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Chapter 10
Biogeography of Root-Associated Fungal Endophytes

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10.1 Introduction

Fungal endosymbionts colonize living roots of all plants and across all surveyed terrestrial ecosystems. Generally considered benign intracellular inhabitants of plant roots, these hidden players inside plants may control plant productivity and community assembly, and thus ultimately the function of ecosystems (Bever et al. 2010, 2012). In addition to the better-known and more extensively studied mycorrhizal symbionts, a diverse group of non-mycorrhizal, nonpathogenic, endophytic fungi also occupies root tissues (Mandyam and Jumpponen 2005; Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011). However, presence of a fungus in the root system does not make it an endophyte (Jumpponen et al. 2011): some superficial inhabitants may be casual colonizers from the soil environment, whereas others are adapted to the root environment—colonizing roots persistently and maintaining some metabolic or molecular interaction with the plant host (Hardoim et al. 2008). As a result, healthy plant roots often host complex and heterogeneous fungal communities (Vandenkoornhuyse et al. 2002; Glynou et al. 2016; Porras-Alfaro et al. 2008)

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that seem abundant in all plants across terrestrial ecosystems (Mandyam and Jumpponen 2005; Sieber and Grünig 2013).

Although the presence of endophytes is widely acknowledged for a range of habitats and hosts (e.g., Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005; Kageyama et al. 2008), the characterization of the root-associated endophyte functions in symbiosis, particularly in natural environments, remains poorly resolved (Mandyam and Jumpponen 2005; Newsham 2011; Mayerhofer et al. 2013). As a result, our understanding of endophyte habitat requirements and their distribution, ecology, diversity, and contribution to plant community feedbacks is currently superficial at best (Mandyam and Jumpponen 2005; Mandyam and Jumpponen 2015). Similarly to the mycorrhizal fungi (see Wilson and Hartnett 1998; Hartnett and Wilson 1999; van der Heijden 2002), inter- and intraspecific variability in host responses may, in part, structure the plant communities (Mandyam et al. 2012), although only sparse empirical evidence exists for such community modulation by the root-associated endophytes (but see Reininger et al. 2012; Aguilar-Trigueros and Rillig 2016). In addition, root-associated endophytes may alter biogeochemical processes, including the breakdown of organic forms of nitrogen (Upson et al. 2009; Mahmoud and Narisawa 2013; Yang et al. 2015). Further, a recent meta-analysis also highlighted their roles in protecting plants against drought and climate warming (Kivlin et al. 2013).

Root endophyte communities include diverse fungi that represent a range of taxa and ecological roles (Mandyam and Jumpponen 2005; Mayerhofer et al. 2013). Some clearly benefit host plants, whereas others may compromise plant performance (Saikkonen et al. 1998; Mandyam and Jumpponen 2015; Bonfim et al. 2016). While some interpret the parenchymatous net and/or the labyrinthine tissue that helotialean endophytes possess when colonizing woody plants (see Jumpponen and Trappe 1998, Lukesová et al. 2015) as a potential site for nutrient exchange, such structures are far from universal and may not form with nonwoody hosts (see Yu et al. 2001). In addition to the common absence of such well-defined, physiological interface that would provide a distinct site for nutrient exchange (Yu et al. 2001), the reported necrotic cytoplasm and cell death evidenced in detailed microscopic investigations of intracellular colonization (Deshmukh et al. 2006; Peterson et al. 2008) further challenge deciphering the host–endophyte interaction. This contrasts with mycorrhizal fungi whose definitions strongly rely on morphological and structural attributes of the fungus–host dual organ (Bonfante 1984, 2001; Smith and Read 2008) and—particularly for the arbuscular mycorrhizal fungi—includes the development of a defined and distinct interface for resource exchange (Bonfante 2001; Genre et al. 2008; Smith and Read 2008).

Distinguishing and identifying the host–fungus interfaces is not a simple task and is additionally complicated by the many organisms that simultaneously inhabit the root tissues. For example, Vági et al. (2014) visualized simultaneous colonization of root tissues and cells by arbuscular mycorrhizal fungi and fungal root endophytes suggesting that endophyte colonization does not necessarily lead to cell death (compare Deshmukh et al. 2006; Peterson et al. 2008). Further, the distinction of plant–fungus interaction may not clearly fall into a single facet of

known ecologies and may be inconsistent even within a strain: Lukesová et al. (2015) observed a helotialean endophyte to form microsclerotia typical to endophytes as well as coils resembling those of ericoid mycorrhizae when colonizing a *Vaccinium* species that is more commonly known to form ericoid mycorrhizae with its fungal partners. It is the combination of these complexities and inconsistencies that crumble the foundations of making simple generalizations about endophytes and their interactions with the host.

Similarly to the complexity of explicitly defining the host–endophyte interface, the functional attributes of these interactions have eluded simple and general functional categorization. Several underlying mechanisms have been proposed to explain host responses to the ubiquitous endophytes (see Mandyam and Jumpponen 2005; Rodriguez et al. 2008; Newsham 2011), and empirical evidence is starting to accumulate for, e.g., endophyte regulation of nutrient uptake, phytopathogen suppression, and control of environmental tolerances. Recently, Yang et al. (2014) observed that endophytic *Phomopsis liquidambari* upregulates genes related to nitrogen uptake and metabolism. These regulatory responses coincided with greater biomass accumulation and nitrogen content in inoculated plants compared to non-colonized controls. Such findings are particularly interesting because of the diverse enzymatic capacities of root endophytic taxa and strains (Caldwell et al. 2000; Mandyam et al. 2010; Knapp and Kovács 2016), which may be crucial for the maintenance of diverse ecosystem functions. Similarly, even though precise mechanisms still often remain unclear (but see review by Hamilton et al. 2012), fungal endophytes present some promising candidates for biocontrol and either antagonize or suppress phytopathogens (see, e.g., Harman et al. 2004; Chen et al. 2016; Terhonen et al. 2014a, b). Finally, endophytes can alter plant ecophysiological performance and thus also the environmental tolerances of their hosts (Kivlin et al. 2013). Recent studies suggest that endophyte inoculation can increase net photosynthesis and water use efficiency, improving drought tolerance (Molina-Montenegro et al. 2016). These findings from independent empirical studies support earlier speculation that endophytic fungi may produce phytohormones or secondary metabolites that promote host performance (Mandyam and Jumpponen 2005), defend against antagonists (e.g., Braun et al. 2003; Hamilton et al. 2012), alter host stress responses, or control host metabolism—particularly carbon and nitrogen metabolism—leading to changes in biomass allocation and/or improved performance (Mandyam and Jumpponen 2014).

As diverse as the root-associated endophyte communities can be phylogenetically and functionally, they appear adapted to the root environment: the endophyte communities are distinct from those that inhabit the adjacent soil and other plant organs (Herrera et al. 2010; Porras-Alfaro et al. 2011, Maciá-Vicente et al. 2012). Yet, a large proportion of the endophyte communities remains poorly known (Mandyam and Jumpponen 2005; Coleman-Derr et al. 2016). These root-associated communities—or at least their studied components—have been proposed to improve plant fitness (Mandyam and Jumpponen 2005; Newsham 2011), albeit the experimental evidence for the mechanisms is rather sparse or inconclusive (but see Aguilar-Trigueros and Rillig 2016) highlighting the potential environmental or

biotic context dependency of host responses (Kivlin et al. 2013; Mandyam and Jumpponen 2015).

Here, we draw from the available data on distributions of root-associated endophyte communities and explore questions examining the primary determinants of those communities. As a result of our current research focus on endophytes and rhizobiomes of grasses and the wide diversity of fungi that have been described as root-associated endophytes, we primarily focus on fungi associated with grasses. We fully acknowledge the findings of recent studies that suggest that endophyte colonization is controlled by biotic (host), edaphic, climatic, or spatial (location) factors (Zubek et al. 2009; Ranelli et al. 2015; Bokati et al. 2016), but propose that different endophyte groups are under different controls or selection pressures (Ruotsalainen et al. 2004; Ranelli et al. 2015). While some effort exists to map and better understand the biogeography of the better understood mycorrhizal endosymbionts—even on global scales (e.g., Öpik et al. 2010; Põlme et al. 2013; Davison et al. 2015)—very little is known about the controls of the distribution of the diverse fungal endophytes that seem universally present in most plant roots (Queloz et al. 2011).

We ask questions about whether or not the efforts to seek universal drivers for the endophyte community assembly are likely to prove productive. We approach these issues from two distinct perspectives:

First (from the whole community perspective): is there evidence for distinct communities across broad geographical scales?

Second: is there any evidence that the most well-studied endophyte taxa (i.e., the helotialean endophytes that commonly colonize the roots of woody plants in temperate and boreal forests and the pleosporalean endophytes that are emerging as the common grass associates in the temperate grassland systems) carry any biogeographic signal?

We acknowledge that the data to evaluate such questions are sparse. Thus, by definition, our discussion is largely speculative. We posit, similarly to Glynou et al. (2016), that the organismal functions should be tightly linked to their habitat and thus the ecological roles can be derived from the location of the focal organisms. If endophyte occurrence is correlated with abiotic (environmental conditions such as precipitation) or biotic (host phenotypes or phylogeny) drivers (see Maciá-Vicente et al. 2008, 2012; Glynou et al. 2016), that may facilitate efforts to elucidate endophyte functional roles.

10.2 Biogeographic Signal in Endophyte Communities

In general terms, geography, dispersal, environment, and organismal interactions determine the current and observable biogeographies (Prosser et al. 2007). However, the Baas-Becking hypothesis (Baas-Becking 1934) posits that microorganisms—including the fungal endophytes—are globally cosmopolitan and have high diversity locally but only limited beta-diversity. This is a result of their great

dispersal potential and large population sizes (Fitter 2005), leading to the environmental selection that the Baas-Becking hypothesis suggests. Clearly, a large body of current evidence challenges the "everything is everywhere, but, the environment selects" hypothesis (Baas-Becking 1934) and implies that, in addition to the environmental drivers, dispersal limitations also control the assembly of root-associated fungal communities (e.g., Peay et al. 2012; Peay and Bruns 2014). The relative importance of the environmental drivers and dispersal limitations may be context dependent and differ among fungal guilds. For example, root endophytes may possess distinct biogeographies (Glynou et al. 2016), whereas aboveground (foliar), and other root-associated (mycorrhizae) plant symbionts may not (Tedersoo et al. 2012; U'Ren et al. 2012). Either this indicates that different drivers control the assembly of the different fungal communities (Tedersoo et al. 2012), that some fungal groups have received more research attention than others, or that our understanding of the process of fungal community assembly is far from complete. To exemplify the last point, we highlight the contrast between the results of Queloz et al. (2011) versus Glynou et al. (2016). Whereas the former—focusing on the *Phialocephala fortinii* sensu lato *Acephala applanata* species complex (*Phialocephala–Acephala* complex, hereafter PAC) characterized by cryptic species—explained that stochastic effects are primarily responsible for PAC community composition, the latter highlighted the strong influence of the local environment in determining root endophyte community composition. Clearly, the jury is out on the importance of environmental or habitat filtering of root endophyte communities.

Detecting a biogeographic signal in the heterogeneous root-associated symbiont communities is a challenging undertaking. Efforts to elucidate the drivers that result in the observed organismal distribution pose an even greater challenge. Glynou et al. (2016) suggested that climatic drivers may be more important than dispersal limitation or soil variables in influencing the assembly of a root-colonizing fungal endophyte community. Additional variables that may include a set of other environmental, historical, or biotic variables were also considered influential under a combined "spatial effect" variable in that research effort. Interestingly, Glynou et al. (2016) observed no evidence for strict distance-decay effect (see Green et al. 2004; Peay et al. 2007) suggesting that it is not the geographic distance—and therefore not dispersal limitation—but instead the site-relevant environmental attributes, and thus the endophyte and host plant environmental tolerances, that are the primary filters that control the endophyte community assembly. These findings are congruent with Kivlin et al. (2014), who similarly concluded that fungal communities in soil and those collected from air currents had no compositional shifts over distance, but rather seemed structured by environmental filtering. Because these authors observed community commonalities among sites that were very distant from each other, it seems that the soil-inhabiting and endophyte communities may distribute propagules abundantly and over great distances. However, contrastingly, Glynou et al. (2016) observed that sites separated by greater distances tended to be more similar than those adjacent to each other, suggesting that some environments may strongly inhibit the establishment of some propagules.

These latter conclusions are similar to the Baas-Becking hypothesis and to empirical results from studies of shoot-colonizing endophytic fungi (U'Ren et al. 2012).

Jumpponen and Egerton-Warburton (2005) attempted to summarize components that define community assembly by liberally adopting Diamond's environmental filtering model (Diamond 1975) for mycorrhizal communities. A similar approach can be used for root-associated endophytes. In this model, local and regional propagule pools represent a transient community, from which persistent community members are selected, possibly based on abiotic filtering (see Kivlin et al. 2014). Only those members from the available pool that can establish under the prevailing environmental conditions may become members of the endophyte community, given that they locate compatible hosts to colonize. Among those that establish, biotic interactions (competition and facilitation) select individuals and species that remain and persist in the community. These persistent community constituents then enrich the local propagule pools with abundant short distance dispersal that can be initiated from the relatively few propagules that had dispersed over larger distances. This model would lead to a core community enriched with locally adapted taxa along with numerous transient components that persist in the system for only limited periods of time under the current prevailing environmental conditions. Although such filter models may overly simplify community assembly and dynamics, they provide a starting point for dissecting processes that lead to biogeographic signals in endophyte communities.

What then constitutes the local or regional propagule pools that permit the long-range dispersal of root-associated endophytic fungi and upon which the environmental selection may act? Many root endophytes rarely sporulate and thus lack the abundant dispersal propagules (Jumpponen and Trappe 1998; Addy et al. 2005) that would best explain the absence of distance-decay effects described in Glynou et al. (2016) and Kivlin et al. (2014). It is possible that the endophytes, or some constituents of the root endophyte community, would share dispersal strategies similar to those of vertically transmitted foliar (clavicipitalean) grass endophytes that colonize the seed and thus the emerging plant at the time of germination (Clay and Schardl 2002; Saikkonen et al. 2004). However, to our knowledge, there is no strong evidence supporting such seed-borne vertical transmission, although some endophytes can be isolated from both above- and belowground tissues, including the seed coat (Redman et al. 2002). In fact, the endophyte communities seem quite distinct between the above- and belowground plant compartments, albeit both may be recruited from the same soil inoculum pool (see Bodenhausen et al. 2013). But, there are some possible exceptions, which show commonalities in composition between roots and shoots (Rodriguez et al. 2009; Herrera et al. 2010; Porras-Alfaro et al. 2014b). Other possible dispersal mechanisms may include vector-mediated propagule transport and deposit. Two lines of evidence support this possible dispersal mechanism. First, some endophytes commonly develop structures that are resistant to the environment, as exemplified by the common microsclerotia of the so-called dark septate endophytes (Jumpponen and Trappe 1998; Currah et al. 1993; Kageyama et al. 2008; Porras-Alfaro et al. 2008). Second, some studies have reported that fungal communities present in the herbivore dung include a

considerable proportion that overlap with root-associated fungal communities (see Hawkins 1996, 1999; Porras-Alfaro et al. 2008; Herrera et al. 2011a and references therein). It is not only the mammalian herbivores that may carry inoculum. Bultman and Leuchtmann (2008) summarized data from clavicipitalean fungi and concluded that insects are likely dispersers of propagules for foliar endophytes. Taken together, herbivore-mediated dispersal combined with the persistent propagules that resist environmental decay may to a degree explain the lack of dispersal limitations. Finally, dispersal mechanisms common in soil-inhabiting fungi (see Kivlin et al. 2014), e.g., wind dispersal combined with adhesion to soil particles, may also underlay the observed broad distribution and effective dispersal of the root endophytes.

10.3 Biogeographic Signal in the Commonly Observed Endophyte Taxa

One challenge in identifying a biogeographic signal in populations of root-associated endophytes is the difficulty of strict and explicit taxon delineations. Currently, the efforts to identify endophyte community constituents are hindered by the lack of a consistent taxonomic and phylogenetic framework. In other words, many of the constituent taxa may still remain undescribed and new to science. Fortunately, recent morphological and molecular systematic work has begun to elucidate these issues for some pleosporalean taxa (Knapp et al. 2015). These studies circumscribed three novel genera that are related to other common endophytes in grassland biomes (Mandyam et al. 2010, Porras-Alfaro et al. 2008) and clearly highlight the lack of understanding of the endophyte taxon distribution even at the coarsest spatial levels. Advances have also been achieved for the helotialean endophyte taxa. For example, the development and use of restriction fragment length polymorphism (RFLP) probes, inter-simple sequence repeat (ISSR) (Grünig et al. 2001), and microsatellite markers (Queloz et al. 2010) have assisted in taxon assignments and spatial and/or temporal dynamics of the cryptic PAC taxa. Combined, these efforts have elucidated spatial dynamics of genotypes over extended periods of time in forest tree plantations (Stroheker et al. 2016) indicating that—once established—endophyte communities shift over space and time and that few genotypes maintain persistent colonization. These studies on defined spatial scales highlight the dynamic nature of endophyte communities and populations and contrast with those that highlight a lack of biogeographic signal in endophyte communities on larger spatial scales (Queloz et al. 2011).

Although the PAC fungi have been successfully assigned to a number of molecularly distinct, but morphologically indistinguishable and thus cryptic species, this is not the case for all root-inhabiting endophytes that still lack tools permitting reliable taxon assignments. The lack of a morphological taxonomic framework, unreliable production of taxonomically indicative morphological

structures (Jumpponen and Trappe 1998; Addy et al. 2005; Sieber and Grünig 2006), and existence of many closely related cryptic taxa that possess some degree of host preference (Grünig et al. 2004, 2008a, b; Queloz et al. 2008, 2010) all complicate taxon identification. Combined, these challenges severely hinder a better understanding of the biogeography of endophytes and their communities.

A further challenge in seeking broad biogeographic patterns of plant-associated organisms is that host plants are not globally distributed. As a result, separating host-mediated effects from environmental and dispersal effects becomes increasingly challenging as the spatial scale increases. Detecting geographic range limits of host-specific fungi would require co-modeling the distribution of the host plants in order to separate dispersal limitation from limitations due to host plant availability. Consequently, many of the existing studies that aim to tackle these challenges focus on different host species that are not present in all sampled locations (e.g., Maciá-Vicente et al. 2008). Those few studies that have succeeded in meeting the challenges presented by distribution of the hosts provide evidence that root-associated endophyte communities colonizing conspecific hosts across larger geographic ranges do indeed shift across large spatial scales and carry a biogeographic signal (Herrera et al. 2010; Glynou et al. 2016).

In those studies, Herrera et al. (2010) compared cultured, root-associated fungal communities of blue grama grass (*Bouteloua gracilis*) along a transect from Mexico to Canada. Although the communities differed in the less common members, many taxonomically related groups commonly occurred at all sites (including fungi in the Pleosporales, Agaricales, and Hypocreales). Because shared members of the dominant groups resulted in communities that were more similar among adjacent sites, the community and geographic distances were negatively correlated—consistent with the distance-decay models (see Green et al. 2004; Peay et al. 2007). Similarly, Glynou et al. (2016) analyzed cultured root endophytes of non-mycorrhizal plants in the genus *Microthlaspi* across 52 plant populations in Europe. These studies revealed that climate—along with geographic controls—best explained endophyte community composition. Corroborating the findings of Herrera et al. (2010), Glynou et al. (2016) also observed a few common taxa in the orders Pleosporales and Hypocreales, and also Helotiales, that altogether represented approximately half of the collected isolates. Taken together, these studies suggest that while common endophytes may occur ubiquitously across large geographic ranges, the communities as a whole can be strongly influenced by environmental drivers. However, additional research efforts are necessary to expand the geographic reaches of studies, even if they must rely on naturalized, non-native taxa such as *Arabidopsis* (e.g., Lundberg et al. 2012; Bodenhausen et al. 2013).

As a comprehensive treatise of the distribution of root endophytes would be an exhausting exercise in futility, we broadly target the commonly observed helotialean PAC endophytes in temperate and boreal forested ecosystems (Grünig et al. 2008b; Queloz et al. 2011) and the pleosporalean endophytes that appear common in grassland ecosystems in both North America (Porras-Alfaro et al. 2008; Mandyam et al. 2010) and Europe (Knapp et al. 2012, 2015). We fully acknowledge that we are likely to combine several biological species into super-taxa. At the same

time, our speculation and broad conclusions are not sensitive to cryptic species or inaccurate taxon delineations. Despite our very broad grouping of taxa commonly observed as endophytes, some patterns still emerge. In a nutshell, while our data use coarse categories for focal taxa, it appears that the members of PAC are commonly and abundantly present in temperate (Ahlich and Sieber 1996; Queloz et al. 2005) and boreal coniferous forested ecosystems (Summerbell 2005; Kernaghan and Patriquin 2011; Vohnik et al. 2013; Terhonen et al. 2014a) as well as in arctic tundra ecosystems (Björbäkmo et al. 2010; Walker et al. 2011; Dean et al. 2013). This pattern is in stark contrast with the near absence of these common fungi in temperate grassland ecosystems in North America (Porras-Alfaro et al. 2008; Mandyam et al. 2010) and Europe (Knapp et al. 2012). Instead of the common helotialean components, these grassland ecosystems host a large pleosporalean component. Interestingly, these pleosporalean isolates, when inoculated on either native or model hosts, produced morphological structures quite similar to those reported for the helotialean fungi from forested systems (Mandyam et al. 2010, 2012; Knapp et al. 2012). Taken together, these observations seem to suggest that there is some level of biome specificity in the constituent taxa that colonize hosts in distinct grassland and forested biomes. Although our discussion here is quite speculative, we propose that the hypothesis of biome specificity serves as a starting point for more detailed studies, including perhaps common garden or reciprocal transfer experiments that would permit better and more rigorous testing.

Our speculation utilized studies that relied exclusively on efforts that isolated fungi into pure culture. We considered this important as only few community-level studies fulfill Koch's postulates, which we consider mandatory to confirm whether any isolate forms endophyte symbiosis (see Jumpponen et al. 2011). However, these pure culture studies are also burdened by a potential shortcoming. There is a question whether the pure culture data are a result of culturing bias (see, e.g., Walker et al. 2011). However, our choices for targeted systems are a result of existing available information, and our cursory synthesis leads to conclusions that while some biogeographic signals may distinguish the forested and grassland systems, we may still be far from being able to argue for biome-specific, root-associated endophyte guilds.

10.4 Drivers of the Root-Associated Endophyte Communities

The few studies that have focused on identifying the drivers that structure root-colonizing fungal communities rather consistently imply some degree of importance for edaphic or climatic conditions, or both (e.g., Bokati et al. 2016; Glynou et al. 2016), in addition to some control by the host taxon (e.g., Bokati et al. 2016). Naturally, edaphic and climatic drivers are not geographically randomly distributed but often strongly correlate with each other leading to complex problems in

selecting the best explanatory models and variables. It is also unlikely that a single strong driver governs the fungal community dynamics. Rather, the community dynamics are near certainly under control of multiple, interacting variables that affect which species successfully colonize host roots. To exemplify, Bokati et al. (2016) recently concluded that soils play a primary role in structuring the root-associated fungal communities in maize, wheat, and their progenitors. Despite the soil's primary role, there were some similarities in the communities that were best explained by host species identity (Bokati et al. 2016). These results are congruent with others that conclude that soil microbiomes are originators of root microbiomes (e.g., Edwards et al. 2015; Zarraonaindia et al. 2015) and biotic and/or abiotic filtering likely takes place during the community assembly of host-associated microbiomes. This soil-driven community assembly would produce root communities that are effectively subsets of soil and rhizosphere communities. Further, although aerial dispersal likely dominates, as perhaps implied from the Baas-Becking hypothesis, other factors, such as vector-mediated dispersal, may also control how endophyte communities assemble. Long-range transmission by insects (as is the case for clavicipitaceous endophytes; Bultman and Leuchtmann 2008) or herbivores (see discussion in Porras-Alfaro et al. (2008); Herrera et al. 2011a) provides evidence that some of the transmission—and thus also assembly—may be vector-driven and not exclusively airborne. However, these alternate dispersal hypotheses are challenging to test given that roots likely filter endophyte communities from the more diverse microbial community in the surrounding soils.

Some data describing fungal communities colonizing Poaceae suggest that many of the grassland species harbor a suite of cosmopolitan root-associated taxa (Herrera et al. 2010; Knapp et al. 2012, 2015). In some cases, some of these taxonomic clades vary over geographic space or environmental conditions (Herrera et al. 2010, 2011b). Recent and unpublished data examining the root communities in five different grass species over geographic distances suggested that there are some modest distinctions among sites (see case study below), but none among hosts. Similarly, provisional microscopy assessment also indicated that the fungal endophytes colonize different grass species at about the same rate, although some of the grasses responded to water amendments by, for example, quickly increasing the proportion of some clades (Herrera et al. 2011b). This evidence suggests that the root-associated communities are not stable in time or across environmental stressors, but may—indeed—rapidly respond to changes in environmental conditions and shift dramatically over very short periods of time. Although speculative, these data suggest that additional research is needed to ascertain the effects of localized edaphic and environmental conditions on root-associated fungal communities, in addition to identifying drivers on broader geographical scales. Similarly, while the colonization frequency by different endophytes may differ among host species (Tejesvi et al. 2013), there appears to be no strict host specificity wherein some endophytes would prove incapable of colonizing a host species or even a guild of hosts in either natural or manipulative experimental settings (Mandyam et al. 2012). Collectively, there is some support for conclusions that the root-associated fungal communities are not specifically bound to any one host but rather are generalists, as suggested in previous synthetic efforts (Jumpponen and Trappe 1998; Mandyam and Jumpponen 2005), and that these

communities may be transient and/or respond to environmental drivers either over spatial or temporal scales.

10.5 Case Study

As a part of an ongoing investigation of root-associated mycobiomes (fungal rhizobiomes) of common graminoids in central and south central United States, we evaluated the use of ITS2 barcodes to identify unknown cultured fungal isolates. Our approach parallels that described in Shokralla et al. (2015), wherein the authors MiSeq-analyzed PCR-amplified cytochrome c oxidase DNA barcodes that spanned 658 bp from 1010 specimens representing eleven orders of arthropods. That approach proved successful, and the authors argued that the use of next-generation sequencing of taxon barcodes permitted superior data generation at reduced cost compared to the more conventionally used Sanger sequencing.

In the course of our ongoing rhizobiome research, we isolated fungi into pure culture from a total of 23 sites located in Colorado, Kansas, Nebraska, New Mexico, Oklahoma, Texas, and Wyoming (Fig. 10.1). Our goal was to establish replicated latitudinal gradients to enable robust generalizations that capture natural and multifactor climatic contexts. We aimed to address three specific hypotheses on root endophyte communities: (1) root endophyte communities are distinct among the host species, i.e., shaped by the host identity (Hartmann et al. 2009; Prescott and Grayston 2013); (2) root endophyte communities decrease in similarity with increasing geographic distance; and (3) root endophyte communities correlate with environmental gradients, i.e., are structured by environmental drivers and thus likely driven by the environmental tolerances of the constituent species (see Jumpponen and Egerton-Warburton 2005). We take this opportunity to evaluate the first two hypotheses with early emerging data from this project to better refine these hypotheses and to provide a basis for future discussion on potential drivers. Although we list three hypotheses here, the sparse data matrices resulting from the initial ITS2 barcode evaluation provide inadequate data to compare models with a great variety of environmental drivers that may be correlated with each other. As a result, we address only the first two hypotheses here.

For this rhizobiome research project, within each of the selected sites, we targeted grasses that are dominant, widely used in restoration, and span the major tribes of Poaceae in Central US grasslands: *Andropogon gerardii* (big bluestem); *Bouteloua gracilis* (blue grama), *B. eriopoda* (black grama), *Buchloe dactyloides* (buffalograss), and *Schizachyrium scoparium* (little bluestem). Many of these grasses also host a variety of root endophytic fungi as indicated by earlier data (Barrow 2003; Porras-Alfaro et al. 2008; Herrera et al. 2010; Jumpponen et al. 2011; Mandyam et al. 2012), making them prime targets for rhizobiome surveys. A total of twelve whole plants were excavated with a transplanting shovel as described in Mandyam et al. (2012), and root systems were sampled for culturing at Western Illinois University (WIU) by Porras-Alfaro's group.

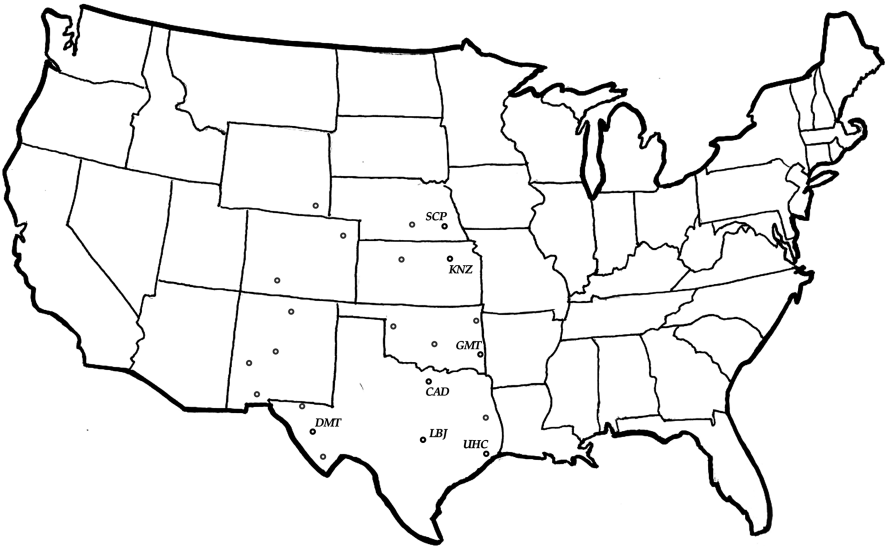

Fig. 10.1 Map of sites included in the current field survey of rhizobiomes in the common and dominant grasses. *Black dots* with site identifiers are those included in the case study here; *gray dots* identify additional samples not included in the preliminary barcode trial analyses. Sites are CAD, Caddo and Lyndon B. Johnson National Grassland, Texas; DMT, Davis Mountains State Park, Texas; GMT, Guadalupe Mountains National Park, Oklaholma; KNZ, Konza Prairie Biological Station, Kansas; LBJ, Ladybird Johnson Wildflower Center, Texas; SCP, Spring Creek Prairie Aubudon Center, Nebraska; UHC, University of Houston Coastal Center, Texas. Additional information on the sites is available in Table 10.1

To culture fungi from excised root tissues, surface-sterilized roots of the replicate individuals were plated on malt extract agar (MEA) on 48-well plates. A subset of the surface-sterilized roots was pressed against the media to confirm the effectiveness of the surface sterilization—these press controls remained largely free of any fungal colonies, indicating successful surface sterilization. Fungi emerging from roots were aseptically transferred to MEA, and representatives of the cultures are currently maintained at the WIU Fungarium and at UNM in cryovials with sterile water.

From >2000 pure cultures generated thus far, we selected 768 early emerging isolates to preliminarily evaluate the utility of barcode analyses. In this experiment, our primary goal was to test the utility and expedience of the barcode identification for a large number of cultures and to assign them to provisional OTUs for more detailed screening. These analyses and conclusions will be further confirmed with Sanger sequence data once the culturing efforts are completed. From the selected pure cultures, DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, Wisconsin) and adjusted to 2 ng μl^{-1} concentration. Similarly to the approach described by Shokralla et al. (2015), we chose a barcode of life locus that has been proposed for fungi (Schoch et al. 2012) and the PCR-amplified internal transcribed spacer 2 (ITS2) locus using primers that flank the target region

(Ihrmark et al. 2012). A total of 20 ng of each template DNA was PCR-amplified in 50 μl reactions using fITS7 (Ihrmark et al. 2012) and ITS4 (White et al. 1990) primers, 192 of each carried 12 bp DNA-tags that differed in a minimum of two nucleotides. The PCR conditions and protocols were identical to those described earlier (Oliver et al. 2015a, b), and except that for expedience, we omitted the primary PCR without the DNA-tagged primers. This approach included 192 pure cultures in each of four MiSeq Libraries, to each of which Illumina TruSeq adapters were ligated using a GeneRead DNA Library I Core Kit (Qiagen, Hilden, Germany; catalog #180432) at the Integrated Genomics Facility at Kansas State University. The four libraries were paired-end sequenced using a MiSeq Reagent Kit v3 (Illumina, San Diego, California) with 2 × 300 cycles in a combined run, from which ~10% of the anticipated total yield—or roughly two million raw reads—were expected for each of the four libraries.

Our barcode libraries yielded a total of 6,495,500 raw sequences across the four libraries (or ~1.6M reads per library); 758 of the 768 isolates (98.7%) yielded some sequence data. The paired raw sequences were contiged and quality screened as described previously (Oliver et al. 2015b) using mothur software (v. 1.33.1; Schloss et al. 2009): sequences with no exact match to primers or DNA-tags, with long homopolymers (>8), or with ambiguous bases were omitted. The sequences from the four MiSeq libraries were then merged to expedite downstream analyses and truncated to 236 bp—a length equal to the shortest resultant read—to facilitate pre-clustering of near identical (99.2% similarity) sequences and reduce potential sequencing bias (Huse et al. 2008). These data were screened for chimeras (uchime; Edgar et al. 2011), and 1,254 putative chimeras were omitted. A total of 4,588,780 reads passed quality screening and included a total of 15,188 nonidentical sequences, suggesting considerable heterogeneity in the dataset characterizing the collection of isolates. These data were used to estimate a pairwise distance matrix (conservative nearest neighbor clustering), based on which the sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity. The OTUs were assigned to putative taxon identities using the Naïve Bayesian classifier (Wang et al. 2007) with UNITE taxonomy reference (http://unite.ut.ee/repository.php). To improve data integrity, rare OTUs (OTUs with sequence count ≤ 10) were omitted from each DNA-tag-identified sample (Brown et al. 2015; Oliver et al. 2015a). This resulted in a total of 740 isolates with sequence data.

Of the 740 isolates that yielded sequence data passing our quality control, a total of 417 (56.4%) resulted in unambiguous single OTUs and thus potential barcode identification. The remaining 43.6% of the isolates resulted in more than one OTU, compromising thus the unambiguous identification. Reasons for the multiple OTUs resulting from presumably monospecific isolates are unclear but may include mixed cultures; multiple divergent ITS copies within an isolate (Thiery et al. 2012; Zhao et al. 2015); PCR-induced mutations (Qiu et al. 2001); stochastic generation of chimeric sequences (Fonseca et al. 2012; Shin et al. 2014) that remained undetected in our screening; cross contamination during DNA extraction, plate manipulation, PCR, or subsequent cleanup steps; polymerase errors (Eckert and Kunkel 1991; Oliver et al. 2015a); DNA-tag switching (Carlsen et al. 2012); and/or sequencing

artifacts (Medinger et al. 2010; Brown et al. 2015). To make use of these data as well, we assumed that the most abundant read for each isolate was the most likely representative of the template DNA of the intended isolate. Our ongoing research efforts include Sanger sequencing to evaluate the value and reliability of the barcoding approach. Regardless of the low percentage of isolates that yielded only one OTU for an isolate, we learned two important lessons from this exercise. First, generating data in a laboratory with an easy and inexpensive access to sequencing is very fast—generating these four libraries ready for data generation required less than 1 week of work in the laboratory. We conclude that this NGS approach serves as an expedient tool for preliminary screening of large isolate collections. Based on these efforts, the most interesting—or most common—isolates can be selected for more detailed analyses.

The 740 isolates retained in our analyses were assigned to a total of 132 OTUs that we presume to represent species level resolution at 97% sequence similarity. These OTUs represented 120 putative species, 71 genera, 48 families, and 26 orders. The close similarity of OTU and putative species numbers suggests that the OTUs likely approximate species level. However, ITS2 barcode-inferred OTUs may fail to resolve some closely related species, such as those exemplified by OTUs assigned to genus *Fusarium* (e.g., *F. oxysporum* and *F. redolens* in our dataset) or its sexual states (genus *Gibberella*) (Geiser et al. 2004). A large majority of isolates belonged to phylum Ascomycota (91.4%) followed by a small proportion of Basidiomycota (6.6%) and a few unclassified OTUs (~2%). On the order level, Hypocreales (36.9%) and Pleosporales (29.5%) dominated, although a few members of Agaricales (5.5%), Eurotiales (4.1%), Sordariales (3.2%), Xylariales (3.1%), and Helotiales (2.8%) were also present. Although the taxon rankings may differ, these order level data corroborate those of others. Glynou et al. (2016) observed that Pleosporales and Hypocreales (also Helotiales) represented a large proportion of isolates acquired from *Microthlaspi*, and Herrera et al. (2010) observed that Pleosporales, Agaricales, and Hypocreales were dominant orders in their isolates from *Bouteloua*. Notably, our data also included some Helotiales, but they were a rather minor component (~ 3% of the isolates). These helotialean isolates represented a few different putative species: uncultured *Lachnum* (14 isolates), *Acephala* (3 isolates), *Chalara* (3 isolates), and *Cryptosporiopsis ericae* (1 isolate). The small proportion of the helotialean taxa that have been confirmed endophytes strongly indicates that helotialean taxa are indeed rare in these grassland systems, supporting our earlier speculation on biome-defined endophyte guilds. Based on the congruence with the observations in Glynou et al. (2016) and Herrera et al. (2010), we conclude that barcode identification has the potential to serve as an expedient method for assigning large numbers of specimens into clusters approximating conspecific groups. However, this approach may suffer from issues emerging from operator errors and limited resolution in available databases.

Overall, in these barcode identified data, consistently with other published studies (e.g., Maciá-Vicente et al. 2008), the most common genera included *Fusarium* (20.0%) and its sexual teleomorph state *Gibberella* (3.1%) for a total of 23.1%

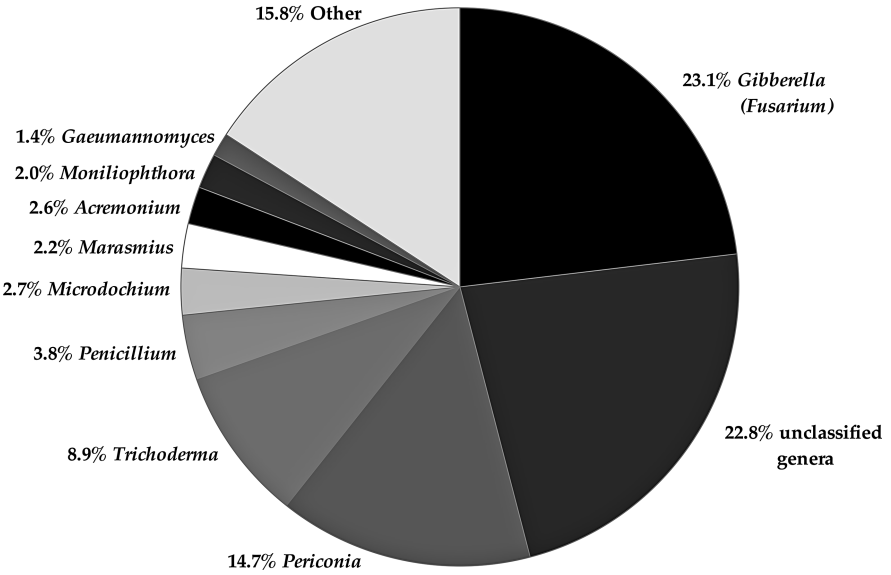

Fig. 10.2 Genera isolated in the current field survey of rhizobiomes (Fig. 10.1) in the common and dominant grasses. The *pie chart* includes ten most common genera—the remaining 61 genera are combined under "other"

of all isolates (Fig. 10.2). The second largest group was a mixture of isolates that lacked generic affinities (unclassified genera; 22.8% of all isolates). These were isolates placed into higher taxonomic levels including Pleosporales (10.1%) and Sordariales (2.0%) plus others that constituted <1% of all isolates. Interestingly, consistent with other studies from grassland ecosystems based on pure culture analyses (see Mandyam et al. 2010; Knapp et al. 2012), OTUs assigned to genus *Periconia* (Pleosporales) were also common and represented the second largest group of isolates with generic affinities in our analyses (14.7%). Other common taxa included some typically common soil and rhizosphere fungi (Fig. 10.2).

From these data, we selected those that permitted inferences on the phylogeography of the rhizobiome. Because the selection of isolates for our barcode trials was not specifically stratified to address these questions, we set some a priori thresholds for sample selection. We omitted all sites that did not yield a minimum of five isolates for a host sampled at that site. This resulted in a data matrix with seven sites and two to four hosts present per site, for a total of 19 observations (Table 10.1). As a result of this selection of samples, the number of included OTUs was reduced to 84.

We converted the sequence counts to presence/absence data and estimated the Bray–Curtis pairwise distances for use in nonmetric multidimensional scaling (NMS) in PC-ORD (v. 6.19; McCune and Mefford 2011). A two-dimensional solution ($k = 2$; Fig. 10.3A) resulted in stress (0.18) significantly lower for each axis than that derived from randomized data ($P < 0.05$). The two axes represented

Table 10.1 Site descriptions, site names, locations, elevations and host species sampled for the analyses of cultured fungal communities in the ITS2 barcode trials

Site	Name	Latitude	Longitude	Elev (m)	Grassland type	Hosts
UHC, TX	University of Houston Coastal Center, Texas	29.40N	95.05W	6	Coastal tallgrass	*Andropogon gerardii* (1), *Schizachyrium scoparium* (1)
LBJ, TX	Ladybird Johnson Wildflower Center, Texas	30.18N	97.87W	2554	Mixed grass	*Andropogon gerardii* (1), *Buchloe dactyloides* (1), *Schizachyrium scoparium* (1)
DMT, TX	Davis Mtns/ Mimms Ranch, Texas	30.63N	104.17W	2660	Desert, shortgrass	*Bouteloua eriopoda* (1), *Schizachyrium scoparium* (1)
CAD, TX	Caddo–LBJ National Grassland, Texas	33.42N	97.63W	272	Mixed grass	*Andropogon gerardii* (2), *Schizachyrium scoparium* (1)
KNZ, KS	Konza Prairie Biological Station, Kansas	39.10N	96.56W	415	Tallgrass	*Andropogon gerardii* (2), *Bouteloua gracilis* (1), *Buchloe dactyloides* (1), *Schizachyrium scoparium* (2)
SCP, NE	Spring Creek Prairie Audubon Center, Nebraska	40.69N	96.85W	406	Tallgrass	*Andropogon gerardii* (1), *Bouteloua gracilis* (1)

The short, *three letter codes* refer to the site names in Fig. 10.1. *Numbers in parentheses* following the host taxon binomials indicate the number of host individuals remaining in the analyses after applying the a priori thresholds for experimental unit retention in the case study. Note that these preliminary analyses did not permit inclusion of nested or interactive terms as a result of low number of included experimental units

10.4% and 53.6% of the variation (Fig. 10.3A). To test for differences among the host species and sites, we used multi-response permutation procedure (MRPP) suitable for unbalanced experimental designs like ours. The MRPP analyses indicated that while the hosts did not differ in the rhizobiomes detected in our isolation effort ($T = –0.923$; $P = 0.1757$), the rhizobiomes at the seven sites did ($T = –2.65$; $P = 0.0066$). These results are consistent with others (Bokati et al. 2016), who concluded that hosts were of lesser importance than the site/soil properties in root-associated fungal endophyte communities. Our subsequent pairwise comparisons indicated that Ladybird Johnson Wildflower Center site in Southern Texas was distinct from other sites ($P \leq 0.0318$) and that Guadalupe Mountains National Park in Texas was distinct from Konza Prairie Biological Station in Kansas ($P = 0.0197$). It is of note that the low number of replicates in these analyses did

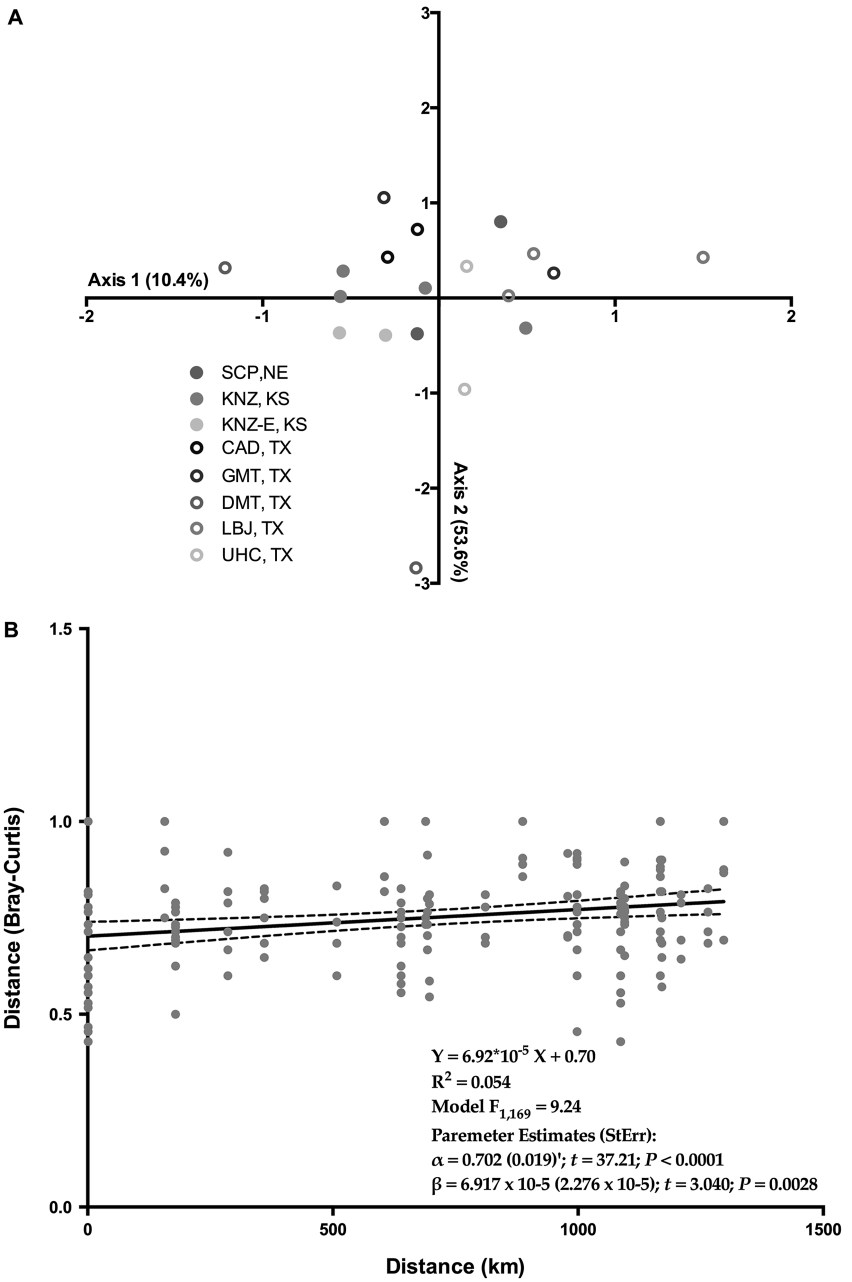

Fig. 10.3 A: Nonmetric multidimensional scaling (NMS) ordination of the 19 observations of cultured communities included in the ITS2 barcode trials. Multiple response permutation procedure (MRPP) indicated that while the host plants do not differ in their isolated fungal communities ($T = -0.923$; $P = 0.1757$), the sampled sites do ($T = -2.65$; $P = 0.0066$). B: The pairwise community distances (Bray–Curtis dissimilarity) plotted against geographic distance between the sites. Regression analyses indicate small (slope $= 6.92 \times 10^{-5}$) but significant ($P = 0.0028$) increase in the community distance with increasing geographic distance

not permit all possible pairwise comparisons. Yet, we argue that our data clearly suggest a geographic distinction among the analyzed rhizobiomes, whereas the host species were of lesser importance.

To further evaluate this geographic distinction, we analyzed the distance-decay effect (Green et al. 2004; Peay et al. 2007) using linear regression analysis of the Bray–Curtis distances and the geographic distances among the included sites (Fig. 10.3B). Interestingly, this analysis indicated a slight but positive correlation (slope $= 6.9 \times 10^{-5} \pm 2.3 \times 10^{-5}$) between the pairwise geographic distances and the pairwise community distances. The effect was rather weak, as indicated by the small slope ($-6.9 \times 10^{-5} \pm 2.3 \times 10^{-5}$) and low R^2 (=0.05) but highly significant ($T = 3.040$; $P = 0.0001$). However, one should bear in mind that our data matrix is preliminary and rather sparse. Thus, additional data are necessary to refine our observations and to shed further light into the dispersal limitations in these communities.

10.6 Challenges of Biogeographical Studies of Root Endophytes

We briefly touched on the challenges of broadscale studies on the root-associated endophytes resulting from poorly defined taxonomic frameworks and potential challenges of locating conspecific hosts over large geographic ranges. Here, we return to some additional challenges that stem from our lack of understanding of the ecology of root-associated fungi and the coinciding poor annotation of references in available databases.

Rhizosphere environments harbor a diversity of fungi with a range of potential interactions with their hosts (Vandenkoornhuyse et al. 2002; Glynou et al. 2016). However, presence of a fungus in the root system does not make it an endophyte. Although there are means to fulfill Koch's postulates to confirm endophytes isolated from roots (Jumpponen et al. 2011), they are rarely employed because the manipulation of symbiotic systems is challenging and tedious. Studies that inoculate acquired fungal isolates back to native hosts (Walker et al. 2011; Mandyam et al. 2012, Lukesová et al. 2015), non-model (Mugerwa et al. 2013; Knapp et al. 2012), or model plants (Mandyam and Jumpponen 2015) have observed indicative fungal morphologies within the roots and permitted simultaneous confirmation of endophytic colonization as well as evaluation of host growth responses to inoculation. These studies establish a model for the effort required for confirmation of endophytic association. Such experiments are particularly demanding with native plants, whose germination rates can be dismal and growth rates painfully slow. Fortunately, recent syntheses that summarize data and conclusions from model and native plant experiments strongly suggest that the model plant systems that are more simple to execute can serve as reasonable proxies to infer

colonization and host responses in native plant systems (Mandyam and Jumpponen 2015).

Both pure culture and direct environmental sequencing studies rely heavily on available reference databases such as UNITE (http://unite.ut.ee/; Tedersoo et al. 2011) or RDP (https://rdp.cme.msu.edu; Cole et al. 2014) for assigning isolates or phylotypes to taxa. However, although these databases and the automated classifiers (e.g., Naïve Bayesian classifier; Wang et al. 2007) make taxon annotations expedient and objective, additional assignments to ecological roles are lagging far behind. Further, transfer of information on the confirmed endophyte taxa to the existing databases usually works with a lag and requires some substantial community involvement. Such efforts are already underway for some groups of fungi (Nilsson et al. 2014) and lay a foundation for database annotations of additional functional roles. Alternatively, independent tools for ecological annotation, exemplified by FUNGuild (Nguyen et al. 2016), are likely to simplify sharing the emerging ecological information, but do similarly rely extensively on third party annotation of the database entries. Concerted community efforts to update and maintain these databases would likely expedite improved use of and greater insight into the data on endophyte communities and their phylogeographies. Some efforts to initiate curated databases for root endophytic fungi are underway (Gábor M. Kovács and Dániel G. Knapp, personal communication). The plans include crosslinking these databases with other fungal and sequence databases with oversight by advisory boards drawn from the community of endophyte researchers.

Another issue is data compatibility. Although ITS regions have been proposed as the primary barcode for fungi (Schoch et al. 2012), some studies have chosen the use of large subunit (LSU) as a target (e.g., Amend et al. 2010; Rigdon et al. 2013) in environmental analyses of fungal communities, whereas others choose alternative markers because of inadequate resolution within their target groups (e.g., Maciá-Vicente et al. 2008). Although many of the examined markers—such as the LSU and ITS regions—yield comparable data (Brown et al. 2014; Porras-Alfaro et al. 2014a), use of the different targets makes direct comparisons across datasets impossible. Similarly, the use of different subregions of the proposed universal fungal barcode—the ITS (Schoch et al. 2012)—compromises such direct comparisons. However, the choice of a marker is not straightforward: some have concluded that the ITS2 region is superior in high-throughput applications (Tedersoo et al. 2015), whereas others have claimed that ITS1 provides a superior resolution for Eukarya (Wang et al. 2015). Additionally, while ITS is commonly used, it is unreliable for distinguishing *Fusarium* species (Geiser et al. 2004), whose identification relies on other loci (e.g., translation elongation factor 1α—TEF-1α) that are also common in phylogenetic analysis within this group (Seifert 2009). As a result, each research group makes decisions on selecting the primary target region, resulting in datasets for which comparisons are possible only based on annotated sequence data at generic levels, at best. We cannot provide a recommendation for the target locus selection here, but wish to draw attention to the problem posed by heterogeneity in accumulated data. A potential solution is the use of multiple marker genes, as is common in phylogenetic studies (e.g., James et al. 2006).

However, single copy genes are difficult to deploy in direct environmental sequencing approaches, and additional genes linearly increase sequencing costs if used to identify collections of pure isolates.

In addition to the problems resulting from marker selection, data generation, and sparse information on the ecology of the fungi that reside within the rhizobiomes, there are gaps in our understanding of the distribution of endophyte taxa. Above, we highlighted two endophyte guilds—grassland endophyte communities that appear to host a large Pleosporales component and the distinct forested ecosystem communities that host a large Helotiales component. This is agreeably quite a coarse resolution to infer either distributions or commonalities within the endophyte communities. Yet, these coarse distinctions at least serve as a starting point for developing more defined hypotheses on the distribution of endophyte communities and their constituent taxa and eventually also the primary drivers that define those communities, be it host species, dispersal limitations, or edaphic and climatic environmental controls. What becomes apparent, however, is the urgent need to execute large-scale field studies to broaden the range of parameters that can be used to select the most likely controls for the assembly of endophyte communities. Although our cursory case study focusing on a subset of pure cultures isolated from grasses from widely distributed field sites strongly suggest distinctions among the sites, it falls short of identifying possible environmental drivers. Our goal—upon completion of the culture-based and culture-independent analyses—is to provide further insight into the primary drivers of the root endophyte communities.

10.7 Conclusions

We summarized some of the data available on the distributions of some of the better-known groups of root-associated fungal endophytes. Without aiming to be comprehensive in our treatise, we arrived at a conclusion—at the coarsest level of resolution—that at least two distinctly distributed guilds of root-associated endophytes seem to exist. One consisting mainly of pleosporalean culturable taxa appears common and perhaps dominant in the grassland ecosystems and another consisting of helotialean culturable taxa seems similarly common in forested and other northern ecosystems. Agreeably, such biome-level coarse syntheses leave many unanswered questions. However, we sincerely hope that the hypotheses that we pose spark greater interest in resolving questions about the distribution of fungal taxa that establish endophyte symbioses with their hosts.

We presented preliminary data that we generated in a trial of high-throughput sequencing of ITS2 barcodes to identify fungi in pure culture libraries. While these trials were only a partial success, they did nonetheless provide a limited dataset that permitted us to identify a number of common root-associated fungi from grassland ecosystems. These data suggested that, while the hosts were seemingly similar in their culturable rhizobiome communities, the sites were distinct. These results beg the obvious questions on the drivers of such distinctions. Our data provided some

support for distance decay, with greater dissimilarities among communities that were more distantly located. However, some of these sites were also located >1100 km apart, and several edaphic and climatic conditions differ among them. Because of the limited data matrices thus far, we did not explore environmental drivers—such as gradients in precipitation or annual mean and maximum temperatures. We hope these data provoke interest in studies that broadly address the composition of endophyte communities and rhizobiomes over large geographical scales. Clearly, there is evidence for geographic distinctions, but the underlying reasons remain open questions.

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