Mechanisms of Invasion and the Microbiome of Introduced Species

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Dedication

For Karen.

Abstract

Invasive species represent a critical threat to ecosystems and ecological communities. Understanding the mechanisms behind their invasion is important for understanding why they invade and the consequences of their invasions. Furthermore, invasive species, like all macroscopic organisms, harbor symbiotic microbes that constitute their microbiomes. Symbionts have been implicated in invasion success of several species, and they also represent an opportunity to learn about community assembly. In this dissertation, I explore the causes and consequences of plant invasion, and the microbiomes that invasive species harbor. In Chapter 1, I explore how two invasive, congeneric beachgrasses (*Ammophila arenaria* and *A. breviligulata*) differently alter plant succession in Pacific Northwest, USA dunes. The newer invader, *Ammophila breviligulata*, occupied a wider distribution across dunes than the established invader, *A. arenaria*, allowing *A. breviligulata* to persist longer through successional time. In Chapter 2, I characterize the above- and belowground symbionts of these two *Ammophila* species and the native *Elymus mollis*. I found that symbiont communities aboveground are most influenced by host species and geographic distance from one another, while those belowground are most influenced by environmental filtering. In Chapter 3, I conducted two experiments to test the mechanisms by which *A. breviligulata* invades dunes dominated by *A. arenaria*. I found that *A. breviligulata* dominates by tolerating competition from *A. arenaria*, and that there is some evidence that *A. arenaria* might limit itself via negative plant-soil feedbacks. Finally, in Chapter 4, I explore the relationship between herbivory and symbiont communities using the invasive wetland

forb *Lythrum salicaria*. I found positive associations between herbivory and symbiont communities indicative of facilitation.

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Introduction

Invasive species represent a critical threat to ecosystems and ecological communities. The establishment of invasive species can transform environments, alter ecosystem services, and radically shift community structure (D'Antonio and Vitousek 1992, Mack et al. 2005). Invasive plants are able to establish themselves in communities via several mechanisms such as positive response to disturbance (e.g. Seabloom et al. 2003, Hillerislambers et al. 2010), escape from natural enemies (Keane and Crawley 2002, Mitchell et al. 2006), or the establishment and/or disruption of mutualisms with pollinators or mycorrhizae (Richardson et al. 2000, Stinson et al. 2006, Dickie et al. 2010). Importantly, the mechanisms by which particular species invade may vary, and these mechanisms have important consequences for invasive species' impacts (Blumenthal et al. 2009, Maron et al. 2013).

One mechanism by which plants invade is through associations with microbes. Like all plants and animals, invasive species associate with diverse assemblages of bacteria, fungi, and viruses (Borer et al. 2013). In some cases, the presence of a particular species of microbe has been linked to plant invasion. For instance, pines (*Pinus contorta*) require the presence of their native ectomycorrhizal fungi to invade forests in New Zealand (Dickie et al. 2010), and introduced tall fescue (*Lolium arendinaceum*) harbors a systemic fungal endophyte (*Neotyphodium coenophialum*) that allows it to invade grasslands and slow succession (Rudgers et al. 2012). Recent studies have also shown infection by horizontally-transmitted fungal endophytes (*Alternaria* sp.) can increase invasion success of spotted knapweed (*Centaurea stoebe*) (Aschehoug et al. 2012, 2014). In other cases, microbes can play more indirect roles in invasion. For instance, the non-mycorrhizal herb garlic mustard (*Alliaria petiolata*) might invade by disrupting arbuscular mycorrhizal networks of native plant species (Stinson et al. 2006, but

see Poon and Maherali 2015). Finally, microbes can influence plant invasion through feedbacks with the soil that promote the growth of the invasive species or hinder the growth of established species (Klironomos 2002, van der Putten et al. 2007, Bever et al. 2012).

While similar examples of notable symbionts abound in the literature, the effects of most symbionts on their hosts are uncharacterized, and many symbiont taxa likely exhibit commensal relationships with their hosts (Rodriguez et al. 2009). The last decade has seen tremendous advances in technology (e.g. 454 sequencing, Illumina MiSeq and HiSeq), bioinformatics pipelines (e.g. MOTHUR (Schloss et al. 2009), QIIME (Caporaso et al. 2010)) and databases (e.g. UNITE (Abarenkov et al. 2010), Greengenes (DeSantis et al. 2006)) that have allowed for more complete characterization of microbial communities. These advancements offer the opportunity to understand the factors influencing how symbionts, and microbes in general, assemble into communities (Saunders et al. 2010, Fierer et al. 2012, Borer et al. 2013). For instance, researchers have long believed that microbial community assembly followed the Baas-Becking hypothesis that 'everything is everywhere and the environment select' (Baas-Becking 1934). Under this hypothesis, microbial taxa are not limited by dispersal, and their communities are structured solely based on the environmental conditions in which they grow best. However, more recent work has showed that microbes can exhibit dispersal-limitation (Peay et al. 2007, Talbot et al. 2014). The extent to which dispersal-limitation is important to microbial communities, and symbiont communities in particular, is an important area of ongoing research. Examining the roles of the environment and dispersal-limitation in shaping symbiont communities associating with invasive species is particularly important for understanding the influence of invasive species on the diversity and structure of microbial communities within hosts.

The research presented in this dissertation aims to improve our understanding of plant invasion and the associated symbiont taxa that comprise invasive plant species' microbiomes. In Chapters 1-3, I explore this subject using the coastal dune system of the USA Pacific Northwest. Dunes in this region were historically characterized by their unstabilized, shifting sands with sparse vegetation (Cooper 1958). Following European colonization, dunes were stabilized for urban development with the introduction of the European beachgrass *Ammophila arenaria*, which came to dominate the West Coast from Southern Canada to Northern Mexico. In the 1930-40s, a second beachgrass, *A. breviligulata*, was introduced to Southern Washington from the Eastern USA (Schwendiman 1977, Seabloom and Wiedemann 1994). Since then, *A. breviligulata* has spread north and south, displacing *A. arenaria* (Seabloom and Wiedemann 1994, Hacker et al. 2012). This rapid spread has led to declines in plant diversity and a reduction in coastal protection from flooding (Hacker et al. 2012, Zarnetske et al. 2012, Seabloom et al. 2013).

In Chapter 1, I used the Pacific Northwest dune system to investigate how the two invasive *Ammophila* grasses differ in the impacts on ecological succession. To do this, I used a long-term dataset of plant community surveys of foredunes in Oregon and Washington (Hacker et al. 2012, Seabloom et al. 2013). I characterized the spatial distribution across the dune and in dune habitats of varying ages for two invasive *Ammophila* species.

In Chapter 2, I characterized the above- and belowground microbiomes of the two *Ammophila* species and the native *Elymus mollis* in order to understand the relative importance of filtering by the abiotic environment and host species and of dispersal-limitation. Because symbionts in the above- and belowground plant tissue experience vastly different environments, host tissue chemistry, and conditions for dispersal, I predicted that these communities should be

uniquely influenced by filtering and dispersal-limitation. To do this, I used a culture-based approach to isolate fungal endophytes growing in the leaves and roots of the three host species, and identified endophytes as operational taxonomic units (OTUs) based on sequence similarity. I also conducted a growth assay of the most common OTUs to investigate whether colony growth rates and/or asexual spore production were predictive of whether an OTU colonizes above- or belowground tissue.

In Chapter 3, I investigated the mechanisms behind *A. breviligulata* invasion. Specifically, I considered how the two *Ammophila* species and the native beachgrass *E. mollis* differed in their responses to disturbance and competition in a field experiment. I then characterized root colonization of arbuscular mycorrhizal and fungal endophytes using culture-based and microscopy approaches in order to test for differences in colonization among host species and associations between colonization and plant biomass. I also considered the influence of pathogens on plant invasion by measuring parasitic nematode abundance for each host species. In a second experiment, I further considered how plant-soil feedbacks might contribute to *A. breviligulata* invasion success in dunes long-dominated by the established *A. arenaria*. In a growth chamber, I inoculated individual tillers of the two *Ammophila* species with filtrates derived from *A. arenaria* soil to investigate how soil microbes might benefit or harm the two species.

In Chapter 4, I explored the interactions between the plant microbiome and herbivory. To do this, I considered endophytes of purple loosestrife (*Lythrum salicaria*), an invasive wetland forb that has been subjected to herbivory from biocontrol agents (two species of *Galerucella* beetles) in Minnesota since 1992 (Malecki et al. 1993). Biocontrol success has been variable across the state, reducing *L. salicaria* populations in some wetlands while failing to establish in

others (Quiram 2013). One proposed idea is that the existence of an endophyte might explain the agents' failure to establish in some sites. This relatively simple system consisting of a near-monodominant plant, its specialist herbivore, and the plant's microbiome presents an opportunity to investigate potential facilitative and antagonistic relationships between herbivory and symbionts. To do this, I characterized endophyte communities using a culture-based approach and quantified the level of herbivory caused by the biocontrol agent in the field at six southeastern Minnesota wetlands. I then explored associations between endophyte communities and biocontrol herbivory in order to detect facilitation or antagonism between these groups of organisms.

Chapter 1 Invasive congeners differ in successional impacts across space and time

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ABSTRACT

Invasive species can alter the succession of ecological communities because they are often adapted to the disturbed conditions that initiate succession. The extent to which this occurs may depend on how widely they are distributed across environmental gradients and how long they persist over the course of succession. We focus on plant communities of the USA Pacific Northwest coastal dunes, where disturbance is characterized by changes in sediment supply, and where the plant community is dominated by two introduced grasses – the long-established *Ammophila arenaria* and the currently invading *A. breviligulata*. Previous studies showed that *A. breviligulata* has replaced *A. arenaria* and reduced community diversity. We hypothesize that this is largely due to *A. breviligulata* occupying a wider distribution across spatial environmental gradients and persisting in later-successional habitat than *A. arenaria*. We used multi-decadal chronosequences and a resurvey study spanning 2 decades to characterize distributions of both species across space and time, and investigated how these distributions were associated with changes in the plant community. The invading *A. breviligulata* persisted longer and occupied a wider spatial distribution across the dune, and this corresponded with a reduction in plant species richness and native cover. Furthermore, backdunes previously dominated by *A. arenaria*

switched to being dominated by *A. breviligulata*, forest, or developed land over a 23-yr period. *Ammophila breviligulata* likely invades by displacing *A. arenaria*, and reduces plant diversity by maintaining its dominance into later successional backdunes. Our results suggest distinct roles in succession, with *A. arenaria* playing a more classically facilitative role and *A. breviligulata* a more inhibitory role. Differential abilities of closely-related invasive species to persist through time and occupy heterogeneous environments allows for distinct impacts on communities during succession.

INTRODUCTION

Ecologists have sought to understand the processes and mechanisms of succession for well over a century (e.g. (Cowles 1899, Clements 1916, Olson 1958, Connell and Slatyer 1977, Pacala and Rees 1998, Pickett et al. 2009)) with a focus on the way in which individual species can shape community composition through time. Early-colonizing species can have disproportionate influence on subsequent community succession due to founder effects (e.g. (Grime 1998)), their ability to capitalize on available resources (Tilman 1985), or their positive responses to disturbance (Connell 1978). Invasive plant species in particular are often uniquely positioned to influence succession because many are well-adapted to the disturbed conditions which initiate succession (D'Antonio and Vitousek 1992, Lichter 1998, Prach and Walker 2011, Moles et al. 2012). In some cases, their high abundance or life-history strategies can also make them ecosystem engineers, capable of transforming habitats and having long lasting effects after they are gone (Hastings et al. 2007), especially if they are capable of changing the disturbance regime themselves (D'Antonio and Vitousek 1992, Moles et al. 2012).

The extent to which early-colonizing invasive species alter succession may depend on how long they can persist in the community. Early-colonizing species tend to capitalize on low competition and high light availability during primary succession, and modify their environment to facilitate later-colonizing species before extirpation (Olson 1958, Connell and Slatyer 1977, Tilman 1985). Species that are able to persist longer may inhibit establishment of later species (Connell and Slatyer 1977). For instance, invasive species could alter the dispersal processes of resident species (McConkey et al. 2012) or directly compete with them for resources (e.g., (Tilman 1985)). Alternatively, invasive species may give way to later successional species or persist in the community but cease to limit establishment of colonizing species (see facilitation

and tolerance models in (Connell and Slatyer 1977, Lubke 2004)). Different invasive species that play inhibitory or facilitative roles will have distinct effects on the ecological succession of the community.

Invasive species could persist longer in communities if they are able to tolerate a wide range of environmental conditions experienced during succession (e.g. (Vitousek and Sciences 2002, Veeneklaas et al. 2013)). Species with broader environmental tolerances likely have wider distributions across spatial and successional gradients (Pulliam 2000, Guisan and Thuiller 2005, Lambdon 2008, Slatyer et al. 2013). Wider environmental distributions could lead to greater impacts on resident species because the invasive species is able to invade more environments and persist within them for longer periods (e.g. (Batten et al. 2006)).

To better understand how invasive species influence resident communities during succession, we compared the distributions of two congeneric, invasive grasses across space and time in coastal dunes along the USA Pacific Northwest Coast. Coastal foredunes in this region, defined as linear ridges parallel and adjacent to the shoreline (Cooper 1958), contain rapidly advancing shorelines in many locations creating replicated chronosequences of herbaceous plant communities of up to 75 years (Figure 1-1a). Furthermore, shoreline change rates vary widely across the region, so it is possible to separate the temporal effects of dune age from the purely spatial gradient created by proximity to the beach. Foredunes are invaded by two early-colonizing, introduced species of *Ammophila* beach grass – the longer established *A. arenaria* which is rapidly disappearing from northern foredunes, and the currently invading *A. breviligulata* which has potentially displaced it (Seabloom and Wiedemann 1994, Hacker et al. 2012). Pairs of invasive, congeneric species such as these can provide insights into the mechanisms and long-term consequences of invasion because they allow for comparisons

between closely-related species occupying similar but not identical distributions (e.g., (Ruesink et al. 2010, Hacker et al. 2012). Whereas both grasses are important foredune stabilizers that can occur in nearly monodominant stands, *A. breviligulata* dunes are associated with lower plant diversity compared to *A. arenaria* dunes (Hacker et al. 2012) and create foredunes of lower height (Zarnetske et al. 2012), increasing the risk of coastal flooding (Seabloom et al. 2013). Using chronosequence data we document shifts in dominance from *A. arenaria* to *A. breviligulata* as well as their associated herbaceous communities through successional time. *Ammophila breviligulata* may outcompete *A. arenaria* on foredunes (Zarnetske et al. 2013), but it is not yet clear whether the former invades by preemptively colonizing new foredunes created by sand deposition or whether it can displace established *A. arenaria* in foredunes and historical dunes further inland (Figure 1-1b-d). Understanding the distributions of the two species may provide insight into how *A. breviligulata* invades. Here we investigate the distributions of two high impact invasive species over two decades to ask the following questions: First, we ask how distributions of these two invaders differ across space and time. We hypothesize that the more recent invader *A. breviligulata* occupies a wider spatial distribution across the dune and persists in later-successional habitats, which has allowed it to replace *A. arenaria* on foredunes (Hacker et al. 2012). Second, we ask how these distributional changes correspond to impacts on resident communities. We hypothesize that the more broadly distributed species will also be associated with lower total species richness and native species abundance across space and through time. To test these hypotheses, we first use a chronosequence study to determine the distribution of both *Ammophila* species along successional and spatial environmental gradients. We then asked whether the *Ammophila* species differ in their associations with native plant cover, total species richness, and soil properties along spatial and successional environmental gradients.

Additionally, we resampled transects after 21 years to determine whether *A. breviligulata* invasion has led to a predictably wider spatial distribution in *Ammophila* across dune cross-sections and whether *A. breviligulata* has displaced *A. arenaria* in backdunes.

MATERIALS AND METHODS

Study System

Coastal dune systems are ideal for understanding how invasive species may affect succession of plant communities because they contain strong spatial gradients in community composition and physical characteristics (Cowles 1899). We define the 'dune gradient' as the spatial gradient beginning at the start of the vegetation (occurring at or slightly seaward of the dune toe) and extending to the heel (Seabloom and Wiedemann 1994) (Figure 1-1a). From toe to heel, there are often increases in nutrient availability and moisture, and decreases in wind, sand scour, and salt spray (Lichter 1998, Lortie and Cushman 2007). Plant communities along dune cross-sections typically begin as a few early-colonizing plants (e.g., *Ammophila*, *Cakile*) that begin stabilizing the sand, and gradually accumulate other grass, forb, and occasionally shrub species towards the dune heels (Wiedemann and Pickart 2004). In the backdune, the grass and forb dominated communities give way to shrubs and forest (Wiedemann and Pickart 2004). For the chronosequence and decadal studies described below, we focus on the relatively-early succession of the herbaceous community in the foredune, and do not address the long-term successional processes associated with forest development in the backdune. Succession of herbaceous foredune communities may influence the populations of several plant species endemic to dunes, and as well as animals such as the endangered Western Snowy Plover (Zarnetske et al. 2010, Hacker et al. 2012). Because new dunes are continuously being formed

via sand deposition, herbaceous foredune plant communities persist through time, even if individual dunes eventually become forested.

In some of the USA Pacific Northwest beaches in Washington and Oregon, the shoreline is expanding seaward with the deposition of wave and wind delivered sand (Ruggiero et al. 2013). This phenomenon creates a chronosequence along dune cross-sections in which inland areas are older than more seaward and recently-formed areas (Lichter 1998) (Figure 1-1). Variation in sand supply across the region (Hacker et al. 2012, Ruggiero et al. 2013) may lead to differences in foredune shape and age. From central Oregon north, high sand supply leads to wider, shorter, and younger foredunes created by the recently deposited sand and are typically dominated by *A. breviligulata* (Psuty 1988, Hacker et al. 2012, Zarnetske et al. 2012). In contrast, from central Oregon south, sites are typically dominated by *A. arenaria* and experience relatively low sand supply which leads to narrower, taller, and older foredunes, the result of small amounts of sand deposition in one location over many years (Psuty 1988, Hacker et al. 2012, Zarnetske et al. 2012).

In response to sand deposition, both *Ammophila* species advance into newly created bare sand habitat and build foredunes through sand capture (Hacker et al. 2012, Zarnetske et al. 2012). The European beach grass, *Ammophila arenaria*, was introduced for dune stabilization in the early 1900's to the US Pacific Northwest Coast, and the American beach grass, *A. breviligulata*, was later introduced to northern Oregon in 1935 from the Eastern USA (Schwendiman 1977). *Ammophila breviligulata* has since spread along the coast throughout the region, sharply reducing *A. arenaria* in Washington and northern Oregon dunes while not yet reaching southern Oregon (Seabloom and Wiedemann 1994, Hacker et al. 2012, Zarnetske et al. 2012). Both beach grass invaders are superficially similar in morphology and growth form, and

are the primary species involved with sand capture and creation of foredunes due to their high tiller density (Seabloom and Wiedemann 1994, Hacker et al. 2012, Zarnetske et al. 2012) However, their distinct growth forms create different shaped dunes as a result (Seabloom and Wiedemann 1994, Hacker et al. 2012, Zarnetske et al. 2012).

Chronosequence Study

We investigated the distributions of *Ammophila* species abundance, native species abundance, and total plant species richness along dune cross-sections from 52 unique transects surveyed in 2006 and 2009 (32 transects surveyed in both years, 2 surveyed only in 2006, 18 surveyed only in 2009; Appendix 1-1). Analysis of total richness allows for an understanding of the accumulation of species through space and time, while that of native plant abundance addresses how *Ammophila* specifically impacts the native vegetation. Plant community composition and dune morphology data were collected along transects running perpendicular to the shoreline, from the foredune toe to its heel in the backdune (see (Seabloom and Wiedemann 1994, Hacker et al. 2012) for transect methodology) (Appendix 1-1). Every 5m along each transect, we visually estimated percent cover of every plant species present within a 50x20cm quadrat, and transects ranged from 40m-325m in length. Permission to access field sites was granted by United States Forest Service, United States Fish and Wildlife, Willapa Bay Wildlife Refuge, Washington State Parks and Recreation Commission, Oregon Parks and Recreation Department, Scott McKenzie Ranch, and Lane County Park.

To determine underlying soil properties of foredunes, we collected soil samples from *A. arenaria* and *A. breviligulata* dominated dunes at three locations along foredunes – the toe, crest, and heel (Figure 1-1a). Soil cores (20.5 long x 2.5 cm diameter) were collected from 21 sites varying in dominant *Ammophila* species, shoreline change rate, and latitude in 2012

(Appendix 1-1). For each location within a site, three replicate samples were taken. Soils were stored at -20˚C following collection, air-dried at 60˚C, homogenized within locations at a site, and sieved through a 500µm mesh to remove debris before analysis. We obtained the following soil properties: percent C, percent N, concentrations of P, K and Na, organic matter, pH, cation exchange capacity.

For each transect surveyed, we also estimated shoreline change rate, a measure of how much habitat has been formed from sand deposition (positive) or lost due to erosion (negative). Shoreline change rate was calculated from two endpoints: 1) proxy-based shorelines derived from 1950-60s era aerial photographs; and 2) datum-based lidar-derived shoreline positions from 2002 (Ruggiero et al. 2013).

For vegetation transects with a net positive shoreline change rate, we used the estimated shoreline change rate to interpolate the approximate ages of the locations of each quadrat after adjusting for the average distance between the shoreline and the start of the vegetation. This adjustment was made to correct for age differences between the shoreline location where the shoreline change rate was measured and the start of the vegetation where transects began. We used a natural log transformation of chronosequence age in all analyses to avoid placing too much weight on older quadrats whose age estimates could be more prone to error. In our dataset, quadrats in *A. breviligulata* dominated dunes (range: 4.00-75.00 years; mean: 20.22) were younger in chronosequence age than those in *A. arenaria* dominate dunes (range: 5.00-55.00 years; mean: 23.47) (t-test d.f. = 133, t=3.23, $p < 0.001$). We defined the dune gradient for each quadrat as the standardized location of each quadrat on the dune ranging from -1 to 1, where -1, 0, and 1 refer to the toe, crest, and heel, respectively (Figure 1-1a). While chronosequence age and dune gradient were positively correlated (Pearson's r = 0.337), most of the variance in age

was uncorrelated with distance allowing for independent test of chronosequence age and dune gradient. We used the proportional cover of each *Ammophila* species per transect to categorize transects as either dominated by *A. arenaria* or *A. breviligulata* (proportion of dominant species cover > 0.5). For each quadrat, we calculated *Ammophila* cover as the sum of the raw percent cover of both *Ammophila* grasses, native cover as the sum of all native species cover, and species richness as the number of species present (excluding *Ammophila*). Total cover per quadrat could exceed 1due to overlapping plant canopies.

To analyze the effects of the dune gradient and chronosequence age on *Ammophila* cover, native cover, and richness, we used hierarchical Bayesian models to determine the significant factors associated with our response variables. We modeled the logit-transformed *Ammophila* cover per quadrat as a function of the shoreline change rate, dominant *Ammophila* species, chronosequence age, dune gradient, and their interactions and quadratic relationships, along with the random effects of transect and year (Appendix 1-2). Similarly, we modeled logit-transformed native cover and untransformed species richness (Poisson distribution) per quadrat as a function of the same predictor variables as well as *Ammophila* cover (Appendix 1-2). A strong effect of dominant *Ammophila* species × chronosequence age was taken as evidence that the two *Ammophila* species differed in their effects through time on the response variable. Likewise, a strong effect of dominant *Ammophila* species × dune gradient was taken as evidence that the two *Ammophila* species affected response variables differently along the dune gradient. For all models, continuous predictor variables were normalized to allow for comparison of estimates. We used a normal prior for all fixed parameters (Kéry 2010). For the random effects of transect and year, we estimated random intercepts with hyperparameters for the mean and standard deviation using normal and uniform priors, respectively (Kéry 2010). Initial parameter values

were drawn from the normal distribution, and the initial variance was drawn from the lognormal distribution (Kéry 2010). We ran 50,000 iterations with 500 iterations of burn-in and a thinning rate of 2. We assessed model convergence using 10 independent chains and the Gelman-Rubin diagnostic statistic. Analyses were conducted using the *R2jags* package (Su and Yajima 2011) in R Version 3.0.2 (R Development Core Team 2013). To visualize results, we created contour plots using the predicted values of generalized linear models with the form *response variable ~ chronosequence + dune gradient*.

For soils, we used analysis of variance to analyze the effect of the dominant *Ammophila* species and the effect of location within the dunes on each soil property.

Decadal study

Using plant community transect surveys from southwestern Washington, USA, we investigated how *Ammophila* cover and plant species richness changed over a 21-year period (1988-2009), during which time *A. breviligulata* became the dominant species at all transects (Appendix 1-1). Specifically, we compared how transects that have switched in dominance from *A. arenaria* in 1988 to *A. breviligulata* by 2009 differed from those that were already *A. breviligulata*-dominant in 1988 and remained so in 2009. We also considered how such switches in *Ammophila* dominance varied in their effects across the dune gradient.

The decadal dataset consisted of 34 transects along the Washington coast that all had some degree of *A. breviligulata* invasion (Appendix 1-1). Because *A. breviligulata* had become the dominant species in all transects by 2009, we classified transects as either 'switched' from *A. arenaria* to *A. breviligulata* dominated or 'established' by *A. breviligulata* in 1988 and remaining so in 2009. We calculated the proportions of *A. arenaria* and *A. breviligulata* as the raw cover divided by the total *Ammophila* cover for each transect. We classified 7 transects as

'switched' in which there was substantial *A. arenaria* cover (proportions of *A. arenaria* ranging from 0.32-1.0 in 1988) and 27 transects as 'established' that were dominated by *A. breviligulata* (proportions of *A. breviligulata* ranging from 0.77-1.0 in 1988). To compare across transects, we standardized each quadrat's location along the dune cross-section using the dune gradient index previously described.

We analyzed the relative *Ammophila* cover and plant species richness using mixed-effects models with linear contrasts. We assigned four groupings based on the factorial combination of *A. breviligulata* invasion (switched vs. established) and year (1988 vs. 2009). Our linear contrasts compared (1) overall effect of year, and the differences within (2) 1988 and (3) 2009 between switched and established transects. Response variables were modeled as response ~ dune gradient + grouping + dune gradient × grouping and we used transect as a random intercept. To reduce observer sampling bias between years, we used the relative cover as the raw cover of *Ammophila* divided by the total plant cover for each quadrat. We used the logit-transformation of these relative cover values to meet the model assumptions. To analyze richness per quadrat, we fit a model of the number of species per quadrat with a Poisson error distribution. We calculated 95% confidence intervals for parameters using the Wald method (Bates et al. 2013).

Backdune Invasion Boundary Study

In their 1988 surveys of the rapidly accreting Long Beach Peninsula, WA, USA, shoreline, Seabloom and Weidemann (Seabloom and Wiedemann 1994) found that while *A. breviligulata* dominated many of the foredunes, backdune areas were dominated by *A. arenaria*. They surveyed the location of this change in dominance, which we refer to as the backdune invasion boundary between *A. breviligulata* and *A. arenaria*. Though *A. breviligulata* has

steadily increased its range latitudinally along the coast (Hacker et al. 2012), this invasion has been thought to be restricted to foredunes where it preemptively colonizes newly formed, early successional dunes, whereas remnant *A. arenaria* stands in the later succesional backdunes are presumed to persist (Seabloom and Wiedemann 1994) (Figure 1-1b-c). However, if *A. breviligulata* is capable of displacing *A. arenaria*, the latter should be reduced in older backdunes and an invasion boundary should no longer exist between *A. breviligulata* and *A. arenaria* dominated areas (Figure 1-1d). We resurveyed the location of this boundary to determine whether *A. breviligulata* could invade and displace *A. arenaria* in stable backdune areas over the 23 year period between 1988 and 2011.

We resurveyed 16 of the 1988 transects and compared the backdune invasion boundaries to those previously observed by Seabloom and Wiedemann (Seabloom and Wiedemann 1994). In 1988, Seabloom and Wiedemann (Seabloom and Wiedemann 1994) measured the distance from the toe of the dune to the point where the species composition changed from *A. breviligulata* to *A. arenaria* on Long Beach Peninsula, WA. Using aerial photos, we determined the GPS coordinates of these transects and the 1988 location of the compositional changes in herbaceous backdunes. In 2011, we visually determined whether such a compositional change had occurred by resurveying each transect extended landward to forest or human development.

RESULTS

Chronosequence study

Ammophila breviligulata occupied a wider span of chronosequence age and dune gradient compared to *A. arenaria* (Figure 1-2; Bayesian model Gelman-Rubin diagnostic statistic < 1.01 for all parameters). *Ammophila* cover towards the heel of the dune was higher in *A.*

breviligulata dunes than in *A. arenaria* dunes as evidenced by the significant quadratic effect of dominant *Ammophila* species × dune gradient (Appendix 1-3). *A. breviligulata* also had higher cover in quadrats of older chronosequence age (Appendix 1-3) as evidenced by the significant effect of dominant *Ammophila* species × chronosequence age^2. *Ammophila* abundance was generally lower with higher shoreline change rate (Appendix 1-3).

The distribution of native cover differed between dunes dominated by *A. breviligulata* versus *A. arenaria* along both the dune gradient and chronosequence (Figure 1-3a). We found a significant 3-way interaction between the dominant *Ammophila* species, chronosequence age, and the dune gradient, indicating that all three factors contribute to native cover. Higher total *Ammophila* cover and the presence of *A. breviligulata* were both associated with less native cover. Native cover was best explained by the dominant *Ammophila* species × dune gradient interaction; *A. arenaria* dunes had higher native cover closer to the shore (i.e., lower dune gradient indices) relative to *A. breviligulata* dunes (Appendix 1-4). Similarly, *A. arenaria* dunes had higher native cover in younger communities (lower chronosequence ages) compared to *A. breviligulata* dunes.

The distribution of total species richness also differed between *A. arenaria* and *A. breviligulata* dunes (Figure 1-3b). There was no significant main effect of the dominant *Ammophila* species on richness (Appendix 1-4), although richness increased with chronosequence age and along the dune gradient. *A. arenaria* dunes tended to have higher richness at lower chronosequence ages than *A. breviligulata* dunes. In contrast, *A. breviligulata* dunes had higher richness at higher chronosequence age. Richness was not associated with *Ammophila* cover.

Soil properties differed according to the dominant *Ammophila* species and the location (toe, crest, heel) along the dune gradient (Appendix 1-5; Appendix 1-6). Percentage of soil C, N and organic matter generally increased moving inland from the beach, and were higher in dune heels of *A. breviligulata* dunes than in those of *A. arenaria* dunes. Concentrations of P, K, and Na and cation exchange capacity generally decreased moving inland from the beach and varied between the dunes with different *Ammophila* species. Soil pH decreased from toe to heel more in *A. breviligulata* than in *A. arenaria* dunes.

Decadal study

Total *Ammophila* cover increased significantly from 1988 to 2009, indicating that it had increased its relative dominance in the plant community (Table 1-1; Figure 1-4a). Transects that switched from *A. arenaria* to *A. breviligulata* had significantly less total *Ammophila* cover than established transects in 1988, but there was no difference in 2009 when all transects were dominated by *A. breviligulata*. This result can be attributed to lower *Ammophila* cover in the backdune of switched transects compared to the established transects in 1988, but again there was no difference in 2009 (Figure 1-4b).

Plant species richness significantly decreased from 1988 to 2009 (Table 1-1; Figure 1-4c). Switched transects had significantly higher richness than the established transects in 1988, but there were no differences between switched and established transects in 2009. We found no significant differences along the dune gradient for any of the linear contrasts (Table 1-1, Figure 1-4d).

Backdune Invasion Boundary Study

We found no evidence of a boundary between *A. breviligulata* and *A. arenaria* dominated communities in backdunes sampled in 2011. Of the 16 resurvey transects, 10 of the 1988

backdune invasion boundaries were now located beyond the foredune and were forested (n=6) or developed (n=4). All of the remaining transects (n=6) were dominated by *A. breviligulata*. We found isolated patches of *A. arenaria* in only 4 transects.

DISCUSSION

The extent to which invasive species persist through time and tolerate varying spatial environments may determine their impact on succession. We have shown that while closely related invasive species overlap in space and time, their resulting distributions are associated with differential native plant cover and richness. Specifically, we found that the newer invader, *A. breviligulata* persisted at higher abundance in the backdunes and at later chronosequence ages than the established invader, *A. arenaria*. These findings show that *A. breviligulata* occupies a wider distribution than *A. arenaria*, and has the potential to have broader impacts on plant species richness, native cover, and soil nutrients through time and space. Furthermore, over the past two decades *A. breviligulata* invasion into foredunes previously dominated by *A. arenaria* has led to a predictable increase in *Ammophila* cover in the backdune, though richness did not significantly differ along dune cross-sections in sites of different invasion history. Finally, we observed that *A. breviligulata* dominates in backdune areas where a boundary between the two species once existed, suggesting that *Ammophila breviligulata* has displaced *A. arenaria* and has the potential to limit native cover and richness beyond the foredune. Our results provide evidence that two closely related invasive species occupy similar yet distinct distributions, with implications for their roles in succession. *A. breviligulata* may play a more inhibitory role in foredune succession than *A. arenaria*, and the former's invasion may ultimately slow herbaceous successional processes.

Invasive species that are widespread and can tolerate a range of conditions may have the greatest impact on succession. They may inhibit colonizing species for long periods of time (Connell and Slatyer 1977), and thus slow the recovery of plant communities after disturbances (Keeley et al. 2005). They may also remove spatial refugia for resident species, potentially causing reductions in population growth rates (Gram et al. 2004, Gilbert and Levine 2013). Several mechanisms could explain why *A. breviligulata* has a wider distribution and potentially larger impacts on species richness than *A. arenaria*. *Ammophila breviligulata* may be a superior competitor for resources (Zarnetske et al. 2013), or could also be a faster colonizer and preemptively colonize new habitat. Species-specific differences in morphology and sand capture ability may favor *A. breviligulata* in the dune heels and backdunes and allow it to displace *A. arenaria* (Hacker et al. 2012, Zarnetske et al. 2013). *Ammophila arenaria*, which tends to grow vertically in response to sand deposition, depends much more on sand burial to achieve high growth than does *A. breviligulata* (Zarnetske et al. 2012). Therefore, *A. breviligulata* could have an advantage in backdunes with low sand burial. Finally, *A. breviligulata* could have more favorable interactions with symbiotic organisms such as arbuscular mycorrhizae (de la Peña et al. 2006) or fungal endophytes (Rodriguez et al. 2008) found in dune systems or experience a greater benefit from natural enemy release from pathogens such as nematodes (Beckstead and Parker 2003, van der Putten et al. 2005) , particularly if these organisms vary in their host specificity or distribution along dunes.

Ammophila breviligulata's strong impact on native cover and richness could be attributed to feedbacks between vegetation and biophysical processes that alter the physical features of a landscape through time (Zarnetske et al. 2012, Durán and Moore 2013). Both *Ammophila* species build dunes by capturing sand and changing the topography of their habitat (Zarnetske et al.

2012), therefore the two species may uniquely alter dune morphologies and the resulting local dune conditions. Spatial gradients may impose an environmental filter that influences where certain species are able to colonize during succession (Aplet and Vitousek 1994, Donnegan and Rebertus 1999). This potentially creates distinct local environmental conditions between dunes dominated by the two *Ammophila* species in terms of soil properties (Appendix 1-6), as well as alter salt spray and wind exposure (Lortie and Cushman 2007). Interestingly, soil in dune heels dominated by *A. breviligulata* was more characteristic of late dune succession (e.g., higher C and N) than *A. arenaria* soils (Olff et al. 1993, Lichter 1998) despite having lower native cover. It is possible that plant-soil feedbacks differ between the *Ammophila* species, leading to higher nutrient content in *A. breviligulata* soils. Additionally, higher nutrient content simply could have a minimal effect on plant communities given that nutrient levels were generally low, and could suggest that traits such as response to disturbance are more important than competition for resources such as nitrogen (e.g. (Brunbjerg et al. 2014)). Positive responses to disturbed environments (e.g. higher wind, sand burial (Hesp 1989)) could be a mechanism by which the two species of *Ammophila* reduce establishment of colonizing species. For instance *A. arenaria* may rely on rapid sand burial to displace colonizing native species the foredune toe and crest (e.g. (Hilton et al. 2005)), but low sand supply in dune heels would lessen its ability to do so. The horizontal growth pattern of *A. breviligulata* (Hacker et al. 2012, Zarnetske et al. 2012) could also contribute to lower richness in dune toes by allowing *A. breviligulata* to rapidly create new dunes devoid of species that have yet to colonize.

Furthermore, *A. breviligulata* may not necessarily differ from *A. arenaria* in its direct interactions with the herbaceous plant community, but rather its wider distribution may augment its ecological effects. For instance, both *Ammophila* species may inhibit the establishment of

colonizing species by leaving fewer unconsumed resources (Tilman 1985, 2004), and high *Ammophila* cover and tiller densities (Hacker et al. 2012, Zarnetske et al. 2012) may also reduce light availability for colonizing species. In our study, we found that the two *Ammophila* species were associated with similar native cover and richness in younger dunes, and only found differences in older dunes where *A. arenaria* cover was reduced. *Ammophila* species' interactions with herbaceous plant communities could be likely mediated via differences in biophysical dune feedbacks (Zarnetske et al. 2012, 2013), and the *Ammophila* species' respective abilities to persist in older, landward parts of the dune.

Following the distributions of invasive species over space and time may provide insights into the classical successional roles they play (*sensu* (Connell and Slatyer 1977)). Specifically, our results suggest that the invasion of the more widely-distributed *A. breviligulata* has shifted the role of *Ammophila* on foredunes from that of facilitator to inhibitor. *Ammophila arenaria* may play a facilitative role as an early colonizer that gives way to later colonizing species through time (*sensu* (Connell and Slatyer 1977)). In addition to occupying a narrower distribution, *A. arenaria* was excluded from herbaceous backdune communities, likely due to displacement from *A. breviligulata* on the shoreside and forest encroachment on the landward side. In contrast, *A. breviligulata* may play a more inhibitory role (*sensu* (Connell and Slatyer 1977)), maintaining dominance over longer periods of successional time and reducing native cover and richness. The shift from a more facilitative to inhibitory role of the dominant species has important consequences for how subsequent species establish, interact, and ultimately form communities. Invasive species, particularly those present at the initiation of succession, that are able to persist longer and tolerate more spatially-heterogeneous environments may have the

greatest impact on the succession of resident communities. We have shown that two seemingly similar invasive species can differ in these regards and play distinct roles in succession.

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Source	*Ammophila* Cover		Species Richness	
	Estimate	Wald 95% CI	Estimate	Wald 95% CI
(Intercept)	**1.26**	**(0.85, 1.67)**	0.00	(-0.22, 0.21)
Dune gradient	-0.24	(-0.89, 0.41)	**0.79**	**(0.62, 0.96)**
2009 vs. 1988	**1.04**	**(0.72, 1.37)**	**-0.10**	**(-0.19, -0.01)**
Switched sites vs. established sites (within 1988)	**-0.97**	**(-1.53, -0.41)**	**0.26**	**(0.03, 0.5)**
Switched sites vs. established sites (within 2009)	-0.08	(-0.56, 0.39)	0.12	(-0.12, 0.35)
dune gradient (2009) vs. dune gradient (1988)	**0.79**	**(0.14, 1.45)**	0.01	(-0.17, 0.18)
dune gradient (switched sites) vs. dune gradient (established sites) (within 1988)	**-1.15**	**(-2.17, -0.13)**	0.04	(-0.21, 0.29)
dune gradient (switched sites) vs. dune gradient (established sites) (within 2009)	-0.51	(-1.32, 0.31)	-0.07	(-0.31, 0.17)

Table 1-1 Results from the decadal study using linear mixed models with independent linear contrasts.

Sites were categorized as 'switched' (A. arenaria dominated in 1988 and A. breviligulata dominated in 2009) or 'established' (A. breviligulata dominated in both 1988 and 2009). Relative Ammophila cover was logit-transformed and analyzed with Gaussian error distribution, and species richness was analyzed using a Poisson error distribution. Estimates for individual contrasts and Wald 95% confidence intervals are shown. Independent contrasts tested (1) the effect between 1988 and 2009, (2) the effect between switched and established sites in 1988 when sites were either A. arenaria or A. breviligulata dominated, and (3) the effect between switched and established sites in 2009 when all sites were A. breviligulata dominated. Terms are bolded as significant when the CI did not contain 0.

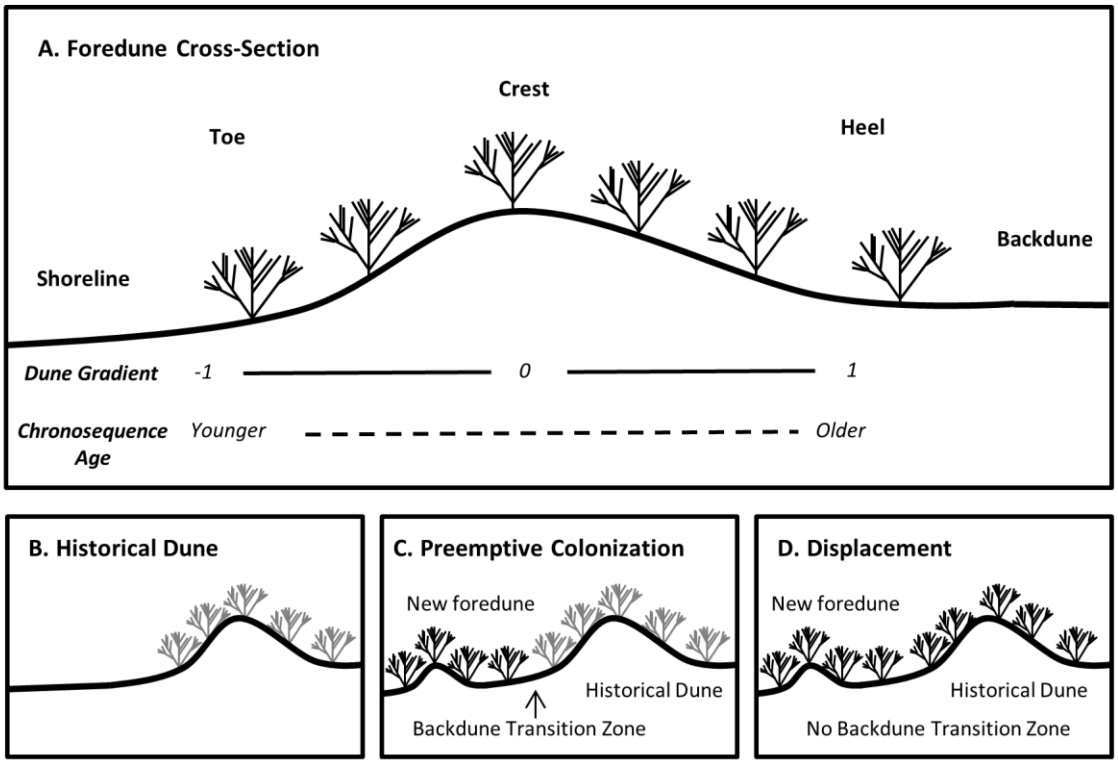

Figure 1-1 Foredune cross-section schematics.
(A) Foredunes contain both spatial and temporal gradients along their cross-sections. Foredune consists of toe, crest, and heel moving from left to right. For the spatial dune gradient, quadrats were assigned values between -1 and 1 for their placement along the dune cross-section. Chronosequence age increased from the dune toe to heel (see main text for details). Shoreline and backdune are shown for orientation. (B) Historical dune consisting of only Ammophila arenaria (gray). (C) Invasion of historical dune by A. breviligulata (black) through preemptive colonization of a new dune. Ammophila arenaria retains its original population, and a backdune invasion boundary delineates the two species' distributions along the dune. (D) Invasion of historical dune by A. breviligulata through displacement of A. arenaria. Note that there is no longer a backdune invasion boundary because the A. arenaria has been locally extirpated.

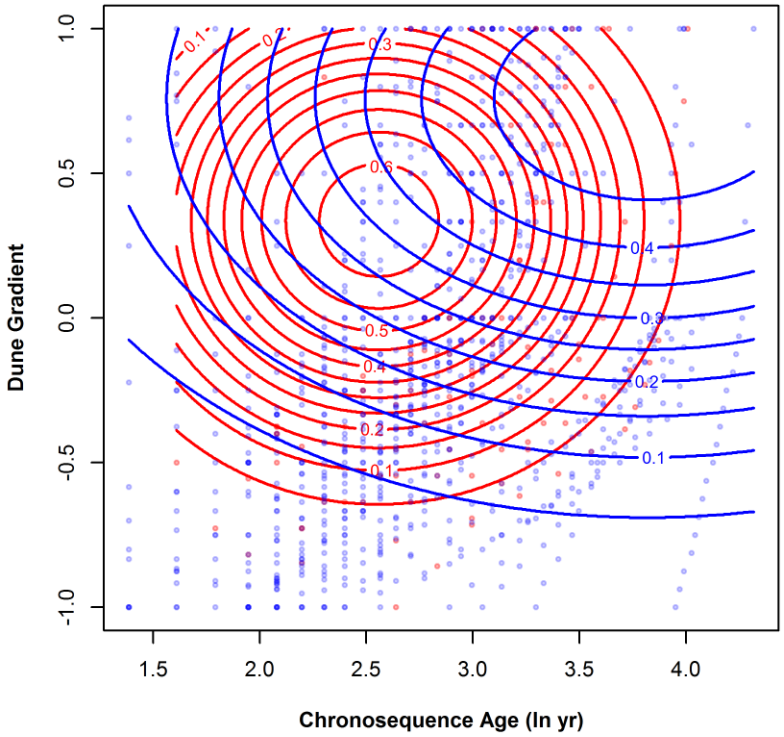

Figure 1-2 Contour plot of Ammophila cover shows distinct species distributions across chronosequence ages and dune gradient

Contours show areas of increasing cover across both gradients. Contours were created using predicted values of a GLM (see main text for details). Chronosequence ages were calculated for each quadrat using aerial photos and shoreline change rates (see main text for details). Dune gradient was calculated for each quadrat based on its standardized location along dune cross sections with the toe, crest, and heel at -1, 0, and 1, respectively. Each point on figure refers to the chronosequence age and dune gradient for an individual quadrat. Sites dominated by *A. arenaria* are shown in red, and those from *A. breviligulata* sites shown in blue.

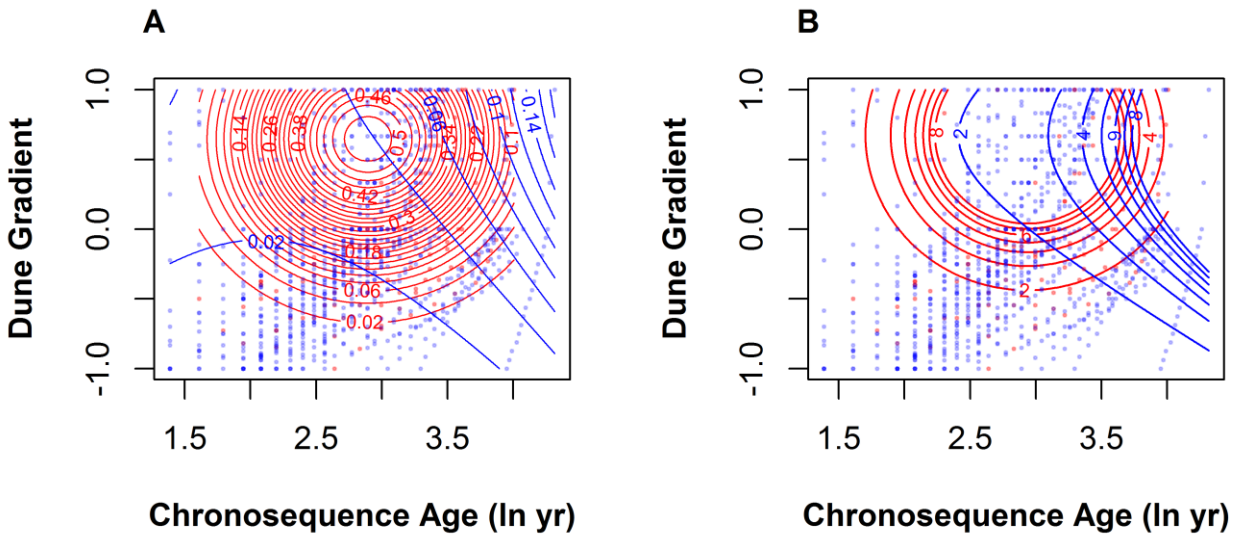

Figure 1-3 Contour plot showing differences in distribution across chronosequence ages and dune gradient in dunes of different *Ammophila* dominance.
Plots of (A) native cover and (B) species richness. Contours show areas of increasing cover or richness across both gradients. Quadrats from sites dominated by *A. arenaria* are shown in red, those from *A. breviligulata* sites shown in blue. See main text and Figure 1-2 caption for additional details.

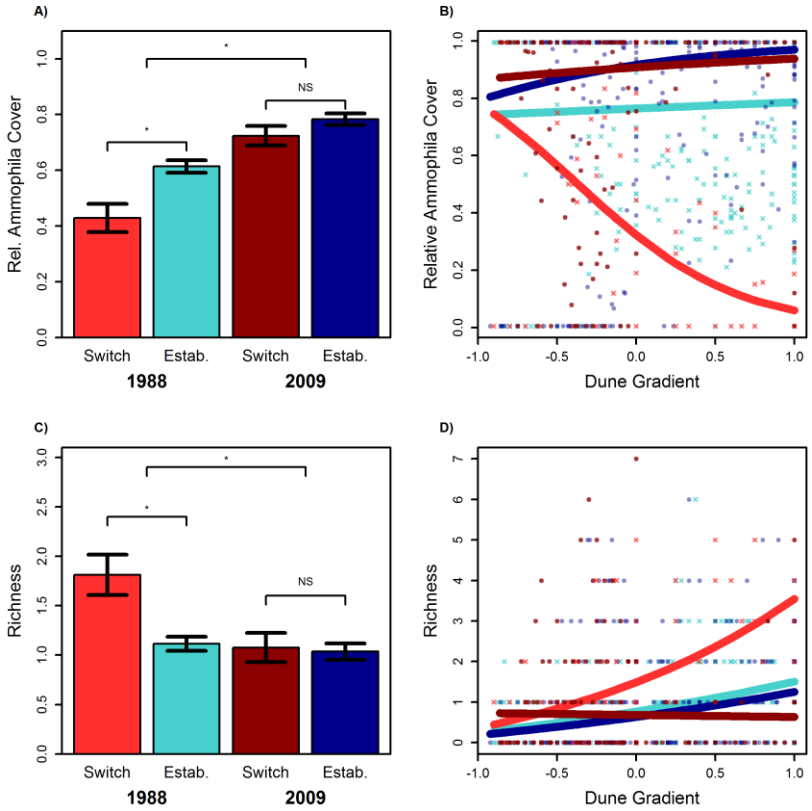

Figure 1-4 Changes in *Ammophila* cover and plant richness over a 21-year period from 1988 to 2009.

'Switched' refers to transects that had substantial *A. arenaria* in 1988 and were dominated by *A. breviligulata* in 2009. "Established' refers to transects that were dominated by *A. breviligulata* in 1988 and 2009. (A) Mean relative *Ammophila* cover ± S.E.; (B) Relative *Ammophila* cover across the dune gradient; (C) Mean plant richness ± S.E.; (D) Plant richness across the dune gradient. Light red denotes switched transects in 1988; light blue denotes established transects in 1988; dark red denotes switched transects in 2009; dark blue denotes established transects in 2009. Three separate linear contrasts shown at different heights in (A) and (C), stars signify significance, NS signifies not significant. For (B), there was a significant difference in *Ammophila* cover in 1988 but not in 2009. For (D), there were no significant differences in richness along the dune gradient within either 1988 or 2009. See Table 1-1 for full models.

Chapter 2 Plant host species and geographic distance affect the structure of aboveground fungal symbiont communities, and environmental filtering affects belowground communities in a coastal dune ecosystem

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Abstract: Microbial symbionts inhabit tissues of all plants and animals. Their community composition depends largely on two ecological processes: (1) filtering by abiotic conditions and host species determining the environments that symbionts are able to colonize and (2) dispersal-limitation determining the pool of symbionts available to colonize a given host and community spatial structure. In plants, the above- and belowground tissues represent such distinct habitats for symbionts that we expect different effects of filtering and spatial structuring on their symbiont communities. In this study, we characterized above- and belowground communities of fungal endophytes—fungi living asymptomatically within plants—to understand the contributions of filtering and spatial structure to endophyte community composition. We used a culture-based approach to characterize endophytes growing in leaves and roots of three species of coastal beachgrasses in dunes of the USA Pacific Northwest. For leaves, endophyte isolation frequency and OTU richness depended primarily on plant host species. In comparison, for roots, both isolation frequency and OTU richness increased from the nutrient-poor front of the dune to the higher-nutrient backdune. Endophyte community composition in leaves exhibited a distance-decay relationship across the region. In a laboratory assay, faster growth rates and lower spore production were more often associated with leaf- than root-inhabiting endophytes. Overall, our results reveal a greater importance of biotic filtering by host species and dispersal-limitation over regional geographic distances for aboveground leaf endophyte communities and stronger effects

of abiotic environmental filtering and locally patchy distributions for belowground root endophyte communities.

Introduction

Microbial symbionts inhabit all plants and animals, with their effects on hosts spanning from mutualism to parasitism (Fierer et al. 2012, Borer et al. 2013, May and Nelson 2014). Because interactions between hosts and their symbionts have important implications for host fitness, it is critical to understand the ecological factors that underlie the assembly of symbiont communities (e.g. Saunders et al. 2010, Fierer et al. 2012). Symbiont communities are influenced by several abiotic and biotic factors that may limit symbiont species' abilities to colonize, persist, and disperse (Arnold 2007, Rodriguez et al. 2009, Vellend 2010, Dini-Andreote et al. 2015, Seabloom et al. 2015). Furthermore, ecological communities, and symbiont communities in particular, are hierarchically structured by processes occurring at regional, local, and within-host scales (Seabloom et al. 2015). Understanding how abiotic and biotic factors can influence communities across scales may provide insights into the processes governing assembly of symbiont communities, and ultimately how they may affect host growth and reproduction.

Ecological filters can determine which individual species are capable of colonizing and persisting in the host, and, in the case of symbionts, these filters can broadly be subdivided into the environment external to the host (hereafter "environment") and host species (Arnold 2007, Saunders et al. 2010). The environment includes abiotic factors such as climate (temperature, humidity, precipitation, etc.) and soil properties (soil type, pH, nutrient availability, etc.) that may influence a symbiont species' ability to persist in that environment (Parrent et al. 2006, Peay et al. 2010, Tedersoo et al. 2012, Blaalid et al. 2014). For instance, desert soil provides a filter for thermophilic fungi that are able to grow at high temperatures (Powell et al. 2012). The biotic filter of the host may include factors such as host defense and host tissue chemistry that influence the symbiont species' ability to infect,

grow, and reproduce within a host (Arnold 2007, Rodriguez et al. 2009). While both the environment and host species contribute to symbiont is not yet clear. Many symbiont species have an apparently broad host range, and such communities may be completely shaped by the environment (e.g. U'Ren et al. 2012). Other symbiont species have a narrower host range and their communities are likely more strongly influenced by host species or genotype than by environment (Bruns et al. 2002, Schardl et al. 2004).

Furthermore, the importance of dispersal-limitation, or the inability of propagules of species to arrive at a given site (Terborgh et al. 2011), has become more widely recognized in microbial communities (Ettema and Wardle 2002, Peay et al. 2007, Talbot et al. 2014). Microbial dispersal-limitations have been long overlooked due in part to the pervasive and influential Baas-Becking hypothesis (Baas-Becking 1934) that "everything is everywhere." While microbes may indeed be dispersal-limited (e.g. Peay et al. 2007, Talbot et al. 2014), the role of dispersal-limitation in structuring microbial communities, particularly symbionts, is not well understood and may vary for different microbial and host species and across different spatial scales. While dispersal itself is notoriously difficult to measure for any organism, dispersal-limitation is inferred from assessments of spatial structure (Ettema and Wardle 2002). At the regional scale, if symbiont species vary in their dispersal, then communities will be more dissimilar from one another with increasing geographic distance and exhibit spatial structure assessed as a distance-decay relationship (Peay et al. 2007, Hanson et al. 2012, Talbot et al. 2014, Beck et al. 2015). However, the lack of a distance-decay relationship could have several causes, such as strong, local filtering by the environment or hosts, or communities composed of highly mobile and less dispersal-limited species.

While the relative strengths of filtering and dispersal-limitation in structuring symbiont communities may vary across host species and environments, there is also variation in communities within a host individual (Seabloom et al. 2015). Symbiont communities are often heterogeneous in composition across host tissues (Petrini 1991, Rodriguez et al. 2009, Costello et al. 2009, Coince et al. 2014), and some component species may provide tissue-specific functions (Newsham 2011, Ridaura et al. 2013). For symbionts of plants, the air mediates symbiont community assembly in aboveground tissue yet provides a relatively weak environmental filter, while soil mediates belowground tissue communities and provides a relatively strong filter (sensu Kivlin et al. 2014). Moreover, above- and belowground plant tissues differ greatly as habitats for microbial growth and reproduction, and if fungal endophyte species have adapted differently to these environments, relative investment in vegetative growth versus spore production may differ among symbionts. For instance, aboveground symbionts might be expected to produce more spores and thus travel greater distances between plants than do belowground symbionts (Gilbert and Reynolds 2005, Kivlin et al. 2014). Conversely, if belowground symbionts colonize hosts via a hyphal network growing through the soil, their hyphae may grow faster than that of aboveground symbionts.

Here, we considered how the assembly of above- and belowground symbiont communities differ with respect to (1) the importance of filtering by environment and host species, (2) filtering-independent spatial structure at the regional scale, and (3) the growth and sporulation of individual symbionts. Our general expectations were that belowground symbiont communities should exhibit greater filtering by the environment and greater spatial structure than aboveground symbiont communities, and that the belowground

symbionts should grow faster and produce fewer spores than those isolated from aboveground communities. A priori, we expected that filtering by host species should not differ between above- and belowground symbiont communities. To investigate these questions, we characterized communities of fungal endophytes for each of three species of beachgrasses along the USA Pacific Northwest coast by culturing and sequencing fungi from asymptomatic plant tissues and delimiting operational taxonomic units (OTUs) based on sequence similarity. Fungal endophytes are a diverse group of fungi primarily belonging to the phylum Ascomycota and inhabiting tissues of all plants without causing disease symptoms (Petrini 1991, Rodriguez et al. 2009). While some endophytes grow systemically throughout the plant and are vertically transmitted, most cause small, local infections and are horizontally transmitted (Rodriguez et al. 2009). We evaluated the importance of abiotic environment (physical location along the dune and soil properties) and biotic environment (host species), in affecting endophyte isolation frequency, OTU richness, and community composition at both local (within sites) and regional (across sites) levels. For both above- and belowground communities, spatial structure was evaluated by determining relationships between community similarity and geographic distance (distance-decay) and differences in turnover in composition across sites (i.e., beta diversity). Finally, we quantified differences in life-history traits of individual symbionts by measuring colony growth rate and asexual spore production of the 25 most commonly occurring endophyte OTUs.

MATERIALS AND METHODS

System

We studied culturable fungal endophyte species in beachgrasses that occurred in dunes along the Pacific Coast of Oregon and Washington, USA. These dunes provide habitat for endemic and federally listed plant and bird species, and the three dominant common grass species studied here—the native Elymus mollis, and the exotic Ammophila arenaria and Ammophila breviligulata introduced to stabilize dunes and protect inland developments from flooding (Zarnetske et al. 2010, Hacker et al. 2012, Seabloom et al. 2013). Dunes contain a strong environmental gradient from the shore to inland areas in which the backsides of dunes tend to be shielded from wind and are composed of more stable soil with higher C, N, and micronutrients than are soils at the front of the dune (Cooper 1958, David et al. 2015b). We categorized local environments as the front, crest, and backdunes (hereafter "dune locations") constituting, respectively, the front slope of the dune, the top of the dune, and the area beyond the back slope of the dune.

Sampling

We sampled from five sites in which all three grass species co-occurred along the Oregon and Washington coast in late August to early September 2011. The five sites included four in Oregon (Pacific City, Sand Lake, Seaside, and Fort Stevens) and one in Washington (Grays Harbor). Distances between sites ranged from 8 to 180 km. Within sites, we sampled along one to four transects, each containing a single 3 × 3 m quadrat in the front, crest, and back of the dune (mean transect length = 48.64 m ± 6.64 S.E.). We selected locations for transects to represent a broad range of relative amount of cover by the three grass species, which we visually estimated for each quadrat. We collected roots and leaves from three individual plants (defined as one or more tillers attached to a single rhizome) and placed them in Ziploc bags until processing.

One soil core (10-cm deep) was taken from each quadrat. Soils were air-dried and analyzed for percent C and percent N at the University of Nebraska Ecosystem Analysis Laboratory (Lincoln, NE, USA) and pH, cation exchange capacity, organic matter, and concentrations of Ca, K, P, Na, S, by A&L Analytical Laboratories (Memphis, TN, USA).

Endophyte Culturing and Identification

Under sterile laboratory conditions, we surface sterilized plant tissue using a rinse in sterile DI water followed by successive baths of 70 % ethanol (1 min), 70 % bleach (3.675 % NaOCl, 2 min), 70 % ethanol (1 min), and a final rinse in sterile DI water. Tissue was cut into ~1.5 mm2 segments and placed onto 2 % malt extract agar (MEA). For each individual plant, 20 sterilized tissue segments were placed in MEA, 10 from leaves and 10 from roots. Cultures were allowed to grow for 4 months before sub-culturing onto separate Petri dishes. Control plates were made by pressing surface-sterilized tissue to the media and checked for any subsequent fungal growth that would indicate the presence of surface fungi.

We extracted total genomic DNA and PCR amplified the ITS rDNA region from each isolate using one of two methods. In the first method, we used SDS buffer and phenol:chloroform extraction (Arnold et al. 2007). The ITS-LSU gene was amplified as a single amplicon using the ITS1F (Gardes and Bruns 1993) and LR3 primers (Vilgalys and Hester 1990) and Takara Taq (Takara Bio Inc. Otsu, Shiga, Japan) (40 μL reaction consisting of 5 μL Takara 10× buffer, 4 μL dNTP (10 μM), 1 μL (10 μM) each of forward and reverse primer, 35 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1.30 min). In the second method, we used the Extract N' Amp ReadyMix kit (Sigma Aldrich Corp., St. Louis, MO, USA) to extract and amplify the DNA (20 μL reaction consisting of 5 μL REDExtract N' Amp buffer, 0.8 μL (10 μM) each of forward and reverse primer, 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min). Sanger

sequencing of amplicons was conducted at Beckman Coulter (Brea, CA, USA). Sequences were edited using Geneious (Kearse et al. 2012) and clustered into OTUs at the 97 % similarity level using the workflow developed by Monacell and Carbone (2014) that uses ITS extractor (Nilsson et al. 2010) to extract the ITS1 and ITS2 regions, MOTHUR (Schloss et al. 2009) to check for chimeras, and ESPRIT (Sun et al. 2009) to cluster sequences based on alignment-free similarity.

Growth Assays

We measured colony growth rates and asexual spore production of the 25 most commonly isolated OTUs (Table 1). Three isolates representing each OTU were randomly selected and plated onto starter cultures on MEA. From the starter cultures, two plugs (0.5-cm diameter) were each plated onto separate MEA culture plates. We measured the diameter of the cultures every 2 days for 14 days when the fastest growing cultures neared the edge of their plates. At the conclusion of the study, we collected the asexual spores by flooding the Petri plates with 10 mL DI water, scraping the spores off the culture, and counting the number of spores in a hemacytometer.

Data Analysis

We used R Version 3.0.2 (R Development Core Team 2013) to conduct all analyses and to generate figures. Data, GenBank accession numbers, and additional sample location information were deposited in the publically available Data Repository for the University of Minnesota (David et al. 2015a).

Filtering

To evaluate the importance of filtering, we examined the relationship of abiotic environmental factors and host species on the frequency at which we obtained fungal endophyte cultures (isolation frequency), OTU richness, and endophyte community

composition at spatial scales of quadrat and site. Because several soil variables were highly correlated, we clustered variables based on Ward's minimum variance method to determine collinearity (Appendix 2-1). Based on this analysis, we included the effects of %N, organic matter, and soil pH as environmental covariates. We also included latitude as an environmental variable to account for regional changes in the relative abundances of the three grass species (Hacker et al. 2012).

We determined the effect of host community by examining the relationship between the neighboring conspecific host cover endophyte abundance and OTU richness. A positive relationship could suggest that the neighboring conspecific hosts serve as source of endophyte inocula. Cover was scaled as the relative proportion of the percent covers of the three grass species in a quadrat (i.e., the relative covers of all three species in a quadrat summed to 1), and the conspecific cover was the cover value for a given host species. We visualized these results using the predicted values of a simplified generalized linear mixed-model with binomial error with site and transect as nested random effects and host species and conspecific host cover as fixed effects.

We estimated endophyte OTU richness, the number of different OTUs present in a sample, at the quadrat, site, and regional levels. At the quadrat level, OTU richness was calculated as the rarefied number of OTUs found within conspecific plant hosts within a quadrat. Richness was rarefied according to the lowest sampling effort (i.e., number of surface-sterilized tissue segments) of a host species within a quadrat. We used this quadrat-level approach to avoid pseudoreplication caused by sampling multiple individuals of the same plant species within a quadrat. At the site-level, we calculated a rarefied OTU richness for each dune location (front, crest, backdune) within sites and pooled across all three host species. At the regional level, we

calculated richness using a similar rarefaction approach, but only estimated one value for each dune location pooled across all sites.

We used the lme4 package (Bates et al. 2015) in R to construct mixed-effects models to analyze the effects of environment and host species on isolation frequency and OTU richness. Transect was nested within site as random effects, and latitude, %N, organic matter, pH, conspecific host cover, dune location, host species, and the interactions conspecific host cover × host species and dune location × host species were fixed effects. All continuous predictor variables were scaled to a mean of 0 and a standard deviation of 1 to allow for comparison of estimates (Burnham and Anderson 2002). Differences in the categorical variables dune location and host species were tested using independent contrasts. Dune location was analyzed first using a linear contrast that tested differences in the response variable between the front and back locations, and second with a quadratic contrast that tested whether the response variable for the crest location was higher or lower than expected along the linear transect. Host species was analyzed with the first contrast comparing *E. mollis* to both the *Ammophila* species, and the second contrast comparing *A. arenaria* to *A. breviligulata*.

For quadrat-level analyses, we used generalized mixed-effect models with binomial error structure to analyze endophyte abundance and a linear mixed-effect models to analyze rarefied OTU richness. Endophyte abundance in roots was quantified as the isolation frequency (i.e., proportion of sterilized tissue segments with an emergent fungus). Because we recovered endophytes from a low proportion of leaves, we analyzed endophyte abundance per leaf as the presence/absence of any endophytes in a leaf. For the richness analyses, only those plants with at least one OTU were used in the analysis to avoid effects

of plants for which no endophytes were recovered and data were natural-log transformed to meet model assumptions. We used a model-averaging approach with the MuMIn package (Barton 2014) in R to identify the important factors in each model. This approach first identified the best models within four AIC units, then averaged the coefficients in these models (Barton 2014). The importance value is a weighted average of the best models in which a term appears. For the site-level analyses, we used linear mixed-effects models to test how dune location and soil properties affected rarefied site-level richness. We used the model-averaging approach previously described and considered the effects of latitude, N, organic matter, pH, and dune location on rarefied species richness using site as a random effect.

To analyze sources of variation in endophyte community composition in leaves and roots, we used the *adonis*() function in the vegan package (Oksanen 2011) to perform permutational multivariate analysis of variance (perMANOVA). We conducted a preliminary analysis to verify that endophyte community composition differed between leaves and roots ($F_{1,115} = 7.48$, $p < 0.001$) and, therefore, analyzed the two communities separately. We modeled endophyte community composition at the quadrat level (for quadrats in which at least one OTU was found) as a function of site, transect within a site, latitude, %nitrogen, organic matter, and pH dune location, host species, and dune location × host species. We then reduced models by sequentially eliminating non-significant terms.

Regional Spatial Structure

To assess distance-decay relationships in leaf and root endophyte community composition at the regional level, we performed multiple regression on distance matrices (MRM). Bray-Curtis community similarity matrices were calculated based on an average of

100 rarefactions of endophyte communities pooled across all individuals in each quadrat. Similarity matrices were regressed against a Euclidian distance of physical distances among quadrats using the ecodist package (Goslee and Urban 2007). We included the distance matrices for differences in %nitrogen, organic matter, and pH to control for effects of soil properties. We analyzed distance-decay for both above- and belowground community composition in two ways: first using a global analysis that included all quadrats, and second using separate analyses to analyze distance-decay patterns for each of the three dune locations. To visualize the error associated with isolation by distance estimates, we used a jackknife approach to calculate a standard error.

To analyze compositional turnover in endophyte communities across spatial scales, we compared the regional-level rarefied OTU richness to quadrat- and site-level richness. We also calculated a multivariate measure of turnover as the dispersion of quadrat-level community composition of each host species in multivariate space (i.e., average distance of points from the group centroid) and tested for differences across groups using the betadisper() function in the vegan package (Oksanen 2011). High compositional turnover is consistent with, but not proof of, dispersal-limitation.

Growth and Sporulation

We determined if endophytes found in leaves and roots differed in the rate of colony growth or sporulation by asking whether differences in these life-history traits predicted the tissue from which an OTU was isolated. For growth assays, we calculated the average growth rate for each individual culture plate as the slope of the linear regression of diameter colony growth against the number of days of growth (mean R^2 for all regressions = 0.98, minimum ^{R2} = 0.88). We calculated a mean growth rate to characterize each OTU as an

average of six slopes (three isolates per OTU × two replicate plates per isolate). Because the assay ended when the first isolates neared the edge of their Petri dishes, growth curves were linear and did not asymptote. We calculated spore production as the total spores estimated per culture divided by the final diameter length of the colony. This metric represented the number of spores produced per unit diameter of the colony. Analysis of total (unscaled) spore production yielded similar results to those obtained using the spore production scaled by diameter. We used a GLM with quasibinomial error to account for overdispersion to analyze the effects of growth rate and spore production on the probability that a given OTU was found in a leaf. We tested the model for significance using an analysis of variance with type II sum of squares using the car package (Fox and Weisberg 2011). To assess model fit, we regressed the observed means as a function of the predicted means.

RESULTS

We isolated a total of 607 fungal cultures out of 5538 surface sterilized plant tissues, 134 of which were isolated from leaves and 473 from roots. From these, we successfully extracted DNA, amplified, sequenced, and clustered 363 isolates into 76 OTUs at the 97 % level of sequence identity, 27 of which were found in leaves, 60 in roots, and 11 in both leaves and roots (Table 1). OTUs primarily belonged to the phylum Ascomycota (70 OTUs), but we also found members of the Basidiomycota (5 OTUs) and the Zygomycota (1 OTU). The most common classes included the Dothidiomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes. Several of the OTUs belonged to genera previously characterized as dark septate endophytes such as *Botryosphaeria*, *Cadophora* (*Phialophora*), *Exophiala*, *Leptodontidium*, *Leptosphaeria*, *Microascus*, *Microdochium*, *Periconia*, *Pleospora*, and

Xylaria (Jumpponen and Trappe 1998, Mandyam and Jumpponen 2005). OTUs related to the genus *Lewia* were the most commonly isolated from leaves, and OTUs related to the genus *Microdochium* were most commonly isolated from roots (Table 2-1).

The OTUs associated with leaf communities were typically shared across hosts and locations (Fig. 2-1a). In total, *E. mollis* harbored 10 of 11 OTUs found more than once in leaves (non-singletons), while the *Ammophila* hosts tended to harbor somewhat smaller subsets of all non-singleton leaf endophytes (8/11 for *A. arenaria*, 6/11 for *A. breviligulata*; Fig. 2-1c). The OTUs associated with roots were more specific to dune locations than were those associated with leaves, as evidenced by the high number of OTUs found in only one dune location (Fig. 2-1b). Root endophyte OTUs exhibited a range of host species association (Fig. 2-1d).

Evidence for Filtering

We first evaluated the effects of filtering on endophyte abundance in leaf and root tissues. Model averaging revealed that the abundance of endophytes in leaves was primarily influenced by host species (Table 2-2). The proportion of leaves from which we obtained at least one endophyte was higher for the native *E. mollis* than either of the *Ammophila* species (Table 2-2, Fig. 2-2. Isolation frequency in roots generally increased from the front to the back of the dune, though isolation frequency in *E. mollis* was lower in the crest relative to the front and backdunes (Table 2-2, Fig. 2-2). Isolation frequency in roots was significantly negatively associated with organic matter and soil pH (Table 2-2). We found that conspecific host cover was positively associated with the proportion of hosts from which at least one leaf endophyte was isolated, and this effect did not differ across host species (Table 2-2, Fig. 2-3).

OTU richness at the quadrat-level increased with soil pH in both leaves and roots, but there were no significant effects of dune location or host species (Table 2-2, Fig. 2-4). At the site-level, we found no influences of any predictor variables on OTU richness of leaves, but higher pH did have a negative effect on OTU richness in roots (Table 2-3, Fig. 2-4).

Our perMANOVA analyses revealed that endophyte community composition in leaves was significantly structured by an interaction between dune location and host species ($F_{4,26} = 1.50$, $R^2 = 0.110$, $p = 0.040$), and all other environmental covariates were dropped from the model (Fig. 2-5a). In contrast, endophyte community composition in roots was significantly structured by dune location ($F_{2,52} = 1.53$, $R^2 = 0.041$, $p = 0.003$) and marginally affected by soil pH ($F_{1,52} = 1.53$, $R^2 = 0.021$, $p = 0.058$) (Fig. 2-5b). For community composition in both leaves ($F_{4,26} = 1.95$, $R^2 = 0.143$, $p = 0.004$) and roots ($F_{4,52} = 2.32$, $R^2 = 0.122$, $p < 0.001$), we found a strong effect of site (Fig. 2-5c, 2-5d).

Evidence for Regional Spatial Structure

We found that root endophyte communities were structured at the local spatial scale whereas the leaf communities showed evidence of regional spatial structure. Endophyte communities in leaves exhibited a significant distance-decay relationship, as quadrats closer to one another tended to harbor more similar endophyte communities (Table 2-4, Fig. 2-6). When separately tested for each dune location, we found that this pattern held for front dunes, but not for the crest or backdune locations (Table 2-4). In contrast, we did not find evidence of a distance-decay relationship in root communities across all quadrats or within quadrats (Table 2-4).

We found higher compositional turnover in endophyte communities of roots than those of leaves. Regional OTU richness in leaves increased from the front to the crest, but plateaued from the crest to the back (solid line in Fig. 2-4a), while richness in roots increased from the front to the back (solid line in Fig. 2-4b). This result, in conjunction with the previously stated result that OTU richness at the quadrat- and site-levels did not change with dune location (dashed and dotted lines in Fig. 2-4), suggests that turnover across sites accounts for the increases in OTU richness from the front to the back of dunes, particularly for communities in roots. Indeed, dispersion (multivariate measure of compositional turnover) was higher in root than leaf communities (permuted $F_{1,173} = 4.893$, $p = 0.021$). For endophyte communities in leaves, there were no differences in dispersion among dune locations pooled across host species (permuted $F_{8,53} = 1.300$, $p = 0.257$) or within-host species ($p > 0.05$ for all three host species). Similarly, endophyte communities in roots showed no differences in dispersion among dune locations permuted $F_{8,104} = 1.441$, $p = 0.190$), although there was a significant increase in dispersion for communities in *A. breviligulata* from the front to the back of the dune (permuted $F_{2,35} = 3.276$, $p = 0.044$).

Growth and Sporulation

The mean hyphal growth rates and spore production of isolates representing OTUs varied greatly (Appendix 2-2). Contrary to our expectations, we found that OTUs with faster growth rates were more likely to be isolated from leaves ($df = 1$, $\chi^2 = 10.7$, $p = 0.001$), while OTUs with greater spore production were less likely to be found in leaves ($df = 1$, $\chi^2 = 31.7$, $p < 0.001$; Fig. 2-7). When we regressed the observed against the expected results, we found the model slightly underestimated the probability that an OTU was found in a leaf (slope = 0.89; adjusted $R^2 = 0.41$). Interestingly, OTU1 (*Microdochium bolleyi*), the

most common endophyte (34% of all sequenced isolates) and typically found in roots, exhibited the fastest growth rate among OTUs predominantly found in roots and was among the highest spore producers (Appendix 2-2).

DISCUSSION

In this study, we investigated the structure of above- and belowground fungal endophyte communities in beachgrasses along the USA Pacific Northwest Coast. We report three key findings. First, host species provided the strongest filter for leaf endophyte communities, whereas the abiotic environment provided the strongest filter for root endophyte communities. Isolation frequency and OTU richness was greatest for endophytes from *E. mollis* leaves than for leaves of *A. arenaria* or *A. breviligulata*, the leaves of which contained subsets of the OTUs found in *E. mollis*. Endophyte isolation frequency in leaves was greater for hosts neighbored by high abundances of conspecific hosts. These results suggest that leaf endophytes have a degree of host association specificity, and thus, their distribution could be limited by the abundance of neighboring hosts as sources of inocula. In contrast, endophyte abundance, richness, and community composition in roots depended more highly on abiotic environment (dune location and pH) than did leaf endophyte communities. Second, evidence for regional spatial structure differed for endophytes in leaves and roots. Community similarity decreased with geographic distance in leaf but not in root endophyte communities, and endophyte turnover in roots had greater turnover across sites than those in leaves. These results suggest that leaf endophyte species are dispersing more widely than are species of the root endophyte communities, which are more locally structured. Third, although we expected that endophytes most often found in leaves should

produce more spores and grow more slowly than those found in roots, we instead found the reverse, a result that could suggest different modes of dispersal between above- and belowground endophytes. Taken together, leaf endophyte communities were more strongly structured by host species and geographic distance, and root communities were more strongly structured by filters of the local environment.

Environmental Filtering Versus Host Filtering

Our findings contribute to our general understanding of the effects of filtering on microbial symbiont communities. Filtering by the environment and host species both play important roles in the assembly of symbiont communities in many systems (Yatsunenko et al. 2012, Borer et al. 2013, Blaalid et al. 2014). Our results suggest that the relative importance of the environment and host species for structuring endophyte communities depends on the plant tissue. The soil environment, particularly soil pH, provided a strong filter for endophyte communities in roots, a result that is similar to those of other recent studies demonstrating strong environmental filters and patchy, local distributions of root-associated fungi (Tejesvi et al. 2010, Blaalid et al. 2014, Botnen et al. 2014). Organic matter was also a predictor of isolation frequency in roots, but surprisingly, the two were negatively associated. Organic matter generally increases with dune succession, particularly in northern dunes dominated by *A. breviligulata* (David et al. 2015b) and is a potential resource for species of facultative endophytes that also live saprobically in the soil (Caldwell et al. 2000). Yet, previous work with the root endophyte *Phialocephala fortinii* showed no change in root colonization when organic matter was added to the soil (Jumpponen et al. 1998). The utilization of organic matter therefore may not necessarily lead to increased root colonization by endophytes. In contrast to the endophyte communities

in roots, our results showed that those in leaves demonstrated weak effects of environmental filtering, and this could be attributable to colonization by airborne inocula. However, we did find that increasing soil pH decreased OTU richness in leaves at the quadrat level, suggesting an indirect effect of the soil environment in shaping these communities.

Implications for Dispersal-Limitation

While the classic Baas-Becking hypothesis (1934) has been rejected by several studies showing evidence for dispersal-limitation in microbial communities (e.g. Peay et al. 2007, Higgins et al. 2014, Talbot et al. 2014), the extent to which plant-associated symbionts are dispersal-limited remains unclear. The results of our study show that above- and belowground symbionts display different regional spatial structure. Aboveground symbiont propagules are likely aerially dispersed over relatively longer distances, resulting in the observation that more similar communities are found in closer physical locations (i.e., distance-decay relationship). Similar patterns of spatial structuring according to geographic distance have also been found among horizontally transmitted leaf endophytes of tropical grasses (Higgins et al. 2014) and temperate forests (U'Ren et al. 2012). In contrast, belowground symbiont taxa are likely highly limited in their dispersal among sites and experience strong effects of environmental filtering, resulting in the observation of high turnover in belowground communities among sites and the absence of a distance-decay relationship. However, we caution that our results, like those of other studies that have sought to identify dispersal-limitation based on spatial structure, could still have been driven by some unmeasured environmental variable not accounted for in our model. For instance, it is plausible that slight differences in precipitation or temperature along our

latitudinal gradient could have accounted for the effect of geographic distance we found in the aboveground endophytes.

Conspecific host species may serve as sources of inoculum for dispersal of endophytes. We found that the probability of endophyte isolation in leaves increased with conspecific host abundance, suggesting that these neighboring hosts supply propagules of endophytes to new, conspecific hosts. Similar effects have been observed in pathogen systems, in which the transmission rate is dependent on the local abundance of compatible hosts (Mitchell et al. 2002, Keesing et al. 2006). This result may be caused by specific endophyte × host species interactions in leaves, or by the presence of favorable microclimate conditions (e.g., wind flow (Zarnetske et al. 2012)) for endophytes in leaves of a particular host species. In contrast, belowground symbionts, whose communities were less influenced by the host species, were subsequently not affected by conspecific neighboring host abundance.

Finally, our results suggest that the mode of dispersal may account for differences in the spatial structure of above- and belowground endophyte communities. Growth rates and spore production reasonably predicted the tissue from which an OTU was isolated, but surprisingly, fast growth and low spore production were associated with leaf infection, not root infection. Although the endophyte OTUs we identified here clearly exhibit a wide range of growth and sporulation rates, we speculate that growth and sporulation may have different roles in above- and belowground plant symbionts. For instance, the faster colony growth rates of leaf symbionts may correlate with competitive ability. In dune systems, windblown sand causes abrasions on leaves (Ogura and Yura 2008) as the host plants capture sand (Zarnetske et al. 2012). It is plausible that sand could deliver hyphae directly

to the leaf, and the hyphae subsequently enter the leaf through these abrasions. Within plants, faster growth may be more beneficial in leaves than in roots, especially if leaves are relatively short-lived compared to roots. In contrast, endophyte species belowground might rely on spores to colonize new hosts, particularly if they are poor competitors in the soil against saprobic fungi or if their hyphal networks are frequently disturbed. Spores may also be important survival structures for symbiont species in belowground communities (Glassman et al. 2015).

Endophyte Diversity in Dune Plants

Dune systems provide an environmentally stressful habitat of strong winds, salt spray, and low nutrient levels for both plants and fungi (Cooper 1958, David et al. 2015b) and, therefore, represent an opportunity to add to our understanding of fungal diversity in these habitats. In one such study, Rodriguez et al. (Rodriguez et al. 2008) found that an endophyte, *Fusarium culmorum*, conferred tolerance to salt stress in *E. mollis*. Though *F. culmorum* was isolated from *E. mollis* individuals growing north of our field sites on islands in the Puget Sound, WA, USA, we did not detect this endophyte in any of our samples. It is also worth noting that we found no instances of *Epichloë* spp., a genus that grows systemically in grasses and may confer resistance to herbivores or drought (Schardl et al. 2004). Research in the Great Lakes region of the USA found that *Epichloë* infects some populations of *A. breviligulata* and is present in commercial varieties used in plantings (Emery et al. 2010). We suspect that *A. breviligulata* was either introduced from a different, non-infected commercial variety, or has since lost its association with *Epichloë*. We did find a high abundance of *M. bolleyi* (OTU1) in all three grass hosts. *Microdochium* has previously been reported to colonize *A. arenaria* (Beckstead and Parker 2003) and is a

common root endophyte of grasses (Mandyam et al. 2013). The effects of *Microdochium* on its host may be positive or negative depending on the genotypes of the fungus and the plant (Mandyam et al. 2013). It is possible that *M. bolleyi* is an opportunistic colonizer in beachgrass, as it exhibited both relatively fast hyphal growth and high spore production.

Conclusions

Our study shows the varying degrees of filtering and regional spatial structure and differences in symbiont growth of above- and belowground symbiont communities. We found that communities of aboveground endophytes were structured according to host species and geographic distance, while communities belowground were structured by environmental filtering. As we attempt to understand the drivers of the microbial symbiont communities associated with plants and animals, it is important to consider how the host tissue exposes symbiont species to different environmental or host-specific filters and could favor species with particular dispersal processes.

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OTU	Total Isolations (Leaf Isolations)	Phylum	Class	Order	Closest BLAST Match (GenBank Accession No.)	Query Length (Coverage)	Similarity %
154	2 (0)	Ascomycota	Dothideomycetes	Botryosphaeriales	Microdiplodia hawaiiensis (GU361956)	428 (89.64)	90.5
13*	7 (6)	Ascomycota	Dothideomycetes	Capnodiales	Cladosporium cladosporioides (JQ724382)	466 (100)	100
157*	6 (1)	Ascomycota	Dothideomycetes	Capnodiales	Cladosporium sphaerospermum (AB572897)	467 (100)	100
145*	10 (10)	Ascomycota	Dothideomycetes	Capnodiales	Penidiella strumelloidea (EU019277)	434 (95.11)	91.9
148	3 (3)	Ascomycota	Dothideomycetes	Capnodiales	Penidiella strumelloidea (EU019277)	443 (97.33)	92.6
170	1 (1)	Ascomycota	Dothideomycetes	Capnodiales	Penidiella strumelloidea (EU019277)	468 (98.73)	95.7
169	1 (1)	Ascomycota	Dothideomycetes	Pleosporales	Drechslera erythrospila (EU552124)	554 (100)	100
41	13 (0)	Ascomycota	Dothideomycetes	Pleosporales	Drechslera nobleae (AY004792)	474 (100)	91.2
151	1 (1)	Ascomycota	Dothideomycetes	Pleosporales	Fusicladium sicilianum (FN549914)	283 (50.25)	85.3
141*	23 (21)	Ascomycota	Dothideomycetes	Pleosporales	Lewia infectoria (EF104194)	513 (99.61)	95.1
158	2 (1)	Ascomycota	Dothideomycetes	Pleosporales	Lewia infectoria (GU584953)	498 (97.46)	100
88*	13 (9)	Ascomycota	Dothideomycetes	Pleosporales	Lewia infectoria (JX421701)	501 (97.46)	98.4
168	1 (0)	Ascomycota	Dothideomycetes	Pleosporales	Ophiosphaerella agrostis (AF191550)	506 (100)	89.8
149*	7 (7)	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeria insignis (AF439485)	503 (99.6)	98.6
166*	4 (4)	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeria nigrans (AF439492)	485 (97.59)	97.7
161	1 (1)	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeria triglochinicola (AF439507)	493 (99.2)	88.6
146*	5 (4)	Ascomycota	Dothideomycetes	Pleosporales	Pleospora herbarum (GU584954)	494 (100)	99.6
25	2 (0)	Ascomycota	Eurotiomycetes	Chaetothyriales	Cladophialophora chaetospira (EU137333)	528 (100)	100
97*	3 (0)	Ascomycota	Eurotiomycetes	Chaetothyriales	Exophiala salmonis (AF050274)	550 (97.68)	97.5
22*	16 (1)	Ascomycota	Eurotiomycetes	Chaetothyriales	Exophiala salmonis (AM176667)	535 (97.46)	95.9
10	4 (0)	Ascomycota	Eurotiomycetes	Chaetothyriales	Exophiala salmonis (GU586858)	561 (99.82)	99.8
94	1 (0)	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillus tubingensis (JQ693399)	514 (100)	100
142	7 (4)	Ascomycota	Eurotiomycetes	Eurotiales	Penicillium brevicompactum (FJ004277)	493 (100)	100
60*	4 (0)	Ascomycota	Eurotiomycetes	Eurotiales	Penicillium canescens (JN585940)	380 (76.31)	100
164	1 (1)	Ascomycota	Leotiomycetes	Helotiales	Cadophora hiberna (AF530463)	459 (100)	92.8
124*	3 (0)	Ascomycota	Leotiomycetes	Helotiales	Cryptosporiopsis rhizophila (AY176753)	472 (100)	100
21	1 (0)	Ascomycota	Leotiomycetes	Helotiales	Dactylaria appendiculata (AY265339)	545 (100)	90.6
34	1 (1)	Ascomycota	Leotiomycetes	Helotiales	Dactylaria appendiculata (AY265339)	531 (100)	95
3*	8 (0)	Ascomycota	Leotiomycetes	Helotiales	Leptodontidium orchidicola (AF214577)	534 (97.8)	100

68	1 (0)	Ascomycota	Leotiomycetes	Helotiales	Leptodontidium orchidicola (FJ665276)	537 (99.08)	97.8
72	2 (0)	Ascomycota	Leotiomycetes	Helotiales	Phialocephala fortinii (EU314682)	479 (100)	99.4
155	2 (2)	Ascomycota	Leotiomycetes	Thelebolales	Thelebolus microsporus (DQ402525)	428 (84.2)	88.6
85	4 (0)	Ascomycota	Leotiomycetes	uncertain	Leohumicola minima (AY706329)	464 (100)	92.5
54*	4 (0)	Ascomycota	Leotiomycetes	uncertain	Leohumicola verrucosa (AY706325)	467 (100)	89.6
70	2 (0)	Ascomycota	Leotiomycetes	uncertain	Meliniomyces bicolor (AJ308340)	470 (100)	98.3
29	5 (0)	Ascomycota	Sordariomycetes	Diaporthales	Phomopsis columnaris (GU934561)	488 (100)	100
18	1 (0)	Ascomycota	Sordariomycetes	Hypocreales	Acremonium cavaraeanum (JF912333)	484 (96.71)	90.2
56	1 (1)	Ascomycota	Sordariomycetes	Hypocreales	Acremonium nepalense (GU586837)	475 (100)	96.2
65*	14 (0)	Ascomycota	Sordariomycetes	Hypocreales	Acremonium strictum (AM924152)	501 (100)	100
78*	5 (0)	Ascomycota	Sordariomycetes	Hypocreales	Acremonium strictum (HM016899)	482 (96.82)	95.1
51	3 (0)	Ascomycota	Sordariomycetes	Hypocreales	Fusarium avenaceum (JX074742)	478 (100)	100
128	1 (0)	Ascomycota	Sordariomycetes	Hypocreales	Fusarium pseudograminearum (JF739304)	468 (100)	100
66	2 (0)	Ascomycota	Sordariomycetes	Hypocreales	Hirsutella rhossiliensis (DQ345567)	501 (100)	100
16*	3 (0)	Ascomycota	Sordariomycetes	Hypocreales	Neonectria ditissima (JF735309)	447 (97.42)	83.2
33	2 (0)	Ascomycota	Sordariomycetes	Hypocreales	Neonectria radicicola (GU934547)	459 (100)	100
37	2 (0)	Ascomycota	Sordariomycetes	Hypocreales	Trichoderma pubescens (EU280121)	516 (100)	99.8
8	1 (0)	Ascomycota	Sordariomycetes	Hypocreales	Trichoderma viride (AF359255)	518 (100)	100
2*	4 (0)	Ascomycota	Sordariomycetes	Magnaporthaceae	Gaeumannomyces cylindrosporus (JF508361)	509 (100)	99.8
103*	3 (0)	Ascomycota	Sordariomycetes	Magnaporthaceae	Gaeumannomyces graminis var tritici (FJ771005)	484 (100)	100
165	1 (0)	Ascomycota	Sordariomycetes	Microascales	Corollospora maritima (JN943388)	533 (96.17)	81
147	1 (0)	Ascomycota	Sordariomycetes	Microascales	Microascus trigonosporus var trigon (AM774156)	402 (82.29)	89.4
119*	4 (0)	Ascomycota	Sordariomycetes	Microascales	Pseudallescheria boydii (JN207435)	356 (67.49)	82
95	1 (0)	Ascomycota	Sordariomycetes	Microascales	Scedosporium apiospermum (JN872195)	250 (47.41)	90.1
62	1 (0)	Ascomycota	Sordariomycetes	Phyllachorales	Plectosphaerella cucumerina (JQ796755)	469 (100)	100
115	1 (0)	Ascomycota	Sordariomycetes	Sordariales	Cercophora coprophila (AY999136)	466 (96.82)	92.4
30	2 (0)	Ascomycota	Sordariomycetes	Sordariales	Chaetomium funicola (FN394680)	495 (100)	100
24	1 (1)	Ascomycota	Sordariomycetes	Sordariales	Kylindria ellisii (EF029190)	360 (74.27)	81.3
143	4 (0)	Ascomycota	Sordariomycetes	Sordariales	Podospora glutinans (AY615208)	496 (100)	98.8
6*	3 (0)	Ascomycota	Sordariomycetes	Sordariales	Podospora minicauda (GQ922539)	473 (100)	97.7

159	1 (1)	Ascomycota	Sordariomycetes	Sordariales	Podospora pleiospora (AY515364)	481 (100)	100
160	2 (2)	Ascomycota	Sordariomycetes	Trichosphaeriales	Nigrospora oryzae (JN211105)	479 (100)	97.9
162	1 (0)	Ascomycota	Sordariomycetes	uncertain	Arthrinium phaeospermum (AJ279447)	526 (100)	99.2
156	1 (0)	Ascomycota	Sordariomycetes	uncertain	Myrmecridium schulzeri (EU041772)	481 (99.17)	96.9
1*	66 (1)	Ascomycota	Sordariomycetes	Xylariales	Microdochium bolleyi (GU934539)	469 (100)	100
153	2 (0)	Ascomycota	Sordariomycetes	Xylariales	Microdochium nivale (AM502260)	468 (100)	100
79*	5 (1)	Ascomycota	Sordariomycetes	Xylariales	Microdochium phragmitis (AM502263)	462 (100)	99.6
144	1 (1)	Ascomycota	Sordariomycetes	Xylariales	Rosellinia pepo (AB017659)	480 (97.88)	80.4
102	2 (0)	Ascomycota	uncertain	uncertain	Chalara piceaeabietis (FR667230)	467 (100)	91.3
9*	19 (1)	Ascomycota	uncertain	uncertain	Dokmaia monthadangii (JN559405)	448 (100)	99.1
5*	18 (0)	Ascomycota	uncertain	uncertain	Xenochalara juniperi (DQ132827)	481 (100)	92.1
104	6 (0)	Basidiomycota	Agaricomycetes	Agaricales	Marasmius tricolor (JN943601)	618 (100)	98.5
150	2 (0)	Basidiomycota	Agaricomycetes	Agaricales	Panaeolus acuminatus (JF908518)	569 (100)	99.8
163	1 (0)	Basidiomycota	Agaricomycetes	Agaricales	Tetrapyrgos subdendrophora (EF175521)	650 (100)	99.4
152	1 (0)	Basidiomycota	Agaricomycetes	Cantharellales	Tulasnella calospora (AB369940)	552 (100)	98.9
39	2 (0)	Basidiomycota	Tremellomycetes	Tremellales	Exidia uvapsassa (DQ241776)	505 (100)	87.4
167	1 (0)	Zygomycota	uncertain	Mortierellales	Mortierella globulifera (JN943800)	584 (99.83)	99.7

Table 2-1 OTU assignments based on alignment-free clustering at the 97 % similarity level.
Columns show the OTU name assigned in this study, the total number of times the OTU was isolated and the number of times it was isolated in leaves, the taxonomy based on the closest BLAST match, query length with coverage, and percent similarity of the query to the BLAST match. [a]Those OTUs used in the growth assay.

Term	Level	Leaves				Roots			
		Iso. Freq.		Richness		Iso. Freq.		Richness	
		Impt.	Est.(S.E.)	Impt.	Est.(S.E.)	Impt.	Est.(S.E.)	Impt.	Est.(S.E.)
(Intercept)			-1.10 (0.17)***		0.76 (0.04)***		-1.82 (0.27)***		1.00 (0.04)***
Latitude		0.12	0.07 (0.16)	0.2	0.04 (0.04)	0.25	-0.28 (0.27)	0.14	0.02 (0.04)
%Nitrogen		0.15	0.13 (0.23)	0.17	0.03 (0.04)	0.45	0.18 (0.12)	0.12	0.01 (0.04)
Organic Matter		0.35	-0.24 (0.21)	0.26	-0.04 (0.04)	1	-0.29 (0.09)***	0.12	0.00 (0.04)
pH		0.15	-0.07 (0.16)	1	-0.12 (0.04)**	0.88	-0.38 (0.14)**	1	-0.18 (0.04)***
Conspecific Host Cover		0.84	0.40 (0.19)*	0.11	-0.02 (0.03)	0.16	0.05 (0.07)	0.14	-0.02 (0.04)
Conspecific Host Cover × Host Species	*E. mollis vs. Ammophila.*	0.05	-0.17 (0.13)	0		0		0	
	A. arenaria vs. A. breviligulata		0.01 (0.23)						
Dune Location	*linear*	0.05	0.02 (0.27)	0		1	0.32 (0.18)	0	
	quadratic		-0.35 (0.26)				0.46 (0.11)***		
Host Species	*E. mollis vs. Ammophila.*	1	0.69 (0.13)***	0		1	0.00 (0.05)	0.07	0.01 (0.03)
	A. arenaria vs. A. breviligulata		0.27 (0.21)				0.01 (0.07)		0.06 (0.05)
Dune Location × Host Species	*Linear: (E. mollis vs. Ammophila)*	0		0		1	-0.29 (0.07)***	0	
	Quadratic: (E. mollis vs. Ammophila)						0.28 (0.08)***		
	Linear: (A. arenaria vs. A. breviligulata)						0.02 (0.12)		
	Quadratic: (A. arenaria vs. A. breviligulata)						-0.19 (0.11)		
	# models within δ=4		13		8		7		6

Table 2-2 Factors influencing quadrat-level endophyte isolation frequency and rarefied richness in leaves and roots based on model averaging

Importance values (*Impt.*) give the weighted proportion of models that a term appeared in with a cutoff of $\delta = 4$ AIC units. Estimates (*Est.*) are the model averaged estimates with the adjusted standard errors shown in parentheses. Only those terms that appeared in models within the $\delta = 4$ have estimates. All continuous variables were scaled prior to analyses to allow for comparison of estimates. Isolation frequency was analyzed with generalized mixed-effects models with binomial error structure. Richness was rarefied and natural-log transformed, and was analyzed using linear mixed-effects models.

Term	Level	Site OTU Richness in Leaves		Site OTU Richness in Roots	
		Impt.	Est.(S.E.)	Impt.	Est.(S.E.)
(Intercept)			2.48 (0.27)***		4.85 (0.41)***
Latitude		0.1	0.18 (0.28)	0.12	-0.38 (0.44)
%Nitrogen		0.1	0.14 (0.21)	0	
Organic Matter		0.08	0.06 (0.21)	0	
pH		0.18	-0.26 (0.20)	1	-1.82 (0.38)***
Dune Location	*linear*	0		0	
	quadratic				
	# models within δ=4		4		2

Table 2-3 Factors influencing site-level endophyte richness in leaves and roots based on model averaging

Sampled plants were pooled by dune location in each site and analyzed using a linear mixed-effects model. See Table 2-2 for details on model averaging

	Leaves			**Roots**	
	Term	*Slope*	*p*	*Slope*	*p*
Across Dune Locations					
	(Intercept)	0.363	0.003	0.146	0.131
	N	-0.032	0.237	-0.020	0.378
	Organic Matter	0.022	0.535	0.004	0.879
	pH	0.000	0.976	-0.007	0.649
	Distance	-0.051	0.001	-0.007	0.27
	Regression	R^2 = 0.064	0.005	R^2 = 0.016	0.538
Within Dune Locations					
Front					
	(Intercept)	1.025	0.003	0.007	0.978
	N	0.003	0.978	-0.085	0.161
	Organic Matter	0.034	0.581	0.065	0.154
	pH	-0.052	0.424	0.087	0.106
	Distance	-0.181	0.003	0.023	0.454
	Regression	R^2 = 0.291	0.008	R^2 = 0.131	0.151
Crest					
	(Intercept)	0.088	0.561	-0.014	0.892
	N	0.019	0.603	-0.046	0.016
	Organic Matter	-0.075	0.059	0.035	0.061
	pH	0.030	0.48	0.003	0.879
	Distance	0.011	0.747	0.022	0.134
	Regression	R^2 = 0.064	0.391	R^2 = 0.111	0.075
Back					
	(Intercept)	0.172	0.332	0.103	0.396
	N	-0.016	0.797	-0.030	0.289
	Organic Matter	0.032	0.557	0.022	0.543
	pH	0.078	0.111	0.021	0.424
	Distance	-0.042	0.298	-0.009	0.664
	Regression	R^2 = 0.121	0.466	R^2 = 0.042	0.614

Table 2-4 Results from multiple regression on distance matrices (MRM) analyses for endophyte community composition in leaves and roots as a function of soil properties and geographic distance

Analyses were conducted across all dune locations and within dune locations.

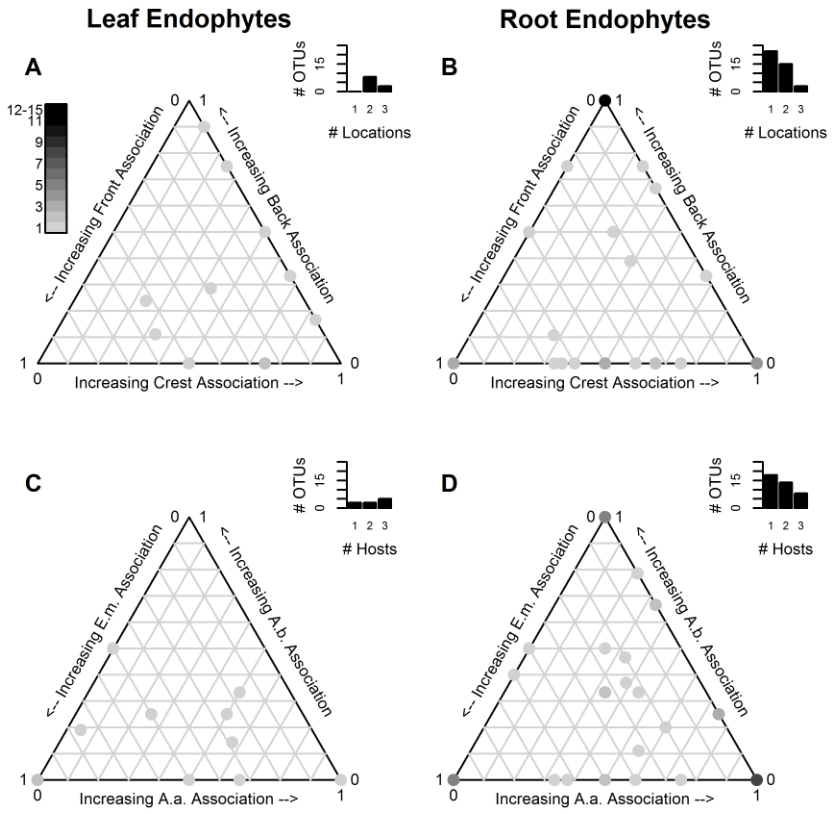

Figure 2-1 OTUs shared across dune locations and host species
Triangle plots showing endophyte OTUs shared across dune locations (a–b) and host species (c–d). Leaf endophytes are shown in (a) and (c), and root endophytes are shown in (b) and (d). Points are shaded based on the number of OTUs they represent. *Inset bar graphs* show the total number of OTUs shared across 1, 2, or 3 locations or host species. For host species, *E.m. Elymus mollis*, *A.a. Ammophila arenaria*, *A.b. A. breviligulata*

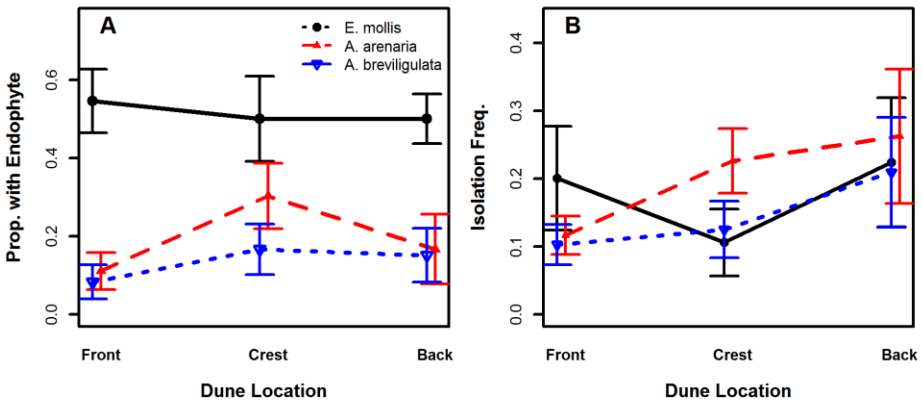

Figure 2-2 Endophyte abundance in leaves and roots.
In (a) leaves, abundance is quantified as the proportion of individuals in a quadrat in which at least one endophyte was found. For (b) roots, abundance is quantified as the isolation frequency (emergent fungi divided by total sterilized tissue segments) of endophytes in a quadrat. Means ± 1 S.E.M. are shown

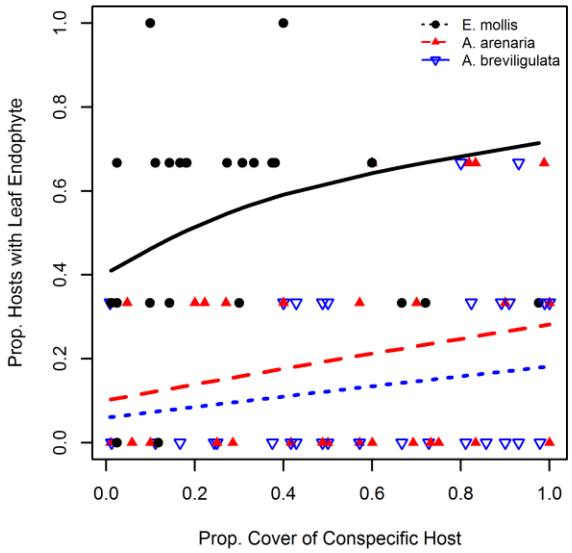

Figure 2-3 Proportion of hosts with at least one leaf endophyte increases with proportional cover of the conspecific host species

Trend lines show predicted values from generalized mixed-effects model with binomial error. *Y-axis* represents the proportion of hosts in a quadrat with at least one leaf endophyte. No significant interactions were found between host species and conspecific cover

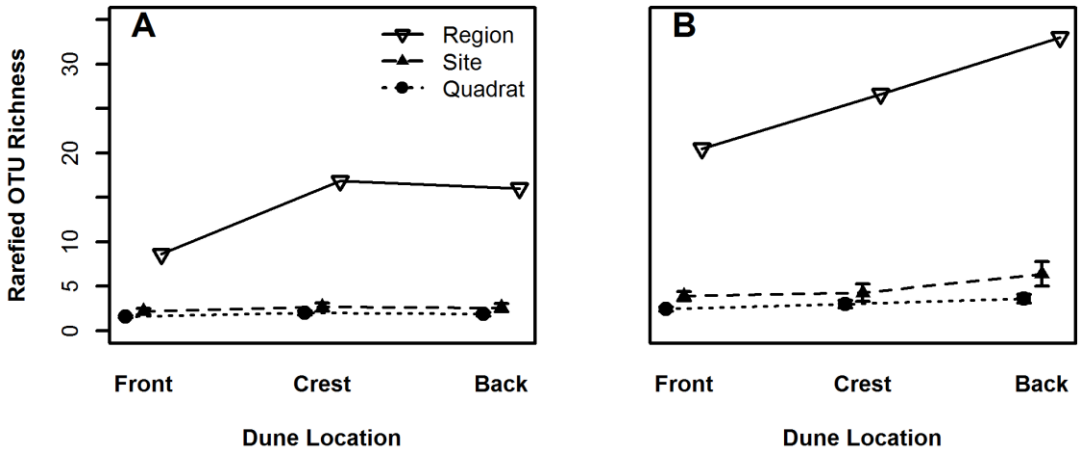

Figure 2-4 Endophyte OTU richness across leaves and roots.
Quadrat, site, and regional rarefied richness are shown on for (a) leaves and (b) roots. Note that these three richness metrics were rarefied to different levels, so richness is not directly comparable among the three levels. No significant differences in quadrat or site richness were found among dune locations (mean ± 1 S.E.M. is shown). Regionally, richness increased from the front to the back of dunes

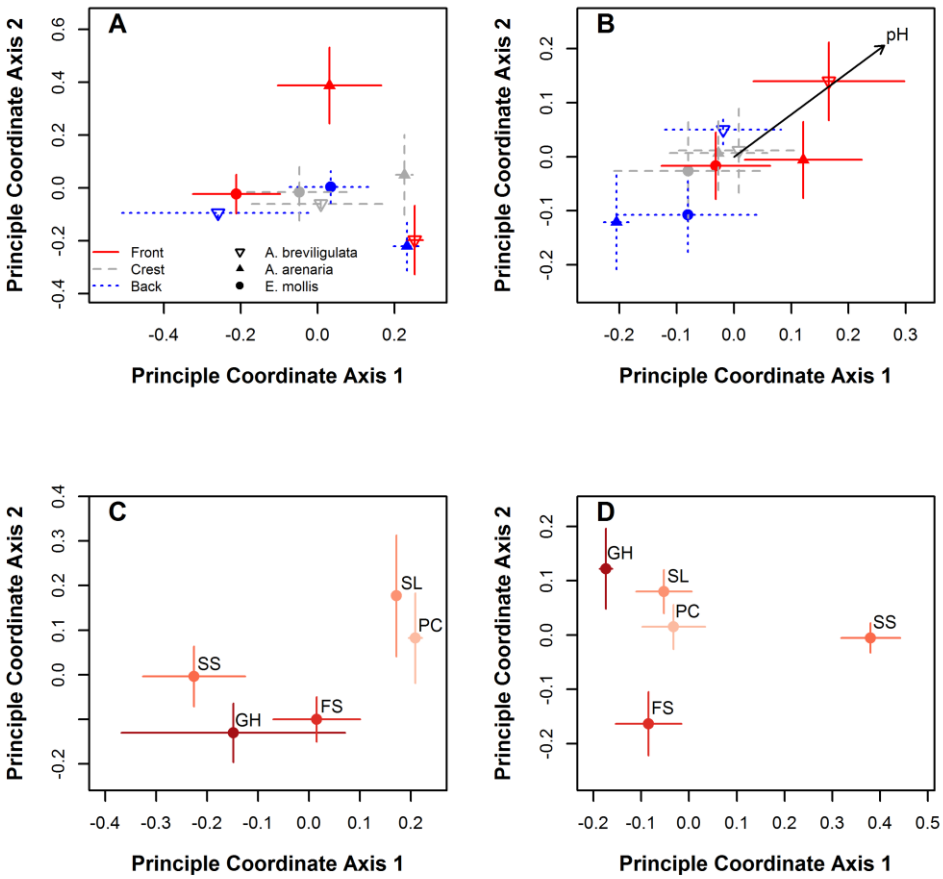

Figure 2-5 Community analyses for quadrat-level endophyte communities
(a,b) Principle coordinates analyses for leaves and roots, respectively, along with significant environmental vectors. Leaf endophyte communities showed a significant interaction between dune location and host species, while root endophyte communities were structured by dune location and pH. Means ±1 S.E.M. of axes 1 and 2 are shown. (c,d) Site means from the same principle coordinates analyses. Sites are shaded according to latitude, with *lighter red* signifying southern sites and *darker red* signifying northern sites. *PC* Pacific City, *SL* Sand Lake, *SS* Seaside, *FS* Fort Stevens, *GH* Grays Harbor. Means ± 1 S.E.M. are shown

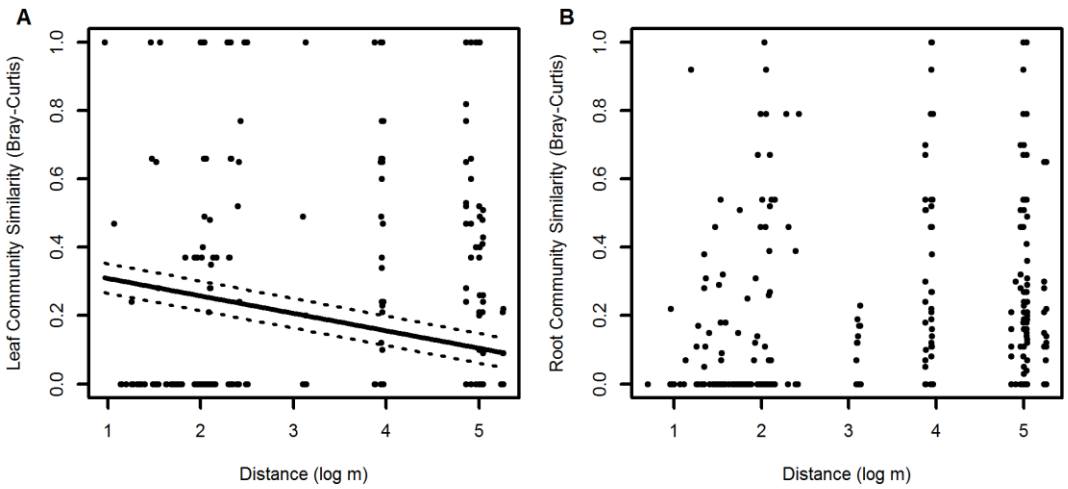

Figure 2-6 Regional Bray-Curtis community similarity as a function of distance in leaves and roots

(a) shows leaf endophyte communities, and (b) shows root endophyte communites. *Points* represent pairwise similarities between quadrats (across all hosts within the quadrat). *Line* shows significant effect of distance (log10 scale) in endophyte communities of leaves, and *dashed lines* show standard error around the slope estimate using a jackknife approach.

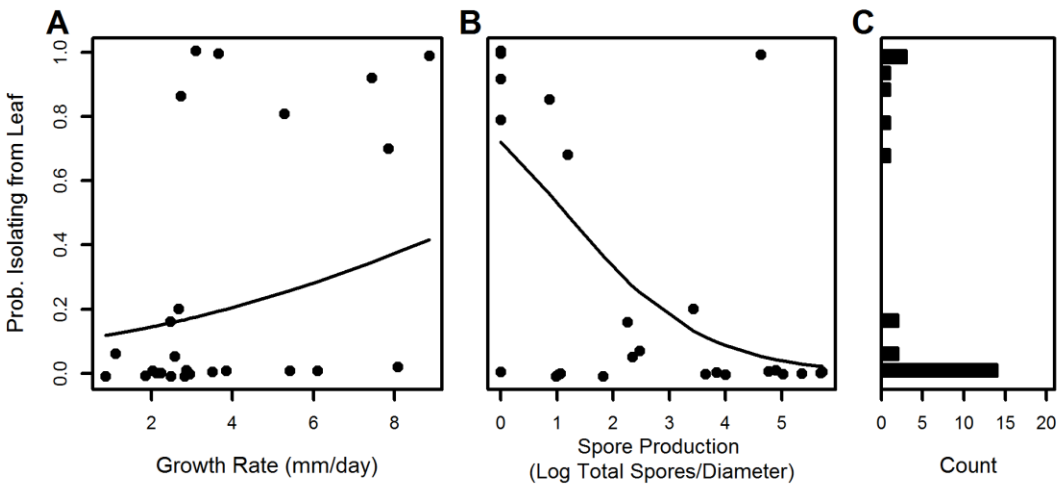

Figure 2-7 Probability that a given OTU is isolated from a leaf as a function of a **growth rate and** b **spore production.**

Each data point represents an individual OTU. Higher probabilities suggest OTUs are more commonly found in leaves, while lower probabilities suggest OTUs are more commonly found in roots. (a) Growth rate for each culture was calculated as the slope of the diameter as a function of the number of days cultures were growing, and the OTU growth rate was calculated as an average of these slopes. (b) Total spore production per plate was scaled by the diameter of the culture when the spores were sampled and averaged for each OTU on a log10 scale. Y-axis values were jittered to more easily visualize overlapping data points. (c) A histogram of the probabilities in (a) and (b)

Chapter 3 Tolerance of competition and beneficial plant-soil feedbacks drive beachgrass invasion in coastal dunes

ABSTRACT

Biological invasions are a threat to ecological communities and ecosystems, yet the mechanisms behind invasions are not fully understood. Specifically, identifying the contexts under which different mechanisms operate and the relative importance of these mechanisms is critical to understanding the causes and consequences of invasion. In this study, we investigate four potential mechanisms behind the ongoing invasion of American beachgrass (*Ammophila breviligulata*) into USA Pacific Northwest dunes dominated by its established, invasive congener, the European beachgrass (*A. arenaria*) – (1) tolerance to competition, (2) facilitation by mutualists, (3) escape from pathogens, and (4) plant-soil feedbacks. We conducted a field experiment to quantify the effects of disturbance and competition on biomass of both invasive *Ammophila* species and on the native beachgrass *Elymus mollis* within established stands of *A. arenaria*. We quantified colonization by fungal symbionts and infection by plant-parasitic nematodes. Next, we conducted a growth chamber experiment to investigate whether soil microbes associated with *A. arenaria* mediate invasion of *A. breviligulata*. We found that biomass of the invading *A. breviligulata* and of the native *E. mollis* were unaffected by competition, whereas *A. arenaria* biomass was greater when grown in disturbance plots than when grown with competition. Root-associated fungi were poor predictors of plant biomass, and plant-parasitic nematodes were infrequently observed. *A. arenaria* achieved greater belowground biomass than *A. breviligulata* when inoculated with a control filtrate, but the addition of microbes equalized the difference in belowground biomass between species. Overall, our results suggest that *A. breviligulata* invasion is driven by its tolerance to competition and positive

effects of plant-soil feedbacks. These results have important implications for other early successional systems, and contribute to our understanding of how ecological contexts influence invasion processes.

INTRODUCTION

Biological invasions can radically transform ecological communities and ecosystem processes. For instance, grasslands in California have been converted from communities dominated by native perennials to communities of invasive annuals (D'Antonio and Vitousek 1992), the understory of many Eastern USA forests has been replaced by invasive European buckthorn (*Rhamnus cathartica*) (Knight et al. 2007), and coastal dunes worldwide have been invaded by European beachgrass (*Ammophila arenaria*) introduced to stabilize dunes for development (Wiedemann and Pickart 1996). The mechanisms underlying the establishment and persistence of invasive plants play a critical role in the interactions among invading and established species and for the consequences of invasion to ecological communities and ecosystem processes. Two broad mechanisms – response to disturbance (e.g. D'Antonio and Vitousek 1992) and facilitative and/or antagonistic interactions with biota (Maron and Vila 2001, Mitchell et al. 2006, Bever et al. 2010) – are commonly evoked to explain large-scale invasions. These mechanisms are not necessarily mutually exclusive and elucidating their relative importance and the conditions under which they are important remains a challenge.

Invading species vary in their response to disturbance, and their responses have implications for the effects on the communities they invade. Here, we define 'invading species' or 'invader' as a species introduced to a given habitat that is rapidly expanding its range, and 'established species' as those native or exotic species present prior to the arrival of the invading species. Some invaders are highly disturbance-dependent relative to the established species, and the invaders' populations decline in the absence of disturbance and in the face of competition (Seabloom et al. 2003, MacDougall and Turkington 2005). Other invaders may invade established communities directly, either through utilization of resources not used by the

established species (Tilman 2004) or through interference competition (e.g. Amarasekare 2002, Hart and Marshall 2012). Disturbance-dependence may have important consequences for the invader's effect on successional communities. For instance, invaders with high disturbance-dependence might be expected to be poor competitors and have relatively short-term effects on a community, while those with low disturbance-dependence that are able to tolerate competition from established species may have greater long-term effects (Prach and Walker 2011, David et al. 2015b).

Invasion may also be mediated through biotic interactions with enemies or mutualists (Klironomos 2002, Mitchell et al. 2006, Pringle et al. 2009). For example, the enemy release hypothesis predicts that the invader is less susceptible to natural enemies in the introduced habitat than are the established species and therefore gains a competitive advantage (Maron and Vila 2001, Keane and Crawley 2002, Mitchell et al. 2006). If the established species have relatively few natural enemies, the effects of enemy release will be limited because the invader gains little competitive advantage over the established species (Beckstead and Parker 2003, Parker and Gilbert 2007). Furthermore, mutualisms with resident symbionts may benefit an invader more than they benefit the established species (Richardson et al. 2000). For instance, associations with arbuscular mycorrhizal fungi (Pringle et al. 2009) or dark septate endophytic fungi in roots (Newsham 2011) often benefit plants by increasing nutrient acquisition. If these symbiotic associations occur more frequently with the invading species than the established species, the invading species could gain a competitive advantage (Pringle et al. 2009). Finally, the long-term buildup of microbes in soil may create feedbacks that facilitate or impede invasion. Microbial effects may often be caused by the accumulation of specific pathogens or mutualists over time, but may also be attributed to the complex soil microbial communities that develop in

the presence of a given plant species (Bever et al. 2010). Thus, plant-soil feedbacks may provide a mechanism of invasion in the case where feedbacks from the established species limit the established species' growth and persistence, while facilitating that of the invading species (Klironomos 2002, Bever et al. 2010).

Coastal dunes of the USA West Coast represent a system particularly well suited for understanding invasion mechanisms because two successive invasions of congenic plant species have occurred there over the past century. In the early 1900s, the European beachgrass *Ammophila arenaria* was introduced for dune stabilization, and rapidly transformed dunes from shifting sand masses with sparse vegetation to stabilized, near-monocultures of beachgrass (Schwendiman 1977). Subsequently, in the 1930-40s, the American beachgrass *A. breviligulata* was introduced from the Eastern USA to the Southern Washington coast and, in a rarely documented instance of nearly complete regional competitive exclusion, has rapidly displaced *A. arenaria* on most of the dunes (Seabloom and Wiedemann 1994, Hacker et al. 2012, David et al. 2015b). Additionally, the *A. breviligulata* invasion has decreased plant diversity and dune height, leading to reduced coastal protection from flooding (Seabloom and Wiedemann 1994, Hacker et al. 2012, Seabloom et al. 2013). *A. breviligulata* may invade by rapidly spreading into newly deposited bare sand on the shoreside of foredunes through the production of horizontal rhizomes (Hacker et al. 2012), yet this mechanism does not explain how *A. breviligulata* also invades and displaces established stands of *A. arenaria* (David et al. 2015b). We hypothesize that *A. breviligulata* invasion into established stands could be due to its tolerance to competition from the established *A. arenaria* or due to differing biotic interactions with enemies or mutualists. The dramatic displacement caused by *A. breviligulata* in a plant community of relatively low

diversity provides a unique opportunity to study and evaluate the importance of mechanisms behind species invasions.

While support for different invasion mechanisms varies across systems, few studies (e.g. Kulmatiski et al. 2006, Maron et al. 2013) have attempted to evaluate their relative importance within a single system. For instance, in early successional habitats, disturbance could play a larger role than plant-soil feedbacks or natural enemies that may have yet to establish. Indeed, plant parasitic nematodes have been implicated in the decline of the *A. arenaria* in late successional dunes in its native range (Van der Putten et al. 1993), and in other habitats, late successional plant species have been found to associate more often with arbuscular mycorrhizal fungi than early successional species (Koziol and Bever 2015). Furthermore, the environmental variability of dunes allows for the opportunity to study how the relative importance of invasion mechanisms may vary over space. Across individual dunes, *A. breviligulata* occupies a wider distribution over space and time, maintaining higher abundances than *A. arenaria* in both young foredune habitat (defined as sand ridges adjacent to the shoreline), and older backdune habitat (defined as the area beyond the foredune further inland) (David et al. 2015b). Because the fore- and backdunes represent distinct habitats with regards to wind, salt spray, nutrients, and diversity of plants and their associated fungi (Cooper 1958, David et al. 2015b, 2015a), different mechanisms may be important to *A. breviligulata* invasion of each habitat.

Here, we used the *A. breviligulata* invasion to evaluate the support for disturbance-dependence and biotic mechanisms of invasion during primary succession. Specifically, we investigated the mechanisms by which *A. breviligulata* has invaded younger foredune and older backdune plant communities dominated by the established, non-native *A. arenaria*. First, we conducted a manipulative field experiment to examine the effects of disturbance and competition

on the performances of both *Ammophila* species and of the native *Elymus mollis* in *A. arenaria*-dominated fore- and backdunes. We characterized the plant-parasitic nematode and root-associated fungal communities in the experimental plots in order to evaluate the roles of natural enemies and mutualists on invasion. Second, we conducted a plant-soil feedback study to evaluate the role of *A. arenaria*-associated soil microbes on the performance of the two *Ammophila* species.

MATERIALS AND METHODS

Experiment 1: Competition and Disturbance Study

For this study, we transplanted tillers of each beachgrass species collected in the field to plots located in foredune or backdune habitat in which plant communities were either disturbed or left intact. Beachgrasses are dispersed onto new beaches as tillers via ocean transport and, while seed germination has been observed, these species primarily rely on rhizomatous growth for propagation (Wiedemann and Pickart 1996). For these reasons, we chose to use harvested tillers rather than grow plants from seed. Tillers of *Elymus mollis*, *Ammophila arenaria*, and *A. breviligulata* were collected in May 2012 from the Pacific City site (see details below) where all three plant species co-occurred. We collected tillers of each species from two sources within this site -- the foredune and backdune – so that pre-existing microbial symbiont communities (David et al. 2015a) were moved with the tiller. Following collection, tillers were soaked in tap water for 10 days to stimulate root growth and increase survival in the field (S. Gerrity, pers. comm.). Tillers were soaked in individual containers to avoid potential dispersal of microbes among different individuals, and were trimmed to a single stem and two leaves to standardize

aboveground size. Pre-treatment wet weight, tiller height, and rhizome length were then measured to estimate pre-treatment dry weight (Appendix 1).

Tillers were transplanted into experimental plots in the field. Each plot (0.6m diameter) contained 6 individuals (3 beachgrass species × 2 tiller sources, 216 total individuals). Experimental blocks contained four plots assigned to one of each habitat × disturbance treatment combinations. Within blocks, two adjacent foredune habitat plots were established on the shoreside slopes of the dunes, and two adjacent backdune habitat plots were established behind dunes at the edge of herbaceous dune habitat at approximately the same latitude as the foredune plots. We assigned one plot in each fore- and backdune pairing to the disturbance treatment and the other to the competition treatment. For plots assigned the disturbance treatment, we removed all above- and belowground plants material 0.6m deep, and the soil was sieved in the field using a 0.635cm screen to remove plant roots and debris. Plots assigned the competition treatment were essentially control plots and were not modified. We replicated blocks three times in each of 3 sites – Pacific City (Bob Straub State Park, Pacific City, OR, USA, 45°10 N, 123°58’ W), Sand Lake (Sand Lake Recreation Area, Cloverdale, OR, USA, 45°17’ N, 123°57’ W), and Cape Meares (Bayocean Peninsula County Park, Tillamook, OR, USA, 45°30’ N, 123°57’ W). *Ammophila arenaria* was the dominant plant species at all three sites.

We harvested the tillers after 11 wk. Approximately 30cm of root tissue from each surviving plant was collected for microscopy and culturing of microbial symbionts. For the subset of plants used for root nematode extraction (see below), we removed all remaining roots, and these roots were weighed after the extraction and included in analyses. The remaining plant material was dried at 60°C for three days, and above- and belowground biomass was recorded.

For roots used in microscopy, we washed roots in tap water and stored them in 50% ethanol until staining. We used the ink and vinegar method (Vierheilig et al. 1998) to clear and stain fungal structures. Roots were soaked in 10% KOH solution and stained using Parker Blue ink (5% ink:acetic acid). We used the line-intersect method (McGonigle et al. 1990) to record the presence of arbuscular mycorrhizae hyphae, dark septate hyphae, and microsclerotia (fungal resting structures) in 40 fields of view (200x) using a compound light microscope (Figure 3-1a-c). We measured the percent colonization of roots as the percentage of fields of view in which a given symbiont was present.

To culture fungal root endophytes, we used a modified approach described in David et al. (2015a). Roots were initially cleaned with tap water and then surface sterilized using successive rinses of 70% ethanol (1 min), 10% bleach (2 min), and 70% ethanol (1 min). Roots were cut into 20 segments (~1.5mm^2) and plated on 2% malt extract agar. We sequenced the ITS region of the rDNA of emergent fungi to assist in identification. Total DNA was extracted and the ITS-LSU region was Polymerase Chain Reaction (PCR) amplified using the Extract N' Amp kit (Sigma-Aldrich, Saint Louis, MO, USA) with primers ITS1F (Gardes and Bruns 1993) and LR3 (Vilgalys and Hester 1990). PCR conditions were as follows: 35 cycles of 95°C for 30s, 54°C for 30s, 72°C for 1min. Amplicons were sequenced using Sanger sequencing at Beckman Coulter, Inc. (Brea, CA, USA). We edited sequences using Geneious (Drummond et al. 2011), and clustered the sequences into operational taxonomic units (OTUs) at 97% similarity using the workflow developed by Monacell and Carbone (2014). We used BLASTn to identify OTUs using the UNITE database (Abarenkov et al. 2010). Our previous work showed that the fungus *Microdochium bolleyi* is the most commonly occurring root endophyte in these beachgrasses (David et al. 2015a), and we consider this fungus in our analyses (Figure 3-1d).

Nematodes were surveyed in soil and in roots. To survey ectoparasitic nematodes residing in the soil, we used the Baermann funnel method for a subset of plots. Soil from outside each pair of disturbance and competition plots was first passed through a 0.635cm mesh screen to remove plant roots and debris in the field. In the laboratory, we extracted nematodes from a total of 200g of soil for each sample over the course of 5 days. To survey endoparasitic nematodes residing within roots, we placed roots in a mist chamber for 7 days and collected nematodes that emerged from a subset of 32 plants in the Sand Lake site. For both protocols, we counted all plant parasitic nematodes in the sample and identified to genus when possible.

All analyses were conducted using R version 3.2.3 (R Core Team 2015), the lme4 package (Bates et al. 2013) and the MuMIn package (Barton 2014). We analyzed plant responses to experimental factors using separate mixed-effects models for each plant species and analyzed log-transformed total biomass, aboveground biomass, and belowground biomass as response variables. We modeled response variables as a function of the log-transformed pre-treatment dry weight, source of the individual, the presence or absence of AMF, dark septate hyphae, microsclerotia, and *Microdochium bolleyi*, disturbance treatment, habitat, and the two-way interactions between habitat, disturbance treatment, and the presence of each symbiont. We used plot, plot pairings, block, and site as nested random effects. Using the dredge() function in the MuMIn package (Barton 2014), we identified and averaged all models within 4 AIC units of the best model. We report the conditional model-averaged coefficients and the importance values (weighted percentage of models within 4 AIC units in which the term appears).

Next, we analyzed responses of the fungal symbionts to the experimental treatments. We analyzed the presence of each symbiont associating with the three host plants by constructing generalized linear models with a binomial error structure using analysis of deviance and Tukey

post-hoc tests to determine significance. We analyzed the effects of the disturbance treatment, habitat, and their interaction on the presence and colonization percentage (when the symbiont was present) using generalized mixed-effects models with binomial error with random variables of host species and plot nested within block nested within site. We included host species as a random effect in these models in order to generalize across host species. Percent colonization of roots was based on observations of presence or absence of symbionts in 40 microscope fields of view (AMF, dark septate hyphae, and microsclerotia) or in 20 sterilized tissue segments (*M. bolleyi*). Significance was determined by removing terms from the model and comparing models using analysis of variance.

Ectoparasitic nematode abundance in soil was analyzed using a generalized linear mixed-effects model with Poisson error using block nested within site as a random effect and habitat as a fixed effect. Endoparasitic nematodes in roots were too infrequent to formally analyze due to lack of statistical power, and we instead report the observed count data.

Experiment 2: Plant-Soil Feedback Study

To investigate the influence of *A. arenaria* soil microbial communities on the growth of the two *Ammophila* species, we conducted a growth chamber experiment in which plants were grown in soil inoculated with filtrates representative of the soil microbial community. We inoculated pots with one of three filtrate treatments – a control (de-ionized (DI) water) and filtrates representative of the foredune or backdune microbial communities. To make the experimental filtrates, we collected soil from the fore- and backdunes at the Pacific City site and passed the soil through a 0.635cm mesh screen. In the laboratory, we mixed 18.2 kg of soil with 5L of DI water and let the mixture soak for 4 hours. The liquid filtrate was poured off and passed through successive screens of 250 µm, 63 µm, and 38µm mesh to remove plant debris,

nematodes, and sand particles. The filtrates contained negligible differences in total dissolved nitrogen, and different fungal and bacterial taxa, suggesting that any effects caused by the filtrates could be attributed to the microbes present and not to differences in nutrient content (Appendix 2).

To evaluate the effects of the soil filtrates on *Ammophila arenaria* and *A. breviligulata* growth, we collected and treated tillers from the fore- and backdune habitats as described above for Experiment 1. Tillers were potted in 25.2cm depth cone-tainers (Steuwe and Sons, Tangent, OR, USA) in a 50:50 mix of Nurserymen's Preferred Playsand (TCC Materials, Mendota Heights, MN, USA) and autoclaved Sunshine Professional Growing Mix potting mix (Sun Gro Horticulture, Agawam, MA, USA). Individual plants were randomly assigned to one of two blocks and one soil filtrate treatment, to give a total of 3 replicates for each species × source × soil filtrate combination in each block. Beginning one day after planting, plants were inoculated with 50mL of their assigned soil filtrate for three days. After 16wk, plants were harvested and separated into belowground, live aboveground, and dead aboveground tissue, dried at 60°, and weighed. We characterized fungal symbionts using the culturing and microscopy techniques described for Experiment 1.

We analyzed the effect of the soil filtrate on plant performance using linear models with two independent contrasts. The first contrast tested for the overall effect of microbes on the plants by comparing the fore- and backdune filtrates versus the control. The second contrast tested for differences between the fore- and backdune filtrates. We also included block, log-transformed pre-treatment dry weight, source of the tiller, plant species, and the interaction between plant species and filtrate contrasts in the model. We performed F-tests to test for overall

treatment differences. We considered the response variables of log-transformed live aboveground biomass, belowground biomass, root:shoot ratio, and total biomass.

RESULTS

Experiment 1: Competition and Disturbance Study

We found that *Ammophila arenaria* grew better in disturbance plots than in competition plots, while *A. breviligulata* and *E. mollis* grew equally well in both treatments. *Ammophila arenaria* had 23% less total biomass and 34% less aboveground biomass (conditional model-averaged coefficients) in the competition treatment compared with the disturbance treatment (Table 3-1; Figure 3-2). In contrast, neither *Elymus mollis* nor *A. breviligulata* showed significant differences between disturbance and competition treatments (Table 3-1; Figure 3-2). *A. arenaria* had 46% less belowground biomass in the backdune compared with the foredune, and neither *A. breviligulata* nor *E. mollis* showed differences in biomass between backdunes and foredunes (Table 3-1). We found no significant interactions between the competition treatment and habitat for any of the three beachgrass species (Table 3-1), indicating that there were no differences in the plant species' responses to disturbance between the early-successional foredune and the late-successional backdune habitats. The source of the tiller influenced final biomass for *E. mollis* and *A. arenaria*. The *E. mollis* tillers from the backdune source had 24% greater total biomass and 28% greater aboveground biomass than foredune source tillers (Table 3-1). *A. arenaria* tillers from the backdune source had 61% lower belowground biomass compared to those from the foredune source (Table 3-1).

The presence of the four symbionts (arbuscular mycorrhizal fungi, dark septate endophytes, microsclerotia structures, and *Microdochium bolleyi*) were not significant predictors

of biomass for any of the three beachgrass species. *E. mollis* aboveground biomass was 29% less when AMF were present, but this result had a low importance value and was only marginally significant (Table 3-1). Belowground *A. breviligulata* biomass in backdunes was negatively associated with the presence of AMF and the endophyte *M. bolleyi*, and positively associated with dark septate hyphae, though these results also had low importance values and were only marginally significant (Table 3-1).

Arbuscular mycorrhizal fungi occurred more frequently in *E. mollis* (present in 74.5% of individuals) than either of the *Ammophila* species (46.8% of *A. arenaria*, 41.5% of *A. breviligulata*) (d.f. = 2, deviance =215.6, $p < 0.001$; Figure 3-3), but did not significantly differ among experimental treatments ($p > 0.05$ for all terms). AMF percent colonization (fraction of microscope fields of view that the symbiont was present) was 64.5% greater in individuals from the competition plots than in those from disturbance plots (Figure 3-3), yet percent colonization was generally low, averaging 11.1% ± 1.1 S.E. of root tissue on individuals where AMF was present. Dark septate hyphae were observed in 22.7% of individuals, though their presence did not differ among plant species (d.f. = 2, deviance = 1.03, $p = 0.310$; Figure 3-3) nor among experimental treatments ($p>0.05$ for all terms). When present, percent colonization by dark septate hyphae was low (7.2% ± 1.6 of root tissue) and did not differ among experimental treatments (Figure 3-3). The presence of microsclerotia differed among plant species (d.f. = 2, deviance = 6.386, $p = 0.041$), occurring in 32.3% of *A. breviligulata* individuals, 20.0% of *E. mollis* individuals, and 12.8% of *A. arenaria* individuals (Figure 3-3). Microsclerotia presence did not differ among experimental treatment groups ($p>0.05$ for all model terms). Percent colonization of microsclerotia when present averaged 9.5% ± 1.2 of root tissue, and decreased by 48.4% in the competition treatment compared with the disturbance treatment (Figure 3-3).

Microdochium bolleyi was isolated from 31.1% of individuals, and its presence did not significantly differ among plant species (d.f. = 2, deviance = 3.13, p = 0.210; Figure 3-3) nor among experimental treatment groups (p>0.05 for all model terms). *M. bolleyi* percent colonization (percentage of surface-sterilized root segments in which the fungus emerged in culture) when present averaged 20.2% ± 0.02 and did not differ among treatments (Figure 3-3).

Plant parasitic nematode abundance in soils and roots was generally low, and the genera included *Helicotylenchus*, *Pratylenchus*, and *Paratylenchus.* Ectoparasitic nematodes in soil were more abundant in backdunes (mean 11.6 ± 3.7 S.E.M. per 200g soil) than in foredunes (mean 2.67 ± 0.90 S.E.M. per 200g soil) (generalized linear mixed-model p < 0.001). Endoparasitic nematodes in roots were also rare (0-3 per sample), although we did find two infestations (1,184 and 57 endoparasitic nematodes, respectively) that we were unable to identify to genus among the nine *E. mollis* root samples surveyed.

Plant-Soil Feedback Experiment

The microbial filtrates increased live aboveground biomass by 7.6% in *A. arenaria* and by 9.5% in *A. breviligulata* relative to the control filtrate, though there were no significant differences between the fore- and backdune filtrates or between *Ammophila* species responses to the filtrate (Table 3-2; Figure 3-4a). The microbial filtrates did differentially affect belowground biomass in the two *Ammophila* species by increasing belowground biomass by 15.5% in *A. breviligulata* relative to the control filtrate, and non-significantly decreasing belowground biomass in *A. arenaria* by 8.1%. No differences were found between fore- and backdune filtrates on belowground biomass (Table 3-2; Figure 3-4b). Similarly, the microbial filtrates also increased the root:shoot ratio of *A. breviligulata* by 9.8% relative to the control filtrate indicating greater belowground investment, and marginally decreased root:shoot ratio in *A. arenaria* by

9.7% (Table 3-2; Figure 3-4c). Finally, there were no significant effects of the filtrate treatments on total biomass of either species (Table 3-2; Figure 3-4d); however the microbial filtrates increased total *A. breviligulata* biomass by 11% relative to the control, and this effect was marginally significant (Table 3-2). Presence of AMF (7.0% of individuals), dark septate endophytes (4.2%), microsclerotia (2.8%), and M. bolleyi (20%) were generally low.

DISCUSSION

In this study we evaluated the roles of disturbance-dependence and biotic mechanisms for the invasion of the Eastern USA beachgrass *Ammophila breviligulata* into primary successional dunes along the USA Pacific Northwest coast. Our results suggest that the invading *A. breviligulata,* compared with the established European beachgrass *A. arenaria,* is less dependent on disturbance for growth and possesses a greater tolerance to competition from established stands of *A. arenaria*. Additionally, plant-soil feedbacks increase belowground growth of *A. breviligulat*a while slightly decreasing belowground growth of the *A. arenaria*. We found little evidence that escape from natural enemies or increased associations with potentially mutualistic fungi play a role in the invasion. *E. mollis* had infestations of nematodes and a higher likelihood of colonization by arbuscular mycorrhizal fungi than did either *Ammophila* species, but there were no differences between *A. arenaria* and *A. breviligulata* in either case. Importantly, we found little evidence that mechanisms of invasion differ between the early-successional foredune and late-successional backdune. Overall, our findings contribute to a broader understanding of how ecological context influences the mechanisms driving biological invasions.

Our results experimentally confirm previous observations that *A. breviligulata* is capable of invading established *A. arenaria* stands, an invasion that has resulted in the decline of native

species diversity and abundance (Hacker et al. 2012, David et al. 2015b). The invading *A. breviligulata* tolerated competition better than the established *A. arenaria*, as evidenced by the former's equal biomass in competition and disturbance plots and the latter's reduced biomass in the competition plots. Importantly, this result implicates *A. breviligulata* as a 'driver' of change in this system, and not solely a 'passenger' that capitalizes on disturbance (sensu MacDougall and Turkington 2005). Greater competitive ability allows a species to invade intact communities and reduce the abundance of established species. For instance, European buckthorn (*Rhamnus cathartica*) grows vigorously in both sun and in shade, allowing it to invade many habitat types in the Eastern USA (Knight et al. 2007). In contrast, invasive species with stronger disturbance-dependence may have relatively less impact on native species despite their community dominance, especially if the native species are limited by other factors such as dispersal (Seabloom et al. 2003, MacDougall and Turkington 2005). While our study did not identify the resource (e.g. light, nitrogen, phosphorous) for which *A. breviligulata* better competes, we suspect that competition for light may play an important role. In primary successional systems, early colonizers such as beachgrass are adapted to low nutrient conditions, but often are shaded out by later successional species (Connell and Slatyer 1977, Chapin et al. 1994).

We also found support for plant-soil feedbacks favoring invasion success. Invasive species are generally thought to exhibit less negative or possible positive feedbacks compared with native species, and these feedbacks may be an underlying cause of invasive species' high abundances (Klironomos 2002). The microbial filtrates in the plant-soil feedback study had the effect of equalizing belowground biomass of the two *Ammophila* species compared to the control filtrate, though this effect was not found for aboveground biomass and was marginally significant for total biomass. We suggest that the species' differential responses to microbes

associated with *A. arenaria* soil could mediate competitive interactions between the two *Ammophila* species. Plant-soil feedbacks allow for both stabilizing mechanisms (increased intraspecific competition relative to interspecific competition) and equalizing mechanisms (minimized fitness difference between species) for coexistence (Adler et al. 2007, Bever et al. 2010). Our results suggest microbes associated with the established *A. arenaria* could allow *A. breviligulata* to initially invade *A. arenaria* stands as a fitness-equalizing mechanism for belowground growth. Once established, *A. breviligulata* could outcompete *A. arenaria* for light and belowground resources, resulting in the displacement of *A. arenaria* (David et al. 2015b).

We found little support that *A. breviligulata* invasion is mediated by plant-pathogenic nematodes. Our findings of low nematode abundances match those of Beckstead and Parker (2003), who reported low abundances of nematodes in coastal dunes of California south of our study system. Furthermore, in a global study of nematodes associated with *A. arenaria*, Van der Putten et al. (2005) showed *A. arenaria* in Oregon harbored a similar diversity of nematode taxa as did neighboring native grasses. From these studies and our present results, it appears unlikely that plant-pathogenic nematodes mediate invasion of either *Ammophila* species. While pathogens are not apparently important in this system, they can influence invasion success (Keane and Crawley 2002, Mitchell et al. 2006). Two recent meta-analyses have begun to reveal the ecological contexts in which pathogens play important roles in invasions. Blumenthal et al. (2009) found that the effect of pathogen release on invasion is low in low nitrogen systems such as our dune system. Heger and Jeschke (2014) found that most support for the enemy-release hypothesis came from comparisons between success of a single species in its native and invaded regions (74.6% of studies supported enemy-release hypothesis), and that comparisons between invasive and native species in invaded regions (29.2%) or between invasive and non-invasive

species in invaded regions (21.8%) supported the enemy-release hypothesis far less frequently. Our results, therefore, follow what these meta-analyses might predict: a comparison among invasive and native species in a low nutrient invaded system should fail to support the enemy-release hypothesis. The USA Pacific Northwest dunes contain low abundances of plant-parasitic nematodes, and therefore nematodes likely do not mediate interactions among plant species.

Similarly, mutualists, and arbuscular mycorrhizal fungi in particular, are often implicated for their roles in plant invasion, yet our findings do not support such a role in *A. breviligulata* invasion. Pringle et al. (2009) identified several mechanisms by which AMF could mediate plant invasion, and our findings reject several of these potential mechanisms. First, non-mycorrhizal invasive species can disrupt mycorrhizal networks, and thereby gain a competitive advantage (e.g. Stinson et al. 2006). However, the invading *A. breviligulata*, like the established *A. arenaria* and native *E. mollis*, appears to be, from our study and others (Koske and Gemma 1997, Emery and Rudgers 2011), facultatively mycorrhizal. The ability for *A. breviligulata* to disrupt AMF networks seems unlikely, particularly when compared with the ability of other factors such as local disturbance that significantly reduce AMF colonization as our findings show. Second, the invading species might be more or less responsive to AMF than the established species, thereby providing an opportunity for feedbacks between plant and fungus to eventually favor invasion. Yet, we found that plant biomass of any of the three grass species was not significantly associated with AMF infection, suggesting little opportunity for such feedbacks to contribute to invasion. Percent colonization on the roots was low, though within the range reported in previous studies of *Ammophila* (Koske and Gemma 1997, Emery and Rudgers 2011). Such low colonization may suggest that AMF in our system are unlikely mediators of plant invasion. Nevertheless, if plant species associate with or respond uniquely to different AMF species, this

could suggest a more complex role for AMF in mediating plant invasion (Richardson et al. 2000, Klironomos 2003, Pringle et al. 2009). Though more work on the potential benefits provided or harm caused by AMF species to their beachgrass host species is needed, it seems unlikely that AMF could mediate *A. breviligulata* invasion in dunes based on our present results.

Furthermore, we found little evidence that fungal endophytes play a role in invasion. We found no significant associations between dark septate hyphae, microsclerotia, or *Microdochium bolleyi* on plant biomass. It is possible that other symbionts that were not measured could mediate invasion. For instance, nitrogen-fixing bacteria (*Burkholderia spp*.) that are found on beachgrass roots can provide nitrogen to plant species found in our study region (Dalton et al. 2004). In other dune systems, aboveground fungal endophytes such as *Fusarium culmorum* and *Epichloë* can also provide benefits such as tolerance to salt stress (Rodriguez et al. 2008) or drought (Emery et al. 2010), respectively, yet our previous work showed that these particular endophyte taxa were not present in our system (David et al. 2015a). Our results do suggest further investigation into the potential role of root endophytes producing microsclerotia on plant fitness, as microsclerotia were found most frequently in roots of *A. breviligulata* compared with the other beachgrass species. Dark septate endophytes, which can produce microsclerotia, are known to provide mutualistic benefits to their hosts, but their effects widely vary across host species or genotypes and endophyte strain (Newsham 2011, Mandyam et al. 2013).

Biological invasions may occur via several mechanisms, and determining the ecological contexts in which different mechanisms are important is critical to our understanding of the invasion process. We have shown that the invasion of *A. breviligulata* into USA Pacific Northwest dunes is likely a result of that species' greater tolerance to competition compared to the established *A. arenaria* that it displaces. Plant-soil feedbacks on *A. arenaria* soil equalize

competition for belowground resources between the two species, perhaps initially allowing *A. breviligulata* to coexist with *A. arenaria* before overtaking it. We found little evidence that soil pathogens or mutualists mediate the invasion. Disentangling the many hypothesized mechanisms for invasion is essential for determining the underlying causes and future consequences of invasion and informing management decisions.

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Term	Total Biomass						Aboveground Biomass						Belowground Biomass					
	ELMO		AMAR		AMBR		ELMO		AMAR		AMBR		ELMO		AMAR		AMBR	
	Impt	Est	Impt	Est	Impt	Est	Impt	Est	Impt	Est	Impt	Est	Impt	Est	Impt	Est.	Impt	Est
(Intercept)		0.27 (0.35)		0.22 (0.24)		-0.15 (0.17)		-0.06 (0.43)		-0.36 (0.30)		-0.37 (0.22)		-1.09 (0.38) **		-0.59 (0.48)		-1.60 (0.34) ***
Pre-trt Biomass (ln-transformed)	1	0.73 (0.15) ***	1	0.50 (0.17) **	1	0.68 (0.12) ***	1	0.79 (0.18) ***	1	0.65 (0.20) **	1	0.63 (0.16) ***	1	0.55 (0.20) *	0.7	0.55 (0.26) +	1	0.76 (0.22) ***
Source (back vs. front)	0.83	0.24 (0.11) *	0.07	-0.06 (0.11)	0.14	0.05 (0.09)	0.79	0.28 (0.13) *	0.21	0.17 (0.13)	0.15	0.10 (0.12)	0.07	-0.01 (0.15)	1	-0.61 (0.16) ***	0.89	-0.35 (0.15) *
AMF Presence	0.51	-0.22 (0.13)	0.19	-0.14 (0.12)	0.22	-0.10 (0.09)	0.59	-0.29 (0.15) +	0.08	-0.03 (0.14)	0.29	-0.15 (0.12)	0.13	0.10 (0.18)	0.26	-0.25 (0.19)	0.23	-0.00 (0.23)
Dark Septate Hyphae Presence	0.38	-0.17 (0.12)	0.06	0.04 (0.14)	0.17	-0.09 (0.12)	0.37	-0.20 (0.14)	0.09	0.07 (0.16)	0.17	-0.12 (0.15)	0.13	-0.10 (0.16)	0.06	0.01 (0.21)	0.14	0.07 (0.23)
Microsclerotia Presence	0.1	-0.00 (0.14)	0.07	0.05 (0.16)	0.27	0.11 (0.10)	0.11	-0.00 (0.16)	0.08	0.01 (0.19)	0.28	0.14 (0.12)	0.07	0.02 (0.18)	0.19	0.18 (0.30)	0.08	0.03 (0.16)
***M. bolleyi* Presence**	0.1	0.05 (0.13)	0.06	-0.00 (0.12)	0.08	-0.05 (0.10)	0.11	-0.04 (0.15)	0.08	-0.04 (0.14)	0.16	-0.11 (0.13)	0.08	0.06 (0.18)	0.19	0.20 (0.18)	0.12	0.10 (0.20)
Effect of Disturbance Trt (Comp vs. Disturbance.)	0.14	-0.08 (0.11)	0.8	-0.23 (0.10) *	0.05	-0.00 (0.10)	0.14	-0.10 (0.13)	1	-0.34 (0.12) *	0.09	0.03 (0.12)	0.25	-0.19 (0.15)	0.06	-0.00 (0.17)	0.31	-0.20 (0.15)
Effect of Habitat (Back vs. Fore)	0.12	0.03 (0.11)	0.17	-0.12 (0.10)	0.21	-0.09 (0.10)	0.11	0.04 (0.13)	0.08	0.02 (0.12)	0.1	-0.07 (0.12)	0.07	-0.03 (0.15)	0.93	-0.46 (0.18) *	0.45	-0.13 (0.19)
Competition × Habitat	0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.00	
AMF Presence × Habitat	0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.12	-0.63 (0.32) +
Dark Septate Hyphae Presence × Habitat	0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.03	0.73 (0.37) +
Microsclerotia Presence × Habitat	0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.03	0.63 (0.51)	0.00	
***M. bolleyi* Presence × Habitat**	0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.00		0.03	-0.57 (0.30) +

Table 3-1 Results from linear mixed-effects models and model averaging with the *dredge()* function in the MuMIn R package.

Best models (within 4 AIC units) were averaged, and importance values and estimates (standard errors) for predictor terms for each species are shown. Importance values were calculated as the weighted proportion of the number of models in which the term appeared. Estimates are only shown for terms that appeared in the best models. All response variables were natural-log transformed. + denotes $p<0.1$, * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$.

		Live Aboveground Biomass		Belowground Biomass		Root:Shoot Ratio		Total Biomass	
Term	Df	Estimate	F	Estimate	F	Estimate	F	Estimate	F
(Intercept)		-0.355+	NA	0.155	NA	-0.203	NA	0.617**	NA
Block	1	0.041	1.547	-0.084	0.038	0.002	0.011	-0.019	0.272
Pre-trt Mass (ln-transformed)	1	0.863***	47.578***	0.601**	31.733***	-0.069	1.455	0.751***	63.35***
Source (back source vs. fore source)	1	0.041	0.212	-0.358**	8.103**	-0.334**	7.268**	-0.149	2.188
Species (AMBR vs. AMAR)	1	-0.330***	11.973***	-0.342**	9.066**	-0.296**	7.048*	-0.356***	17.767***
Filtrate (Microbes vs. Control)	2	0.076+	4.293*	-0.081	0.501	-0.097+	0.295	-0.001	1.666
Filtrate (Back vs. Fore)		0.103		0.004		-0.012		0.077	
Species × Filtrate (Microbes vs. Control)	2	0.019	0.168	0.236**	4.589*	0.195*	3.242*	0.111+	2.284
Species × Filtrate (Back vs. Fore)		-0.055		-0.079		-0.075		-0.097	
Residuals	60								

Table 3-2 Results from linear models of plant-soil feedback study

Results from linear models of plant-soil feedback study. All biomass data were natural-log transformed. + denotes p<0.1, * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001

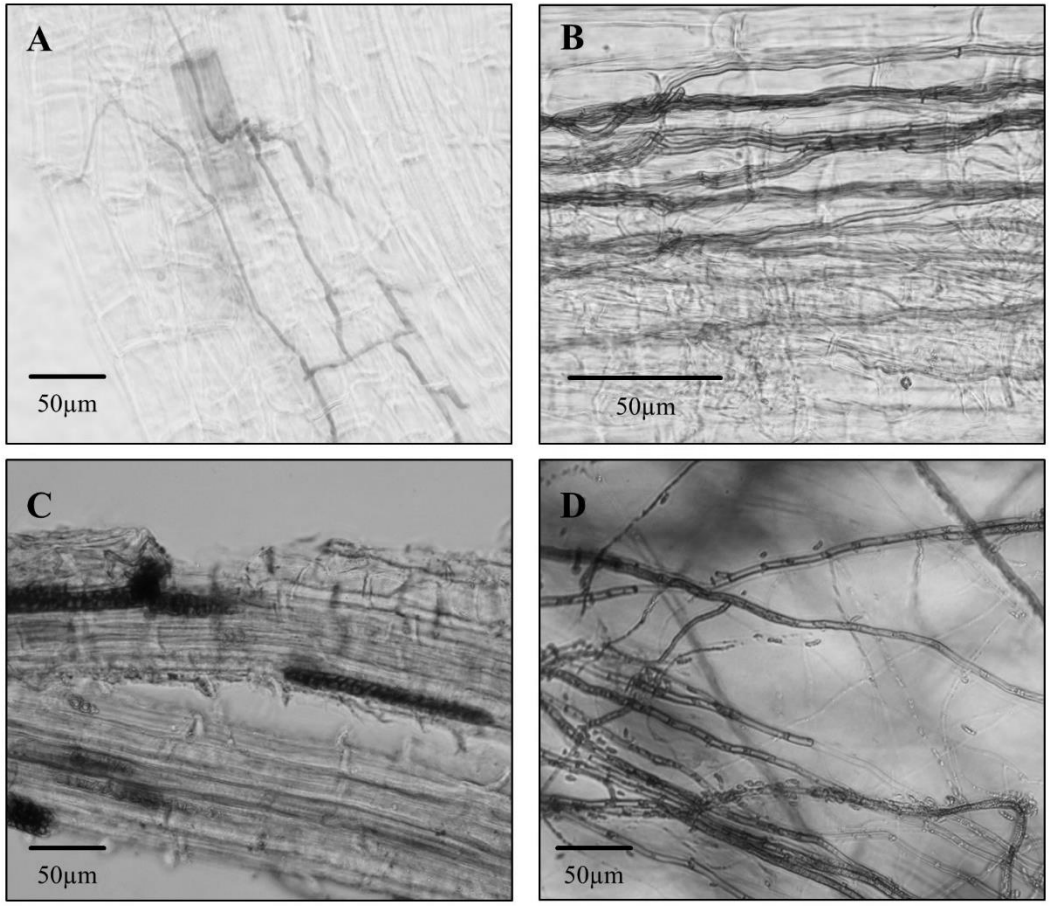

Figure 3-1 Images of common fungal root symbionts.
(A) Arbuscular mycorrhizal fungi growing with *Ammophila arenaria* root stained with blue Parker ink (200x), (B) dark septate hyphae growing within *A. breviligulata* root (400x), (C) microsclerotia growing within *A. breviligulata* root (200x), and (D) *Microdochium bolleyi* hyphae and conidia when grown in 2% malt extract agar media (200x). Photo credit: A. S. David.

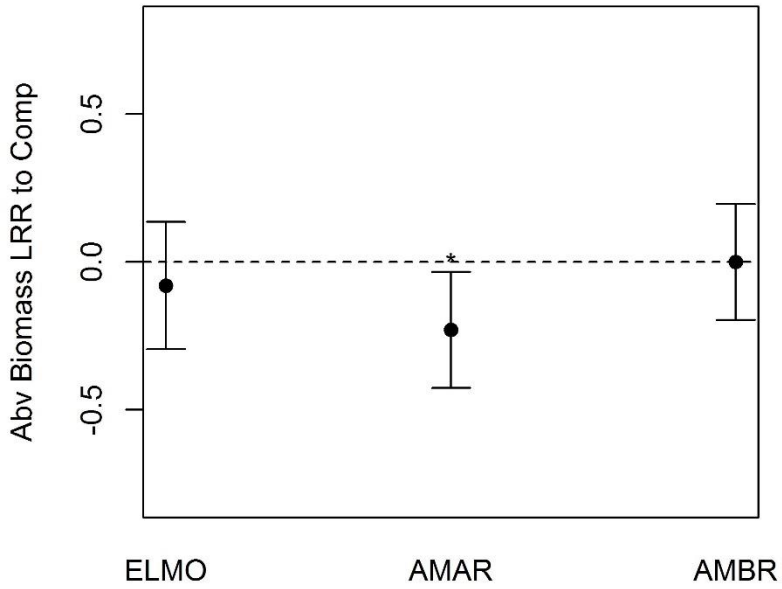

Figure 3-2 Log-ratio responses of total biomass to competition treatment in the field experiment.
Positive responses for *Elymus mollis* (ELMO), *Ammophila arenaria* (AMAR) and *A. breviligulata* (AMBR) signify the plants produced more biomass in the competition treatment, negative responses indicate more in the disturbance treatment. Error bars show 95% confidence interval. * denotes $p<0.05$.

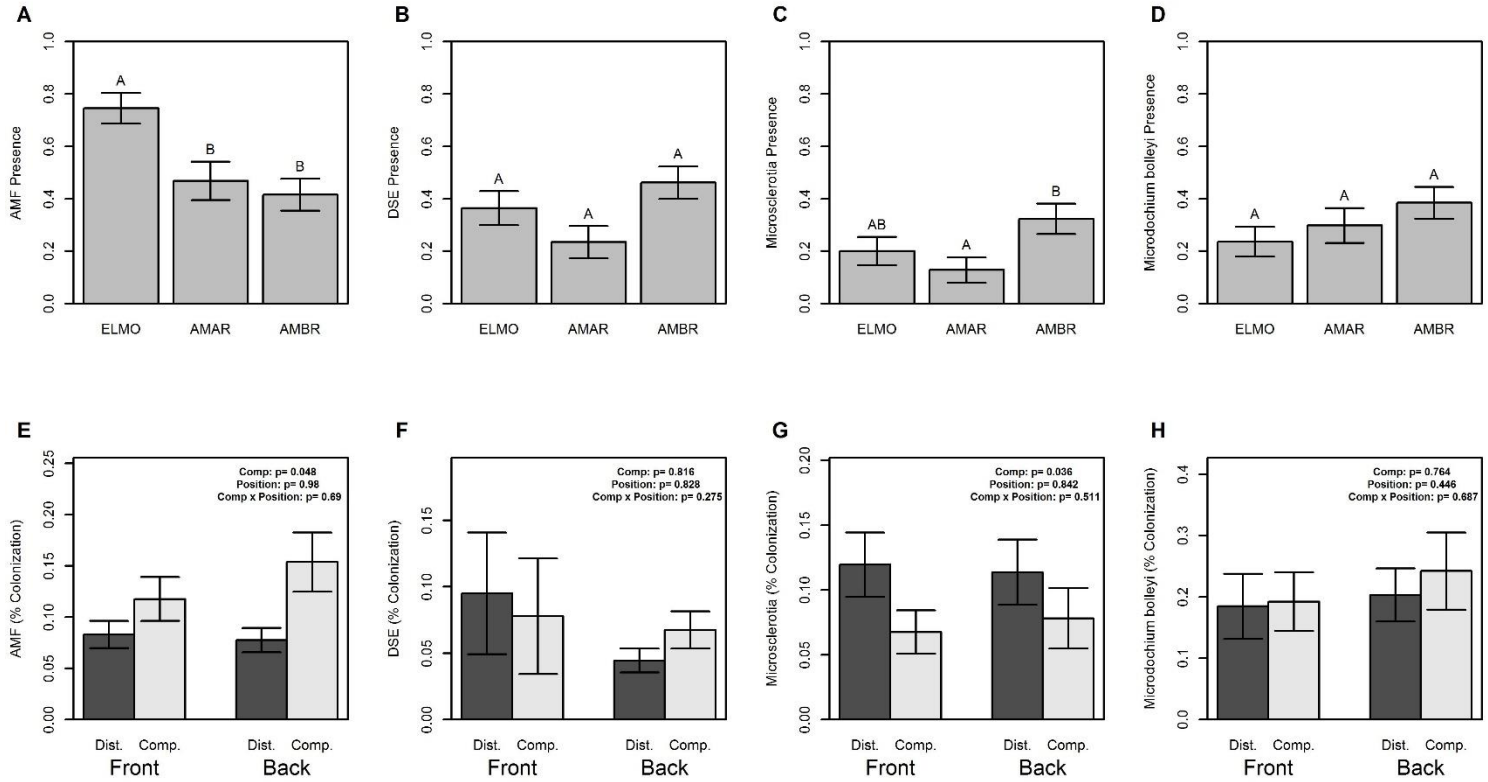

Figure 3-3 Responses of symbionts in the field experiment.
Panels A-D show the presence/absence ±1 binomial S.E.M. of arbuscular mycorrhizal fungi, dark septate endophytes, microsclerotia, and *Microdochium bolleyi* in *Elymus mollis* (ELMO), *Ammophila arenaria* (AMAR) and *A. breviligulata* (AMBR). Panels E-H show the percent colonization ± 1 S.E.M. of the same symbionts when present (plants without the given symbiont were excluded from analyses) in each disturbance treatment (disturbance or competition) × habitat (fore- or backdune) combination

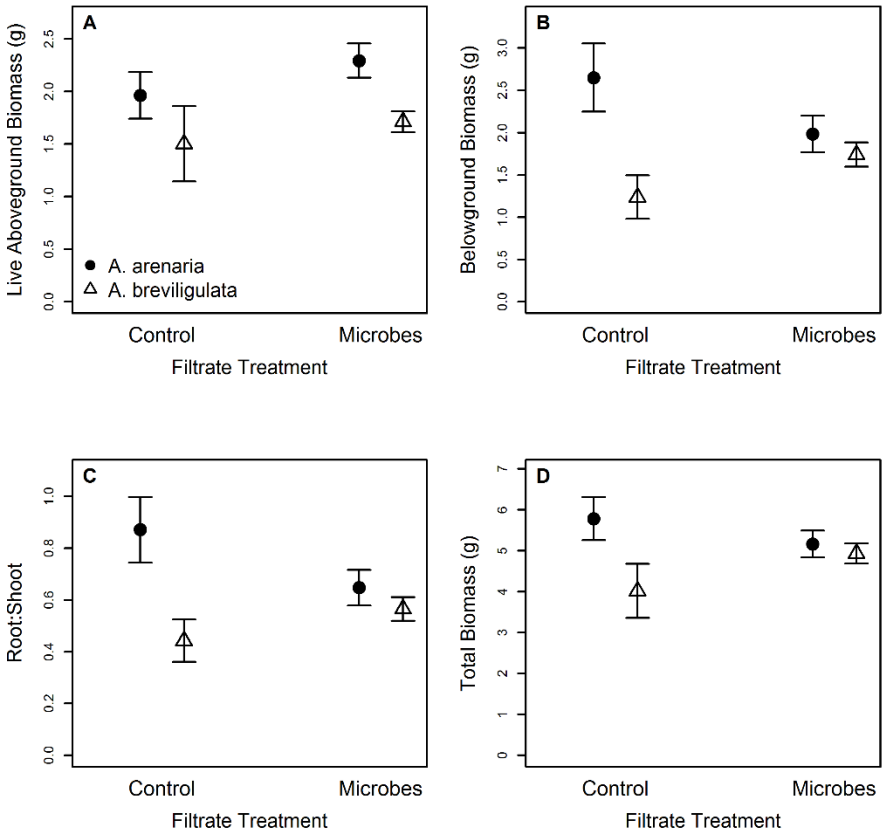

Figure 3-4 Results from growth chamber experiment.
A) Aboveground biomass, B) belowground biomass, C) root:shoot ratio, and D) total biomass. Plots show the effect of microbes (foredune and backdune filtrates) compared with the control filtrate. Points show mean ± 1 S.E.M. See Table 2 for details of statistical tests.

Chapter 4 Quantifying the associations between fungal endophytes and biocontrol-induced herbivory of invasive purple loosestrife (*Lythrum salicaria* L.)

David AS, Quiram GL, Sirota J, Seabloom EW. 2016. Quantifying the associations between fungal endophytes and biocontrol-induced herbivory of invasive purple loosestrife (Lythrum salicaria L.). Mycologia. doi: 10.3852/15-207.

ABSTRACT

Fungal endophytes are one of several groups of heterotrophic organisms that associate with living plants. The net effects of these groups of organisms on each other and ultimately on their host plants depend in part on how they facilitate or antagonize one another. In this study, we quantified the associations between endophyte communities and herbivory induced by biological-control in the invasive *Lythrum salicaria* at various spatial scales using a culture-based approach. We found positive associations between herbivory damage and endophyte isolation frequency and richness at the site-level, and weak, positive associations at the leaf-level. Herbivory damage was more strongly influenced by processes at the site-level than were endophyte isolation frequency and community structure, which were influenced by processes at the plant- and leaf-levels. Furthermore, endophytic taxa found in low herbivory sites were nested subsets of those taxa found at high herbivory sites. Our findings suggest that endophyte communities of *L. salicaria* are associated with, and potentially facilitated by, biocontrol-induced herbivory. Quantifying the associations between heterotrophic groups may ultimately lead to a clearer understanding of their complex interactions with plants.

INTRODUCTION

Fungal endophytes, a diverse group of organisms found living within tissues of all major plant lineages, are one of several groups of heterotrophic taxa that utilize living plants for resources or reproduction (Petrini 1991, Bennett 2013, Borer et al. 2013). Endophytes may interact with other heterotrophic groups such as mycorrhizal fungi, pathogens, or herbivores, and these interactions may ultimately scale up to community or ecosystem-wide effects (Van der Putten et al. 2001, Wardle et al. 2004, Bennett 2013, Busby et al. 2015). In particular, herbivores that consume plant material may interact positively or negatively with endophytes, and the net effects of these interactions have important consequences for herbivore and endophyte communities, as well as for the host plant (e.g. Van der Putten et al. 2001; De Vos et al. 2006; de la Peña et al. 2006; Rudgers and Clay 2008; Humphrey et al. 2014).

Positive or negative associations between endophytes and herbivores observed in natural settings may be suggestive of facilitation or antagonism caused by one or more direct or indirect mechanisms. For instance, facilitation between herbivores and endophytes could arise if a plant is unable to defend against both, as has been recently reported between herbivores and bacteria inhabiting leaf surface (Humphrey et al. 2014). Herbivores may facilitate the entry of endophytes that require natural openings or wounds to colonize (Petrini 1991, Hatcher 1995, Quadt-Hallmann 1997), or may contribute to the dispersal of endophytes among host individuals (Devarajan and Suryanarayanan 2006). Antagonism of herbivores by endophytes is well-documented, and is often attributed to the production of secondary metabolites (Albrectsen et al. 2010, Van Bael et al. 2012, Yang et al. 2012). For example, clavicipitaceous endophytes (e.g. *Epichlöe*) can alter herbivore communities and reduce herbivory through production of alkaloids

(Schardl et al. 2004, Rudgers and Clay 2008). Conversely, herbivory can also induce plant defenses that reduce endophytes' abilities to infect plants (De Vos et al. 2006).

The spatiotemporal scale at which endophyte-herbivore interactions are considered may also be important for determining the strength and direction of the interaction. At the leaf-level, facilitation or antagonism could arise via direct contact between endophyte and herbivores (Schardl et al. 2004, De Vos et al. 2006, Rudgers and Clay 2008). At the plant-level, either might trigger host defenses that indirectly facilitate or antagonize the other (Humphrey et al. 2014). Herbivores and endophytes may also easily disperse between closely-spaced leaves of the same individual plant, and therefore individual plants could exhibit some degree of homogeneity with regards to herbivory and endophyte community (Grünig et al. 2002, Gripenberg and Roslin 2005, Barber and Marquis 2011, Cordier et al. 2012). At the site-level, metapopulations or metacommunities of herbivores and endophytes may indicate habitat or climate suitability (Tack et al. 2010, Blaalid et al. 2014, Higgins et al. 2014). Finally, sites experiencing similar histories with respect to herbivory might contain similar endophyte taxa if local populations of these taxa have increased through time.

In natural settings, the strength and direction of endophyte-herbivore interactions may leave their imprint on the direction of their correlation and spatial structure (*sensu* McIntire and Fajardo 2009). By observing patterns between endophytes and herbivores, we may find evidence of facilitation or antagonism between these groups. Moreover, endophyte communities – often structured by environmental conditions or host preference (Arnold 2007, Saunders et al. 2010, Zimmerman and Vitousek 2012, David et al. 2015) – could be structured in the context of herbivory. We considered two ways in which this effect might be inferred using observational data. First, endophyte communities may exhibit patterns of non-randomness across leaves,

individual plants, or sites. Non-randomness patterns could suggest facilitative or antagonistic interactions among endophyte species (e.g. Pan and May 2009), or a common response to an external factor such as herbivory. Second, changes in endophyte community structure along a gradient of herbivory damage could arise either from the addition of new species to the existing community or from a compositional turnover in species. It is therefore useful to evaluate whether endophyte communities within the context of herbivory are nested – that is, the extent to which the taxa inhabiting sites (or individual plants or leaves) of lower endophyte richness are comprised of subsets of the taxa inhabiting sites of higher endophyte richness (Almeida-Neto and Guimaraes 2008). For instance, if herbivory were positively associated with diversity of endophytes, are the taxa found in leaves of low herbivory (and hence leaves of low endophyte diversity) distinct from those found in leaves of high herbivory (and high endophyte diversity), or are the taxa of the former a subset of the latter? A more complete compositional turnover between leaves exhibiting low versus high herbivory might suggest systemic effects of herbivores on endophyte communities. In contrast, if endophyte communities of leaves exhibiting low herbivory are perfectly nested within those exhibiting high herbivory, this finding might suggest there is a base community of endophytes found in all leaves, and that the remaining endophytic taxa colonize after herbivory.

Systems with an invasive plant species and an introduced biological control agent are useful for investigating these questions because they contain a simplified food web consisting of a specialized plant-herbivore interaction. Furthermore, novel plant-herbivore-microbe interactions could promote or reduce the impact of herbivory on the invasive plant in the introduced range relative to the native range (Bennett 2013). However, it is not yet understood how biological control agents may interact with endophytes residing in the invasive plant they

were introduced to control (Hayes et al. 2013). It has been proposed that endophytes may have an antagonistic relationship with herbivore biocontrol agents (Evans 2008, Newcombe et al. 2009) and that these interactions may vary over space and time (Tscharntke et al. 2008).

In this study, we sampled endophytes from leaves of the invasive wetland perennial forb *Lythrum salicaria* L. (purple loosestrife) in sites experiencing varying degrees of herbivory caused by leaf-chewing beetles (*Galerucella pusilla* and *G. calmariensis*) introduced as biological control agents in Minnesota, USA (Quiram 2013). Using a culture-based approach, we characterized endophyte communities in six wetlands composed of near monocultures of *L. salicaria* and histories of high or low levels of *Galerucella* spp. herbivory (Quiram 2013) to address the following questions: (i) what is the relationship between herbivory damage and endophyte abundance and richness at different spatiotemporal scales? (ii) at what spatiotemporal scale is each influenced? (iii) do endophyte communities exhibit patterns of non-randomness suggestive of facilitation or antagonism among endophyte species? (iv) are endophyte communities nested along a gradient of herbivory, suggestive of a base community of endophytes that accumulates species with herbivory? While this observational study cannot determine causality, identifying patterns between endophytes and herbivory does provide insights into their interactions.

MATERIALS AND METHODS

Study System

Lythrum salicaria was first introduced to North America in the early 1800s and is considered an invasive species in wetlands throughout the United States and Canada (Malecki et al. 1993). *L. salicaria* typically grows in dense monocultures that degrade wetland structure and

function, outcompete native plants, and endanger wildlife (Malecki et al. 1993). Beginning in 1992, two leaf-chewing beetles, *Galerucella pusilla* and *G. calmariensis*, were introduced as biological control agents to consume *L. salicaria* in the USA (Malecki et al. 1993). Since then, *Galerucella* in Minnesota have had variable success in establishing and subsequently controlling *L. salicaria* (Quiram 2013). We characterized endophyte communities from six sites in southeastern Minnesota within a 75km radius that ranged in their biological control efficacy (Appendix 4-1). Three sites – Circle Lake (*CL*), Pig's Eye (*EY*), and Winona (*WI*) – were chosen for their historically high herbivory levels, and three sites – Dodge Center Nature Preserve (*DC*), Hall's Marsh (*HM*), and Pottery Pond (*PP*) –for their historically low herbivory levels (Quiram 2013). Historical herbivory was defined based on 10 years of survey data of biocontrol agent presence and feeding damage to *L. salicaria* leaves completed between 1998 and 2010 (Quiram 2013). Historically high herbivory sites were defined as wetlands with abundant biocontrol agents and 50% or greater leaf damage characteristic of *Galerucella* species feeding, and historically low herbivory sites were defined as wetlands with few or no biocontrol agents present and minimal leaf damage (Quiram 2013).

Sampling

In June 2011 we sampled endophyte communities from leaves of 16 *L. salicaria* plants within each site. Of these 16 plants, ten were randomly selected along a 30m transect, and six were haphazardly chosen along the transect, three of which exhibited relatively low herbivory and three of which exhibited relatively high herbivory for the site. This design allowed us to sample across a broad range of plant herbivory within sites. Endophytes are typically cultured from healthy, undamaged leaves (e.g. Arnold et al. 2003, Zimmerman and Vitousek 2012, David et al. 2015), yet this was not possible at these sites because most leaves exhibited some degree of

herbivory. We instead selected two leaves for culturing that exhibited the least amount of herbivory for each plant from the mid-to upper-level of the stem. These leaves were photographed prior to culturing to measure the leaf-level herbivory. Two additional, haphazardly chosen leaves from each plant were photographed in the field to measure plant-level herbivory.

Leaf Damage

Photographs of leaves from the field were analyzed for damage using ImageJ software (Abràmoff 2004). We measured the total leaf area and total area damaged in order to calculate percent herbivory for each plant. Where leaf margins had been removed by herbivores, we approximated the previous tissue assuming a lanceolate cordate shape typical of *L. salicaria* leaves (Mal et al. 1992).

Culturing

To characterize endophyte communities, we used a culture-based approach. While culture-based approaches may have biases towards certain groups of fungi, particularly fast-growers, and against others such as obligate biotrophs, other methodologies such as next-generation sequencing have their own biases towards other groups of fungi (Arnold et al. 2007, Nguyen et al. 2014). Despite these biases, studies involving comparisons of endophyte diversity or community structure have found similar results using both culture-dependent and independent methods (Arnold et al. 2007, Pan and May 2009).

We sampled endophytes from two leaves of each plant. Leaves were surface sterilized with a rinse in sterile, deionized water, followed by successive baths in 70% ethanol (1 min), 70% bleach (3.675% NaOCl; 2 min), 70% ethanol (1 min), and a second rinse in sterile DI water (David et al. 2015). Following surface sterilization, leaves were cut into 5 segments (total area ~11mm^2) and were placed in Petri dishes containing one of three types of nutrient media. For the

10 randomly sampled plants, leaf segments were placed on 2% potato dextrose agar, 2% malt extract agar, or 10% malt extract agar (2 leaves × 3 media types × 5 segments per plate = 30 total segments per plant). Three media types were used for this subset of samples to determine whether fungal richness depended on the media used, but we found no evidence for this (d.f. = 2, $\chi^2 = 1.14$, $p = 0.565$). For the other six plants collected (representative of low and high site herbivory), segments were plated on only 2% malt extract agar (2 leaves × 1 media type × 5 segments per plate = 10 total segments per plant). We plated tissue from the interior sections of plant leaves away from chewed sections and leaf margins to avoid sampling fungi which may not have established within the leaf. We verified surface sterilization effectiveness with leaf prints of sterilized tissue pressed upon 2% malt extract agar plates (Fröhlich et al. 2000). Cultures were monitored for two months following plating, and emergent fungi were subcultured onto separate Petri dishes with 2% malt extract agar.

Endophyte classification

We sequenced fungal isolates and classified them into operational taxonomic units (OTUs). DNA was extracted from isolates using the REDExtract N' Amp Kit (Sigma Aldrich Corp., Saint Louis, MO, USA). We amplified the internal spacer region and a portion of the large subunit as a single fragment with the ITS1-F (Gardes and Bruns 1993) and LR3 primers (Vilgalys and Hester 1990) using 20µL reactions (10µL REDExtract N' Amp Mix, 0.8µL (10µM) each of forward and reverse primers, 4.4µL water, and 4µL DNA template; PCR conditions consisted of an initial denaturation step at 95°C for 3min, 35 cycles of 95°C for 30s, 54°C for 30s, 72°C for 1min, and an additional 10min extension time at 72°C). We sequenced the amplified DNA using the ITS1-F primer which resulted in ~700 bp fragments. Sequences were trimmed using Geneious Pro 5.5.7 (Kearse et al. 2012) to exclude regions with >5% error

per base and to allow a maximum of ten low quality bases and six ambiguities. We clustered sequences using a workflow (Monacell and Carbone 2014) that extracted the ITS1 and ITS2 regions using ITS extractor (Nilsson et al. 2010), checked for chimeras with UChime (Edgar et al. 2011), and clustered sequences based on alignment-free similarity with ESPRIT (Sun et al. 2009) and MOTHUR (Schloss et al. 2009). We used the 97% similarity for all subsequent analyses, but obtained similar results when OTUs were clustered at the 95% and 90% similarity levels. We assigned taxa to our OTUs using the naïve Bayes classifier in MOTHUR (minimum confidence allowed = 0.60) implemented through QIIME (Caporaso et al. 2010). We classified taxa based on the accessions within a combined database of the UNITE (Abarenkov et al. 2010) and the Emerencia databases (Ryberg et al. 2009). We calculated species accumulation curves to evaluate how well our sampling captured overall endophyte OTU richness and richness within each of the six sites.

Data Analysis

Leaf-level and plant-level herbivory were highly correlated (Pearson's $r = 0.825$, $t = 20.1$, d.f. = 190, $P < 0.001$), and therefore we only included leaf-level herbivory in subsequent analyses. Site-level herbivory was calculated as the average plant-level herbivory of the 10 randomly selected plants (excluding those plants specifically chosen for their low or high herbivory) within a site. We analyzed site-level differences in herbivory using ANOVA, and we used a t-test to assess whether measured site-level herbivory differed between sites with low and high historical herbivory.

We calculated endophyte isolation frequency (a measure of abundance) as the number of emergent fungi isolated from each leaf or site divided by the total number of surface-sterilized leaf segments analyzed for that leaf or site. We used generalized linear mixed-effects models

with binomial error to investigate the relationships between isolation frequency and site- and leaf-level herbivory damage using the nested random effects of historical herbivory, site, and (for the leaf-level analyses) plant. Significance was determined by removing the herbivory damage term and comparing models using ANOVA, and we report χ^2 test-statistics and p-values from these model comparisons in the results. For those leaves containing at least one OTU, OTU richness was analyzed using similarly nested linear mixed-effects models with maximum likelihood at the leaf- and site-levels. OTU richness was scaled by the sampling effort and log-transformed. For both isolation frequency and OTU richness, we then conducted separate analyses for each site in order to determine if among-site patterns were also detected within sites. We also investigated the effect of leaf-level herbivory on the probability of isolating the two most common endophytes (OTU1 and OTU14) using logistic generalized linear mixed-effects models with analysis of deviance to test for significance.

To investigate the influence of varying spatiotemporal scales, we used variance components analysis to quantify the variation of leaf-level herbivory, isolation frequency, and endophyte community explained at the hierarchical levels of historical site herbivory (high or low), site, plant, and residuals. We interpreted the residuals as the variance explained by leaves because each leaf represented one sampling unit. Herbivory damage was analyzed using a nested mixed-effects model with restricted maximum likelihood. Isolation frequency was analyzed as a nested generalized linear mixed-effects model with binomial error. Endophyte communities were analyzed with permutational analysis of variance (100,000 permutations) using historical herbivory, site, and plant as fixed-effects, and the sum of squares were used to determine proportion of variance explained.

We investigated endophyte community structure in two ways. First, we considered whether endophyte communities exhibited non-randomness indicative of facilitation or antagonism among endophyte OTUs. We analyzed the number of checkerboard units – i.e. the number of times an OTU was only present in a first plant but not a second, and a second OTU was present in a second plant but not the first – and compared it to a fixed column – fixed row null model using the 'swap' algorithm (Stone and Roberts 1990). We tested for non-randomness at the levels of the leaf, plant, site, and plants within a site. Second, we investigated nestedness of endophyte communities along the herbivory damage gradient and evaluated the nestedness metric based on overlap and decreasing fill (NODF) of the endophyte community matrix at the levels of leaf, plant, and site, and plants within sites (Almeida-Neto and Guimaraes 2008). NODF has significant advantages over other nestedness indices in that it is a more conservative test less prone to Type I error (Ulrich and Gotelli 2007, Almeida-Neto and Guimaraes 2008). To test for significance of nestedness, we compared the NODF index to simulated random communities using the *r1* null hypothesis which uses the species marginal frequencies as probabilities (Wright 1998) and, unlike fixed-total null hypotheses, avoids Type II error (Ulrich and Gotelli 2013).

All statistical analyses were conducted with R Version 3.0.2 (R Development Core Team 2013) and the vegan (Oksanen et al. 2013) and lme4 packages (Bates et al. 2015). Sequences were deposited in GenBank under accession numbers KT290958-KT291128.

RESULTS

We found significant differences in leaf herbivory damage across sites ($F_{5,186} = 17.6$, $P < 0.001$). However, site-level herbivory damage differences did not match our *a priori* predictions

based on consistent historical herbivory levels observed at the study sites between 1998 and 2010 (Quiram 2013); there was no effect of historical herbivory on measured site-level herbivory damage ($t = 0.422$, d.f. = 4, $P = 0.695$). In particular, site *EY* had the lowest amount of herbivory damage despite having historically high herbivory, while the historically low herbivory site *DC* had higher herbivory higher than expected. While the sites used in this study had been observed to have consistent historical herbivory levels over 12 years, *Galerucella* populations can vary temporally and the impacts of biological control programs on *L. salicaria* populations can continue to change 10-20 years after the initial release of the agent (Blossey et al. 2001, Lindgren 2003).

In total, we isolated 235 emergent fungi (10.9% isolation frequency). Of these, we successfully sequenced the ITS1 and ITS2 regions of 171 isolates and classified them into 36 OTUs. The species accumulation curve did not plateau for most sites (Appendix 4-2), suggesting our sampling may not have fully captured the total richness in the system. Of the 36 OTUs, 18 were isolated only once, and 7 were isolated two times. The most common taxa were *Alternaria* and *Penicillium*, and the orders Pleosporales (Dothideomycetes) and Eurotiales (Eurotiomycetes) were particularly dominant (Table 4-1). All OTUs were from the phylum Ascomycota, with the exception of three OTUs from the order Agaricales in the phylum Basidiomycota.

We found positive relationships between herbivory damage and endophyte isolation frequency at the site-level (d.f. = 1, $\chi^2 = 37.9$, $p < 0.001$) and plant-level (d.f. = 1, $\chi^2 = 15.3$, $p < 0.001$; Figure 4-1a-b). However, the *CL* site had a much higher herbivory damage than the other sites; we therefore reanalyzed the data without this site, and we still found a significantly positive relationship at the site-level (d.f. = 1, $\chi^2 = 33.2$, $p < 0.001$) but not at the plant-level (d.f. = 1, $\chi^2 = 2.43$, $p = 0.119$). Within sites, there was a strong positive relationship between leaf-level

herbivory damage and endophyte isolation frequency at the *CL* site (d.f. = 1, χ^2 = 7.16, p = 0.007), but no significant relationships within any other site (p > 0.05 for all other sites; Figure 4-1b). Similarly, we found a significant positive relationship between herbivory damage and OTU richness at the site (d.f. = 1, χ^2 = 6.49, p = 0.011) and a marginally-significant positive effect at the leaf-level (d.f. = 1, χ^2 = 3.44, p = 0.064; Figure 4-1c-d). The site-level analysis was robust to removal of the high herbivory *CL* site (d.f. = 1, χ^2 = 5.53, p = 0.019). There were no significant differences between leaf-level herbivory damage and OTU richness within sites (site *PP* had insufficient data to run analysis, all other sites p > 0.05) Figure 4-1d). The probability of isolating the most common OTU, OTU1 (*Alternaria sp.*) increased with herbivory damage (d.f. = 1, χ^2 = 8.56, p = 0.003), but we found no relationship for the second-most common OTU, OTU14 (*Penicillium sp.*) and herbivory damage (d.f. = 1, χ^2 =1.72, p = 0.190).

The variance components analysis revealed that herbivory damage and endophyte communities were mostly explained at the plant-level, while endophyte isolation frequency was mostly explained at the leaf-level (residuals) (Figure 4- 2). A substantial amount of variation for herbivory damage was also explained at the site-level, while the site-level did not explain much of the endophyte isolation frequency or community variation. Historical herbivory explained little variance for any of the response variables.

Endophyte communities did not show clear evidence of non-randomness at the plant- or site-levels, as the *C*-score from the checkerboard analyses did not significantly differ from that of the null models (Table 4-2). At the leaf-level, we found significantly fewer checkerboard units than would be expected from random, which is indicative of positive associations among endophyte taxa (Table 4-2).

Endophyte communities were nested along the herbivory damage gradient at both the site-level and plant-level, such that sites or plants with less herbivory damage contained endophyte communities that were subsets of those in sites or plants with high herbivory damage (Table 4-3, Figure 4-3). This finding was robust to removal of the common OTU1 at the site-level (Null NODF = 30.0 NODF = 20.9, $z = 3.43$, $p<0.001$), but not at the plant-level (Null NODF = 4.38 NODF = 3.87, $z = 0.816$, $p= 0.405$). Both the site-level NODF (Null NODF = 30.0 NODF = 21.0, $z = 3.54$, $p<0.001$) and the plant-level NODF analyses (Null NODF = 12.5 NODF = 9.14, $z = 2.21$, $p= 0.040$) were robust to removal of the second-most common OTU14. We did not find evidence of nestedness at the leaf-level, potentially due to low matrix fill caused by low endophyte richness within individual leaves Table 4-3). There was evidence of within-site nestedness for the sites exhibiting the highest herbivory damage (*CL* and *DC*), indicating that endophyte communities in low herbivory plants were subsets of those in high herbivory plants within these two sites (Table 4-3).

DISCUSSION

In this study we investigated the association between two heterotrophic groups – fungal endophytes and herbivores – in a natural setting at varying spatiotemporal scales. We report four key findings. First, herbivory damage and endophyte abundance, as measured by isolation frequency, were positively associated at the site-level and plant-level. There were generally no associations between isolation frequency and herbivory damage within sites, with the exception of a positive association found at the site (*CL*) experiencing the highest herbivory damage. The endophyte-herbivory relationship was likely influenced by the most common OTU, OTU1 *Alternaria sp.*, whose probability of occurrence increased with herbivory. Second, we found

evidence that herbivory damage occurred at a larger spatiotemporal scale than endophytes. Herbivory damage was structured at the site- and plant-level, while endophytes (isolation frequency and community) were structured at the plant- and leaf-levels. Historical herbivory of the site accounted for little of the variation for either herbivory or endophytes. Third, endophyte communities showed non-randomness in the checkerboard analysis at the leaf-level indicative of overall positive associations among taxa, but no evidence of non-randomness at the plant- or site-levels. Fourth, endophyte communities were nested along a gradient of herbivory damage, with taxa in low herbivory sites and plants representing nested subsets of those taxa found in high herbivory sites and plants. Taken together, we find no support for antagonism between endophytes and herbivory, and instead find support for facilitation. However, while support for facilitation was evident across sites, there was less evidence of facilitation within-sites, which we discuss below.

Our results contribute to a developing understanding of how endophyte communities are structured in natural settings. Recent work has shown strong influences of the surrounding environment and dispersal (Saunders et al. 2010, Zimmerman and Vitousek 2012, Higgins et al. 2014, David et al. 2015), yet less attention has been directed towards the ways in which herbivores, in conjunction with environment and dispersal, might shape communities. While vertically-transmitted clavicipitaceous endophytes are known to impede herbivory through the production of compounds such as alkaloids (Schardl et al. 2004) and alter herbivore communities (Rudgers and Clay 2008), such antagonism could be favored because of the close ecological and evolutionary relationship those endophytes have with their plant hosts. Most endophytes, however, are horizontally-transmitted and consist of local infections within their host (i.e. Type III endophytes, Rodriguez et al. 2009), which could suggest there is less selective advantage

towards antagonizing herbivores. Many studies considering endophyte diversity focus on leaves exhibiting no herbivory to avoid sampling potentially non-endophytic fungi that have opportunistically colonized a wounded leaf (e.g. Arnold et al. 2003). However, our results suggest that herbivores could play important roles in structuring endophyte communities, and studies that avoid sampling leaves exhibiting herbivory might underestimate endophyte diversity.

Though our observational study does not resolve whether it is endophytes or herbivores that drive this positive association, our results do lend credence to the idea that endophyte communities do not influence herbivory in this system. Herbivory was structured at the site- and plant-levels, while endophyte isolation frequency and communities were structured and the plant- and leaf-levels (residual). Given this spatial structuring and the finding that herbivory and endophytes were positively correlated with one another, it seems unlikely that endophytes are inhibiting biocontrol agents from feeding on *L. salicaria* and therefore an unlikely cause for biocontrol failure at historically low herbivory sites.

An alternative hypothesis that endophytes colonize or increase within-leaf abundance where herbivory has already occurred is supported by our findings, though this would need to be confirmed experimentally. If herbivores facilitated endophytes, then we might have expected a positive relationship to hold both across and within sites. Instead, our results showed that the relationships between herbivory and endophyte isolation frequency, richness, and nestedness were most strongly supported across sites rather than within sites. This could be interpreted as evidence that herbivores and endophytes do not interact, but are both influenced by the same site-level factor. For instance, frequency of water inundation can reduce *Galerucella* (Yeates et al. 2011) and endophyte abundance (Dolinar and Gaberscik 2010). However, this result could also be attributed to at least two alternative causes that are still consistent with the hypothesis

that herbivores cause changes in endophyte assemblages. First, low variation in herbivory within the sites for which we found no herbivory-endophyte relationship could obscure detection of a within-site relationship. Further experimental studies that directly manipulate herbivory could determine if this is the case. Second, there may be a threshold at which herbivory begins to facilitate endophyte taxa. For instance, if herbivory facilitates endophytes through the creation of wounds in the plant, sufficient wounding might be necessary before endophytes are able to capitalize and increase their abundance.

Our study also contributes to a growing understanding of the communities of endophytes residing within invasive plants (Shipunov et al. 2008, Aschehoug et al. 2012, David et al. 2015). Though these endophyte communities are often overlooked, invasive plants are known to harbor distinct endophyte communities in their introduced habitat as compared to their native habitat (Shipunov et al. 2008), suggesting they interact with endophytes with which they have not necessarily evolved. In *L. salicaria*, we found endophyte communities to be generally sparse, but the communities typically contained *Alternaria* and *Penicillium*. Both are high spore-producing, cosmopolitan genera, whose members may be pathogenic, saprobic, or endophytic and produce a variety of mycotoxins (Pitt 2002, Thomma 2003, Sandberg et al. 2014). Endophytes acquired in the introduced range of other invasive plant species have been shown to increase competitive ability or reduce seed predation (Newcombe et al. 2009, Aschehoug et al. 2012). While neither of the two common OTUs in our study exhibited the negative isolation frequency-herbivory damage relationship that would suggest they defend *L. salicaria* from herbivory, congeners of these OTUS are known to increase competitive ability of invasive forbs (Aschehoug et al. 2012) or reduce disease severity (De Cal et al. 1997).

The work we present here is, to our knowledge, the first study to examine the interactions between a biological control agent of an invasive plant and its endophyte communities. The intentional introduction of a biological control agent may have important short- and long-term effects on ecological systems, particularly for those species with which the agent directly and indirectly interacts (David et al. 2013). In our system, there may be short-term alterations in endophyte community composition, but there could also be long-term effects if ecosystem functions such as leaf decomposition are altered (e.g. Purahong and Hyde 2010; but see Osono 2006). The roles of most endophytes and other plant-associated symbiotic microbes in ecological systems are still poorly understood, yet their communities are susceptible to anthropogenic changes (Olsrud et al. 2009, Kivlin et al. 2013, Rudgers et al. 2014). Future work should focus on the ways in which endophyte communities may be altered by anthropogenic changes, and how such changes alter interactions between other heterotrophic groups and host plants.

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OTU	No. Isolations	Phylum	Class	Order	Family	Taxon Assignment	Confidence
OTU28	4	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Cercospora sp	1.00
OTU7	4	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium pseudocladosporioides	0.97
OTU26	2	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Stenella lythri	1.00
OTU3	1	Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	Aureobasidium pullulans	0.99
OTU12	1	Ascomycota	Dothideomycetes	Myriangiales	Elsinoaceae	Sphaceloma bidentis	0.65
OTU1	74	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria sp	1.00
OTU22	2	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	Leptosphaeria microscopica	1.00
OTU9	5	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Lewia infectoria	0.88
OTU2	2	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Nimbya sp	0.93
OTU31	1	Ascomycota	Dothideomycetes	Pleosporales	Incertae sedis	Periconia sp	1.00
OTU19	1	Ascomycota	Dothideomycetes	Pleosporales	Incertae_sedis	Peyronellaea sp	0.82
OTU15	1	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeria pontiformis	0.73
OTU10	3	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeria sp	0.99
OTU21	6	Ascomycota	Dothideomycetes	Pleosporales	unidentified	Pleosporales sp	0.99
OTU6	1	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Pyrenophora triticirepentis	1.00
OTU32	2	Ascomycota	Dothideomycetes	Pleosporales	Massarinaceae	Saccharicola bicolor	0.99
OTU35	1	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Stagonospora pseudocaricis	0.94
OTU20	6	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus insuetus	0.99
OTU34	4	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium funiculosum	0.98
OTU14	23	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium sp	1.00
OTU30	1	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium steckii	1.00
OTU23	2	Ascomycota	Leotiomycetes	Helotiales	Incertae_sedis	Cadophora luteoolivacea	0.77
OTU33	1	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Sclerotiniaceae sp	1.00
OTU24	1	Ascomycota	Sordariomycetes	Diaporthales	Diaporthaceae	Phomopsis sp	0.92
OTU11	2	Ascomycota	Sordariomycetes	Microascales	Halosphaeriaceae	Halosphaeriaceae sp	1.00

OTU27	1	Ascomycota	Sordariomycetes	Microascales	Halosphaeriaceae	Halosphaeriaceae sp	0.82
OTU36	1	Ascomycota	Sordariomycetes	Sordariales	unidentified	Sordariales sp	1.00
OTU13	2	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	Biscogniauxia mediterranea	1.00
OTU5	5	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	Hypoxylon submonticulosum	1.00
OTU17	1	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	Nemania sp	0.67
OTU8	1	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	Seimatosporium discosioides	0.91
OTU29	1	Ascomycota	Sordariomycetes	Xylariales	unidentified	Xylariales sp	1.00
OTU16	1	Ascomycota	unidentified	unidentified	unidentified	Ascomycota sp	1.00
OTU25	1	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Coprinellus micaceus	1.00
OTU4	5	Basidiomycota	Agaricomycetes	Polyporales	Meruliaceae	Irpex lacteus	1.00
OTU18	1	Basidiomycota	Agaricomycetes	Russulales	Peniophoraceae	Peniophora cinerea	0.89

Table 4-1 Operational taxonomic units (OTUs) with their overall isolation frequencies and taxonomic assignments. OTUs were clustered based on 97% similarity of the ITS1 and ITS2 regions. Isolations were measured as the number of emergent fungal cultures that clustered into that OTU. Taxonomy was assigned with the RDP classifier in MOTHUR using the combined UNITE and Emerencia databases. Confidence is the bootstrap confidence score (minimum allowed 0.60)

	N	Null C-score	C-score	z-statistic	*P*	Null NODF	NODF	z-statistic	*P*
Across Sites									
Site	6	0.981	0.989	-0.335	0.836	33.836	23.609	3.964	**0.001**
Plant	96	7.005	6.570	1.997	0.081	15.116	11.173	2.463	**0.031**
Leaf	192	10.084	9.395	2.800	**0.020**	9.272	8.622	0.554	0.560
Within Sites									
DC	16	0.168	0.174	-0.625	0.652	15.227	9.385	2.175	**0.049**
HM	16	0.108	0.099	1.545	0.186	6.738	5.875	0.381	0.76
PP	16	0.035	0.038	-0.610	1.00	0	3.546	-1.848	0.195
CL	16	0.479	0.453	0.842	0.333	20.93	15.449	2.167	**0.045**
EY	16	0.143	0.144	-0.123	1.00	5.556	10.182	-1.473	0.171
WI	16	0.224	0.220	0.314	0.783	17.949	15.684	0.744	0.451

Table 4-2 Non-randomness and nestedness of endophyte communities along an herbivory gradient at different spatial scales. Non-randomness was tested using checkerboard analysis and the fixed-fixed null hypothesis generated by the 'swap' algorithm. Nestedness was tested using the nestedness metric based on overlap and decreasing fill (NODF) index and the *r1* null hypothesis. Binary matrices were sorted by rows from high herbivory to low herbivory, and by columns from most abundant to least abundant OTU. For the three among-site analyses, all samples were grouped by either site, plant, or leaf. For the six within-site analyses, samples were grouped by plant within each site. P-values bolded where significant.

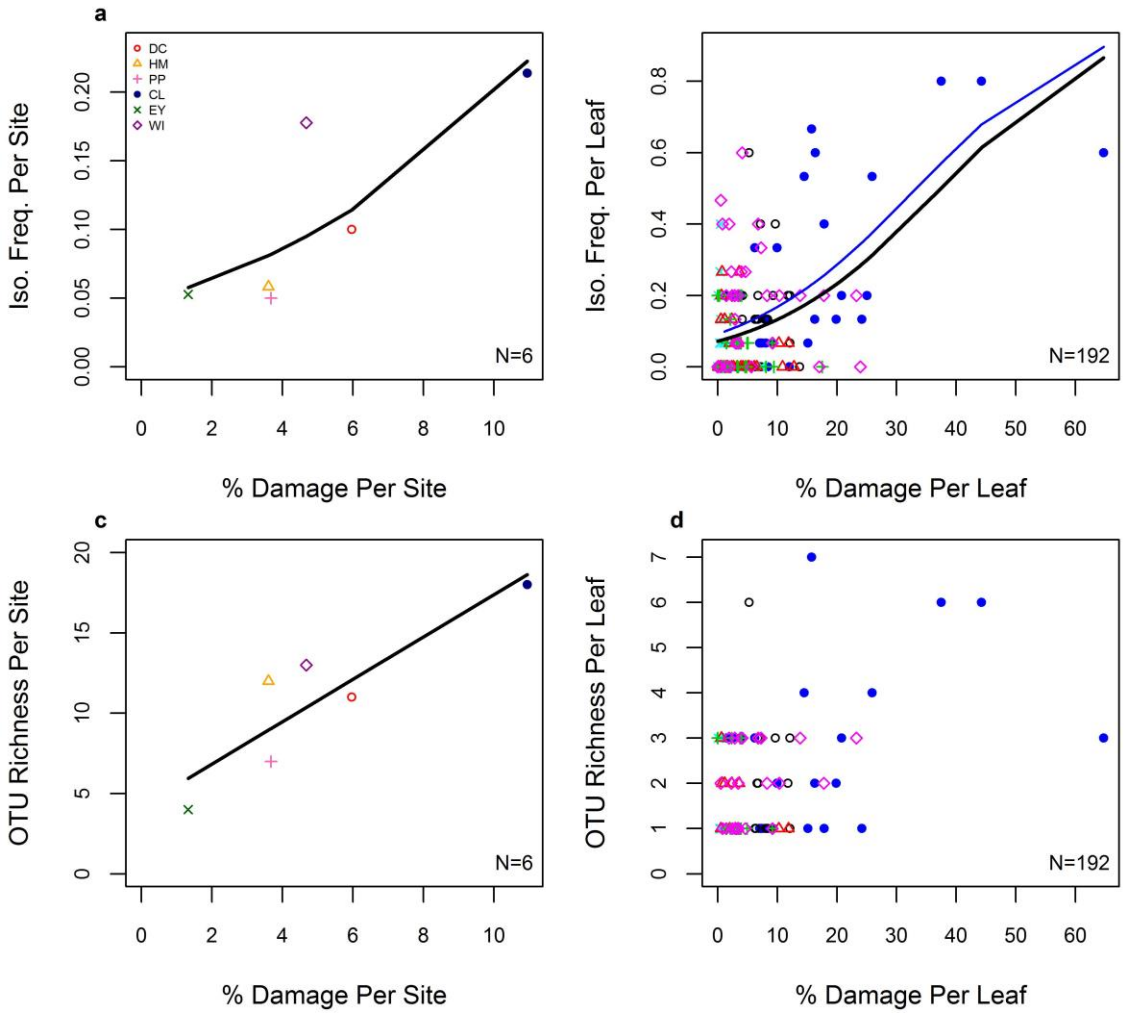

Figure 4-1 Associations between endophytes and herbivory damage.
Total endophyte isolation frequency at the level of (a) the site and (b) the leaf, and OTU richness shown at the level of (c) the site and (d) the leaf. Thick solid black line shows overall significant trends using the predicted, backtransformed values of univariate generalized linear (a, b) or linear models (c). Significant within-site associations are shown in color. Because individual leaves were sampled with either 5 or 15 surface-sterilized tissue segments and thus were not directly comparable, the points in (d) are scaled to be the expected richness per leaf for 15 segments.

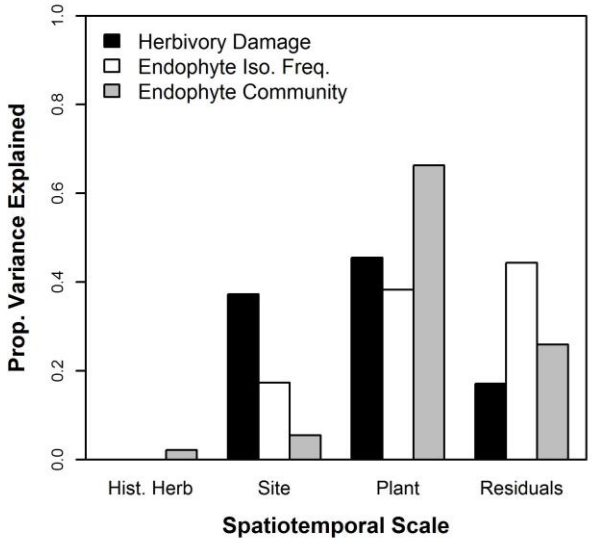

Figure 4-2 Proportion of variance explained for leaf herbivory, endophyte isolation frequency, and endophyte community at the spatiotemporal scales of historical herbivory, site, plant, and leaf (residuals).
Variance components were calculated from a linear mixed-effect model with restricted maximum likelihood for leaf herbivory, a generalized mixed-effects model with binomial error for endophyte isolation frequency, and permutational ANOVA for endophyte community.

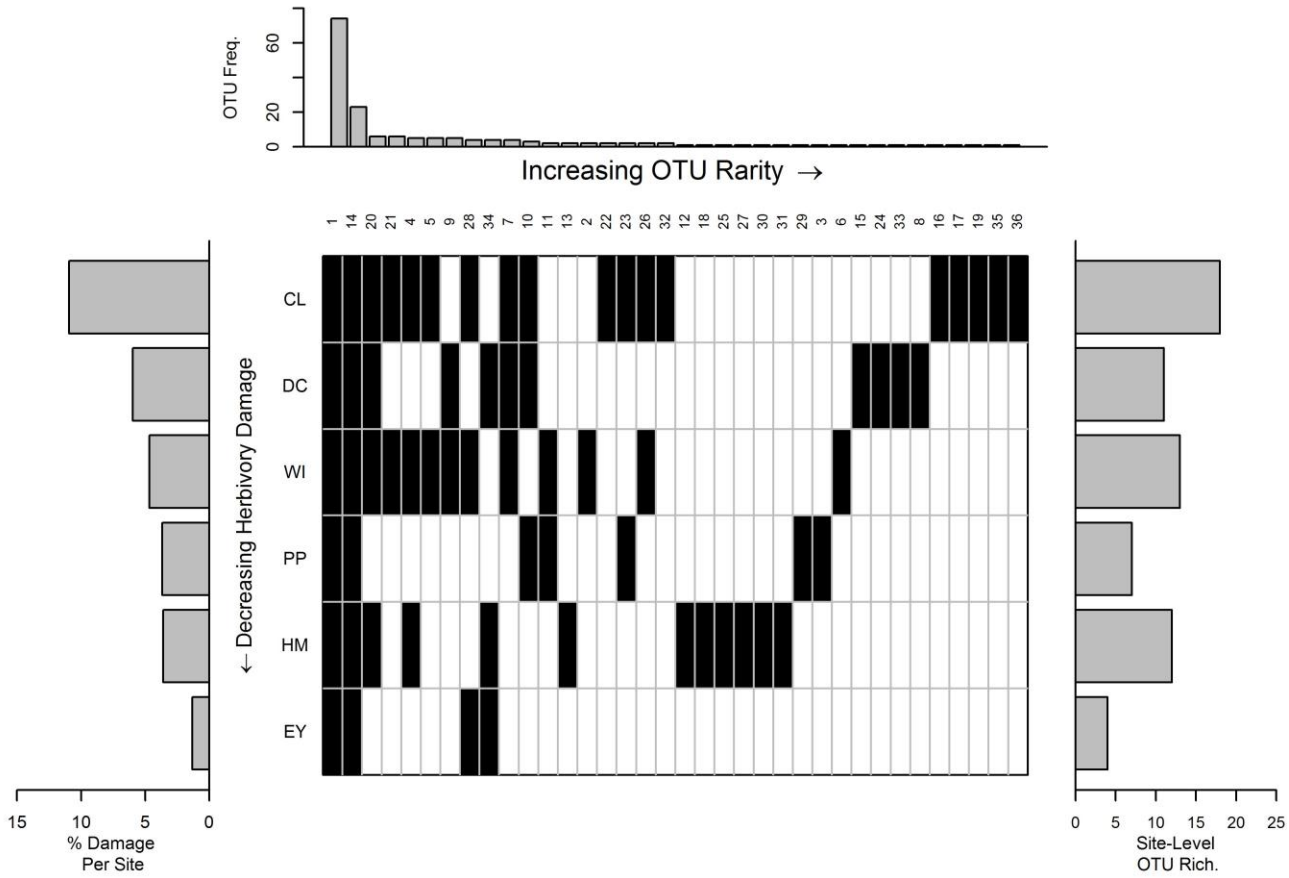

Figure 4-3 Nestedness of endophyte communities along a gradient of plant herbivory using the nestedness metric based on overlap and decreasing fill (NODF).

Plot shows the presence of individual OTUs (top x-axis) in each site (y-axis). OTUs are sorted by increasing rarity from left to right, with the frequency of each OTU shown in the top panel. Sites are sorted by decreasing herbivory from top to bottom shown in left panel, and the OTU richness of each site is shown on the right panel.

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Appendix 1 Supplemental material for Chapter 1

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Appendix 1-1 Transects used for chronosequence study, soil study, and decadal study. The proportion of *A. breviligulata* and the proportion of *A. arenaria* (not shown) sum to 1.

Transect	Littoral Cell	UTM Northing (Zone 10T)	Shoreline Change Rate (m/yr)	Prop. *A. breviligulata*				Chronosequence Study (2006 and 2009)	Soil study (2012)
				1988	2006	2009	2012		
GH82	Columbia River	5189593	1.7	0	0.68	0.56		x	
GH18	Columbia River	5189352	1.5	0	0.55	0.8		x	
GH17	Columbia River	5189059	1.6	0	0.79	0.98		x	
GH09	Columbia River	5187765	0.6	1	0.97	1		x	
GH06	Columbia River	5186818	-0.1	0.89	0.83	1	1		x
GH05	Columbia River	5183438	1.1	0.93	0.85	1	1	x	x
GH13	Columbia River	5183283	1.2	0.86	1	0.86		x	
GH01	Columbia River	5182372	2.2	0.94	1	1		x	
GH12	Columbia River	5180716	6.1	0.91	1	1		x	
GH07	Columbia River	5179641	11.5	1	1	1		x	
GH03	Columbia River	5179506	11.1	0.95	1	1		x	
GH11	Columbia River	5179023	9.7	0.77	1	1	0.79	x	x
GH04	Columbia River	5177364	-2.1	0.83	1	1			
GH20A	Columbia River	5177061	-2.6	0.21	0.85	1			
GH20	Columbia River	5176872	-8.6	0.1	1	0.87			
LBP06	Columbia River	5163832	4.6			0.99		x	
LBP05	Columbia River	5163508	4.8			0.91		x	
LB49	Columbia River	5163387	4.9	0.94	1	1		x	
LBP07	Columbia River	5162745	4.3			1		x	
LBP08	Columbia River	5162235	3.8			1		x	
LB08	Columbia River	5161953	3.9	0.79	0.71	1	1	x	x
LB11	Columbia River	5161378	3.5	0.8	1	1	1	x	x
LB15	Columbia River	5160850	3.4	1	1			x	
LB1020	Columbia River	5156020	3.1	0.83	1	1		x	
LB06	Columbia River	5154359	2.7	0.93	0.99	1		x	

LB01	Columbia River	5154131	2.7	0.83	1	1		x	
LB14	Columbia River	5151538	2.5	1	1	1		x	
LB12	Columbia River	5151254	2.2	0.68	1	0.95	1	x	x
LB05A	Columbia River	5148967	3.8			1		x	
LB05	Columbia River	5148482	3.8	1	1	1	0.58	x	x
LB02	Columbia River	5147332	4	1	1	1	0.72	x	x
LB03	Columbia River	5144911	5.1	1	1	1		x	
LB09	Columbia River	5142778	5.3	0.64	1	1		x	
LB07	Columbia River	5138252	5.9	0.84	1	1		x	
LB35	Columbia River	5134045	4.8	1	1	1		x	
LB36	Columbia River	5132039	3.8	1	1			x	
LB37	Columbia River	5130347	3.2	1	1	1	1	x	x
LB2842	Columbia River	5129357	1.6	1	1	1			
LB32	Columbia River	5128977	1.5	1	1	1		x	
EASTJETTY	Columbia River	5119128	0.8			0	0.71		x
FS02	Columbia River	5117392	0.2		0	0		x	
IREDALE43	Columbia River	5114402	0.3			0		x	
KIM44	Columbia River	5111068	0.6			1	1	x	x
RILEA45	Columbia River	5107099	3			1		x	
FS03	Columbia River	5105783	2.5		0.59	1	1	x	x
FS01	Columbia River	5100274	1.8		0.92	1		x	
DELRAY46	Columbia River	5099952	1.5			1	0.82	x	x
DELRAY46A	Columbia River	5099556	1.8			1		x	
SEASIDE	Columbia River	5094724	0.8			0.91		x	
NB04	Rockaway	5060094	1				0		x
NB03	Rockaway	5059198	0				0		x
PC01	Neskowin	5004502	0.3		0	0		x	
SB03	Newport	4939934	1.9		0	0		x	
SNJ03	Coos Bay	4874857	1.8				0		x
SSJ01	Coos Bay	4873812	2.5				0		x

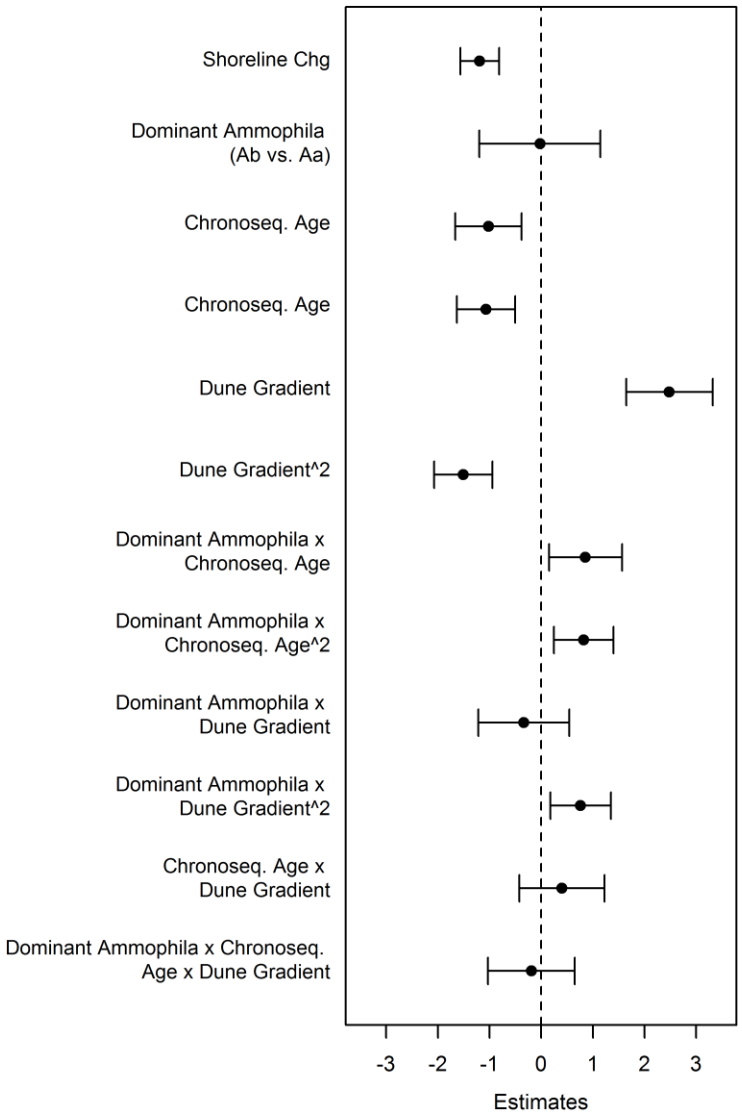
SSJ03	Coos Bay	4872546	0.5				0		x
SILT03	Coos Bay	4857458	0.2			0		x	
DONR06	Coos Bay	4855442	0.2			0		x	
TKNR05	Coos Bay	4851487	-0.4			0	0		x
TKNR04	Coos Bay	4851067	0.6			0	0	x	x
BANNR03	Bandon	4768995	0.7			0		x	
BANNR06	Bandon	4768482	1.4				0		x
BANNR02	Bandon	4768307	1.6			0		x	
BANNR01	Bandon	4766756	0.2			0		x	
MCKNR02	Port Orford	4739800	0.5			0		x	

*2006 data used in decadal study because 2009 data were unavailable

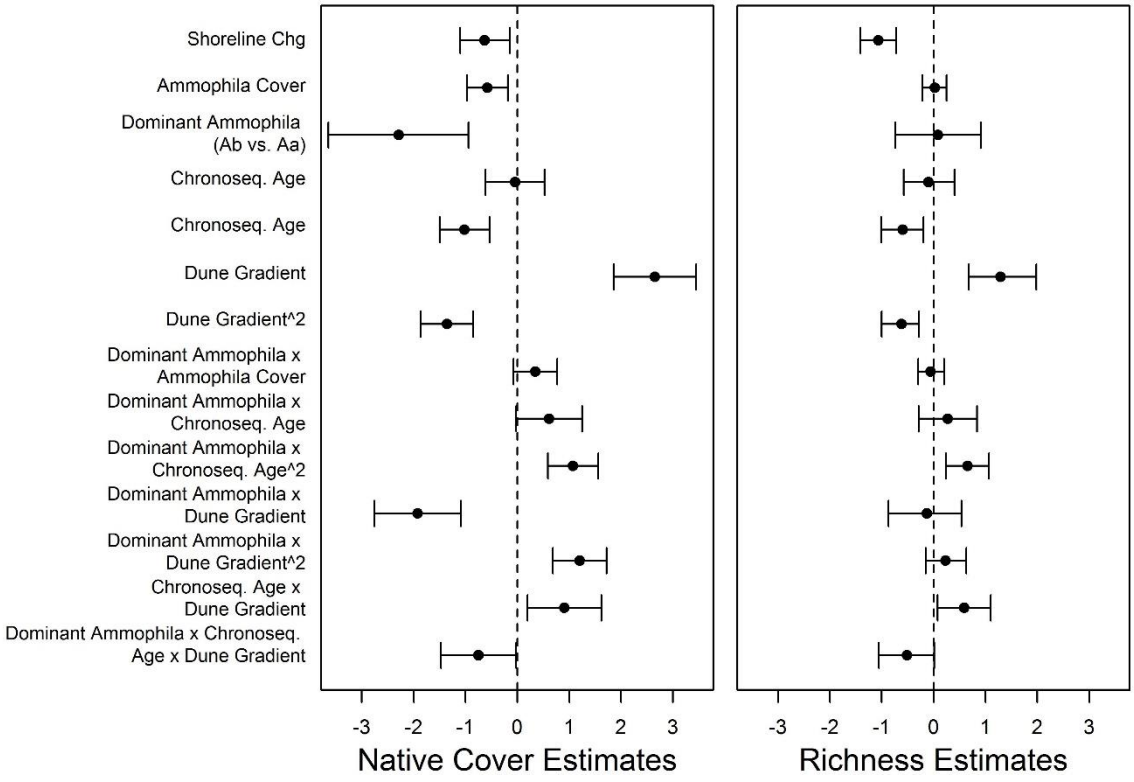
Appendix 1-2 Bayesian models evaluated in the chronosequence study in 2006 and 2009. Response variables are measured on a per quadrat basis, and all cover values are raw values that may sum to greater than 1. Shoreline change rate refers to the distance per year that a shoreline of a particular transect advances towards the coast. The dominant *Ammophila* species refers to transects dominated by either *A. arenaria* or *A. breviligulata*. Chronosequence age refers to the time since the bare ground under a quadrat was formed. Dune gradient refers to the standardized index along a dune cross-section where quadrat occurs.

Response	Error Structure	Model
Ammophila cover (logit transformed)	Gaussian	~ (1|transect) + shoreline change rate + dominant *Ammophila* species + chronosequence age + chronosequence age^2 + dune gradient + dune gradient^2 + dominant *Ammophila* × chronosequence age, + dominant *Ammophila* × chronosequence age^2 + dominant *Ammophila* × dune gradient + dominant *Ammophila* × dune gradient^2 + chronosequence age × dune gradient + dominant *Ammophila* × chronosequence age × dune gradient
Native cover (logit transformed)	Gaussian	~ (1|site) + shoreline change rate + *Ammophila* cover + dominant *Ammophila* species + chronosequence age + chronosequence age^2 + dune gradient + dune gradient^2 + dominant *Ammophila* × chronosequence age, + dominant *Ammophila* × *Ammophila* cover + dominant *Ammophila* × chronosequence age^2 + dominant *Ammophila* × dune gradient + dominant *Ammophila* × dune gradient^2 + chronosequence age × dune gradient + dominant *Ammophila* × chronosequence age × dune gradient
Species Richness	Poisson	

Appendix 1-3 Parameter estimates and 95% confidence intervals based on the posterior distribution of a hierarchical Bayesian model of *Ammophila* cover along chronosequence age and dune gradient from 2006 and 2009. Estimates are standardized for comparisons, and CIs that did not contain zero (shown as dashed vertical line) were interpreted as significant. Model parameters converged (Gelman-Rubin Diagnostic Statistic < 1.01) See Appendix 1-2 for full model details.



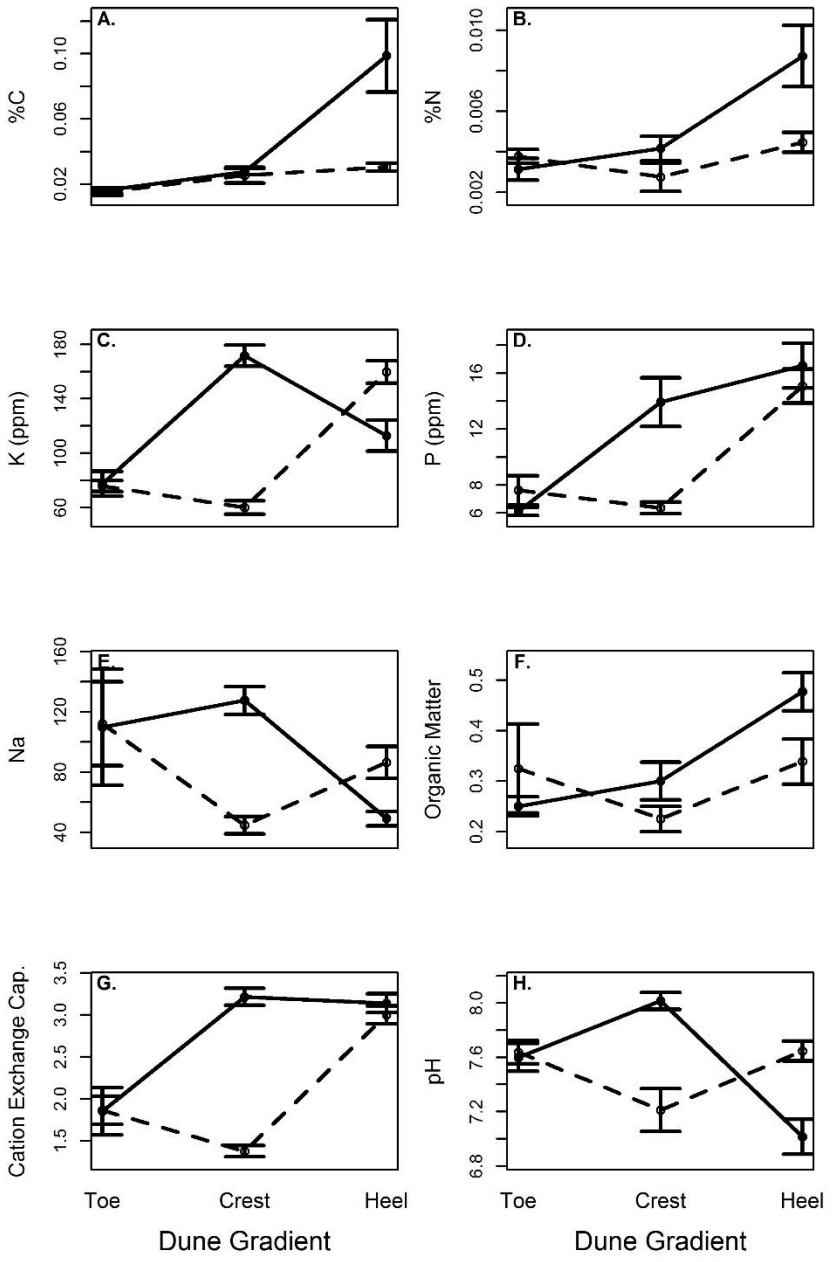
Appendix 1-4 Parameter estimates and 95% confidence intervals based on the posterior distribution of a hierarchical Bayesian model of (A) native cover and (B) species richness along chronosequence age and dune gradient in 2006 and 2009. Estimates are standardized for comparisons, and CIs that did not contain zero (shown as dashed vertical line) were interpreted as significant. Model parameters converged (Gelman-Rubin Diagnostic Statistic < 1.01) See Appendix 1-3 for full model.



Appendix 1-5 Effects of *Ammophila* community type and dune gradient on soil properties in 2012 using ANOVA. Dunes were either dominated by *A. arenaria* or *A. breviligulata*. Dune location was categorical and represented the toe, crest, and heel of foredunes.

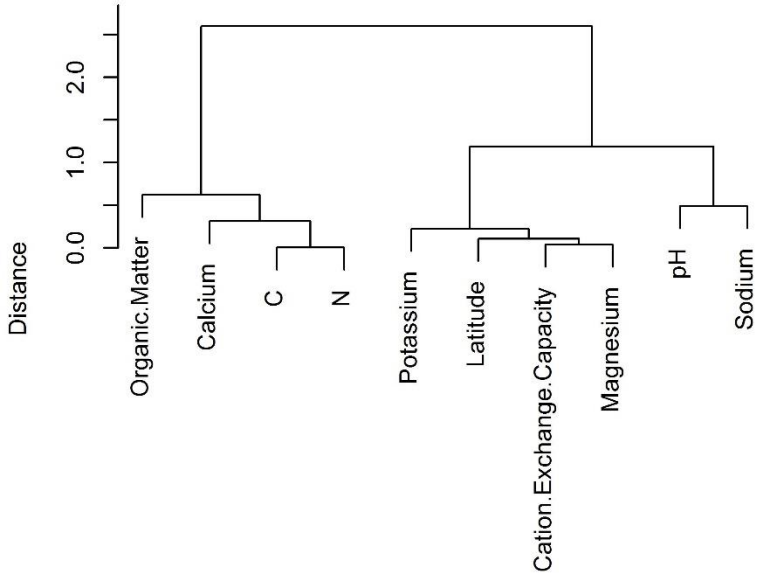
		%C		**%N**		**P**		**K**		**Na**		**Organic Matter**		**Cat**
Source	*d.f.*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*
Ammophila Community (Ab vs. Aa)	1	38.174	0.000	6.312	0.015	51.514	0.000	106.653	0.000	1.009	0.319	9.910	0.003	147.
Location	2	15.248	0.000	0.371	0.691	0.393	0.677	13.158	0.000	20.893	0.000	1.943	0.153	1.
Ammophila Community × Location	2	1.613	0.208	3.103	0.053	0.935	0.398	2.914	0.062	0.924	0.403	4.223	0.019	2.
Residuals	57													

Appendix 1-6 Soil properties in *Ammophila* species dunes along dune gradient. Solid lines show *A. breviligulata* dune soils, dashed lines show *A. arenaria* dune soils. Error bars showed mean ± 1 S.E. Plots show (a) percent C, (b) percent N, (c) concentration of P, (d) concentration of K, (e) concentration of Na, (f) organic matter, (g) cation exchange capacity, and (h) pH. See Table 1-1 for significance.

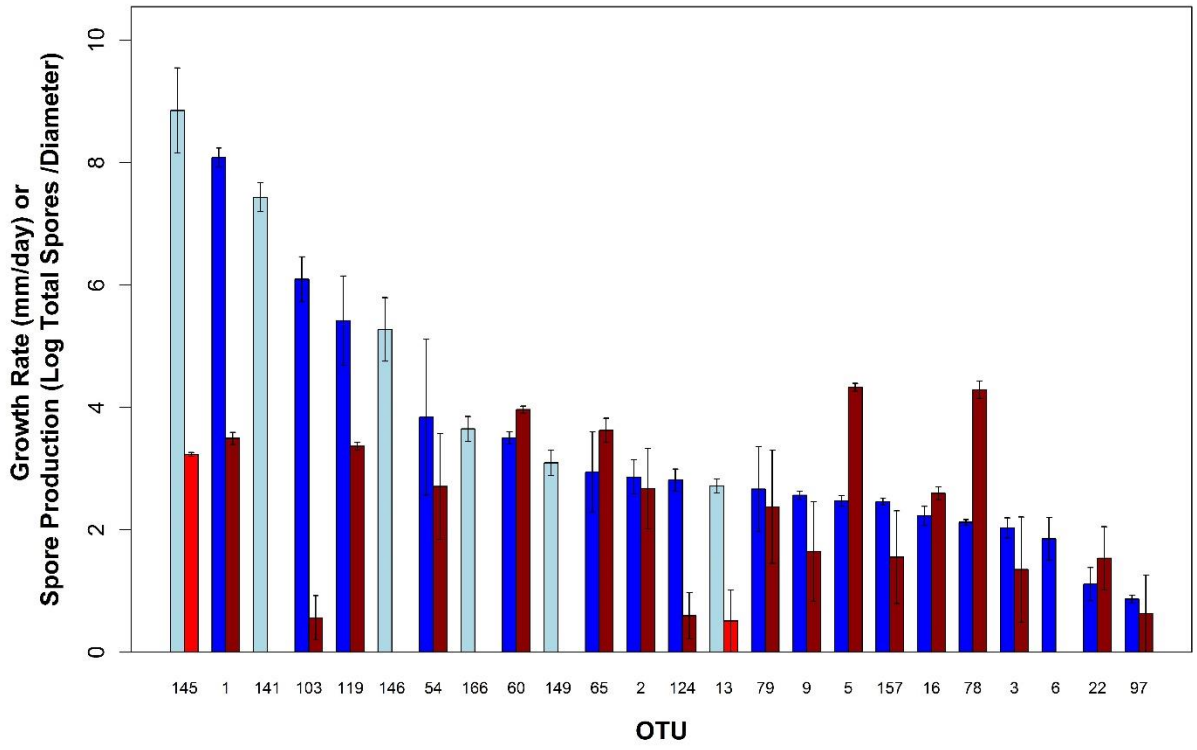


Appendix 2 Supplemental material for Chapter 2

Appendix 2-1 Cluster dendrogram of soil properties and latitude using Ward's minimum variance method. Variables clustered into 4 groupings, and from these the following soil properties were chosen for analyses: organic oatter, %N, latitude, and pH.



Appendix 2-2 Growth rate (blue) and spore density (red) of OTUs. Leaf OTU data (>50% isolations of OTU from leaves) are shown with lighter shades of blue or red, root OTU data (>50% isolations of OTU from roots) are darker shades. OTUs are shown sorted by decreasing growth rates. Shown are means ± 1 S.E.M. OTU details are found in Table 2-1.



Appendix 3 Supplemental material for Chapter 3

Appendix 3-1: Estimation of pre-treatment dry weights in Chapter 3

We collected additional tillers to create a model to estimate dry weights of tillers prior to the experiment. We collected 15 tillers of Elymus mollis, Ammophila arenaria, and A. breviligulata as previously described in the Methods and recorded their wet weights, rhizome lengths, and heights. After drying at 60°C for 3 days, we recorded total dry weight. We then modeled the total dry weight as a function of species, wet weight, rhizome length, and height, and the interactions between species and the three measured variables ($R^2 = 0.904$). We used the model to predict pre-treatment dry weights of each tiller using the following formula:

Pre-treatment dry weight = -1.334 + 0 × height - 0.022 × rhizome length + 1.512 × log (wet biomass) + 0.334 × *A. breviligulata* + 0.834 × *E. mollis* + -0.004 × height × *A. breviligulata* + 0.012 × height × *E. mollis* + 0.019 × rhizome length × *A. breviligulata* + 0.058 × rhizome length × *E. mollis* + -0.156 × log (wet biomass) x *A. breviligulata* + -1.148 × log (wet biomass) × *E. mollis*

Appendix 3-2: Analysis of soil filtrates used in Chapter 3 Experiment 2

For each of the three soil filtrates, we analyzed total dissolved nitrogen content and fungal and bacterial communities.

Total Dissolved Nitrogen

We filtered each filtrate onto a 0.2µm filter using a vacuum pump in order to remove cells and any other debris. We analyzed total dissolved nitrogen using a Shimadzu TOC-L Analyzer (Shimadzu Corp., Kyoto, Japan). From the TDN concentration, we calculated that we applied approximately 0.035mg, 0.16mg, and 0.36mg of total N to individual pots in the control, foredune, and backdune filtrates, respectively. Based on these low numbers, the effect of nitrogen addition from the filtrates on plant growth is likely negligible.

Community Analysis

We used the filters from the previously described filtering technique to concentrate all organisms present in the inocula. We filtered 40mL of control inocula, and 20mL of the foredune and backdune inocula. A larger amount of control inocula was used to increase our chances of sequencing microbes. We extracted DNA using the Qiagen DNEasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and included a control extraction with no filter to account for any contamination present during the extraction or sequencing processes. We amplified the fungal ITS2 region using modified 5.8SR forward (Vilgalys & Hester 1990) and ITS4 reverse (White *et al.* 1990) primers with Illumina adapters, and we amplified the bacterial V4 region using modified 515F forward and 806R reverse primers (Caporaso *et al.* 2012). For both ITS2 and V4, we amplified DNA using Kapa HiFidelity Hot Start Polymerase (Kapa Biosystems, Inc., Wilmington, MA) (denaturing at 95°C for 5min, 25 cycles of 98°C for 20s, 55°C for 15s and

72°C for 1min). Following the first round of amplification, PCR products were diluted 1:100, and 5µL of the diluted product were used in the second round of amplification to add indices and flow cell adapters (10 cycles of the same PCR conditions). Pooled library was denatured with NaOH and diluted to 8pM in Illumina HT1 buffer with 10% PhiX. The library was sequenced with Illumina MiSeq Kit v3 with 600 cycles at the University of Minnesota Genomics Center.

Sequencing of the fungal ITS2 region yielded 368,252 raw reads, and bacterial V4 region yielded 229,433 raw reads. Samples were subsequently demultiplexed and quality filtered. We used cutadapt (Martin 2011) to remove the adapter sites from the ends of the reads, and Trimmomatic (Bolger *et al.* 2014) to trim low quality regions from the ends of the reads and remove reads <150bp. We used MOTHUR (Schloss *et al.* 2009) to remove remaining reads with ambiguous base pairs and QIIME (Caporaso *et al.* 2010) to concatenate fasta files. Reads were dereplicated, clustered at 97% and checked for chimeras using USEARCH (Edgar 2010).

Fungal ITS2 OTUs were screened against the UNITE database (Abarenkov *et al.* 2010), and bacterial V4 OTUs were similarly screened against the Greengenes database (DeSantis *et al.* 2006). OTUs that shared <60% similarity with a fungus or bacterium in the respective database were removed. Dereplicated OTUs were mapped on to all sequences, and were identified according to the UNITE database using QIIME. The control extraction showed 4 OTUs with greater than 10 reads for fungal ITS2 and 36 OTUs for bacterial V4, and all reads that mapped on to these OTUs were removed from the dataset. The final number of reads in the control, foredune, and backdune filtrates are shown in Table S1 and Table S2. We calculated raw OTU richness as the total number of OTU found in each filtrate, the rarefied richness as the expected

richness per 675 reads for fungal ITS2 and 2,225 reads for bacterial V4, and the extrapolated richness using the Chao estimator in the vegan package (Oksanen *et al.* 2013).

Fungal communities varied among the filtrate treatments. The backdune filtrate had the highest OTU richness in every metric, followed by the foredune filtrate, and then the control filtrate (Table S1). Sequencing captured most of the diversity, as evidenced by the similarity between the observed and Chao estimated OTU richness. The backdune filtrate had relatively more reads map onto the phylum Ascomycota reads than the foredune or control filtrate, and relatively fewer reads match fungi of unknown taxonomic placement (Figure S1). Interestingly, the foredune filtrate had a larger proportion of Glomeromycota, the phylum to which arbuscular mycorrhizal fungi belong.

Bacterial communities similarly increased in OTU richness from the control to the foredune to the backdune filtrates (Table S2). Proteobacteria dominated all three filtrates, particularly the control filtrate. The foredune filtrate had higher proportions of Actinobacteria and Bacteroidetes than the backdune filtrate, and the backdune filtrate had higher proportions of TM7, Verrucomicrobia, and Planctomycetes than the foredune filtrate.

Table 1 Fungal communities present in three experimental microbial filtrates used in the plant-soil feedback experiment. Raw OTU richness, rarefied OTU richness (rarefied to 675 reads), and Chao extrapolated richness shown for the control, foredune, and backdune filtrates. Up to 20 of the most abundant taxa (total reads/percentage of filtrate reads) are shown for each filtrate. Taxa were determined using the UNITE database (Abarenkov *et al.* 2010).

Filtrate	**Control**	**Foredune**	**Backdune**
Raw Richness	8	136	484
Rarefied Richness (675 reads)	8	79	207
Chao Extrapolated Richness	9	154	486
Total Reads	677	87058	123491
Most abundant taxa	uncultured fungus (335/49.5%)	uncultured fungus (21048/24.2%)	Rhizopogon occidentalis (7875/6.4%)
	uncultured fungus (146/21.6%)	uncultured fungus (8788/10.1%)	Penicillium novae-zeelandiae (7447/6%)
	Dioszegia hungarica (125/18.5%)	Pyronemataceae sp (8139/9.3%)	Oidiodendron sp (6502/5.3%)
	uncultured Malassezia (34/5%)	Agaricales sp (6127/7%)	Pleosporaceae sp (2887/2.3%)
	Geomyces auratus (31/4.6%)	Pyronemataceae sp (3805/4.4%)	Agaricomycetes sp (2669/2.2%)
		uncultured fungus (3109/3.6%)	Penicillium novae-zeelandiae (2328/1.9%)
		uncultured Rhizophydium (2558/2.9%)	uncultured fungus (2219/1.8%)
		uncultured Glomus (2512/2.9%)	Penicillium roseopurpureum (1987/1.6%)
		uncultured fungus (2253/2.6%)	Entophlyctis helioformis (1872/1.5%)
		Chytridiomycota sp (1997/2.3%)	Penicillium cairnsense (1815/1.5%)
		Pyronemataceae sp (1663/1.9%)	uncultured Chytridiomycota (1810/1.5%)
		Ceratocystiopsis brevicomis (1483/1.7%)	Umbelopsis sp (1670/1.4%)
		Pleosporaceae sp (1278/1.5%)	Chytridiomycota sp (1629/1.3%)
		uncultured fungus (1226/1.4%)	uncultured fungus (1339/1.1%)
		uncultured fungus (974/1.1%)	Pilidium concavum (1332/1.1%)
		Ciliophora sp (929/1.1%)	uncultured fungus (1332/1.1%)
		Botrytis caroliniana (841/1%)	Oidiodendron sp (1329/1.1%)

		Aspergillus calidoustus (699/0.8%)	Umbelopsis sp (1241/1%)
		Cantharellus decolorans (658/0.8%)	uncultured Helotiales (1208/1%)
		Pyronemataceae sp (607/0.7%)	Sordariomycetes sp (1035/0.8%)

Table 2 Bacterial communities present in three experimental microbial filtrates used in the plant-soil feedback experiment. Raw OTU richness, rarefied OTU richness (rarefied to 2225 reads), and Chao extrapolated richness shown for the control, foredune, and backdune filtrates. 20 of the most abundant taxa (total reads/percentage of filtrate reads) are shown for each filtrate. Taxa were determined using the Green Genes database (DeSantis *et al.* 2006).

Filtrate	Control	Foredune	Backdune
Raw Richness	58	1224	1723
Rarefied Richness (2225 reads)	58	524	854
Chao Extrapolated Richness	63	1259	1790
Total Reads	2229	83186	54373
Most abundant taxa	Comamonadaceae spp. (1223/54.9%)	Comamonadaceae spp. (2617/3.1%)	Sphingomonas spp. (699/1.3%)
	MLE1-12 spp. (146/6.6%)	Sphingomonadaceae spp. (2058/2.5%)	EW055 spp. (593/1.1%)
	Caulobacteraceae spp. (119/5.3%)	TM7-1 spp. (1924/2.3%)	Candidatus Rhabdochlamydia spp. (523/1%)
	Caulobacteraceae spp. (81/3.6%)	Sphingobacteriaceae spp. (1376/1.7%)	Sphingomonadaceae spp. (507/0.9%)
	Perlucidibaca spp. (58/2.6%)	Sphingomonadaceae spp. (1373/1.7%)	iii1-15 spp. (419/0.8%)
	Dietzia spp. (46/2.1%)	Flavisolibacter spp. (1363/1.6%)	Pseudomonas spp. (364/0.7%)
	Corynebacterium spp. (46/2.1%)	Kaistobacter spp. (1292/1.6%)	Oxalobacteraceae spp. (358/0.7%)
	Moraxellaceae spp. (40/1.8%)	Ellin6067 spp. (1220/1.5%)	Chlamydiales spp. (334/0.6%)
	Alphaproteobacteria spp. (36/1.6%)	Methylotenera mobilis (1129/1.4%)	TM7-1 spp. (323/0.6%)
	Alphaproteobacteria spp. (34/1.5%)	Kaistobacter spp. (1126/1.4%)	Xanthomonadaceae spp. (321/0.6%)
	Ellin6513 spp. (31/1.4%)	Flavobacterium spp. (1111/1.3%)	Coxiellaceae spp. (308/0.6%)
	Pseudomonas spp. (29/1.3%)	Kaistobacter spp. (1075/1.3%)	Candidatus Xiphinematobacter spp. (306/0.6%)
	Pedobacter spp. (27/1.2%)	Comamonadaceae spp. (1054/1.3%)	Rhodoplanes spp. (303/0.6%)
	Sphingomonas spp. (27/1.2%)	TM7-1 spp. (1044/1.3%)	TM7-1 spp. (296/0.5%)
	Sediminibacterium spp. (26/1.2%)	mb2424 spp. (1012/1.2%)	Sphingobacteriaceae spp. (285/0.5%)
	Actinomyces spp. (25/1.1%)	Rhodobacter spp. (1002/1.2%)	Acidimicrobiales spp. (284/0.5%)
	Lysobacter spp. (21/0.9%)	Comamonadaceae spp. (1002/1.2%)	Chitinophagaceae spp. (270/0.5%)
	Comamonadaceae spp. (16/0.7%)	Pseudomonas spp. (945/1.1%)	Janthinobacterium spp. (262/0.5%)
	Sinobacteraceae spp. (16/0.7%)	Comamonadaceae spp. (930/1.1%)	Oxalobacteraceae spp. (258/0.5%)

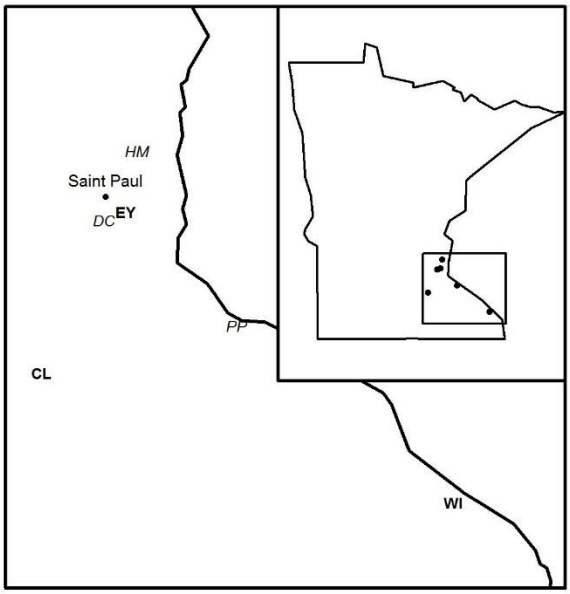
	Friedmanniella spp. (15/0.7%)	Micrococcaceae spp. (882/1.1%)	Ellin329 spp. (257/0.5%)

Appendix 4 Supplemental material for Chapter 4

Appendix 4-1: Location of sampling sites. Historical grade was assigned as low herbivory (L) or high herbivory (H).

In the map, low historical herbivory sites are italicized, and high historical herbivory sites are bolded.

Site Name	Historical grade	Latitude	Longitude
Dodge Nature Center (DC)	L	44° 52' 51" N	93° 06' 22" W
Hall's Marsh (HM)	L	45° 05' 12" N	92° 58' 09" W
Pottery Pond (PP)	L	44° 33' 55" N	92° 32' 56" W
Circle Lake (CL)	H	44° 25' 28" N	93° 21' 56" W
Pig's Eye (EY)	H	44° 54' 18" N	93° 0' 51" W
Winona (WI)	H	44° 02' 18" N	91° 38' 59" W


HM
Saint Paul
DC
EY
PP
CL
WI

Appendix 4-2: Species accumulation curve for endophytes sampled in individual *Lythrum salicaria* plants for each site.

Solid lines show sites with historically low herbivory, dotted lines show sites with historically high herbivory. Gray line shows overall species accumulation curve across all sites.

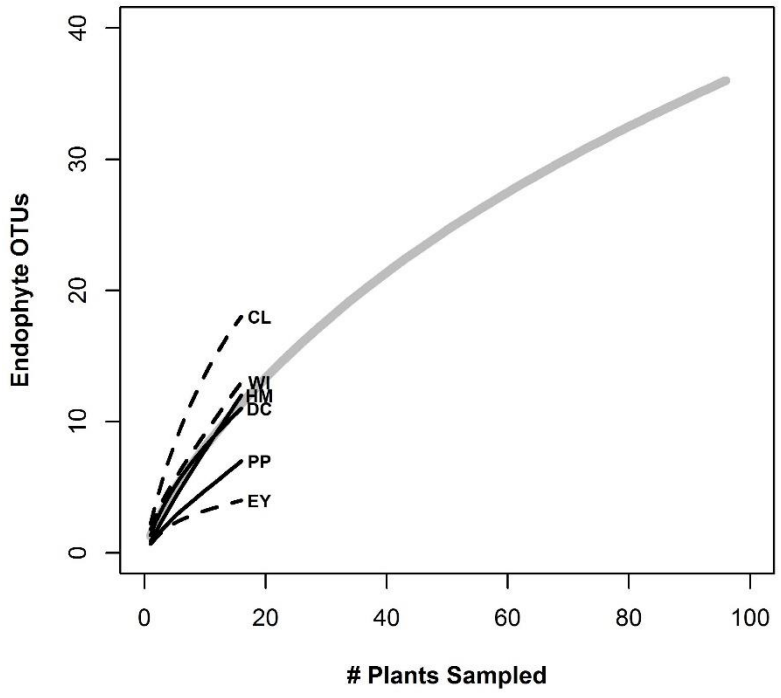


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Figure 1 Fungal taxonomic profiles using sequencing of the ITS2 region of the three microbial filtrates used in the plant-soil feedback experiment. The number in parentheses in the legend shows how many OTUs were found across all filtrates in each order or phylum.

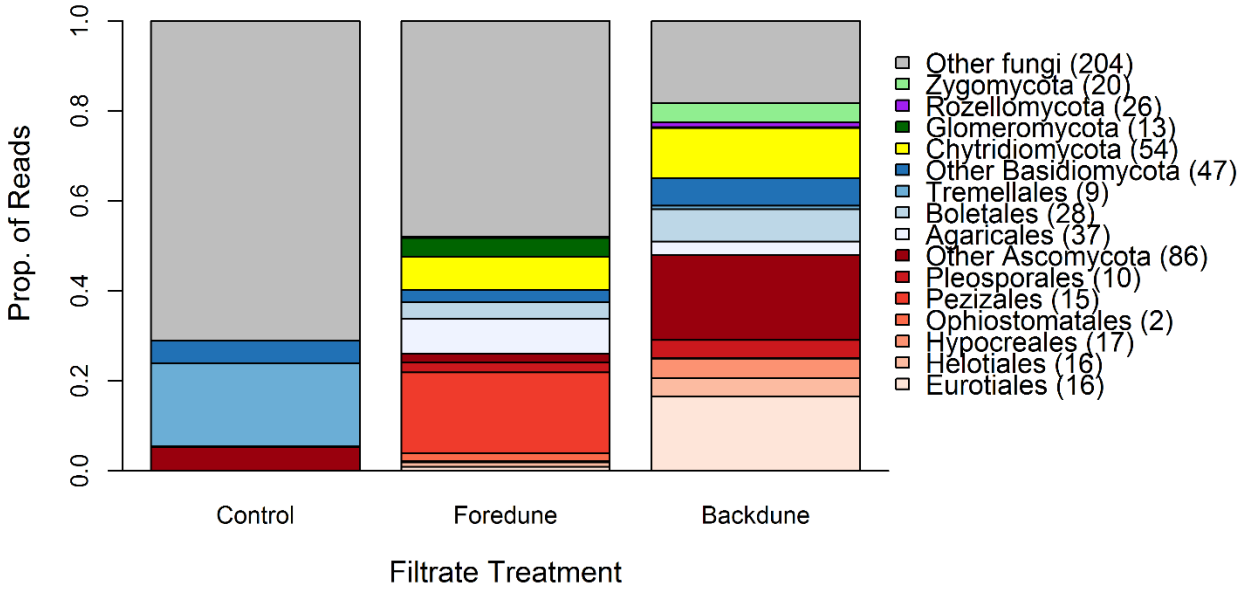

Fig 2 Bacterial taxonomic profiles using sequencing of the V4 region for the most common phyla of the three microbial filtrates used in the plant-soil feedback experiment. The number in parentheses in the legend shows how many OTUs were found across all filtrates in each phylum.