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## Review

# Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers

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### ABSTRACT

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Fungal endophytes, a diverse group of primarily ascomycetous fungi defined functionally by their occurrence within asymptomatic photosynthetic tissues of plants, occur in all major lineages of land plants and in natural and anthropogenic communities ranging from the arctic to the tropics. Because of the tremendous diversity they encompass, ecological questions regarding the interactions of endophytes with the plants in which they live - and with other organisms that in turn interact with endophyte-plants symbiota - are difficult to address. The goals of this review are to highlight progress, challenges, and frontiers in the study of foliar endophyte diversity, with the ultimate goal of encouraging research that both bridges the gap between, and advances, research in alpha taxonomy and ecology. I focus on four themes that are reflected in the recent and rapidly expanding literature regarding endophyte biology: (1) the taxonomic and ecological distinctiveness of endophytes relative to other nonpathogenic plant-associated fungi; (2) the insights that can be gained from studies that consider genotypes as the relevant unit of biological organization, especially in the context of traditional species-level taxonomy and robust phylogenetic methods that tie these genotypes and species together in an explicit evolutionary context; (3) the context-dependency of endophyte communities, highlighting the importance of both the identify of host plants and the geographic location in which plants occur; and (4) the complexity of the endophyte-pathogen-saprotroph continuum, and the challenges and exciting frontiers that lie in understanding the evolutionary relationships and ecological lability of fungi that exhibit these ecological modes. I argue that never before has the study of endophytic fungi been more exciting or more tractable, and that the potential for endophyte researchers to inform diverse areas of biology has never been greater.

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## 1. Introduction

Endophytic fungi, a polyphyletic group of highly diverse, primarily ascomycetous fungi defined functionally by their occurrence within asymptomatic tissues of plants, are found

in above-ground tissues of liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns, and seed plants from the arctic to the tropics, and from agricultural fields to the most biotically diverse tropical forests. Their cryptic lifestyle, ubiquity, and richness within individual plants, coupled with emerging

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evidence of their often-overlooked ecological importance, have inspired growing enthusiasm regarding these little-known fungi over the past four decades. In particular, David Hawksworth's much-discussed estimates of fungal diversity at a global scale (Hawksworth 1991, 2001) engendered tremendous enthusiasm for understanding endophyte diversity. Since 1991, a growing number of surveys – built on the pioneering work of Carroll (e.g., Carroll & Carroll 1978; Carroll 1986, 1988), Fisher (e.g., Fisher et al. 1986; Fisher & Petrini 1990), Petrini (e.g., Petrini & Müller 1979; Petrini et al. 1979; Petrini et al. 1982; Petrini 1985, 1986, 1991), Sieber (Sieber 1989), Stone (e.g., Stone 1986), and others (e.g., Bloomberg 1966; Espinosa-Garcia & Langenheim 1990; Johnson & Whitney 1989; Legault et al. 1989a,b; Rodrigues & Samuels 1990; Spurr & Welty 1975) – have generated a great deal of interest regarding the contributions of these cryptic microfungi to fungal systematics, plant and fungal ecology, evolutionary biology, and applied research ranging from biological control to bioprospecting. This interest is manifested in journal publications focusing on the abundance and/or diversity of fungal endophytes associated with above-ground tissues of non-grass hosts, which have increased in frequency from ca. 1.2 papers/yr (1971-1990) to >15 papers/yr (2001-early 2007; Web of Science search, February 2007). These totals include only English-language papers and exclude numerous outstanding contributions in edited volumes, theses, dissertations, and published abstracts (e.g., Stone 1986; Petrini 1986; Carroll 1991; Stone et al. 2000) as well as review papers (e.g., Saikkonen et al. 1998; Saikkonen et al. 2004).

Concomitant with the explosion of research on endophyte diversity has been a rapid increase in work focusing on interactions with host plants, ultrastructural and colonization studies, characterization of novel metabolites, and other emergent topics related to endophytic symbioses (from 0.8 papers/yr in the 1970s to >200 papers/yr over the past six years). This body of literature has highlighted the ways in which endophytes associated with foliage of most plants differ taxonomically, ecologically, and in terms of diversity relative to the special clavicipitaceous endophytes of grasses (reviewed by Saikkonen et al. 1998; Clay & Schardl 2002). Together, these studies have established the prevalence of horizontal transmission (Arnold & Herre 2003), the timelines that underlie colonization of new leaves (Herre et al. 2007), the phylogenetic relationships of endophytes (Higgins et al. 2007), their sensitivity to environmental perturbations (Helander et al. 1993; Lappalainen et al. 1999), their biochemical diversity (Strobel & Daisy 2003; Gunatilaka 2006; Zhang et al. 2006), and the previously overlooked ways in which they influence the ecological phenotypes of the plants they inhabit. For example, Pinto et al. (2000) showed that symptomless infections by *Colletotrichum musae* and *Fusarium moniliforme* decrease photosynthetic efficiency in maize and banana. Bing and Lewis (1991) showed that the entomopathogen *Beauveria bassiana* can grow as an endophyte and protect maize against an important herbivore (European corn borer, *Ostrinia nubilalis*). Redman et al. (2002) showed that a horizontally transmitted endophyte can provide thermal tolerance for plants. Arnold et al. (2003) showed that diverse assemblages of endophytes decrease lesion formation and leaf death due to *Phytophthora* sp. in the chocolate tree, *Theobroma cacao*, whereas Arnold

and Engelbrecht (2007) demonstrated that seedlings of that species lose water more quickly under severe drought when endophytes are present. The subtlety of these interactions is notable: plants typically show no observable change in growth rate, biomass accumulation, root:shoot ratio, or other easily quantifiable characteristics following inoculation (Arnold 2002). Yet when plants are challenged by biotic or abiotic stressors, the importance of endophytes becomes clear. Together, these studies represent the tip of a very large iceberg: only a tiny fraction of plant-endophyte symbioses has been assessed to understand the costs and benefits to hosts of harboring these fungi in their photosynthetic tissues.

One of the exciting and challenging aspects of endophyte ecology lies in understanding those costs and benefits in the context of the nearly bewildering diversity of foliar endophytes. A powerful tool in this endeavor consists of placing these diverse endophytic associations in an evolutionary and ecological context (Thompson 2005). It has long been recognized that the outcomes of microbe/host interactions can be influenced by the genetic diversity of symbionts, the ways in which they are acquired from the environment, their ability to co-colonize individual hosts, their direct and indirect interactions, and their own evolutionary history, encapsulated by the genomic architecture associated with pathogenicity or other ecological modes (see Herre et al. 1999; Loftus et al. 2005; Maynard-Smith & Szathmary 1997). Inasmuch as recent works have shown that there are few golden rules for predicting the directionality of species interactions along the mutualist-parasite continuum, it has become clear that understanding the context of those interactions is critical for interpreting their ecological and evolutionary significance (Agrawal et al. 2007). An important aspect of that context lies in understanding the evolutionary origins of symbioses. Steps in this direction are being taken with large-scale phylogenetic analyses of endophytes (Arnold et al. 2007b). More fundamentally, however, knowledge is still needed regarding endophyte diversity at the levels of genotype, species, clades, and function: despite an accumulation of numerous studies, we still lack a comprehensive understanding of the diversity of fungi capable of forming endophytic symbioses with plants in any given ecosystem.

The goals of this paper are to highlight progress, challenges, and frontiers in the study of foliar endophyte diversity. Far from an academic question, understanding endophyte diversity has implications for ecologists seeking to understand basic aspects of plant-fungal associations; agriculturalists seeking to apply endophytes in biological control programmes or exploring the genes or genomic features that underlie pathogenicity; bioprospectors searching for efficient methods to uncover novel secondary metabolites; mycologists interested in elucidating the structure of the fungal tree of life; and evolutionary biologists seeking to understand the evolutionary origins, stability, and future of species interactions.

Although only a small portion of the growing literature on endophytes can be reviewed here, several emergent properties of endophyte communities are becoming clear, with major implications for understanding fungal diversity at levels from the genotype to ecological function. This review focuses on four central theses. First, foliar endophytes are largely distinct from other nonpathogenic, plant-associated fungi. In some cases, leaf endophytes represent or are dominated by different

major lineages of Ascomycota (e.g., compared to endo-, ecto-, and ectendomycorrhizal fungi); in other cases, they represent distinctive species or genotypes whose close relatives colonize roots or other tissues. Second, the relevant level of biological organization for estimating the diversity and specificity of endophyte communities may lie below the species level – that is, at the level of genotype – and will also be informed by phylogenetic studies that provide an evolutionary perspective at the family and ordinal level. Third, foliar endophyte communities are shaped both by the identity of host plants and the geographic location in which those plants occur. Communities typically include a mix of common and often generalist species, and rare species whose host specificity and geographic structure are difficult to assess. One major challenge lies in disentangling rarity and specificity; another lies in understanding the evolutionary implications of context-dependent endophyte assemblages. Fourth, some foliar endophytes may also act as pathogens and saprotrophs, in some cases attacking or decaying hosts with which they do not form endophytic associations. However, the frequency and nature of overlap among these communities is not yet clear, with inferences currently limited by methodological issues and by the ecological lability of many fungi. Finally, it is clear that the foundation provided by earlier studies, combined with recent technological and methodological advances and the increased accessibility of distinctive biomes, place us at the cusp of significant steps forward in our understanding of endophyte biology. In the final section of this paper, I highlight several frontiers in elucidating the diversity – and thus the ecological and evolutionary context – of endophytic symbioses.

## 2. The distinctiveness of foliar endophytic fungi relative to other avirulent plant associates

### Foliar endophytic and root-inhabiting communities

Leaves and roots represent the most dynamic interfaces between plants and their environment. Fungi that inhabit these biologically active tissues may share characteristics that allow them to grow and persist in an ever-changing biochemical milieu, or in the context of rapidly changing gene expression as host tissues grow and age. At the same time, fungi colonize roots in a context fundamentally distinct from fungi in the phyllosphere: whereas rhizosphere fungi occur in a matrix that, in many cases, contains moisture, organic material, and low to no light, foliar endophytes move about in large part on air currents, and must germinate and penetrate foliage while coping with desiccation, UV irradiance, vibration, and a lack of a surrounding matrix containing potentially useful nutrients (Juniper 1991). How have major lineages, species, or genotypes of fungi partitioned plant tissues such as roots and leaves in the evolution of avirulent plant-fungus associations? Given the prevalence of Ascomycota among foliar endophytes, I will focus on that phylum, which also includes mycorrhizal, dark-septate, and other root-colonizing fungi.

Mycorrhizal Ascomycota – including ericoid, ecto-, and ectendomycorrhizal fungi – are ecologically important associates of diverse plants from the tropics to high latitudes (Grimaldi et al. 2005; Egger 2006). In the absence of explicit

comparisons of mycorrhizal and foliar communities in the same plants, circumstantial evidence indicates that mycorrhizal Ascomycota are both taxonomically distinct from foliar endophytes at lower taxonomic levels (genera, species), and are dominated by different major lineages of Ascomycota relative to foliar endophytic fungi.

The mycorrhizal habit is especially well represented among the Pezizales (Pezizomycetes), including species of *Geopyxis*, *Helvella*, *Terfezia*, *Tuber* and others (Tedersoo et al. 2006). Pezizalean endophytes occur with some frequency at higher latitudes and at high altitudes in the temperate zone, but rarely dominate foliar endophytic communities (Higgins et al. 2007; Arnold & Lutzoni 2007). The commonly recognized mycorrhizal genera are not known from leaves, which instead harbor endophytes with taxonomic affinities for free-living species of *Peziza* and *Aleuria* (Higgins et al. 2007).

Similarly, helotialean fungi (Leotiomycetes) such as the well-studied *Hymenoscyphus* (*Rhizoscyphus*) *ericae* aggregate are common as mycorrhizal fungi (Vrålstad et al. 2002). Long recognized for its ability to form ericoid mycorrhizae, *H. ericae* also can establish ectomycorrhizal associations with Pinaceae and some angiosperms, indicating flexibility in its symbiotic phenotype (Egger 2006). However, neither *Hymenoscyphus* nor other known leotiomycetous mycorrhizal fungi have been recovered as leaf endophytes, despite the prevalence of Helotiales and the related rhytismatalean fungi in foliage of Pinaceae and other hosts (e.g., Ganley & Newcombe 2006; Stefani & Berube 2006; Arnold et al. 2007a).

The recent description of *Pseudotulostoma volvata* (Elaphomycetales; Henkel et al. 2006) and previous records of mycorrhizal Onygenales (*Myxotrichum*, *Gmnyascella*, and *Pseudogymnoascus*; see Monreal et al. 1999) confirm that some Eurotiomycetes form mycorrhizal symbioses, but records of mycorrhizal and endophytic fungi within this class are relatively few. Jumpponen (2003) recorded ectomycorrhizal Chaetothiales along a receding glacial front and Allen et al. (2003) recorded *Capronia* (Herpotrichiellaceae, Chaetothiales), an occasional genus among endophytes in tropical angiosperms (Arnold et al. 2007b), among ericoid mycorrhizal associates of salal in southwestern Canada. However, no records to date indicate other genus- or species-level overlaps between eurotiomycetous mycorrhizal fungi and foliar endophytes.

Together, the Dothideomycetes and Sordariomycetes contain the majority of foliar endophyte species (Arnold et al. 2007b). These classes account for more than 75 % of endophytes in sites ranging from the arctic to the tropics, although their abundance relative to one another changes markedly as a function of latitude (see below; see also Arnold & Lutzoni 2007). While few dothideomycetous genera have been recorded as mycorrhizal fungi, the ubiquitous fungus *Cenococcum geophilum* has been observed both as a foliar endophyte and as a mycorrhizal associate (see LoBuglio et al. 1996; Arnold et al. 2007a). Here the importance of species concepts, strain-level identification, and phylogenetic analysis is brought into sharp relief: the ecological implications differ markedly should it be the case, as suggested by morphology and BLAST results based on fast-evolving nuclear markers, that strains of *C. geophilum* can exist both as foliar endophytes and mycorrhizal symbionts. The ability of individual genotypes to manifest both symbioses has not been confirmed.

In turn, mycorrhizal Ascomycota are rare among the Sordariomycetes, which instead are dominant members of south-temperate and tropical endophyte communities (Arnold & Lutzoni 2007) and are especially common as endophytes of liverworts (Davis *et al.* 2003). Xiao and Berch (1996) recorded *Acremonium strictum* (Hypocreales) as a mycorrhizal associate of salal, but noted that its association was atypical: root colonization under laboratory conditions was slow, and the fungus was capable of growing on and sporulating from above-ground tissues. Collado *et al.* (1996) found *A. strictum* in above-ground tissues of oaks, suggesting a wide array of ecological modes for this apparently ubiquitous fungus. Indeed, some authors have recorded it as an opportunistic pathogen of humans and other mammals (e.g., Miyakis *et al.* 2006), highlighting the difficulty inherent in assigning a given ecological role to a given fungal species (see also Arnold *et al.* 2007b).

In the absence of other well-documented mycorrhizal Sordariomycetes, this species-rich class appears to be the converse of the Pezizomycetes in relative abundance of endophytic vs. mycorrhizal species. Interestingly, both lineages were recently reconstructed as descending from saprotrophic ancestors in six-gene phylogenetic analyses by James *et al.* (2006). Whether the Pezizomycetes arose and/or diversified belowground in the context of mycorrhizal associations, and the Sordariomycetes diversified aboveground and thus have greater affinities for foliar pathogens and endophytes, remains to be evaluated. Framing such questions based on ancestral state reconstructions represents an exciting new direction in the study of fungal evolution and ecology, and argues for the inclusion of newly recovered endophytic and mycorrhizal fungi in large-scale phylogenetic analyses (see Arnold *et al.* 2007a,b; Higgins *et al.* 2007).

Jumpponen and Trappe (1998) and Jumpponen (2001, 2003) reviewed the biology of dark-septate endophytes (DSE), the biotrophic fungi that infect asymptomatic roots and are distinguished as a functional group on the basis of their melanized hyphae. The ecological roles and diversity of these primarily ascomycetous fungi are still being described, but it is clear that they represent a variety of lineages within the Pezizomycotina with concentrations in the Pezizales (Pezizomycetes), Helotiales (e.g., *Acephala*, *Cado-phora*, and *Phialocephala*; Leotiomyces), and Pleosporales (Dothideomycetes; Jumpponen 2001). DSE and mycorrhizae can co-occur in the same plant tissues (Urceley 2002) but represent distinctive taxa (Bergemann & Garbelotto 2006). While species lists are relatively few, Addy *et al.* (2005) discussed several common Ascomycota that, with the exception of *Exophiala* (Chaetothyriales), are not known among foliar endophyte communities. DSE share with foliar endophytes a biotrophic, horizontally transmitted lifestyle, although endophytes found in foliage are rarely heavily melanized in *planta*. Plants containing DSE often contain large numbers of foliar endophytes as well (see Jumpponen 2001; Higgins *et al.* 2007). The apparent lack of overlap between these communities is of interest in understanding the distinctive features of root vs. shoot colonization and the rhizosphere vs. phyllosphere environments.

Recent studies have examined non-mycorrhizal, non-DSE fungi within roots and in some cases, compared them with assemblages of fungi in aerial tissues (e.g., Suryanarayanan &

Vijaykrishna 2001; Kumar & Hyde 2004). Because these root-inhabiting fungi lack the distinctive structures or distinctive morphology of mycorrhizal symbionts and DSE, this pool of taxa represents the most likely overlap with foliar endophyte communities. Gotz *et al.* (2006) recorded a variety of common endophytic genera, including *Colletotrichum* and *Cylindrocarpum*, from roots of potato. The species recovered in that study have not yet been recorded from leaves, although it is possible they have been recovered and not positively identified. Similarly, Halmeschlager and Kowalski (2004) recovered common endophyte genera such as *Alternaria*, *Aureobasidium*, *Fusarium*, *Phoma*, and *Xylaria* from roots of living oaks in Europe. Notably, these genera represent some of the most ubiquitous and generalist foliar endophytes (Hoffman & Arnold 2007; Davis *et al.* 2003). However, in explicit comparisons of foliar and root endophyte communities, Suryanarayanan and Vijaykrishna (2001) found little overlap despite the fact that their study plant, *Ficus benghalensis*, has aerial (above-ground) roots. Similarly, Kumar and Hyde (2004) found little overlap between root and leaf-inhabiting communities in *Tripterygium wilfordii*, although *Pestalotiopsis* sp. – common as foliar endophytes in many tropical plants (Arnold 2002) – were common in roots in that study.

#### Foliar and wood-inhabiting endophytes

Relative to woody tissues, leaves are shorter-lived, more biochemically dynamic, more environmentally variable, more critical for photosynthesis, and more subject to damage by sucking and chewing herbivores (Arnold 2002). Accordingly, endophytes inhabiting foliage are under a suite of selective pressures distinct from those facing xylem endophytes, or endophytes associated with tissues such as inner bark. The implications of these differences have not been explored, and may elucidate general patterns in life history, specificity, host-benefits, and potential to act as saprophytes for wood- vs. foliage symbionts. For example, are endophytes of foliage more likely to be host-specific than wood-inhabiting endophytes, reflecting (1) the biochemical diversity evident among leaves of different species and (2) the high degree of competition likely in these readily colonizable tissues (Arnold 2002)? Or might endophytes of foliage represent less-specialized, facultative saprotrophs, reflecting the relatively ephemeral nature of the leaves they inhabit? Phylogenetically controlled comparisons for xylem- and leaf-associated endophytes would be especially enlightening in this regard, and ancestral state reconstructions may show different evolutionary origins for leaf- vs. wood-inhabiting fungi.

Overlap of fungal communities between leaf blades and petioles has been assessed in several studies (e.g., Lodge *et al.* 1996; Taylor *et al.* 1999). Typically some overlap is seen between petiole and blade communities, although relative abundances of key taxa may differ (Lodge *et al.* 1996). For example, Taylor *et al.* (1999) found that Xylariaceae were more common in blades than in petioles, although Ascomycota dominated both tissue types.

In contrast, fungi that form latent infections in trunks or branches of most dicotyledonous trees include numerous Basidiomycota as well as Ascomycota (Chapela & Boddy 1988). Fisher *et al.* (1994) explicitly compared twig- and leaf-associated

endophytes in *Quercus ilex*. Among the commonest endophytes, ca. 70 % were found only in foliage, while the remainder were found in both foliage and twigs. No twig-specific taxa were recovered. In that study, both communities were dominated by Ascomycota, including species of *Alternaria*, *Aureobasidium*, *Cladosporium*, *Phoma*, *Phomopsis*, *Sordaria*, and *Xylaria* in leaves, as well as *Colletotrichum*, *Nodulisporium*, *Phyllosticta*, and two sterile mycelia from both leaves and twigs. Similarly, Simeto et al. (2005) recovered the common leaf-endophytic genera *Aureobasidium*, *Botryosphaeria*, and *Cytospora* from stems of *Eucalyptus* in Uruguay. Santamaría and Diez (2005) found a greater proportion of endophyte species in only leaves or twigs rather than in both, but recovered a greater species richness from twigs than from leaves. That study, which focused on a deciduous tree (*Populus tremula*), included springtime samples and thus may have underestimated overlap between leaf- and twig-inhabiting communities by recovering relatively few foliar endophytes early in the season (see Arnold et al. 2003). These studies hint at the importance of considering leaf life history (deciduous or evergreen/persistent) in comparing communities of foliar and twig- or wood-inhabiting fungi. Together, they suggest that leaf- and wood-inhabiting communities share some species, although the balance of evidence suggests that leaves, when fully infected (late in the season), harbor an additional suite of distinctive endophytes.

Although these and other studies have noted overlap between endophytes of woody tissues and leaf laminae, none to date has used molecular tools to assess whether the same genotypes are present in each tissue. To address this, members of my research group assessed fungal communities associated with the scale-like leaves of *Cupressus arizonica* (Cupressaceae), and with woody twigs that immediately subtend these photosynthetic tissues (Arnold, Bhakta and Hoffman, unpubl. data). Fifty-three isolates were recovered from foliage (21 isolates) and twigs (32 isolates) in an arboretum in southern Arizona. All isolates were sequenced for a fast-evolving nuclear marker (nuclear ribosomal internal transcribed spacer; see below) and separated into functional taxonomic units based on 1 % sequence divergence, which allows for sequencing error while conservatively estimating genotype boundaries (Gallery et al. 2007). Fifteen of 18 genotypes (83 %) were found in only in twigs or foliage, but not in both tissue types. An unidentified dothioraceous species and two species of the ubiquitous genus *Phoma* were recovered from both tissue types, with one genotype of *Phoma* representing the most common isolate in both foliage and twig samples. Foliage-only fungi included species of *Preussia* (Sporormiaceae, Pleosporales, Dothideomycetes), *Cladosporium* (Mycosphaerellaceae, Dothideomycetes *incertae sedis*), and *Thielavia* (Chaetomiaceae, Sordariales, Sordariomycetes). Twig-only genotypes represented *Alternaria* (Pleosporaceae, Pleosporales), *Pringsheimia* (Dothioraceae), and a third *Phoma* (*incertae sedis*). Although dominated by the same species, similarity between the foliar and twig communities was low given the proximity of these tissues (Jaccard's index, based on presence/absence of nonsingletons = 0.166, Bray-Curtis index, based on relative abundance of nonsingletons = 0.339). Given the same sampling effort, endophytes from foliage were richer in this coniferous host (12 species; 95 % CI = 6.0-17.9 species; bootstrap estimate = 15.4 species) and more diverse (Fisher's

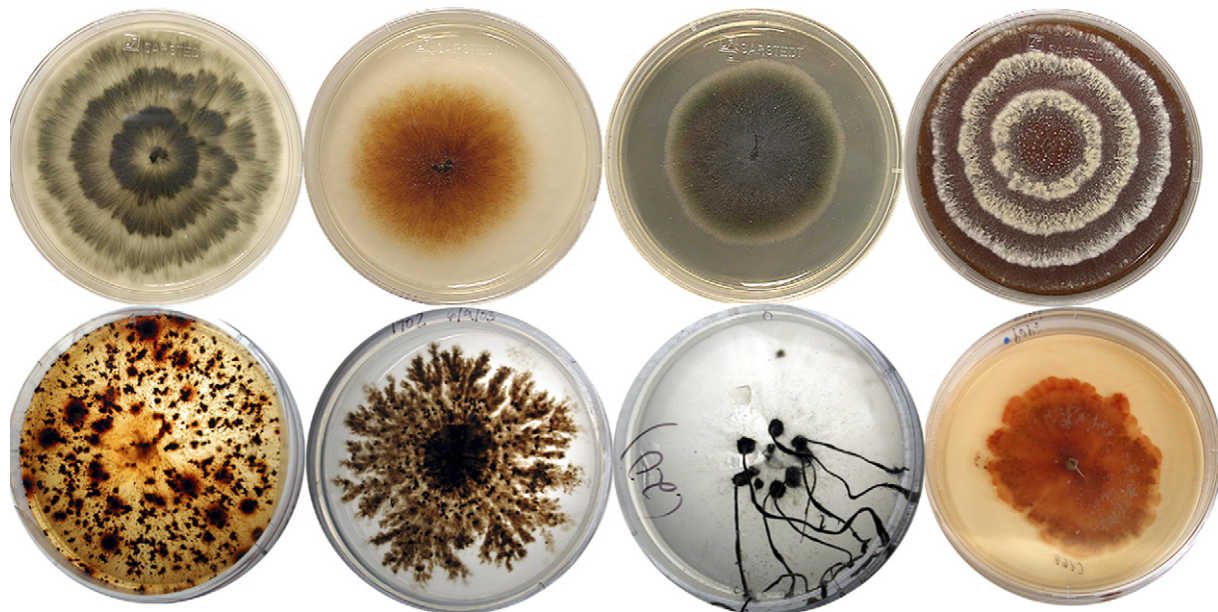
alpha = 11.6, Shannon index = 2.2) than were endophytes from twigs (9 species; 95 % CI = 4.0-14.0 species; bootstrap estimate = 11.7 species; Fisher's alpha = 4.16, Shannon index = 1.2).

Higher diversity in foliage vs. twig samples was unexpected and contrasts with recent work in the palaeotropics (Kumar & Hyde 2004) and temperate Europe (Santamaría and Diez 2005), although this finding was consistent with samples from *Betula* in the temperate zone (lower richness but higher diversity in foliage vs. twigs; Barenge et al. 2000). Notably, several studies have shown that twig endophytes may be less diverse than other endophyte communities in the same hosts (cf. inner-bark endophytes; Tejesvi et al. 2005). In general, the dominance of different fungi in distinctive above-ground tissues, coupled with otherwise distinctive communities specific to each tissue type, appears to be a consistent theme in the angiosperms and conifers examined to date (e.g., Bettucci & Saravay 1993; Taylor et al. 1999; Kumar & Hyde 2004; Kaneko & Kaneko 2004).

### Foliar endophytes and seed-associated fungi

The vast majority of evidence indicates that cultivable endophytes associated with foliage of woody plants move about via contagious spread (horizontal transmission) rather than maternal inheritance (vertical transmission; see Bayman et al. 1998; Fröhlich et al. 2000; Kaneko & Kaneko 2004). Supporting data come from studies that have raised endophyte-free seedlings under sterile conditions (Arnold and Engelbrecht 2007), placed sterile seedlings in the field to observe colonization by ambient fungi (Arnold & Herre 2003), measured infection frequency in leaves as they age (Arnold et al. 2003), or observed a high diversity of endophytes in tissue, suggesting that such communities more likely represent colonization from the external environment rather than vertical transmission *en masse* (e.g., Fröhlich et al. 2000). Coupled with records of high diurnal inoculum potential in sites such as tropical forests (Arnold 2002), and even higher rates of nocturnal inoculum input (Gilbert & Reynolds 2005), these studies highlight the remarkable ability of numerous and highly diverse fungi to move about in the air column, germinate on leaf surfaces, and penetrate and persist within living foliage (Fig 1).

Despite the prevalence of horizontal transmission, cultivable endophytes are occasionally recovered from seeds. Do these fungi represent vertically transmitted symbionts? If so, they are typically more diverse, and are present at a lower frequency, than traditional views of vertical transmission would suggest. For example, despite extensive sampling that recovered over 2000 endophytic isolates from foliage of *Pinus monticola*, Ganley and Newcombe (2006) recovered only 16 endophytes from 750 surface-sterilized seeds. While vertical transmission of symbionts is occasionally imperfect (see Lipsitch et al. 1996; Moran & Dunbar 2006), an infection frequency of ca. 2 % would be surprisingly low for maintaining a viable and ecologically important association. Interestingly, Ganley and Newcombe (2006) found that the dominant foliar endophytes were absent from seeds, which instead contained a variety of less-common foliar fungi. This result points to the possibility for alternative strategies or life histories among the diverse endophytes associated with a given plant species.



**Fig. 1** – Assortment of ascomycetous endophytic fungi recovered from foliage of angiosperms and conifers in North America and Panama. These diverse fungi grow readily in culture and can be maintained in culture collections at little cost. With recent technological advances, the growing popularity of culture-free methods promises to discover ever-greater diversity and to expand our understanding of the structure of the fungal tree of life. One particularly useful aspect of culture-free methods may be to show that particular fungi are present in an environment, thus leading endophyte researchers to optimize culturing conditions as a means to capture those fungi *in vitro*. Such efforts are critical for establishing voucher specimens, which in turn can be used to empirically assess species interactions, as raw materials for bioprospecting or biological control, and as the basis for future research in systematics or genomics. Because many endophytes do not sporulate in culture, and thus are classified only as *mycelia sterilia*, they are not welcome at most established culture collections. Depositories are needed to house and maintain vouchers of these fungi, and to curate their ecological data (site of origin; host plant; season; tissue type). Both the specimens themselves and the data regarding their recovery have tremendous intrinsic value.

The marked disparity in abundance between cultivable leaf- and seed-associated fungi in *Pinus* appears to be a repeated pattern. Arnold *et al.* (2003) failed to recover endophytes from seeds of *Theobroma cacao* in a study of the diverse endophytes associated with foliage of that species. However, Posada and Vega (2005) recorded *Penicillium oxalicum* in leaves, stems, and roots of seedlings raised under sterile conditions, which they suggested to be a vertically transmitted endophyte. Rodrigues and Menezes (2005) recovered *Fusarium* spp. from seeds of cowpea, Washington *et al.* (1999) found *Phomopsis castanea* to be seedborne in chestnut, and several earlier studies occasionally recorded at least occasional vertical or seedborne transmission of endophytes in plants such as Douglas fir (*Pseudotsuga menziesii*; Bloomberg 1966), *Casuarina equisetifolia* (Bose 1947), and *Quercus garryana* (Wilson & Carroll 1994). Interestingly, most of these studies recovered multiple endophyte species from seeds, which contrasts with the single-genotype model of vertical transmission posed by the clavicipitaceous endophytes of grasses (Clay & Schardl 2002). Whether diverse seed-inhabiting fungi represent true cases of vertical inheritance *via* growth from maternal tissues, or instead represent infections contracted by fruits or seeds via contagious spread, should be evaluated.

When Gallery *et al.* (2007) screened surface-sterilized seeds of a tropical tree (*Cecropia insignis*) using culture-independent

methods, they recovered diverse Ascomycota that were genotypically consistent with foliar endophytes from the same forest. Notably, seeds did not yield any fungi in culture in that study. Culture-independent methods such as environmental PCR may be especially important for recovering vertically transmitted fungi, which would be especially recalcitrant to cultivation should their maternal inheritance indicate an obligate association with their hosts.

#### **Foliar endophytes and epiphytes**

While the interior of leaves experience dramatic shifts in temperature and other conditions, the exterior surfaces of leaves can be considered much more extreme in terms of abiotic stress. Petrini (1991) suggested that endophyte communities often contain a variety of classically epiphytic species, including *Alternaria alternata*, *Cladosporium cladosporioides*, *Epicoccum pupurascens* and others, which facultatively enter leaf tissues at the onset of leaf senescence. This observation, coupled with the formation of subcuticular but not truly endophytic infections that may persist through typical methods of surface sterilization, obscures the boundary between phylloplane and endophytic fungi. Interestingly, phylloplane taxa such as those listed above, as well as cosmopolitan genera *Phoma*, *Aureobasidium*, and *Chaetomium*, were especially common as

endophytes in recent surveys of desert plants (Hoffman & Arnold 2007), leading those authors to speculate that these ubiquitous fungi may be especially likely to establish endophytic infections when exterior conditions are inhospitable.

Understanding the distinctiveness of fungal endophytes relative to epifoliar fungi requires explicit comparisons of these two communities, but studies examining them in the same host and season are rare. Legault *et al.* (1989a, 1989b) isolated fungi from foliage of *Pinus banksiana* and *P. resinosa* in Québec, Canada, and showed that in contrast to relatively host-specific endophytes, epiphytic fungal communities mostly consisted of generalist saprotrophs. Santamaría and Bayman (2005) compared endophytic and epiphytic fungal communities in coffee plants in Puerto Rico, and found a higher diversity of endophytes than of epiphytes. That study documented striking differences in composition of superficial and internal communities of fungi associated with individual leaves, highlighting the remarkable diversity of fungi that occur in close proximity, but in distinctive ecological milieus, on and within leaf tissues.

Interestingly, epifoliar and endophytic fungi may have distinctive biological properties that highlight functional or ecological differences between these guilds. Members of my research group compared the antimicrobial activity of epibiotic fungi from leaf surfaces and the external surfaces of lichens against that of endobiotic fungi (endophytes and endolichenic fungi; Arnold and Cook, unpubl. data). Taking into account the phylogenetic relatedness of fungal isolates and scaling inhibition by intrinsic growth rates, we found that fungi from the outside surfaces of hosts ( $N = 37$  species) were better inhibitors of test strains than fungi from host interiors ( $N = 116$  species;  $F_{1,151} = 5.2422$ ,  $P = 0.0234$ ). This result was initially surprising given the tremendous richness of secondary metabolites, including bioactive agents, isolated recently from endophytic fungi (reviewed by Strobel and Daisy 2003; Gunatilaka 2006; Zhang *et al.* 2006). However, it is useful to interpret these empirical findings through the lens of evolutionary biology. Endobiotic fungi, which must penetrate and persist within host tissues, are more likely to exhibit a degree of specificity with regard to their hosts relative to the sometimes transient fungi that may be isolated from external surfaces. If this is true, then endophytes may manifest their antimicrobial activity – or produce notable secondary metabolites – only in the presence of elicitors or substrates from the organisms they inhabit. Previous studies have shown that both the growth rate of endophytic fungi in culture, and the outcomes of interspecific interactions *in vitro*, can be altered markedly by including extracts from different plant taxa in cultivation media (Arnold & Herre 2003; Arnold *et al.* 2003). Bioprospecting studies will likely benefit from using plant defensive chemicals, stress hormones, or at the least, cellulose-based media to seek bioactive metabolites from endophytic fungi.

### 3. Understanding diversity and specificity: below the species level

Petrini (1991) pointed out that the relevant level of biological organization in endophyte biology may lie below the species

level – at the level of genotypes, or in the terminology of plant pathology, *formae speciales*. This would come as no surprise to plant pathologists, who have long recognized the ability of different infraspecific pathovars (in bacteria) or biotypes/*formae speciales* (in fungi) to associate with different hosts, and manifest fundamentally different interactions with different host species (see Agrios 2005).

The implications of strain-level specificity have been largely overlooked in endophyte biology, wherein the typical level of biological organization in surveys is species. Various defined in fungi, endophyte species are typically delimited on the basis of traditional morphological taxonomy or more limited insights from mycelial morphotypes. However, the holy grail of counting species – a typical approach for macroecologists – may underestimate the true number of distinct biological types of endophytes, each of which may have its own evolutionary trajectory, host interactions, and degree of host specificity. The ideal, represented by fungi that are characterized morphologically to species, and then genotyped at multiple loci, should be a focus of endophyte biologists in years to come: only then can we reconcile the relationship of traditionally delimited species with underlying or cryptic biological diversity. Indeed, evidence from a variety of fungi (e.g., the *Gibberella fujikuroi* species complex; Nierenberg & O'Donnell 1998) underscores the need to look within recognized species for the relevant units of biodiversity and to gain an understanding of host- and geographic specificity.

Based on this reasoning, members of my research group used molecular data to examine the diversity and host specificity of endophytes associated with a suite of ecologically important hosts in the biotically diverse woodlands of the Madrean Archipelago, an area of 'sky islands' defined by relatively mesic, cool mountains that rise from arid lowlands in the Sonoran and Chihuahuan deserts in the southwestern USA (Hoffman *et al.* unpubl. data). We isolated endophytes from apparently healthy, mature leaves of *Quercus* spp. (Fagaceae), *Pinus ponderosa* (Pinaceae), *Cupressus arizonica* (Cupressaceae), and *Platycladus orientalis* (Cupressaceae) in and near Tucson, AZ in late summer 2006 ( $N = 16$  leaf segments/leaf, 3 leaves/individual, 3 individuals/species; methods follow Arnold *et al.* 2007b). All fungi were genotyped using the fast-evolving nuclear ribosomal internal transcribed spacers and 5.8 s gene (ITS rDNA), a ca. 400–800 base-pair region frequently used in fungal systematics at the species level. ITS rDNA data can obscure species boundaries in some clades, include non-orthologs in some taxa, and exhibit different rates of evolution among different fungal lineages (Jacobs & Rehner 1998; Lieckfeldt & Seifert 2000; Kim & Breuil 2001). However, ITS rDNA data are useful in several regards. First, comparisons of sequence divergence within recognized species, estimated empirically for four common genera of endophytes (*Botryosphaeria*, *Colletotrichum*, *Mycosphaerella*, and *Xylaria*) indicate an average of ca. 1–2 % difference in sequence composition among conspecifics (U'Ren *et al.* 2007). Thus, ITS rDNA genotypes provide some resolution of lineages below the species level for some of the commonest endophyte taxa. Moreover, the ease with which ITS rDNA sequence data can be obtained and the existence of large, phylogenetically referenced databases of ITS rDNA sequences for endophytes (e.g., 4092

sequences for endophytes isolated from the tropics to the arctic; see Arnold & Lutzoni 2007) underscore the utility of this region for providing a first, if limited, approximation of genotypic differences among samples.

In our study, 56 distinct genotypes were recovered among 197 isolates (Fisher's  $\alpha = 26.10$ ; Shannon index = 2.94). All were filamentous Ascomycota, including Pezizomycetes, Eurotiomycetes, Sordariomycetes, Dothideomycetes, and Leotiomyces. Overall, 53 genotypes were isolated from only a single host genus (95%), suggesting a high degree of host specificity. However, when species boundaries were inferred on the basis of morphospecies, traditional species concepts (for the small subset of isolates that sporulated in culture), or very conservative species boundaries based on 90% ITS rDNA similarity (Arnold et al. 2007a), estimates of host specificity diminished by more than half. Whether this is a general pattern remains to be seen: when Pandey et al. (2003) took a similar approach to measure the genetic variation within an apparently ubiquitous endophyte (*Phyllosticta capitalensis*) associated with different tropical trees in India, they found no variation among isolates from plants in different families and different forest types. Nevertheless, the resolution provided by genotypes, especially in the context of additional loci that may uncover cryptic phylogenetic structure within groups that show no ITS rDNA variation, will be an important tool for endophyte biologists.

#### 4. Understanding diversity and specificity: above the species level

In the study outlined above, we observed no meaningful, species-specific differences in the communities of endophytic fungi associated with seven sympatric species of *Quercus* (Hoffman et al. unpubl. data). Only one oak endophyte was found in foliage of another non-oak host. Does this suggest some degree

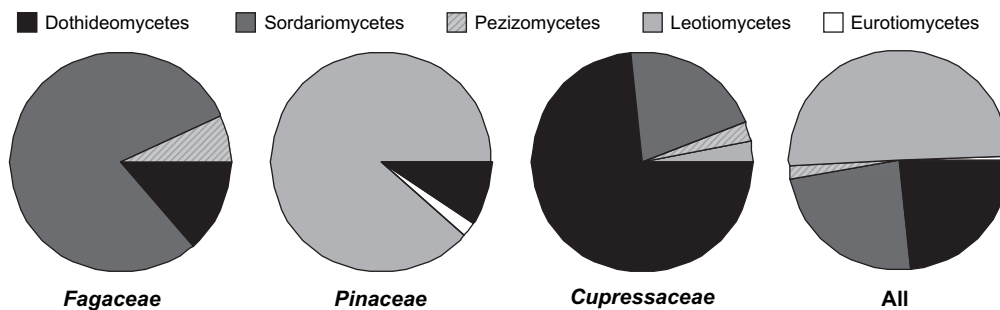
of host specificity at the level of plant genus, or perhaps at a higher taxonomic level? It is impossible to say without further sampling. However, several studies have shown that especially in the case of conifers, some lineages of fungi appear with greater frequency, or only, in plants representing particular families. For example, *Lophodermium* spp. (Rhytismataceae, Leotiomyces) appear with frequency in Pinaceae, but are absent from other sympatric conifers (e.g., *Juniperus* sp., Cupressaceae) or angiosperms (Arnold et al. 2007b).

Inasmuch as infraspecific lineages may be important for understanding the true nature of endophyte diversity, so too will lineages above the species level: have clades of fungi co-diversified with particular plant lineages? Coadaptation is not evident for plants and Ascomycota as a whole (Arnold et al. 2007b; Higgins et al. 2007), but it is possible that for at least some lineages, signals of strict-sense coevolution will be evident at the family or generic level. This will be resolved as endophytes continue to be integrated into phylogenetic analyses.

In our study, endophyte communities associated with *Quercus* spp. were dominated by one genotype that accounted for 54.5% of isolates (*Biscognauxia* sp.). Similarly, one species accounted for 58.6% of isolates from *P. ponderosa* (*Lophodermium* sp.). Sordariomycetes were especially common among oak endophytes, but communities of endophytes associated with other hosts were dominated by different taxa: the Leotiomyces (*Pinus ponderosa*) or Dothideomycetes (*Cupressus*, *Platyclusus*) (Fig 2). Differences in the relative importance of these classes in different plant lineages appear to be a repeated pattern (Arnold & Lutzoni 2007; Arnold et al. 2007a,b; Higgins et al. 2007).

#### 5. Context-dependence and the structure of endophyte assemblages

Implicit in estimates of fungal diversity at a global scale are assumptions regarding the similarity in fungal communities



**Fig. 2** – The phylogenetic context of endophyte symbioses: relative abundance of five classes of Ascomycota among endophytes isolated from three different plant families in southeastern Arizona, USA. Hosts representing the Fagaceae (*Quercus* spp.; N = 44 isolates, dominated by the Sordariomycetes), Pinaceae (*Pinus ponderosa*; N = 111 isolates, dominated by the Leotiomyces), and Cupressaceae (*Cupressus arizonica* and *Platyclusus orientalis*; N = 42 isolates, dominated by the Dothideomycetes) differ markedly in the relative abundance and dominance of each class. The predominant classes listed here also dominate endophyte communities associated with these host families in other sites, including mesic semideciduous forest (North Carolina; all families) and boreal forest (Québec; Pinaceae and Cupressaceae) (Arnold et al. unpubl. data). What are the phylogenetic or evolutionary constraints underlying the endophyte community associated with particular families of plants? What is the appropriate taxonomic scale for seeking evidence of host/endophyte coevolution? The data presented here represent a first step toward addressing these questions, which are critical for understanding the evolution of the endophytic habit.

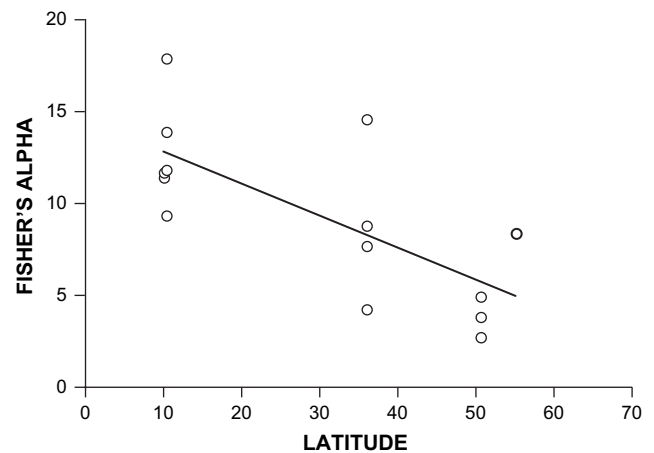
among different hosts and geographic regions. In general, however, very little is known regarding the turnover in endophyte species composition among biogeographic regions. A major challenge lies in comparing surveys conducted in different areas, which often rely on different culturing media, tissue fragment sizes, treatment of tissue following harvesting, and species concepts. For example, Fröhlich and Hyde (1999) used potato dextrose agar for initial isolations, whereas Lodge *et al.* (1996) used 1.5 % malt extract agar (MEA) amended with antibiotics, and Arnold *et al.* (2000, 2001, 2003) preferred 2 % MEA without antibiotics. Little is known about the pervasive effects of these and other methodological differences in shaping conclusions about endophyte diversity.

One recent study avoided these issues by applying consistent methods to sample endophytes from representative angiosperms across a broad latitudinal gradient ranging from northern boreal forest to tropical forest (Arnold & Lutzoni 2007). That work provides evidence for several latitudinal gradients, which highlight the distinctiveness of endophyte communities in different sites. First, tropical angiosperms harbor a higher diversity of endophytes than do those at higher latitudes (Fig 3). Second, phylogenetic composition of endophyte communities changes as a function of latitude: tropical communities were represented by fewer classes and were dominated by Sordariomycetes, whereas boreal communities were represented by many classes and dominated by Dothideomycetes (Arnold & Lutzoni 2007). Third, host generalism was more prevalent in the tropics, while stronger host affinity was seen among boreal endophytes. Together, these data highlight the different symbiotic environment encountered by plants as a function of their occurrence in boreal, temperate, or tropical forests, and illustrate the challenges underlying extrapolative estimates of endophyte diversity based on particular sites/host species.

Latitude, while a correlate of these properties of endophyte assemblages, does not provide information regarding mechanisms that underlie differences in community structure. Many studies have shown that the abundance, diversity, and species composition of endophytes are influenced by microhabitat and microclimatic conditions (e.g., Arnold & Herre 2003, Petrini 1986), although the relative importance of these factors, as well as factors such as local plant diversity, has not been evaluated explicitly.

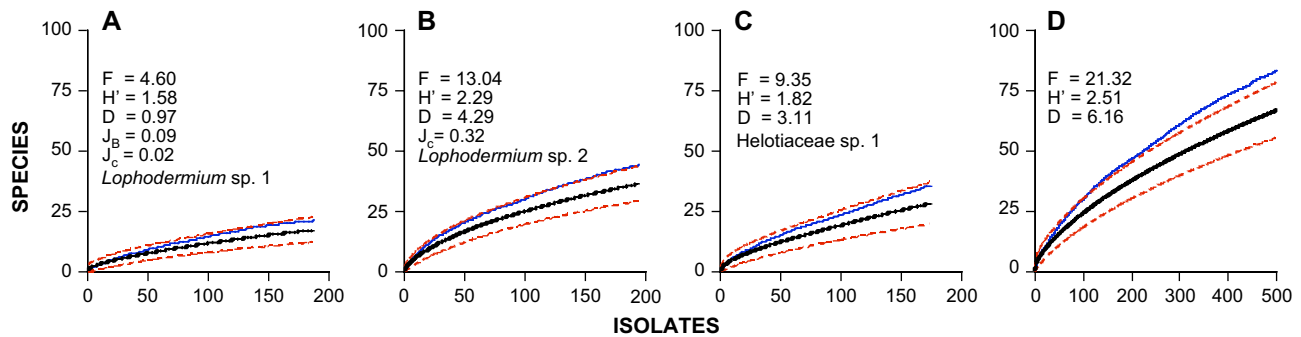
The study by Arnold and Lutzoni (2007) sampled representative angiosperms in forest understories of each site, but did not consider phylogenetic structure among the focal plant hosts. Several studies have provided strong evidence of species turnover among sites for phylogenetically controlled comparisons, including studies of congeneric palms in Australia vs. Brunei Darussalam (Fröhlich and Hyde 1999) and a single species of *Quercus* (*Q. ilex*) in Majorca and Switzerland (Fisher *et al.* 1994). A strength of these studies is that the fungi recovered were identified to species, but the potential for underlying differences due to distinctive genotypes in each site was not assessed.

To control for phylogenetic relatedness while assessing site-to-site differences at the genotype level, members of my research group examined communities of endophytes associated with closely related *Pinus* spp. in three geographic



**Fig. 3 – The geographic context of endophyte symbioses: latitudinal gradient of diversity.** Using consistent methods, foliar endophytes were isolated and genotyped from foliage of 14 representative species of angiosperms, ferns, and lycophytes at four sites: lowland tropical forest at 9.9°N latitude (six species; Barro Colorado Island, Panama), semideciduous temperate forest at 35.6°N (four species; Duke Forest, Durham, North Carolina, USA), and boreal forest at 50.3° and 54.8°N (three species, Mingan Archipelago, Québec, Canada; one species, Schefferville, Québec, Canada) (Arnold & Lutzoni 2007). Focal plant species: *Gustavia superba* (Lecythidaceae), *Heisteria concinna* (Olacaceae), *Laetia thamnia* (Flacourtiaceae), *Ouratea lucens* (Ochnaceae), *Theobroma cacao* (Malvaceae), *Trichilia tuberculata* (Meliaceae), Panama; *Acer rubrum* (Aceraceae), *Magnolia grandiflora* (Magnoliaceae), *Huperzia* sp. (Lycopodiaceae), and *Polystichum acrostichoides* (Dryopteridaceae), North Carolina; *Empetrum nigrum* and *Vaccinium vitis-ideae* (Ericaceae) and *Dryas integrifolia* (Rosaceae), Mingan Archipelago; and *Huperzia selago* (Lycopodiaceae), Schefferville. Plants were selected on the basis of their frequency in the understory in each site. Future studies would benefit from phylogenetically controlled comparisons or common-garden experiments in which members of the same plant species, genus, or family could be examined along a similar latitudinal gradient.

localities that differ markedly in plant diversity, annual rainfall, and other factors: semiarid montane ecosystems in southeastern Arizona, USA; a mesic, mixed-canopy forest in North Carolina, USA; and southern boreal forest near Moisie River, Québec, Canada. We found that endophytes associated with *Pinus* in each locality differed markedly in richness, diversity, and species composition, and in the identity of the dominant endophyte species (Fig 4). Similar patterns have been observed for conspecifics growing in different geographic localities (e.g., *Platyclusus orientalis* in a mesic forest and arid desert; Hoffman & Arnold 2007), and at smaller spatial scales (e.g., in coastal vs. inland sites in Puerto Rico; Bayman *et al.* 1998). Together, these results highlight the context dependency of endophyte communities and the challenges implicit in estimating endophyte diversity.



**Fig. 4 – The geographic context of endophyte symbioses: distinctive communities of endophytes associated with foliage of *Pinus* spp. in three geographically and ecologically distinct sites: (A) Madrean sky island archipelago, southeastern Arizona, USA, 32.3°N; (B) mesic semideciduous forest, Duke Forest, Durham, North Carolina, USA, 35.6°N; (C) southern boreal forest, Moisie River, Québec, Canada; 50.3°N (Arnold et al., unpubl. data). Panel D shows the cumulative results when data from three sites are combined. Each figure shows the observed species accumulation curve smoothed by rarefaction in EstimateS (Colwell 1996) for endophytic fungi (thick solid line), a 95 % confidence interval about that observed curve (dashed line), and the bootstrap estimate of total species richness (thin solid line). Diversity indices are given as Fisher’s alpha (F), Shannon index (H’), and Simpson’s index (D). Similarity between sites is given by Jaccard’s index of similarity (J) calculated for non-singleton genotypes only, with subscripts indicating site-to-site comparisons based on presence/absence data. The taxonomic affinity of the most common genotype in each site is listed. Together, these data show that congeneric trees growing in different environments form symbioses with endophyte assemblages that differ in diversity, dominant species, and species composition relative to other sites. Even though sampling approached statistical completeness in each site, the final panel shows that the sampling presented here was insufficient to capture the total richness of endophytes associated with these *Pinus* species. These data illustrate the remarkable turnover in endophyte species among three sites despite the close relationship among the host species, and the importance of sampling multiple sites/environments – which may differ in richness by a factor of two given the same sampling effort – for extrapolative estimates of diversity.**

## 6. Endophytes, pathogens, saprotrophs: communities of uncertain overlap

Even if a given endophyte is capable of infecting multiple host species, its interactions may demonstrate functional or ecological specificity: an endophyte from one host species, while capable of colonizing a second, may not interact with both hosts in a similar manner. A wealth of plant pathology literature shows that fungal pathogens with complex life cycles inhabit different host species at different life stages, and that their interactions with those hosts differ markedly (Agris 2005). Perhaps most notably, surveys occasionally recover endophytes that are conspecific with known pathogens (Arnold *in press*, but see Ganley & Newcombe 2003). Whether such endophytes represent avirulent strains of otherwise pathogenic species (see Freeman & Rodriguez 1993), strains that are capable of pathogenicity under conditions of host stress or which have long latent periods (Carroll 1988; Fisher & Petrini 1992; Stanosz et al. 2001), strains that are virulent but which are poor competitors relative to other leaf-colonizing fungi (see Arnold et al. 2003), or strains that are virulent pathogens of other hosts remains to be evaluated. Exploring these functional aspects of endophyte symbioses represents an exciting frontier in the study of endophyte diversity. A particularly exciting area of research lies in understanding the potential for viral or bacterial endosymbionts of endophytes to drive the pathogenic or avirulent phenotype.

More generally, the endophyte literature is rich with definitions of endophytism (e.g., Petrini 1986, 1991), and with

a concomitant lack of clarity regarding the evolutionary and ecological relationships among endophytes, pathogens, and saprotrophs. Several studies have highlighted at least partial taxonomic congruence between communities of endophytes and saprotrophs (e.g., Fröhlich and Hyde 1999). However, survey data are often insufficient for estimating the overlap between endophyte and decay communities: these communities are rarely sampled to the point of statistical completion, such that comparisons between them are limited in scope. Careful attention to the sufficiency of sampling is a critical prerequisite for comparing endophyte and saprotroph communities.

A review of the literature suggests that a significant number of fungi exhibit multiple ecological roles, such as the human pathogen and soil saprotroph *Coccidioides posadasii*. Similarly, fungi such as *Chaetomium globosum* are known as endophytes, saprotrophs, and pathogens (Arnold et al. 2007b). Although it is not yet clear whether the same genotypes can play each of these roles with equal success, the ecological lability of these species is remarkable. Understanding the mechanisms behind that lability represents one among many frontiers in endophyte biology.

## 7. Frontiers in the study of endophyte diversity

### Examining endophytes associated with early-diverging lineages of green plants

Most studies have focused on endophytic fungi of angiosperms and conifers. Surveys of early-diverging lineages of plants are

important for elucidating the evolutionary history of the endophytic symbiosis. Interestingly, [Davis et al. \(2003\)](#) show that cultivable endophytes associated with early-diverging lineages such as liverworts contain members of highly derived lineages of Ascomycota (e.g., Xylariales) rather than early-arising fungal taxa (see [James et al. 2006](#)). Similarly, studies of endophyte-like endolichenic fungi, which associate with green-algal photobionts within lichen thalli, often include Xylariales, as well as an assortment of other Sordariomycetes and Dothideomycetes ([Suryanarayanan et al. 2005](#); [Arnold et al. 2007b](#)). However, recent studies have shown that even though endolichenic fungi do not consistently comprise early-diverging fungal lineages, they frequently give rise to endophytic taxa ([Arnold et al. 2007b](#)). Together, these studies indicate the need not only to survey early-diverging lineages of green plants (including green algae and early embryophytes), but to integrate the recovered fungi into large-scale phylogenetic analyses. A study like that of [Hibbett et al. \(2000\)](#), which focused on the evolutionary stability of the ectomycorrhizal symbiosis in the Basidiomycota, would be especially insightful, and will help address the degree to which endophytism represents a pathway toward diversification in the species-rich Ascomycota.

#### **Increased use of robust phylogenetic analyses**

While data to date suggest that cultivable endophytes do not generally represent new major lineages of fungi – instead containing distinctive genera and especially species of known families and orders – ongoing integration of endophytes, including *mycelia sterilia*, into phylogenetic trees is sorely needed. Such efforts will allow researchers to explore the diversification of fungal clades as a result of, or in parallel with, the endophytic symbiosis, the evolutionary origins of endophytism, and the degree to which endophytic fungi can clarify the structure of the fungal tree of life.

In this context, the importance of robust phylogenies cannot be overstated. For example, in a recent study of unculturable endophytes associated with *Pinus taeda* in the southeastern USA, [Arnold et al. \(2007a\)](#) found no evidence of novel orders or classes of fungi when molecular data were analyzed using a rigorous phylogenetic approach (parsimony analyses with ambiguously aligned regions of the nuclear ribosomal large subunit excluded, two methods of assessing branch support, and a conservative backbone constraint). However, when those data were re-analyzed using commonly implemented, rapid search methods such as neighbor-joining, numerous major lineages comprised only of endophytes were recovered. With such an analysis in hand, an enthusiastic researcher could easily and mistakenly assume the existence of new lineages. Such spurious clades – almost certainly an artifact of inadequate phylogenetic sampling – serve to highlight the importance of careful, thorough analyses with explicit assessments of support for the resulting topology. Multilocus analyses and methods that take into account both models of evolution and phylogenetic uncertainty are especially needed ([Arnold et al. 2007a,b](#)). Special care need be taken in culture-free studies, in which chimeric sequences could also overinflate the apparent phylogenetic distinctiveness of endophytic fungi (see [O'Brien et al. 2005](#), in which chimeric sequences were explicitly addressed).

#### **Improving congruence of sampling methods**

Differences in isolation methods – from the selection of cultivation media to the area of individual tissue fragments, the time since harvesting of tissue, or the cultivation conditions – have the potential to influence conclusions about endophyte infection frequency, diversity, and species composition. For example, [Arnold \(in press\)](#) showed that random subsamples from leaves of a tropical treelet (*Laetia thamnia*, Flacourtiaceae), placed into culture after leaves had been surface-sterilized and incubated at ambient temperatures for 1, 24, 48, 72, or 96 h, differed markedly in species composition and the relative abundance of focal endophyte taxa. Similarly, [Gamboa et al. \(2003\)](#) showed that tissue fragment size is negatively correlated with estimates of endophyte species richness. In turn, mycologists have long recognized the selectivity of culture media, and underlying all endophyte surveys is the knowledge that biases are inherent in the culturing process. Moreover, studies often differ in the methods used to identify species, the reliance on morphotypes for organizing sterile mycelia into taxonomic units, the diversity indices used, and the completeness of sampling.

Together, these issues limit the comparability of alpha taxonomic studies conducted by different research groups. A group of researchers in disparate parts of the globe, or intensively sampling one biogeographic area, could greatly advance our understanding of endophyte biology by applying consistent sampling methods across hosts or sites, as well as the same criteria in delimiting taxonomic boundaries. [Arnold and Lutzoni \(2007\)](#) took this approach in comparing endophyte abundance and diversity over a broad latitudinal gradient (nearctic to neotropics), sampling endophytes from diverse plant lineages and sites using a consistent suite of methods and genotypes to delimit functional taxonomic units. Could studies such as this provide a comparative basis for innovative sampling efforts around the globe, providing a first understanding of beta and gamma diversity among endophytes?

#### **Developing new sampling methods**

Microbiologists have long recognized that as much as 99 % of earth's microbial diversity may be unculturable. While the relative importance of unculturable species is not yet clear for endophytes, several studies highlight the degree to which culture-based methods are complementary to culture-free methods such as environmental PCR. In one study, the estimate of endophyte diversity nearly doubled when culture-independent methods were used in conjunction with thorough culture-based sampling ([Arnold et al. 2007a](#)). In that study, four times as many Basidiomycota were recovered by environmental PCR as were found by culturing, highlighting an unexpected difference in the proportional representation of phyla among leaf endophytes.

In general, development of culture-independent methods, especially in the context of ever-improving tools that allow longer and more informative sequences to be obtained from the environment, promises much in the future of endophyte studies. However, a cautionary note is warranted: in addition to the problems faced by chimeric sequences,

the potential problems of PCR bias remain poorly understood. Arnold *et al.* (2007a) frequently recovered diverse endophytic Sordariomycetes in culture from foliage of *Pinus taeda*, but when subsamples of the same tissues were used in environmental PCR, no members of that class were recovered despite successful recovery of all other major lineages of nonlichenized Ascomycota. Were the sordariomycetous taxa represented by very low biomass in leaves, and thus relatively difficult to capture using cloning – but easily isolated due to very rapid growth on a nutrient-rich culture medium? The interplay of culturing bias and cloning bias remains to be understood, and more specific examples are needed in which both methods are applied to subsamples of the same foliage. As part of these efforts, clone libraries should be established and curated, robust phylogenetic analyses should be the rule, and improving culturing methods to eventually capture those fungi found by cloning alone should be an important goal.

Finally, endophyte biologists should assess the potential applications of new technologies in searching for unknown fungi and their ecological roles. Methods of interest may include pyrosequencing, gene chip development for well-delimited systems (e.g., agricultural fields), quantitative real-time PCR for assessing biomass of particular genotypes, and explicit comparisons of RNA vs. DNA to assess fungal activity in the foliar environment. These approaches promise numerous new insights into the diversity and ecology of endophytic fungi, but are entirely untapped to date.

#### **Development of depositories for unknown cultures**

Endophyte surveys in disparate parts of the world frequently identify sporulating isolates using keys from other regions, reflecting the limited availability of taxonomic resources for microfungi at a global scale. Lessons from macrofungi tell us that morphologically consistent fungi in different biogeographic regions frequently represent different species (see Cantrell & Lodge 2001). In the absence of data regarding the geographic range of endophytes, researchers are limited in their ability to predict the utility of non-local keys in adequately identifying their isolates. This can lead to two potential errors: (1) assigning the same name to fungi that are not truly conspecific; and (2) restricting many studies to identifications only at the genus (or morphospecies) level. Imprecise species identifications based on nonphylogenetic matches with publicly available sequence databases also are of very limited use, and sometimes are misleading (Arnold & Lutzoni 2007).

The resulting lack of resolution limits our ability to address fundamental questions in endophyte ecology (beta diversity, host specificity, ecological roles of particular taxa), and is compounded by the large number of sterile isolates typical of most biodiversity surveys (e.g., Arnold *et al.* 2000): *mycelia sterilia* are grouped on the basis of cultural morphology and are not comparable among hosts or sites. Indeed, morphotypes frequently underestimate diversity when compared with taxonomic groups based on molecular data, and will ‘split’ and ‘lump’ taxonomic groups with nearly equal frequency (Arnold *et al.* 2007a). Evidence suggests that morphospecies should be treated with caution in estimating functional taxonomic units and, where possible, should be avoided at all costs.

These issues, coupled with an emerging perspective that the biologically relevant level of organization for endophytes may lie below the species level, argue strongly for several steps forward. In particular, depositories such as public culture collections are needed for vouchers of pure, but sometimes unnamed, endophyte isolates. Quality control in such depositories would restrict accepted samples to those with ecologically relevant annotations (geographic region of origin, host plant taxonomy, microhabitat). Ideally, such depositories could gather genotypic information for all isolates, providing phylogenetically referenced identification to organize the collection, as well as a preliminary tool for identifying novel strains. Information could be made available to researchers in a format similar to that of GenBank, such that new fungi from the environment could be compared to known strains using rapid phylogenetic analyses. In this way, such fungi would be available for further work regarding bioprospecting, systematics, or other applied or ecological studies. The value of mycological herbaria and culture collections for named fungi cannot be underestimated. Endophyte biology would benefit dramatically from similar infrastructure.

#### **“Omics” in endophyte biology**

The explosive growth of evolutionary, comparative, and community genomics over the past decade, coupled with constantly growing enthusiasm regarding the insights that proteomics, metabolomics, and secretomics can provide (Greenbaum *et al.* 2001), have set the stage for new directions in endophyte research. Given the rapidly decreasing cost associated with genome sequencing, it seems reasonable to envisage genome sequencing of many endophytes in the years to come. Phylogenetically controlled comparisons that specifically contrast the genomes of endophytes and closely related species or genotypes that manifest other ecological modes (e.g., saprotrophy, pathogenicity) would allow new insights into the fundamental or realized differences between these guilds of fungi. Conspecific pairs within *Colletotrichum*, *Fusarium*, *Botryosphaeria*, *Alternaria* and others could be especially enlightening in this regard. What factors underlie the apparently avirulent lifestyles of endophytes? Do they lack, downregulate, or fail to upregulate the genetic architecture needed to cause disease in their hosts? Does quorum sensing play a role in the transition to virulence? Comparative genomics in an evolutionary framework would provide comprehensive insight into these and related questions.

Similarly, assessing the proteome, metabolome, and other classes of cellular contents and products now is possible not just for single species, but for pairs of organisms united in symbiosis. Comparative work using sterile and inoculated plant tissues could address the ways in which plants permit or tolerate colonization by these obligate heterotrophs, even in cases in which carbon is a limiting factor (e.g., the tropical forest understorey; Arnold 2002). Evidence from experimental inoculations suggests that when sterile plants are colonized by endophytes, systemic resistance is not induced (Arnold *et al.* 2003). Do endophytes avoid activating the defenses of their hosts? How do host responses to abiotic stress differ in the presence and absence of endophytes? Moving endophytes into the era of modern biotechnology will provide a new vision

of the functional diversity of endophytic fungi and their relatives, and will benefit from large collections of carefully referenced voucher specimens whose phylogenetic relationships are known.

## 8. Conclusions

Understanding the ecology, evolution, and importance of fungal endophytes is a daunting prospect given the tremendous number of fungi capable of forming endophytic associations and their uniqueness relative to other plant-associated microbes. However, never before has the future of endophyte biology been more compelling. By expanding surveys to include early-diverging lineages of plants, we will gain insight into the evolution of endophytic symbioses over the history of green plants. By integrating endophytes into robust phylogenies, we will shed light on the evolution of mutualism, virulence, and other ecological modes in the most species-rich phylum of fungi, and will provide new resolution for the fungal tree of life. By making alpha-taxonomic studies comparable with one another, we will take major steps forward in understanding broad patterns in endophyte distributions, diversity, and host specificity. By integrating surveys of culturable and unculturable fungi, we will understand for the first time the ecological interplay of these cryptic symbionts in shared tissues, and the degree to which our conclusions regarding endophyte ecology – based almost entirely on culturable endophytes – are generally applicable. By creating permanent depositories we can dramatically expand the potential contributions of all endophyte surveys, and create an invaluable resource for future work. By integrating endophytes into comparative genomics and related areas of research, we will begin to address the mechanisms of virulence in evolutionarily relevant comparisons. Uniting these steps forward is an interdisciplinary approach that will rely on the interplay of surveys, hypothesis-driven research, and classical training in mycology. By taking into account the lessons provided by mycologists who have puzzled out the diversity of endophytic fungi for hosts surveyed thus far, we can begin to assess, in an ecologically realistic context, the ecological importance of these cryptic microfungi at multiple trophic levels. At risk of falling into the trap of ending this review with a call to “do it all,” arguably there is no better time to be an endophyte biologist than the present – and the years to come.

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