Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. Mycologia. 89:525–530.

Stone J.K., Bacon C.W., White J.F. Jr. 2000. An overview of endophytic microbes: endophytism defined. In: Bacon C.W., White J.F.,

editors. Microbial endophytes. New York: Marcel Dekker. p. 3–29.

Suryanarayanan T.S., Thirunavukkarasu G.N., Hariharan G.N., Balaji P. 2005. Occurrence of non-obligate microfungi inside lichen thalli. Sydowia. 57:120–130.

Swofford D.L. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland (MA): Sinauer Associates.

Taylor J.W., Spatafora J., O'Donnell K., Lutzoni F., James T., Hibbett D.S., Geiser D., Bruns T.D., Blackwell M. 2004. The fungi. In: Cracraft J., Donoghue M.J., editors. Assembling the tree of life. Oxford: Oxford University Press. p. 171–194.

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