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Competitive avoidance not edaphic specialization drives vertical niche partitioning among sister species of ectomycorrhizal fungi

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Summary

- Soil depth partitioning is thought to promote the diversity of ectomycorrhizal (EM) fungal communities, but little is known about whether it is controlled by abiotic or biotic factors.
- In three bioassay experiments, we tested the role of vertical soil heterogeneity in determining the distributions and competitive outcomes of the EM sister species *Rhizopogon vinicolor* and *Rhizopogon vesiculosus*. We planted *Pseudotsuga menziesii* seedlings into soils that were either a homogenized mix of upper and lower depths or vertically stratified combinations mimicking natural field conditions.
- We found that both species colonized the upper or lower soil depths in the absence of competition, suggesting that their distributions were not limited by abiotic edaphic factors. In competition within homogeneous soils, *R. vesiculosus* completely excluded colonization by *R. vinicolor*, but *R. vinicolor* was able to persist when soils were stratified. The amount of colonization by *R. vinicolor* in the stratified soils was also significantly correlated with the number of multilocus genotypes present.
- Taken together, our findings suggest that the differential vertical distributions of *R. vinicolor* and *R. vesiculosus* in natural settings are probably attributable to competition rather than edaphic specialization, but that soil heterogeneity may play a key role in promoting EM fungal diversity.

Introduction

The niche represents a foundational concept in ecology (Hutchinson, 1957). It underpins much of the ecological theory about the maintenance of species diversity (Hutchinson, 1959; Chesson, 2000) as well as responses to changing environmental conditions (Clavel *et al.*, 2011). Ecologists have long distinguished the fundamental and the realized niche, with the former representing the full range of environmental conditions under which a species can persist and the latter being the actual conditions in which that species is found in nature (Begon *et al.*, 2005). The realized niche is typically considered a subset of the fundamental niche as a result of factors such as competition or predation, although for some mutualistic interactions, the realized niche may be larger than the fundamental niche of each species (Bruno *et al.*, 2003). Niche partitioning has frequently been invoked to explain the coexistence of species that utilize similar resources in a given environment (Schoener, 1974). While niche partitioning can apply to any species with overlapping resource requirements, more closely related species have long been thought to experience greater competition than those that are more distantly related because of phylogenetic constraints on traits involved in resource acquisition (Darwin, 1859). Observational and experimental work in many systems has confirmed that closely related species can often compete intensely for common resources (Begon *et al.*, 2005; Kennedy, 2010; Burns & Strauss, 2011) and that their spatial distributions reflect a minimization of overlap in fundamental niche space (Diamond, 1975; Davies *et al.*, 1998; Pickles *et al.*, 2010).

Similar to other groups of organisms, the high species diversity observed in many ectomycorrhizal (EM) fungal communities has been attributed to both spatial and temporal niche partitioning (Bruns, 1995; Koide *et al.*, 2007). For the former, many studies have found that the horizontal distributions of EM fungal species at local spatial scales are highly patchy (Horton & Bruns, 2001). Along with horizontal spatial variation, studies from a variety of different forests have indicated that EM fungal distributions vary vertically in soil as well (Taylor & Bruns, 1999; Dickie *et al.*, 2002; Rosling *et al.*, 2003; Genney *et al.*, 2006; Lindahl *et al.*, 2007; Anderson *et al.*, 2014; Taylor *et al.*, 2014). The consistent demonstration of shifts in species composition with depth suggests that vertical partitioning is common in EM fungal communities, but the mechanisms responsible for depth-related variation are not well understood (Dickie & Koide, 2014; Bahram *et al.*, 2015). It is possible that species may have specialized on different forms of nutrients, such as organic or inorganic nitrogen (Abuzinadah & Read, 1986), which change in relative abundance in different soil horizons (McKane *et al.*, 2002; Hobbie *et al.*,

2014). Alternatively, the vertical variation in EM fungal communities may not be attributable to resource heterogeneity, but rather to differential tolerances for environmental conditions. A number of abiotic conditions change with soil depth, such as temperature, water content, and oxygen availability, and many of these factors have been shown to significantly influence EM fungal community composition in analyses of horizontal distribution patterns (Erland & Taylor, 2002).

Along with changing abiotic resources and conditions, there are important differences in biotic factors with soil depth that may also play a role in driving the differential vertical distribution of EM fungi. It is widely recognized that the plant root density declines with increasing depth (Jackson *et al.*, 1996), and Peay *et al.* (2011) found evidence that EM fungal community composition was strongly influenced by horizontal gradients in rooting density, with long- and short-distance exploration type species (*sensu* Agerer, 2001) being more common in areas of lower and higher root densities, respectively. Although it has yet to be demonstrated for EM fungi, Newell (1984) showed that preferential invertebrate grazing significantly altered the vertical distribution of two saprotrophic fungal species. These results suggest that hyphal predation may also be an important factor influencing the vertical distributions of EM fungi. It is equally possible that vertical stratification in EM fungal communities could be the result of competitive avoidance, as multiple studies suggest that EM fungi strongly compete for soil nutrients and host root tip occupation (Kennedy, 2010). Given the wide range of possible mechanisms, experimental tests are needed to disentangle the relative importance of abiotic versus biotic factors in driving the vertical distribution patterns of EM fungi.

Most studies investigating the vertical distributions of EM fungi have taken a broad view by sampling the complete EM fungal community. However, recent research by Beiler *et al.* (2010, 2012) mapped the fine-scale spatial structure of EM root tips colonized by the sister species *Rhizopogon vinicolor* and *Rhizopogon vesiculosus*. Those studies found patterns of vertical depth differentiation when the two species co-occurred, with *R. vesiculosus* occupying deeper mean soil depths and *R. vinicolor* occupying shallower mean soil depths. These *Rhizopogon* species share a sympatric range within the Pacific Northwest of North America (Kretzer *et al.*, 2005; Beiler *et al.*, 2010, 2012; Dunham *et al.*, 2013), are obligate EM symbionts of a single host species, *Pseudotsuga menziesii* (Douglas-fir) (Molina *et al.*, 1999), and, when present at the same site, are often sampled as both EM root tips and sporocarps at nearly equal frequencies (Kretzer *et al.*, 2005; Beiler *et al.*, 2010; Dunham *et al.*, 2013). Both species produce long-distance exploration-type extramatrical hyphae, but the density of *R. vesiculosus* hyphae is greater than that of *R. vinicolor* at small scales (20 cm range). Specifically, Beiler *et al.* (2012) showed that hyphal aggregations of *R. vesiculosus* were up to two times larger in diameter than those produced by *R. vinicolor*. *Rhizopogon vesiculosus* also produces larger average genets (*c.* 18 m diameter) than *R. vinicolor* (*c.* 6 m diameter) (Kretzer *et al.*, 2005; Beiler *et al.*, 2010; Dunham *et al.*, 2013). Collectively, the larger clone size and more pervasive exploratory hyphae suggest that *R. vesiculosus* may be more efficient at colonizing root tips through vegetative expansion (although it may also possibly reflect an intrinsically longer life span), while *R. vinicolor* may be more reliant on spore-based colonization to maintain local persistence.

The sympatric distribution, close evolutionary relationship, and obligate reliance upon a single host species suggest that high levels of competition may be present between these two species. However, the relatively equal abundances of the two species at a single site suggest that these species may possess differences in their realized niches that minimize competitive interactions. The goal of the current study was to experimentally investigate the ecological factors driving the observed patterns of vertical depth differentiation for *R. vinicolor* and *R. vesiculosus*. We performed three related growth chamber seedling bioassay experiments designed to separate the effects of depth-associated edaphic factors, interspecific competition, and their interaction. The results focused on distinguishing between two hypotheses: the fundamental niches of *R. vinicolor* and *R. vesiculosus* do not overlap and resources and/or conditions at different soil depths specifically suit the growth of each species; the fundamental niches of *R. vinicolor* and *R. vesiculosus* overlap and their realized niches are determined through competitive interactions. Because of previous microsatellite-enabled research in this study system (Kretzer *et al.*, 2005; Beiler *et al.*, 2010), we were also interested in assessing how the number of genotypes of each *Rhizopogon* species influenced colonization dynamics when in competition.

Materials and Methods

Field site description

The soil and fungal inocula used in this study were collected at a field site on the north slope of Mary's Peak in the Oregon Coast Range, USA (44°32.1′N, 123°32.1′W; maximum elevation = 516 m). This site has previously been used in ecological and population genetic studies of *Rhizopogon vinicolor* Smith & Zeller and *Rhizopogon vesiculosus* Smith & Zeller and the two species are known to occur on this site at near equal frequencies (Kretzer *et al.*, 2005; Dunham *et al.*, 2013). Site conditions are described in detail in Dunham *et al.* (2013).

Soil collection and preparation

Rhizopogon vinicolor produces genets that are distributed at shallower soil depths (median sample depth = 4–6 cm) than those of *R. vesiculosus* (median sample depth = 8–13 cm) (Beiler *et al.*, 2012). In view of this *a priori* knowledge, we chose to sample soil according to depth rather than horizon. In spring 2012, we sampled soil from 11 locations, which spanned our collection site (samples being located *c.* 50 m apart). At each location, we harvested three soil cores (15 × 5 cm) spaced 0.5 m apart. Cores were divided into an upper depth (0–7.5 cm from surface) and a lower depth (7.5–15 cm from surface) at the time of harvest. Both parts of each core were individually passed through a 2-mm soil sieve (no. 10; Fisher Scientific Co., Pittsburg, PA, USA) to remove fine roots and then pooled by depth. To eliminate

resident EM inoculum, each set of pooled soils was autoclaved four times for 90 min at 20 psi and 121°C, with 24-h intervals at 25°C between autoclave sessions. A portion of sterilized upper depth soils was mixed in 1 : 1 ratio with sterilized lower depth soils to create a third soil type that was an equal blend of the two depths. Sterile soils of each type (upper, lower, or blended) were mixed in a 1 : 1 ratio with sterile coarse sand (Monterrey no. 3; Cemex, Marina, CA, USA) to improve soil aeration during the seedling bioassays.

EM fungal inoculum collection and preparation

Field collections of *Rhizopogon* sporocarps occurred between May and August 2011. Four mature sporocarps of both *R. vinicolor* and *R. vesiculosus* were used in preparing EM fungal inoculum. These sporocarps were chosen from collections separated by at least 20 m in the field to increase the probability that different fungal individuals produced them. The surface tissue of sporocarps was removed and a small section of gleba was subjected to DNA extraction for species identification. The remaining gleba tissue was submerged in 30 ml of deionized water (dH_2O), homogenized using an immersion blender, and filtered through cheesecloth to remove tissue fragments. The resulting spore slurry was brought up to a 50 ml volume and stored at 4°C. A total of eight slurries, each containing the spores of a single sporocarp, were created. Spores in each of the slurries were quantified using a hemocytometer (Hausser Scientific, Horsham, PA, USA) and slurries of each species were pooled in equal spore proportions to create a master inoculation solution for each species.

Seed preparation

Seeds of *Pseudotusga menziesii* (Mirb.) Franco were obtained from Silvaseed Company (Roy, WA, USA). To remove any endogenous EM fungal inoculum, seeds were surface-sterilized by being placed in a 10% hydrogen peroxide (H_2O_2) solution for 15 min and stirred at 400 rpm. Seeds were rinsed twice in dH_2O and soaked in fresh dH_2O for 24 h that was stirred at 200 rpm. Any seed that was found floating on the surface after soaking was discarded and the remaining seeds were prepared for germination by cold stratification. Seeds were wrapped in sterilized cheesecloth and soaked in dH_2O for another 24 h. Excess water was removed from the cheesecloth seed pouch by shaking. The seed pouch was then put into a ventilated plastic bag and incubated at 4°C for 28 d.

Planting set-up

Pseudotsuga menziesii seedlings were grown in 'Cone-tainer' planting cells (SC7 stubby; Stuewe and Sons, Tangent, OR, USA). The bottom of each 15-cm-deep planting cell was lined with a small volume of synthetic pillow stuffing (*c.* 3 cm in depth to retain the soils but still provide drainage) and then filled with 80 ml of soil. Initially, three cold-stratified *P. menziesii* seeds were placed on top of the soil and each cell was topped with 10 ml of autoclaved coarse sand (to reduce the chance of cross-contamination during watering). Seedlings were inoculated by premixing *Rhizopogon* spore slurries into each treatment. Each cell received a total of 1×10^7 spores from either one *Rhizopogon* species or both species as a dual inoculation, with each treatment being replicated 10 times.

Experimental design

The study was comprised of three experiments (Fig. 1). Experiment 1 used single species inoculations to determine the role that edaphic properties play in determining the fundamental niches of both *Rhizopogon* species. Experiment 1 contained four treatments; hereafter, referenced as treatments 1.1, 1.2, 1.3 and 1.4. In treatments 1.1 and 1.3, the soil in each cell was layered to mimic natural conditions, with 40 ml of soil from the lower depth (i.e. 7.5–15 cm) at the bottom of the cell and 40 ml of soil from the upper depth (i.e. 0–7.5 cm) at the top of the cell. With the small addition of pillow filling, the depths of soils in the planting cell were close but not perfectly matched with their position in the field (i.e. the 0–7.5 cm field soil was present in the top 6 cm of the planting cell and the 7.5–15 cm field soil was present in the bottom 6 cm of the planting cell). In treatment 1.1, *R. vinicolor* was inoculated into the upper 40 ml of soil and the lower depth soil was not inoculated (matching the higher vertical position of this species in natural settings). In treatment 1.3, *R. vesiculosus* was inoculated into the lower 40 ml of soil and the upper depth was not inoculated (matching the lower vertical position of this species in natural settings). Treatments 1.2 and 1.4 were designed to assess whether certain properties of the soils collected from the upper or lower depths were required for EM fungal

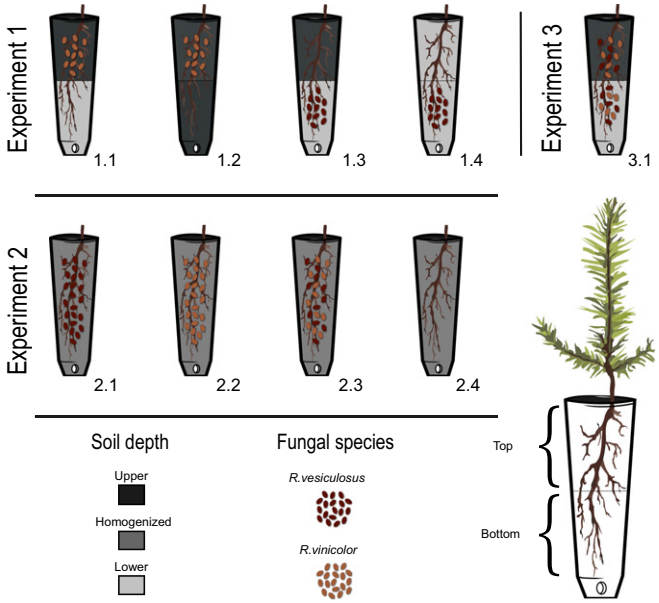

Fig. 1 Design of the three experiments testing the relative importance of abiotic and biotic factors in determining the differential vertical soil distributions of *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* when grown on *Pseudotsuga menziesii* seedlings.

colonization at depths where each species was less typically observed in nature. Treatment 1.2 matched treatment 1.1 except that the entire cell was inoculated with soils from the upper depth (i.e. all soils were from 0–7.5 cm, with *R. vinicolor* spores only being inoculated in the upper 40 ml of soil). Similarly, treatment 1.4 matched treatment 1.3 except that the entire cell was inoculated with soils from the lower depth (i.e. all soils were from 7.5–15 cm, with *R. vesiculosus* spores only being inoculated in the lower 40 ml of soil). Experiment 2 was conducted to determine how interspecific competition apart from edaphic variation may delineate the realized niche of these *Rhizopogon* species. As such, the soils used in the second experiment in both the single- and two-species treatments were a blended mix of upper and lower depth soils. The spore inoculum from each species was mixed into the soil throughout the cell (no position-specific inoculation and/or layering as in experiment 1). Treatment 2.1 contained EM fungal inoculum of only *R. vesiculosus*, treatment 2.2 contained EM fungal inoculum of only *R. vinicolor*, and treatment 2.3 contained EM fungal inoculum of both species. A negative control, treatment 2.4, was included to ensure that endogenous EM fungal inoculum in field soil was eliminated by autoclaving and that exogenous EM spores were not present in the growth chamber where the seedlings were grown. Experiment 3 was designed to investigate the competitive behavior of the two *Rhizopogon* species in conditions more representative of those encountered in the field compared with experiment 2. Specifically, it combined both soil stratification (mimicking natural conditions) and a two-species inoculation at both soil depths. For this experiment, spores of both species were first inoculated into upper and lower depth soils separately. Then 40 ml of inoculated lower depth soil was added to each cell followed by 40 ml of inoculated upper depth soil layered on top. This final experiment contained one treatment; hereafter, referred to as treatment 3.1.

Growing conditions

All three experiments were established on 21–22 May 2012 and placed in an AL-66 growth chamber (Percival Scientific, Perry, IA, USA) set at 14 h 20°C: 10 h 12°C, day: night cycle. Growth cells were kept evenly moist by hand watering for 3 wk, after which each replicate was thinned to a single seedling and then watered every 3 d. Throughout the experiment, cells were periodically rotated within the growth chamber to eliminate effects associated with internal environmental heterogeneity.

Harvest protocol

All three experiments were harvested on 13–14 December 2012. Each seedling was cut at the soil surface. Soil from each replicate cell was physically divided into two portions, upper and lower, where each portion consisted of 40 ml of soil. Soils from the upper and lower depths of each replicate seedling were combined within a treatment (e.g. all the upper depth soils from all the replicates in treatment 1.1 were combined into one plastic bag and all the lower depth soils from all the replicates in treatment 1.1 were combined into a different plastic bag) and stored at 4°C until further processing. Roots from the upper and lower soil depths of each seedling were rinsed in tap water and stored in dH_2O at 4°C for root-tip processing. Using a dissecting microscope, all live EM root tips were removed from each half of the seedling's root system (hereafter referred to as top and bottom, respectively) and placed into 1.5-ml tubes. Tubes were flash-frozen in liquid nitrogen and then lyophilized. The remaining portion of fine roots (< 2 mm diameter) for each portion of the seedling's root system (i.e. top and bottom) was separated and individually oven-dried at 60°C for 48 h (we avoided including all roots > 2 mm diameter because of their lack of receptivity to EM fungal colonization). All samples were individually weighed and colonization by EM fungi was calculated as (EM root biomass/(EM root biomass + non-EM fine-root biomass)) × 100. Given the identical morphologies of EM root tips of these two species, this biomass-based approach provides for a standard metric for comparisons across species. To avoid confusion with counting-based methods, we refer to the amount of seedling colonization as percentage EM root biomass, not percentage EM colonization. Seedling root density was calculated by dividing the total dry fine-root mass (mg) by the volume (ml) of soil sampled.

EM fungal molecular identification

DNA was extracted from EM root tips using the 'dilution protocol' of the PHIRE Plant Direct PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. DNA was extracted from sporocarp tissue using the fastDNA kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's protocol. PCR of all extractions was performed using the PHIRE Plant Direct PCR Kit following the manufacturer's protocol. The species identities of root tips and sporocarps were confirmed by amplifying the internal transcribed spacer (ITS) rDNA region using the primers ITS1F and ITS4 (Gardes & Bruns, 1993) and then digesting PCR products using the enzyme *Alu*I. Restriction digests were visualized and scored directly on 2% agarose gels stained with ethidium bromide. Four EM root tips (two tips from the top and bottom portions of the seedling root system, respectively) were identified to species from each replicate of treatments 1.1, 1.2, 1.3, 1.4, 2.1 and 2.2. Twenty root tips (10 tips from the top and bottom portions of the root system, respectively) were identified to species from each replicate of treatments 2.3 and 3.1. In addition, six microsatellite loci were used to identify multilocus genotypes (MLGs) of *R. vinicolor* (Rv15, Rv46, Rv53, Rve3.21, Rve2.77 and Rve1.34) and five microsatellite loci were used to identify MLGs of *R. vesiculosus* (Rve2.10, Rve2.14, Rv02, Rve1.34 and Rve2.44) (Kretzer *et al.*, 2005) in treatments 2.1, 2.2 and 3.1. Genotyping of EM root tips was performed for 10 root tips (five tips from the top and bottom portions of the root system, respectively) from each replicate. As a consequence of some mortality of seedlings during the experiments (14 of the total of 90 replicates were lost,

with mortality being relatively evenly scattered across treatments; Table 1), only 541 of the 820 possible root tips were sampled and subsequently identified.

Soil parameter measurement

We assessed carbon (C), nitrogen (N), and pH for both the upper (0–7.5 cm) and lower (7.5–15 cm) depth soils (post-autoclaving). Total soil C and N (as a percentage by mass) were measured at the Crop and Soil Central Analytical Laboratory at Oregon State University using a CNS-2000 Macro Analyzer (Leco Corp., St Joseph, MI, USA). Measurement of soil pH was conducted by suspending soil in sterile distilled water (dH_2O) in a 1 : 2 w/v ratio (soil : dH_2O). The mixture was shaken thoroughly and incubated at room temperature for 20 min. A symphony pH meter (VWR, Radnor, PA, USA) and an Orion Ross pH electrode (ThermoFisher Scientific, Waltham, MA, USA) were used to measure pH. Soil C and N were measured at the beginning and end of the experiment, with the pre-experiment analysis having three upper and three lower soil depth replicates and the post-experiment analysis having two upper and four lower soil depth replicates ($n = 12$). Soil pH was measured from the upper and lower soil depths at the beginning of the experiment only ($n = 6$ per depth; $n = 12$).

Statistical analyses

Differences between soil depths (upper versus lower) and times (pre- versus postexperiment) for total C and N were determined using a two-way ANOVA. Differences in soil pHs were analysed using a two-sample *t*-test. Differences in seedling root densities and percentage EM root biomasses for all treatments and positions (i.e. top vs bottom) combinations were analysed across the three experiments using one-way ANOVAs. Post-hoc Tukey tests were used to determine significant differences among treatment means. Differences between the abundances of *R. vinicolor* and *R. vesiculosus* in the two-species treatments (2.3, 3.1) were compared using chi-square analyses (with a Yates correction) for both the top and the bottom portions of seedling roots. For those analyses, we interpreted interspecific competition to be occurring if the colonization patterns differed significantly from a 1 : 1 ratio. While this ratio is conservative, it is based on previous similar studies of competition among other *Rhizopogon* species, which have typically found that competitively superior species have equivalent amounts of colonization in single- and two-species treatments and competitively inferior species have significantly lower colonization in two- than single-species treatments (Kennedy, 2010). In the MLG analyses, the probability of observing a given MLG was calculated as the expected frequency of that genotype given all potential pairings of alleles from parent sporocarps using Punnett squares. As neither *R. vinicolor* nor *R. vesiculosus* is known to produce asexual propagules or possess a high rate of secondary homothallism (Dunham *et al.*, 2013), we assumed allelic recombination in the F_1 populations occurred by crossing monokaryotic hyphae germinated from individual basidiospores. Differences in the frequency of MLGs of the two species across replicates were analysed using Wilcoxon rank sum tests. Differences in the expected heterozygosity (Nei, 1978) and evenness of MLGs on seedlings colonized by both *R. vinicolor* and *R. vesiculosus* were analysed with two-sample *t*-tests. Finally, differences in the relationship between the number of MLGs and the percentage of EM root biomass of each species in treatment 3.1 were analysed using simple linear regressions. As MLGs of *R. vesiculosus* were present on all replicates at both depths in treatment 3.1 but MLGs of *R. vinicolor* were only present on some replicates at either depth, we randomly selected an equivalent number of *R. vesiculosus* replicates to match those of *R. vinicolor* ($n = 8$). All statistics were run using R (www.r-project.org) and considered significant at $P < 0.05$.

Table 1 Ectomycorrhizal root biomass (%) on *Pseudotsuga menziesii* seedlings by treatment and position across the three experiments

Experiment	Treatment	Position	*n*	Mean ± SE	Significance
1	1	Top	7	35.20 ± 4.88	abc
1	1	Bottom	7	13.83 ± 3.52	bc
1	2	Top	8	23.36 ± 7.40	abc
1	2	Bottom	8	22.59 ± 4.60	abc
1	3	Top	8	27.42 ± 7.17	abc
1	3	Bottom	8	18.71 ± 2.74	ab
1	4	Top	10	27.85 ± 5.79	abc
1	4	Bottom	10	16.88 ± 3.55	ab
2	1	Top	8	34.80 ± 5.26	abc
2	1	Bottom	8	20.29 ± 5.62	ab
2	2	Top	6	38.96 ± 4.62	abc
2	2	Bottom	6	25.29 ± 4.83	ab
2	3	Top	9	45.94 ± 5.85	a
2	3	Bottom	9	32.02 ± 4.66	abc
3	1	Top	9	21.66 ± 5.96	abc
3	1	Bottom	9	9.65 ± 3.08	c

n, seedling replicates harvested within each treatment (differences as a result of mortality during the experiments). Position represents roots located in the upper 40 ml of soil and lower 40 ml of soil. Values with different letters represent significant differences based on a Tukey HSD test.

Results

Soil parameters, root density, and percentage EM root biomass

Soils from the upper depths (0–7.5 cm) had significantly higher total C and N contents than those from the lower depths (7.5–15 cm) (C by depth: $F_{1,8} = 15.74$; $P = 0.004$; N by depth: $F_{1,8} = 5.96$; $P = 0.041$) (Supporting Information Table S1). These patterns held in both the pre- and post-experiment samples (C by time and N by time; both $P > 0.05$), suggesting that soil C and N concentrations stayed relatively constant by depth within treatments. Although the overall range of soil pH was small (5.04–5.42), it varied significantly by depth ($t = -28.32$; $P < 0.001$), being consistently higher in the lower depth soils. Seedling root density was variable both within and between experimental treatments, ranging from 0.5 to 3.77 mg ml^{-1} across all replicates (Table S2). There was, however, no significant difference in root density between the top and bottom

positions of seedling roots in any of the treatments except in treatment 1.2, which showed significantly increased root density in the bottom position (Tukey test; $P < 0.05$). Like seedling root density, seedling percentage EM root biomass also varied considerably across replicates in the three experiments, ranging from 0 to 72%. Although there was a significant difference in percentage EM root biomass across all positions and treatments ($F_{15,114} = 3.45$; $P < 0.001$), within each experiment, differences among the top and bottom positions of the seedling roots in all treatments were not significant (Tukey tests; $P > 0.05$; Table 1).

EM species abundance

All single-species treatments (treatments 1.1–1.4, 2.1 and 2.2) were colonized only by the species used as inoculum and no EM fungal colonization was observed on all of the negative control seedlings (treatment 2.4) throughout the duration of the experiment. In both of the two-species treatments (treatments 2.3 and 3.1), *R. vesiculosus* was the most prevalent species detected on EM root tips. In experiment 2, where soils were a blend of upper and lower depths, *R. vesiculosus* was the complete competitive superior, preventing any colonization by *R. vinicolor* (Fig. 2). This colonization pattern was significantly different from a 1 : 1 ratio in both parts of the seedling roots (top: $\chi^2 = 90$; $P < 0.001$; bottom: $\chi^2 = 90$; $P < 0.001$). In experiment 3, where upper and lower depth soils were layered to mimic natural positions, *R. vesiculosus* was still the dominant competitor at both positions (top: $\chi^2 = 36.89$; $P < 0.001$; bottom: $\chi^2 = 36.04$; $P < 0.001$), representing 83% of all the EM root tips sampled (Fig. 3). However, *R. vinicolor* was found to represent 0–59% of the percentage EM biomass depending on the individual seedling replicate. No clear pattern of vertical depth partitioning was observed, as there was no significant difference in the mean percentage EM root biomass of each species between the top and bottom portions of the seedling roots (*R. vinicolor*: $t = 0.84$; $P = 0.791$; *R. vesiculosus*: $t = 1.45$; $P = 0.913$).

EM genotype occurrence

A total of four and three unique parental MLGs were included in the experiment for *R. vinicolor* and *R. vesiculosus*, respectively. The parent sporocarps AM-OR11-051 (*R. vinicolor*) and AM-OR11-058 (*R. vesiculosus*) possessed unique alleles at the loci Rv 53 and Rve 1.21, respectively, that were both completely absent from samples of the F_1 EM root-tip generation. This latter result suggests that these two parent sporocarps probably did not contribute alleles in mating and EM colonization. Of the 78 *R. vinicolor* and 154 *R. vesiculosus* EM root tips genotyped, a total of 28 unique MLGs were detected for *R. vinicolor* and eight were detected for *R. vesiculosus*. Based on both a lower number of parental strains and lower allelic heterozygosity for *R. vesiculosus* (Table S3), this represented 80% of the total possible genetic diversity for *R. vesiculosus*, but only 9% of the total possible genetic diversity for *R. vinicolor*. Despite the greater overall richness of *R. vinicolor* MLGs, there was no significant difference in the mean genotypic diversity ($t = 0.884$; $P = 0.398$) or evenness ($t = -0.636$; $P = 0.535$) of either species among individual seedling replicates (Table S3). The frequency of occurrence of MLGs across replicates, however, varied significantly between species ($W = 31.5$; $P = 0.020$) (W is the test statistic calculated for this test), with *R. vesiculosus* having seven MLGs (87.5% of the detected MLGs) present on multiple replicates (with an

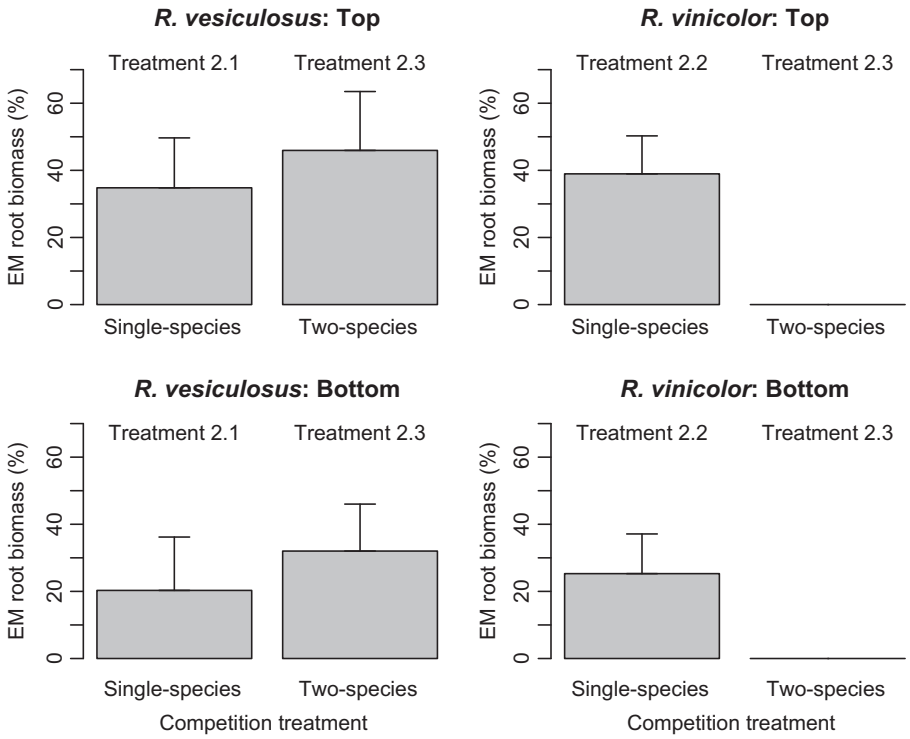

Fig. 2 Colonization patterns (% ectomycorrhizal (EM) root biomass) of *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* on *Pseudotsuga menziesii* seedlings in single- and two-species treatments in experiment 2. 'Top' and 'Bottom' refer to roots located in the top 40 ml of soil and bottom 40 ml of soil, respectively. Errors bars represent ± 1 SD.

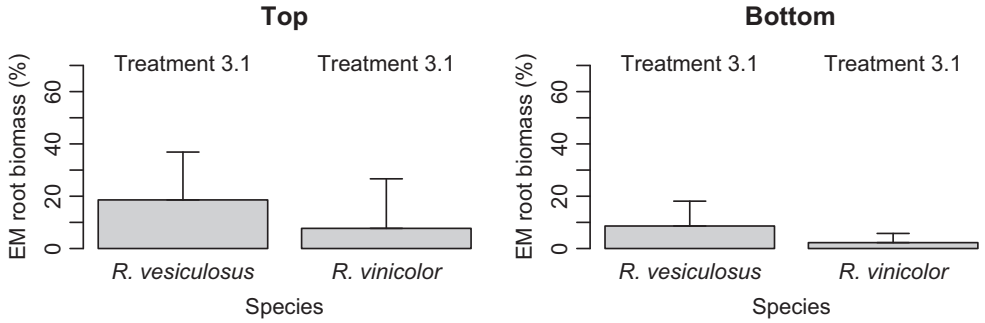

Fig. 3 Colonization patterns (% ectomycorrhial (EM) root biomass) of *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* on *Pseudotsuga menziesii* seedlings in experiment 3. 'Top' and 'Bottom' refer to roots located in the top 40 ml of soil and bottom 40 ml of soil, respectively. Errors bars represent ± 1 SD.

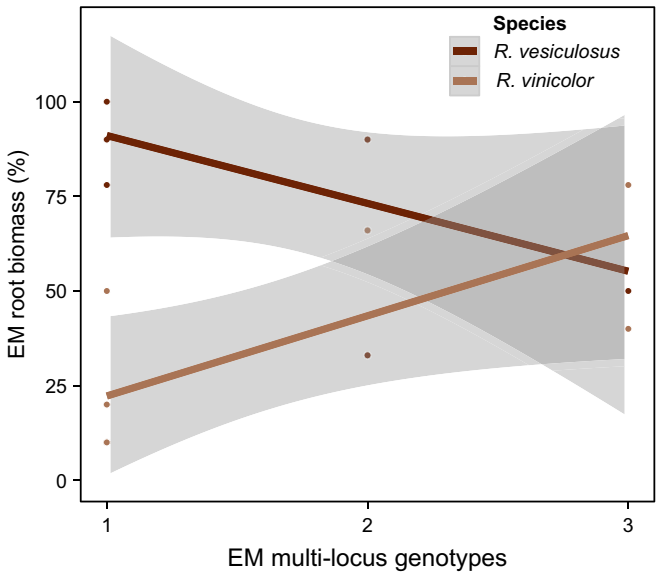

Fig. 4 Proportion of percentage ectomycorrhizal (EM) root biomass by the number of multilocus genotypes (MLGs) for *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* in experiment 3. Grey areas represent 95% confidence intervals.

average of 6.6 repeated detections of the same MLG from independent replicates) and *R. vinicolor* having only five MLGs (17.9% of the detected MLGs) present in multiple replicates (with an average of 2.2 repeated detections of the same MLG from independent replicates). On seedlings colonized by both species in treatment 3.1, there was a significant positive linear relationship between the number of *R. vinicolor* MLGs and the total percentage of EM root biomass it occupied ($r^2 = 0.512$; $P = 0.046$; Fig. 4). By contrast, there was no significant relationship between the number of *R. vesiculosus* MLGs present and the total percentage of EM root biomass it occupied ($r^2 = 0.291$; $P = 0.167$).

Discussion

Our experimental results indicate that the fundamental niches of *R. vinicolor* and *R. vesiculosus* do not appear to be significantly different. This was best evidenced in experiment 1, where both species were able to colonize the upper and lower soil depths in the absence of a competitor. In treatments 1.1 and 1.3, the nutrients and pH in soils differed significantly between the upper and lower depths, being higher in C and N content and lower in pH in the upper depth. Because *R. vinicolor* and *R. vesiculosus* had equivalent percentage EM root biomasses at both depths, these results indicate that the vertical distributions of these two species are not strongly dependent on resource availability (at least for total C and N, and see further discussion of autoclaving below) or soil pH (across the limited range present in this experiment). In treatments 1.2 and 1.4, the soils in the upper and lower positions of each planting cell were identical in terms of nutrient availability and pH, so any differences between positions would be related to changes in environmental conditions. Although we did not specifically measure environmental conditions in this experiment, the equivalent colonization of *R. vinicolor* and *R. vesiculosus* between depths in treatments 1.2 and 1.4 suggests that differences in abiotic conditions are also probably not a primary determinant of the vertical partitioning of these two species. The colonization patterns we observed in experiment 1 were similar to the field study of Beiler *et al.* (2012), who found that, when *R. vesiculosus* was absent from lower soil depths, *R. vinicolor* was able to colonize roots deeper in the soil profile. Similarly, *R. vesiculosus*, while consistently more prevalent at lower soil depths, was also able to successfully colonize roots in upper soil depths. Taken together, the differential vertical distributions of *R. vinicolor* and *R. vesiculosus* observed in both natural and laboratory settings are not consistent with strong edaphic specialization.

In comparison to experiment 1, the results from experiment 2 suggest that the differential vertical distributions of these *Rhizopogon* species may be the product of competitive avoidance. In our second experiment, *R. vinicolor* and *R. vesiculosus* had comparable amounts of colonization in the single-species treatments (2.1 and 2.2), but in the two-species treatment (2.3), *R. vinicolor* was completely absent. This result differed from previous studies of other *Rhizopogon* species, which have observed strong competitive effects but not complete exclusion (Kennedy & Bruns, 2005; Kennedy *et al.*, 2007a,b). It is possible that the greater strength of the negative interaction between *R. vinicolor* and *R. vesiculosus* may be attributable to the close evolutionary relationship of these species. Other studies have demonstrated that more closely

related species can compete more strongly than those that are more distantly related (Slingsby & Verboom, 2006; Burns and Strauss, 2011). However, the results of a number of recent studies, including one on EM fungi, have shown that phylogenetic relatedness does not drive the magnitude of competitive interactions (Cahill *et al.*, 2008; Fritschie *et al.*, 2014; Kennedy *et al.*, 2014; Venail *et al.*, 2014). Those findings suggest this phylogeny-based explanation may not be as strong as initially thought. It is also possible that the strength of the competitive effect observed in experiment 2 was a result of differences in the mode of root-tip colonization by *R. vesiculosus* and *R. vinicolor*. The significantly greater recovery of the same MLG of *R. vesiculosus* on different root tips from a single seedling compared with *R. vinicolor* is consistent with individuals of the former species spreading more rapidly via mycelium. Similarly, Beiler *et al.* (2012) demonstrated that densities of hyphal aggregations of *R. vesiculosus* were consistently higher than those of *R. vinicolor*. If the competitive interactions among these species are driven mainly by rapid spread to new root tips by *R. vesiculosus* and some active takeover of tips colonized by *R. vinicolor*, competitive exclusion would be a likely outcome. Support for this alternative explanation comes from similar seedling-based studies showing that differential rates of mycelial spread and active takeover events can both be important determinants of EM fungal competitive outcomes in laboratory settings (Wu *et al.*, 1999; Kennedy *et al.*, 2011).

In experiment 3, we found that two-thirds of the seedling replicates were colonized by both *R. vinicolor* and *R. vesiculosus*. The difference between this result and the complete exclusion of *R. vinicolor* in the two-species treatment in experiment 2 suggests that soil environmental heterogeneity may play a central role in promoting competitive coexistence among these species. Environmental heterogeneity has long been considered a key factor in the maintenance of diversity in ecological communities (Hutchinson, 1959), particularly for species with high overlap in fundamental niche space (Begon *et al.*, 2005). The mechanism by which vertical soil heterogeneity promotes coexistence between *R. vinicolor* and *R. vesiculosus* is not yet clear, as experiment 1 indicated that the two species can perform equally well in soils from upper and lower depths. Our MLG analyses suggest that greater soil variation may facilitate more independent root-tip colonization events by *R. vinicolor*. Priority effects have been demonstrated to be an important factor determining competitive outcomes among several other *Rhizopogon* species (Kennedy & Bruns, 2005; Kennedy *et al.*, 2009) and our observed correlation between colonization and MLGs in *R. vinicolor* is consistent with an increasing strength of priority effects through the colonization of a greater proportion of available root tips. Alternatively, if the competitive dynamics between these two species is based on active root-tip takeover, heterogeneous soil conditions may slow the growth of *R. vesiculosus* sufficiently to allow *R. vinicolor* to persist. This persistence, however, may only be temporary and it is entirely possible that, had experiment 3 been run for a longer time period, it would have had the same result as experiment 2 (i.e. exclusion of *R. vinicolor*). Given this possibility, additional work is clearly needed to better understand the specific extent to which soil heterogeneity promotes EM fungal coexistence.

Compared with field distributions of these two *Rhizopogon* species, we did see some important differences in our results, particularly in experiment 3. In that experiment, we found that *R. vinicolor* was able to successfully colonize lower depths when *R. vesiculosus* was also present, which was never observed by Beiler *et al.* (2012). We believe that this may be an artifact of using planting cells, which have a small volume and make root density higher than is typically seen in field conditions. Kennedy *et al.* (2007b) observed that the outcome of competition in similar-sized planting cells favored the weaker EM fungal competitor, which parallels the results obtained here. The reason for the general exclusion of *R. vinicolor* at depth in the field may also be related to preferential growth of *R. vesiculosus* at lower depths, where water availability is probably higher during dry summer months (Beiler *et al.*, 2012). The absence of any water stress in our study probably minimized any preferential growth patterns associated with depth, but field-based studies monitoring both water availability and species distributions with depth are needed to better test this possible explanation. Along with these caveats regarding experiment 3, we recognize that many aspects of our growth chamber-based seedling bioassays do not reflect the environmental heterogeneity that would probably influence coexistence between these species in field settings. Larger gradients in biotic factors such as root density and tree age as well as abiotic factors such as soil pH, which were relatively homogenous within treatments in our experiments, have been shown to influence both EM fungal composition (Twieg *et al.*, 2007; Peay *et al.*, 2011) and competitive interactions (Mahmood, 2003) and may dampen the competitive dominance of *R. vesiculosus* in natural settings. Additionally, interactions with other members of the EM fungal community in *P. menziesii* forests could also affect the competitive dynamics between these two species (Twieg *et al.*, 2007). With specific regard to experiment 1, our use of autoclaved soils probably altered differences between upper and lower depth soils in terms of both the forms and amounts of C and N compared with what would be present in natural settings. This greater homogeneity may have allowed both species to more effectively colonize both depths, so caution should be applied in minimizing the role that nutrient gradients may play in delimiting EM fundamental niche space. Finally, while our MLG results suggest that genotypic diversity may play a role in determining competitive outcomes among EM fungi, additional experimentation is needed with more genetically variable parent populations.

The recent study by Taylor *et al.* (2014) demonstrated that vertical depth partitioning among closely related species was a common phenomenon across multiple EM fungal lineages and those authors concluded that the hyperdiverse nature of their study system was probably driven by soil-mediated niche partitioning. Their clear demonstration of preferential colonization of different soil depths by closely related EM fungi fulfills one of the four criteria necessary to demonstrate the importance of niche partitioning in promoting species coexistence (Silvertown, 2004).

Our results, while limited to just two species, also fulfill a second criterion; niche shifts in response to experimental manipulation of competitors. The third test involves demonstrating tradeoffs in performance across niche axes, and the well-documented variation among EM fungal species in the ability to use organic versus inorganic forms of N, for example, satisfies this criterion (Koide *et al.*, 2011, and references therein). Knowledge about the fourth test, which necessitates that intraspecific competition is stronger than interspecific competition, is currently lacking for EM fungi. It appears, however, that the local spatial distributions of EM fungal species, both horizontally and vertically, probably reflect classic explanations of community coexistence based on niche partitioning. Determining the spatial scale at which EM fungal community richness patterns transition from those based on niche partitioning to other factors such as dispersal limitation or climatic effects (Talbot *et al.*, 2014; Tedersoo *et al.*, 2014) is an important future research direction.

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Author contributions

P.G.K., A.B.M., D.M.D., and J.W.S. designed the research, A.B.M. conducted the fieldwork, A.B.M. and P.G.K. executed the experiments, P.G.K., A.B.M., D.M.D., and J.W.S. harvested the experiments, A.B.M. conducted the molecular analyses, P.G.K., A.B.M., and J.W.S. analyzed the data, and P.G.K. wrote the manuscript and produced the figures and tables, with edits from A.B.M., D.M.D., and J.W.S.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Total carbon (% by mass), total nitrogen (% by mass), and pH for the autoclaved soils used in the three experiments

Table S2 *Pseudotsuga menziesii* seedling root density (g dry biomass ml^{-1} soil) from top and bottom portions of roots in each treatment in the three experiments

Table S3 Allelic diversity by microsatellite locus for *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* sporocarps and ectomycorrhizal root tips

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