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Measuring ectomycorrhizal fungal dispersal: macroecological patterns driven by microscopic propagules

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Abstract

Dispersal plays a prominent role in most conceptual models of community assembly. However, direct measurement of dispersal across a whole community is difficult at ecologically relevant spatial scales. For cryptic organisms, such as fungi and bacteria, the scale and importance of dispersal limitation has become a major point of debate. We use an experimental island biogeographic approach to measure the effects of dispersal limitation on the ecological dynamics of an important group of plant symbionts, ectomycorrhizal fungi. We manipulated the isolation of uncolonized host seedlings across a natural landscape and used a range of molecular techniques to measure the dispersal rates of ectomycorrhizal propagules and host colonization. Some species were prolific dispersers, producing annual spore loads on the order of trillions of spores per km^2. However, fungal propagules reaching host seedlings decreased rapidly with increasing distance from potential spore sources, causing a concomitant reduction in ectomycorrhizal species richness, host colonization and host biomass. There were also strong differences in dispersal ability across species, which correlated well with the predictable composition of ectomycorrhizal communities associated with establishing pine forest. The use of molecular tools to measure whole community dispersal provides a direct confirmation for a key mechanism underlying island biogeography theory and has the potential to make microbial systems a model for understanding the role of dispersal in ecological theory.

Keywords: biogeography, dispersal limitation, historical contingency, islands, macroecology, metacommunities, mutualism, *Pinus muricata*, succession

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Introduction

Dispersal of reproductive units across space and time is a fundamental biological challenge for all organisms and determines the emergent properties of many natural systems (Levin *et al.* 2003). As dispersal is both an ecological strategy and a mechanism for gene flow, it provides a link between multiple scales of biological organization. For example, at the ecological scale, differences between species in dispersal ability may give rise to successional dynamics; at the population scale, restricted gene flow may give rise to local adaptation; and at the evolutionary scale, dispersal barriers and vicariance give rise to biogeographic realms.

At the ecological scale, theoretical and empirical work suggest that the degree of dispersal–connectivity in a metacommunity can determine the structure of local assemblages (MacArthur & Wilson 1967; Hubbell 2001; Mouquet & Loreau 2003). In addition, the degree to which dispersal is stochastic is likely to be important to predicting community assembly (Gleason 1926). This is in part because ecological interactions are strongly affected by arrival order (Fukami & Morin 2003;

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Kennedy *et al.* 2009). Whole community measurements of dispersal are difficult at ecologically relevant spatial scales though, and many tests of dispersal-based community ecology theories are laboratory-based (Cadotte *et al.* 2006), theoretical (Mouquet & Loreau 2003) or indirect (Diamond 1972; Lomolino 1984; Peay *et al.* 2010a). While these approaches have yielded great insight, field experiments determining the spatial scale of dispersal limitation, its effects on community assembly and the degree to which species dispersal outcomes are predictable are critical to a complete understanding of community dynamics.

Understanding dispersal is particularly important in the global change era, as the ability of plants and animals to disperse is thought to be key for their future persistence (Walther *et al.* 2002; Loarie *et al.* 2008). However, there is a growing realization that many plant or animal species rely on cryptic organisms such as fungi and bacteria (Perry *et al.* 1990) as symbiotic partners to successfully complete their life cycle (Stachowicz 2001). Unfortunately, despite the importance of dispersal and a decade of active research using modern molecular tools, there is still no clear consensus on the role of dispersal in the ecology and evolution of microbes (i.e. fungi, bacteria and protists). Studies of these organisms have found both similar and different macroecological patterns to those of plants and animals (Finlay 2002; Whitaker *et al.* 2003; Peay *et al.* 2007; Queloz *et al.* 2011; Fierer *et al.* 2011). The increased interest in microbial dispersal has been fuelled in part by the recognition of the key role of fungi and bacteria in driving biogeochemical cycles (Falkowski *et al.* 2008) and in shaping plant and animal community structure (Gilbert 2002; Lips *et al.* 2006). However, these studies are mostly based on indirect statistical inference from spatial patterns of community structure (Green *et al.* 2004; Telford *et al.* 2006) rather than by direct observation of the dispersal process.

Fungi are a critical component of the diversity and function of all terrestrial ecosystems. They produce billions of passively dispersed microscopic propagules (spores) and kilometres of hyphae per gram of soil (Buller 1909; Bååth & Söderström 1979). Relative to many other cryptic organisms, fungal biologists have established robust species concepts (Taylor *et al.* 2000), corresponding molecular markers (Smith *et al.* 2007) and documented natural histories that make them good models for understanding microbial dispersal.

In this study, we apply an island biogeography framework to a unique vegetation matrix to understand how dispersal and colonization shape the community dynamics of a key guild of root-associated symbionts, the ectomycorrhizal fungi. Because ectomycorrhizal fungi are host-specific, we are able to identify 'islands' of suitable host vegetation within a patchy landscape matrix dominated by nonhost plants. Previous studies in this system have shown that the patterns of community structure conform to predictions from island biogeography. In particular, species richness increases with patch size (Peay *et al.* 2007) and decreases with isolation (Peay *et al.* 2010a). In this study, we use molecular tools to experimentally test the dispersal mechanism hypothesized to drive the isolation effect in island biogeography. To do so, we established experimental islands where we measured spore dispersal and ectomycorrhizal colonization of host seedlings across distances ranging from <1 m to >5 km away from potential spore sources. We hypothesized that diversity and abundance of both ectomycorrhizal spores and colonists would decrease with increasing isolation from spore sources. In addition, because previous work has shown that ectomycorrhizal fungi differ in dispersal-related traits, for example spore reactivity (Nara 2009), spore longevity (Bruns *et al.* 2009) and fruit body production (Gardes & Bruns 1996), we hypothesized that there would be strong differences between species in dispersal success. Together, these results would provide direct confirmation for a key mechanism in island biogeography theory, would demonstrate that dispersal limitation is an integral component of microbial community assembly, and would indicate the importance of dispersal-related functional traits in determining the ectomycorrhizal niche.

Materials and methods

Study site

The study was conducted at Point Reyes National Seashore (PRNS), located in west Marin County, California (38°04′ N by 122°50′ W). The experiment was repeated in two seasons, 2008–2009 and 2009–2010. PRNS belongs to the Mediterranean climate of coastal California, with mild, wet winters and cool, dry summers. Mean annual temperature at the coast is around 11 °C, with January averages around 10 °C and September averages around 13.5 °C. Mean annual precipitation is about 43 cm at the coast and occurs almost exclusively in the winter months.

The coastal vegetation matrix at PRNS is ideal for studying the dispersal of ectomycorrhizal fungi. *Pinus muricata* D. Don (Bishop Pine), an ectomycorrhizal host plant, is a closed cone pine that requires high-intensity fires for seed release. As a result of this autecology, it tends to form even-aged, monodominant stands. Near the coast, stands of *P. muricata* intergrade with grasslands and scrub characterized by *Baccharis pilularis*, *Toxicodendron diversiloba* and *Rubus ursinus*, all of which are

nonectomycorrhizal host plants. Because *P. muricata* is the only ectomycorrhizal host in many areas of the coastal scrub, these patches of *P. muricata* represent islands with respect to their ectomycorrhizal colonists and are the only sources for ectomycorrhizal spores in the surrounding landscape.

Experimental design

To measure spore dispersal and colonization across the landscape, we established sixteen 'experimental islands' in 2008 and seventeen in 2009 (16 original sites plus one additional; Fig. 1). Each experimental island consisted of twelve initially uncolonized seedlings placed in a rack on top of a plastic tray to prevent contact with the ground (Fig. 1). Each island was enclosed in chicken wire to prevent browsing or trampling by large mammals. The experimental islands were placed in locations chosen to represent a gradient of distances away from potential spore sources, which we defined as large patches of *P. muricata* approximately >0.2 ha. Distances were originally assessed using park service GIS vegetation maps with further refinements based on ground-truthing and close inspection of 2004 aerial photographs and imagery available on Google Earth. Final distances ranged from 0.5 m to 5.4 km. Greater details on the use of different distance metrics and mapping are available in Peay *et al.* (2010a). While wind direction is important for spore dispersal (Galante *et al.* 2011), it was not possible for us to take into account at the spatial and temporal scale of our experiment.

To populate the experimental islands, seedlings were freshly germinated from surface-sterilized seed collected from multiple *P. muricata* trees at PRNS and grown for *c.* 1 month in sterile soil in a growth chamber, until being placed in the field after the onset of winter rains (Year 1 on 29 October 2008 and Year 2 on 16 October 2009). Each year, seeds were germinated at the end of August and planted into a 1:1 mixture of sterile sand and autoclaved field soil collected from a local site (Tomales Point). Negative control seedlings were maintained in the growth chamber throughout all the stages of the experiment to ensure no ambient fungal inoculum from the growth chamber or planting soils confounded our results (none was found). Seedlings in the field were watered once a week to minimize seedling mortality in the experiment during periods when there was no natural precipitation. Seedlings were left in the field throughout the rainy season (October–April) when the vast majority of mushrooms are produced. At the end of the rainy season (Year 1 on 12 April 2009 and Year 2 on 20 April 2010), seedlings were returned to their original growth chamber until they could be harvested (<1 month).

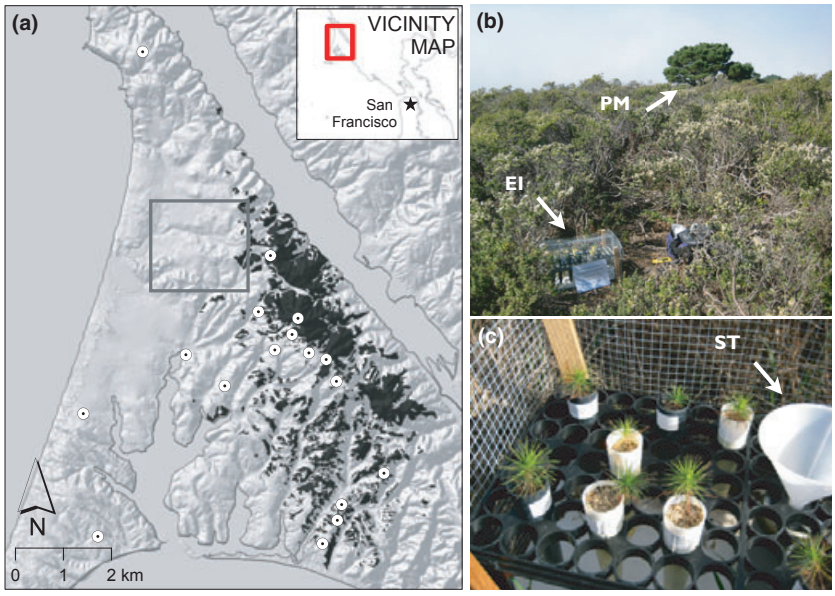

Fig. 1 Experimental set-up used for measuring ectomycorrhizal dispersal and colonization. (a) GIS Map of Point Reyes National Seashore (PRNS) with the location of experimental islands shown as white circles and the distribution of *Pinus muricata* patches shown in black. The grey box bounds the 4-km^2 area modelled in Fig. 6. (b) Shows a caged experimental island (EI) in the foreground surrounded by the non-ectomycorrhizal plant *Baccharus pilularis*. In the background is a lone individual of *Pinus muricata* (PM), an ectomycorrhizal host plant. (c) Close view of the experimental island showing the *P. muricata* bait seedlings, which were initially established free from mycorrhizal colonization in sterile soil, and the spore trap (ST) used to collect dry and wet spore deposition.

Seedling harvest

At the time of harvest, we assessed colonization intensity (CI) of ectomycorrhizal fungi on individual seedlings, measured either as the proportion of root length (Giovannetti & Mosse 1980) or as the proportion of fine roots colonized by ectomycorrhizal fungi. This was calculated using a random subsampling of each root system and using the gridline intersect method (proportion of root length) or by counting the number of colonized and uncolonized root tips (proportion fine roots colonized). Because the two metrics of CI were highly correlated (data not shown), we present only the data on proportion root length colonized. We also kept track of the number of uncolonized versus colonized seedlings at each experimental island as a measure of the extensiveness of ectomycorrhizal colonization (CE).

To determine the identity of ectomycorrhizal colonists, we examined the entire root system of each seedling and selected two representative root tips of each ectomycorrhizal morphotype encountered. DNA was extracted from each root tip and the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA genes sequenced in a single direction using the primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) following the methods of Peay *et al.* (2009). Sequences were combined into OTUs using a 97% sequence similarity cut-off in the program Geneious v5.3.6 (Biomatters, Auckland NZ) and taxonomic identity assigned by comparing with matches to the National Center for Biotechnology Information (NCBI) GenBank and with vouchered specimens from previous studies in the area. The remaining root and shoot system were dried at 65 °C and weighed to determine total biomass. A small number of seedlings died before harvest (~4%) and were excluded from further analysis.

Experimental spore inoculation curves

To establish the relationship between spore number and seedling colonization under controlled conditions, we performed laboratory inoculations of seedlings with spores obtained from fruiting bodies of *Suillus pungens* and *Thelephora terrestris*. These species were chosen because they were the most common fungal colonists in the study, we were able to obtain sufficient spores and we have developed successful inoculation protocols. Inoculation with another common colonist, *Tomentella sublilacina*, was not feasible and thus it was excluded from this portion of the study. Freshly germinated seedlings were inoculated at twenty spore concentrations following a twofold dilution series from 8.9×10^4 to 1.7×10^{-1} spores/mL of soil and allowed to grow for 4 months (*T. terrestris*) and 6 months (*S. pungens*) in a greenhouse following the methods of Bruns *et al.* (2009). Colonization was measured as the proportion of twelve replicate seedlings colonized at each spore concentration.

Rain water spore trap design and harvest

A simple spore trap was established at each experimental island concurrent with seedling deployment. Rainwater was collected with a 16-ounce plastic funnel (12 cm diameter) inserted into the lid of a 1-L mason jar. Mason jars were sterilized, covered in duct tape and contained 20 g of Chelex resin sealed in a 50-μm mesh pouch to prevent any *in situ* growth and curtail the activity of DNases. Water samples were collected approximately once a month (or when full) throughout the duration seedlings were in the field. At collection, all liquids in the spore trap were mixed well and then filtered through cheesecloth to remove large particulate matter and debris. The filtered liquids were centrifuged at 12 800 ***g*** for 10 min, and the supernatant was decanted. Any remaining liquids and pellet were further centrifuged at 15 000 ***g*** for 5 min in a 2.0-mL microcentrifuge tube, the supernatant decanted and the resulting pellet resuspended in 500 μL of 2× CTAB and stored at −20 °C until used. DNA was then extracted from 100 μL using the protocol described by DeSantis *et al.* (2005) with modifications found in Amend *et al.* (2010a). This protocol was designed specifically for extraction and amplification of bacteria and fungal propagules from filters used with high-volume air samplers (DeSantis *et al.* 2005).

Spore quantification

We used quantitative PCR (qPCR) to determine the number of spores of three key species in each spore sample. Based on preliminary data from the first year of the experiment, taxon-specific primers were designed for the three most abundant ectomycorrhizal species found on seedling roots; *Suillus pungens* (SP1F 5′-GC TGTAGCTGGCCCCCTG-3′, SP1R 5′-GCCAAAGGCCT TGTCGC-3′); *Thelephora terrestris* (TT1F 5′- GGACCC TGTCTTCCTTCT -3′, TT1R 5′-GACCAAAGGTTACCTG GCAGACAACA -3′); and *Tomentella sublilacina* (TS1F 5′-CGTAGTTCTANGGTCTGGGAGACCT -3′, TS1R 5′- GG GAGCTGACCGCAGGCCAGCAAAT -3′). Primers were designed manually by comparing the alignments of the ITS1 and ITS2 regions with closely related species in the program Geneious v5 (Biomatters, Auckland NZ). All candidate primers were checked for quality using the program Primer3. PCR reactions were run in a Real-Time PCR system (Applied Biosystems 7300) with

5 μL DNA, 4.7 μL H_2O, 10 μL iTAQ™ SYBR® Green Supermix with ROX (Bio-Rad, Hercules, CA, USA) and 0.15 μL of each primer at 50 μm concentration. Cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 65 °C for 1 min (63 °C in the case of *T. sublilacina* primers), finishing with 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Spore standards were made from multiple fruit body collections at PRNS and were stored and extracted using the methods described above for spore traps. A standard curve ranging from 10^1 to 10^6 spores/μL was derived by regressing $\log^{10}$ spore number versus threshold cycle number, and the curve was then used to estimate spore number from unknown samples. Samples with positive amplification but with melt curve values outside the range of the standards were excluded as nonspecific amplification. Fit of the standard curves was above an r^2 value of 0.90 within runs (generally higher) and across all runs was 0.90, 0.75 and 0.75 for *S. pungens*, *T. terrestris* and *T. sublilacina*, respectively. All PCR reactions were run in triplicate. The coefficient of variation across replicate runs was 0.64, 0.97 and 0.66 for *S. pungens*, *T. terrestris* and *T. sublilacina*, respectively. To reduce the influence of extreme values, we chose to use the median result of all replicates for our statistical analyses.

Spore diversity estimates using next-generation sequencing

Extracted DNA from each spore trap was amplified using modified versions of ITS1F and ITS4B primers (Gardes & Bruns 1993). We chose to use basidiomycete-specific primers because ascomycete ectomycorrhizal fungi are relatively rare in this system, and these primers allowed us to reduce coamplification of other nonectomycorrhizal ascomycetes that are highly abundant in spore trap samples based on preliminary sequencing. Primers were modified by adding the standard Roche A & B adapter tags for Lib-L sequencing and Roche recommended 10 base pair barcodes. Primers were thus A-Barcode-ITS1F and B-ITS4B. Amplification, quantification and pooling were carried out using the methods of Amend *et al.* (2010b) and sequenced at the Duke University Genomics Facility in two ¼ plate runs. Sequences were cleaned using the QIIME pipeline (Caporaso *et al.* 2010) to remove all sequences with ambiguous bases, primer mismatches, homopolymers >10 bp and length <300 or >700 bp, reducing the total number of sequences from 324 545 to 203 002. We then extracted the ITS1 spacer using the ITS extractor (Nilsson *et al.* 2010), clustered at ≥96% using UCLUST 1.22 as implemented in the pipeline QIIME 1.2.1 (Caporaso *et al.* 2010) and extracted representative sequences for each OTU. Representative OTUs were then searched against the UNITE and GenBank databases. Sequences with strong matches to known ectomycorrhizal fungi were separated into taxonomic groups (e.g. Thelephorales and Boletales), aligned against each other, closely inspected and re-searched against GenBank to eliminate potential chimeras and spurious OTUs because of sequencing error. In the final stage, all sequences with ≥95% homology were combined into OTUs.

Statistical analysis

We used a variety of statistical models to estimate the effects of increasing isolation from spore sources on the ectomycorrhizal species richness, colonization and total biomass of bait seedlings, as well as ectomycorrhizal spore richness. To account for site-to-site variation, we chose to use a linear mixed effects model that included a random *Site* effect, following Zuur *et al.* (2009), and using the R package nlme (Pinheiro *et al.* 2009). Mean richness and CI were predicted using $\log_{10}$-transformed distance to forest (*Isolation*), a categorical fixed effect for sampling year (*Year*) and the random *Site* effect. No interaction terms were included. An identical model was used to predict average richness of ectomycorrhizal spores per spore sample in relation to *Isolation*, *Year* and *Site*. To model the change in the proportion of seedlings colonized (CE) at each site, we used a nonlinear mixed effects model implemented by glmmPQL in the MASS package (Venables & Ripley 2002) using the binomial error function. Predictor variables for the models included also included the effects of *Isolation*, *Year* and *Site*. To see how changes in the abundance of ectomycorrhizal fungi affected seedling biomass, we regressed individual seedling biomass against CI and *Year* using a linear mixed effects model with a random *Site* effect.

To see how spore concentration changed with increasing isolation from spore sources, we fit a power law model of the form $y = cx^z$, where y is the number of spores, c is a constant, x is the distance from spore source and z is the estimated rate of decrease. While a number of mathematical approaches have been used to model dispersal (Nathan & Muller-Landau 2000; Levin *et al.* 2003), power laws have a number of advantages. They are simple to fit as linear models with log transformation, where $\log(y) = \log(c) + z\ \log(x)$. In addition, such models have a long history of use in plant pathology and have been shown to provide a good fit to dispersal of microscopic propagules likes spores and pollen (Fitt *et al.* 1987). We estimated the slope of the power law model using $\log_{10}\ x + 0.0001$ transformation of spore numbers, expressed in units of spores per cm^2 per day, regressed against $\log_{10}$ distance (m) away from

forest edge (*Isolation*). We fit separate models for each species that included a random effect to account for variation between sites (*Site*) and fixed effects for *Isolation* and *Year*. No interaction terms were included in the model.

To extrapolate our measurements across the landscape, we created a 1-m^2 resolution raster GIS layer of distances from pine forest (straight line distance function in raster calculator) across a 4-km^2 portion of mixed pine forest and coastal scrub at PRNS (Fig. 1) using ArcGIS v. 9 (Esri, Redlands, CA, USA). We fit the same statistical model to our qPCR results, but adjusted to spores per m^2 per day (to fit the grain of our GIS maps) and Log_{10} *Isolation* + 0.1 m (to allow us to model forested areas with a distance of 0). We then applied the linear regression parameters from the fitted model for Year 1 to the Log_{10} x + 0.1 transformed values for each cell of the raster distance layer using the raster calculator function in ArcGIS. The resulting spore load raster layer was Exp_{10} transformed, and all cell values summed using zonal statistics to obtain the total landscape spore load. This value was then multiplied by 180 days (our approximate sampling duration) to obtain the annual spore loads for the area.

To see how spore concentrations related to colonization in our field observations, we compared with single-species inoculations under controlled conditions. For field and greenhouse seedlings, we calculated the relationship between the log_{10}-transformed total number of spores per seedlings (either inoculated or estimated from spore traps) and the proportion of seedlings colonized (either at a site or per inoculation level) using logistic regression with the glm function in R and the binomial error family. The observed relationship between spore loads and seedling colonization from the greenhouse was plotted against observed values for seedling colonization and spore load from the field study.

Significance of individual model parameters was assessed using an *F*-test (linear mixed effects models) and are presented as *F*-values with numerator and denominator degrees of freedom, or with a *t*-test (nonlinear mixed effects models). Results are considered significant at $P < 0.05$.

Results

All aspects of mycorrhizal seedling colonization were highest for seedlings located at the forest edge and decreased continuously as experimental islands became more isolated from spore sources. While we observed some annual and site-level variability, the intensity of root colonization (*Isolation* effect $F_{1,15} = 17.39$, $P = 0.001$, *Year* effect $F_{1,15} = 9.32$, $P = 0.008$) and proportion of seedlings colonized per site (*Isolation* effect $t = -3.36$, $P = 0.003$, *Year* effect $t = -3.37$, $P = 0.004$) always declined with increasing distance from potential spore sources (Fig. 2a,b). Changes in colonization had consequences for seedling fitness, as total seedling biomass was positively correlated with ectomycorrhizal CI (*Colonization* effect $F_{1,360} = 252.32$, $P < 0.001$, *Year* effect $F_{1,360} = 395.98$, $P < 0.001$, Fig. 2c), approximately doubling between uncolonized and highly colonized seedlings.

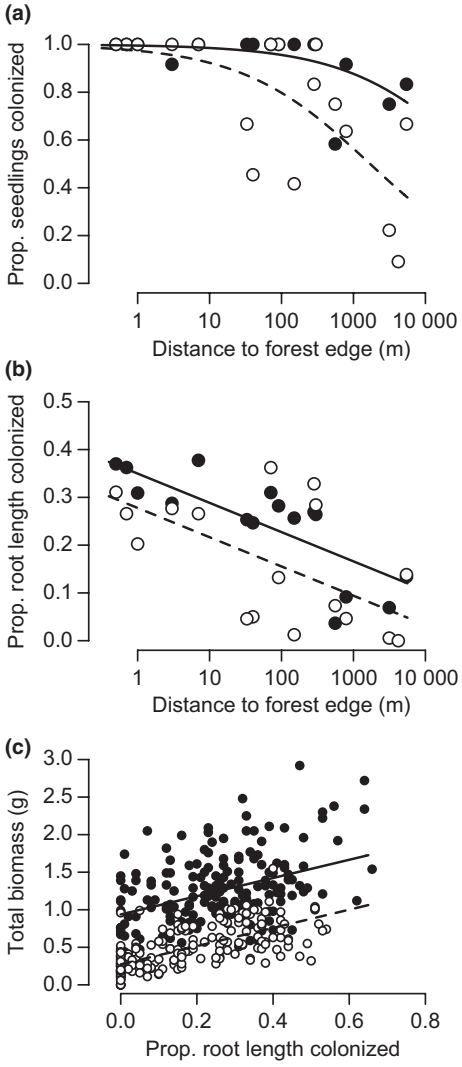

Fig. 2 Effects of isolation on seedling mycorrhizal status and growth. Panels show (a) the fraction of seedlings colonized at a site decreases with increasing isolation from potential spore sources; (b) intensity of ectomycorrhizal colonization, measured by the proportion of total root length colonized, decreases with increasing isolation from spore sources; (c) total seedling biomass increases with increasing ectomycorrhizal colonization. Filled and open symbols show Year 1 and Year 2, respectively.

Table 1 Ectomycorrhizal species observed to colonize *Pinus muricata* seedlings by spore dispersal

Species name	No. seedlings	Top blast match (accession nos)	% ID	No. bases	Accession nos
Thelephora terrestris	218	*Thelephora terrestris* (HM189966)	99	577/578	JN858072
Suillus pungens	192	*Suilllus pungens* (L54094)	99	574/577	JN858071
Tomentella sublilacina	42	*Tomentella sublilacina* (AY880929)	99	529/534	JN858074
Suillus quiescens	11	*Suillus quiescens* (GQ249402)	99	649/651	JN858077
Suillus brevipes	9	*Suillus brevipes* (FJ845440)	99	626/634	JN858079
Suillus pseudobrevipes	7	*Suillus volcanalis(=pseudobrevipes)*(GQ249398)	99	644/648	JN858073
Laccaria cf. proxima	5	*Laccaria proxima* (GU931707)	99	604/605	JN858078
Rhizopogon occidentalis	5	*Rhizopogon occidentalis* (DQ822821)	100	652/652	JN858084
Rhizopogon vulgaris	4	*Rhizopogon vulgaris* (DQ822823)	99	648/654	JN858081
Hebeloma rivulosum	3	*Hebeloma rivulosum* (HQ179682)	98	572/586	JN858080
Paxillus involutus	2	*Paxillus involutus* (JF899567)	100	590/590	JN858075
Thelephoraceae sp. D6	1	Uncultured Thelephoraceae (AY880928)	99	628/631	JN858076
Suillus tomentosus	1	*Suillus tomentosus* (FJ845441)	99	623/625	JN858082
Hebeloma velutipes	1	*Hebeloma velutipes* (AF430254)	98	650/664	JN858083

Actual measurements of spore deposition rates demonstrated that the observed patterns of seedling colonization were driven by dispersal limitation. *Suillus pungens*, *Thelephora terrestris* and *Tomentella sublilacina* were the three most abundant ectomycorrhizal fungi that colonized seedlings, together accounting for 93% of sequenced root tips (Table 1). There was a large amount of variation in spore dispersal rates between sites and between species, with estimated annual input averaging 2360 spores per cm^2 for *S. pungens* (range = 6–29 979), 227 spores per cm^2 for *T. terrestris* (range = 0–3171) and 16 spores per cm^2 for *T. sublilacina* (range = 0–153). A power law model of deposition rates into our spore traps based on qPCR results showed decreasing spore abundance with increasing distance from *Pinus muricata* patches. This decrease was broadly concordant with species-specific seedling colonization rates (Fig. 3). While the three species differed significantly in absolute spore production, the estimated rate of decrease overlapped across species (mean ± SE for *S. pungens* = −0.65 ± 0.10, *T. terrestris* = −0.54 ± 0.12, *T. sublilacina* = −0.72 ± 0.16). Both field- and greenhouse-derived spore–colonization curves showed increasing colonization with increasing spore loads (Fig. 4). However, model parameters varied significantly for both *S. pungens* ($Slope_{Greenhouse}$ = 1.50 ± 0.10

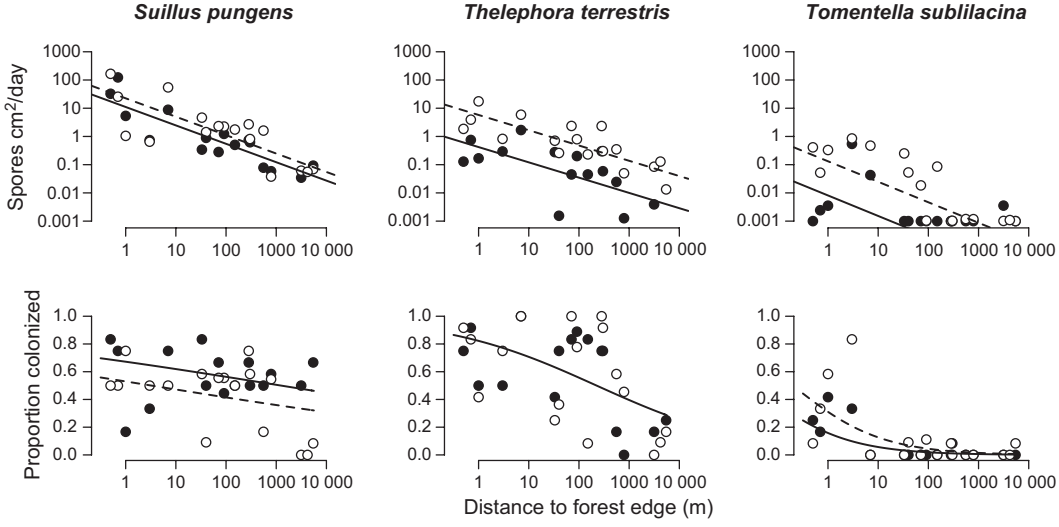

Fig. 3 Spore dispersal rates for three ectomycorrhizal species decrease as a power law with increasing distance from potential spore sources and with concomitant decreases in seedling colonization. Top row shows changes in spore dispersal rate as a function of distance from forest. Bottom row shows proportion of seedlings colonized as a function of distance from forest. Filled and open symbols show raw data from Year 1 and Year 2, respectively. Solid line shows regression for Year 1 or the overall effect (if there is no significant *Year* effect) and the dashed line for Year 2. Model effects with $P < 0.10$ are shown.

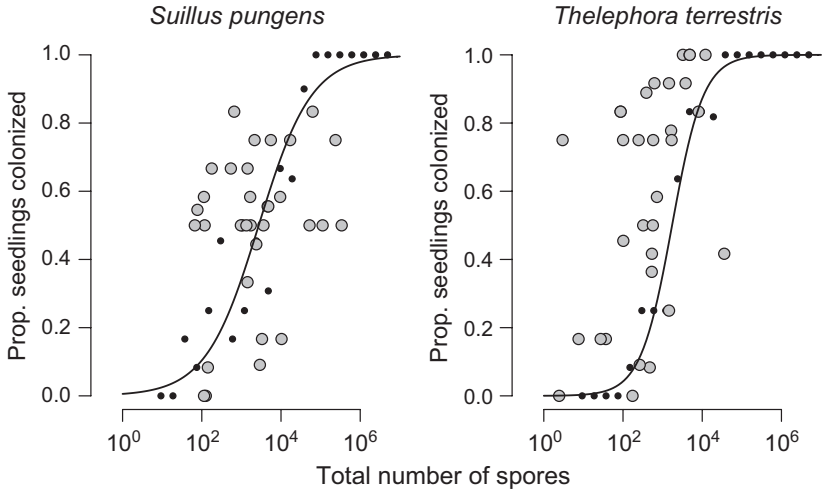

Fig. 4 Comparison of field data with spore–colonization curves derived from a controlled greenhouse inoculation experiment for two species of ectomycorrhizal fungi. Data points show the proportion of seedlings colonized for greenhouse inoculations (black) and field grown seedlings (grey) plotted against the number of spores inoculated (greenhouse) or qPCR estimated spore loads (field) per seedling. Lines show the fitted spore inoculation curves derived from the greenhouse trials. While there is broad concordance between the two data sets, higher variability in the field data indicates that other biological processes, in addition to spore load, may influence colonization.

SE and $Slope_{Field} = 0.25 \pm 0.18$ SE) and *T. terrestris* ($Slope_{Greenhouse} = 0.64 \pm 0.11$ SE and $Slope_{Field} = 2.52 \pm 0.36$ SE). For *S. pungens*, at high spore concentrations, field colonization was lower than expected based on greenhouse inoculation of comparable spore numbers. For *T. terrestris*, field colonization was generally higher than expected based on greenhouse inoculations. Equations used to model daily spore deposition across a 4-km^2 area of mixed vegetation (Fig. 6) were $SP\ \log_{10}(Spores\ m^2 + 0.0001) = 5.08–0.65 \times \log_{10}(Isolation + 0.1\ m)$, $TT\ \log_{10}(Spores\ m^2 + 0.0001) = 3.65–0.\ 54 \times \log_{10}\ (Isolation + 0.1\ m)$, $TS\ \log_{10}(Spores\ m^2 + 0.0001) = 1.95–0.72 \times \log_{10}\ (Isolation + 0.1\ m)$. Landscape modelling estimated total spore deposition at $\sim 8 \times 10^{12}$ spores per km^2 for *S. pungens*, 2.5×10^{11} for *T. terrestris* and 7×10^9 for *T. sublilacina*.

Across the 2 years of the study, 14 species of ectomycorrhizal fungi were observed to colonize seedlings (Table 1). Average colonizer richness across all seedlings was 1.4 species (range = 0–4). There was variability in richness of individual seedlings within sites (average within site standard deviation = 0.60 species and coefficient of variation = 0.67). The mean number of species colonizing each seedling decreased with isolation (*Isolation* effect $F_{1,15} = 26.9$, $P = <0.001$, Fig. 5a), varying from ~2 species per seedling at the forest edge to <1 beyond 1 km. There was not a significant change in ectomycorrhizal colonizer richness across the 2 years of the study (*Year* effect $F_{1,15} = 1.02$, $P = 0.33$, Fig. 5a).

Spore diversity of ectomycorrhizal fungi was far greater than realized diversity on seedlings, with 166 ectomycorrhizal species observed from 13 families via 454 pyrosequencing (Table 2). Mean ectomycorrhizal richness per spore sample (covering *c.* 1 month each) was six species (range = 0–21). All of the species sequenced directly from seedling roots more than once were also observed via spore dispersal, but many large, common and diverse groups of ectomycorrhizal fungi were never observed on seedling roots even though they were detected in the pyrosequenced samples. For example, six species of *Amanita*, 14 species of *Russula/Lactarius*, 19 species of *Inocybe* and 41 species of *Cortinarius* (Table 2) were detected as sequences from spores, but were never found on seedlings. The average number of ectomycorrhizal fungi observed in each spore sample also decreased significantly with isolation across both years (*Isolation* effect $F_{1,15} = 55.\ 72$, $P < 0.001$, *Year* effect $F_{1,15} = 8.\ 84$, $P = 0.01$, Fig. 5b).

Discussion

Our results show that dispersal plays a critical role in community assembly of this group of important plant symbionts. We found that ectomycorrhizal fungi have the potential to disperse and colonize over multiple kilometres (Fig. 2). However, a significant proportion of bait seedlings remained uncolonized at distances of a kilometre or greater and only one species, *Suillus pungens*, consistently colonized seedlings at those greater distances. The rapidity with which spore loads decreased from the forest edge into the surrounding landscape is apparent from the quantified spore loads

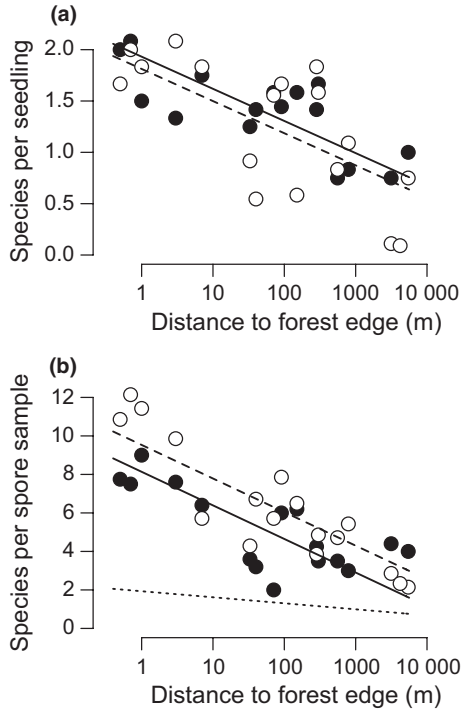

Fig. 5 Immigration rate of fungal colonists and spore load decrease as a function of distance from potential spore sources. (a) The immigration rate of ectomycorrhizal colonists, expressed as the average number of species per year on experimental bait seedlings. Filled symbols and solid line show data from Year 1 and open symbols and dashed line from Year 2. Effects with $P < 0.10$ are shown. (b) The average dispersal rate of ectomycorrhizal species into spore trap samples (sampled approximately once per month) at each site for Years 1 and 2. Filled symbols and solid line show data from Year 1 and open symbols and dashed line from Year 2. Dotted line at the bottom shows regression for seedlings richness in Year 1 of panel (a) for comparison. Effects with $P < 0.10$ are shown.

from three targeted species (Fig. 3). While our 'sporescape' modelling for *S. pungens* (Fig. 6) shows annual spore loads on an area of mixed vegetation to be an astronomical 8 trillion spores per km^2, this is put in perspective when considering that conservative estimates of spore production indicate that 8000 fruiting bodies could account for this output and that fruiting by *Suillus* can reach local densities equivalent to 1×10^5 fruit bodies per km^2 (Dahlberg & Stenlid 1994). If anything we are probably underestimating spore loads inside *P. muricata* stands given the location of our spore traps and the fact that the vast majority of spores fall directly beneath the mushroom cap (Li 2005; Galante *et al.* 2011). Despite the fact a single species can produce an astronomical numbers of spores across the landscape, the reduction in spore loads are still sufficient to cause concomitant decreases in species richness (Fig. 5) and seedling colonization with increasing distance from source vegetation. Together, these results provide strong experimental evidence for the ecological significance of dispersal limitation in fungal communities. Prodigious spore output does not mean unlimited dispersal; rather, it is the evolutionary consequence of the extreme difficulty and low probability of successful dispersal by fungal propagules.

Dispersal limitation is a critical mechanism in a wide range of ecological theories that attempt to link larger-scale processes to local community dynamics (MacArthur & Wilson 1967; Ricklefs 1987; Hubbell 2001; Mouquet & Loreau 2003; Leibold *et al.* 2004). Our finding of community-wide dispersal limitation at the kilometre scale suggests that regional or landscape context is critical to understanding the local dynamics of microbial communities. The current study is the third in a series where we have applied an island biogeography framework to study ectomycorrhizal fungi across a fragmented landscape of host tree patches, or 'tree islands'. Our previous studies have found patterns consistent with the predictions of island biogeography theory, namely that species richness increases strongly with island size (Peay *et al.* 2007) and decreases with isolation from propagule sources (Peay *et al.* 2010a). These studies provided indirect evidence of the potential importance of macroecological processes, such as immigration and extinction, in microbial communities. The current study provides a direct confirmation that immigration rates do decrease with isolation, that the decrease in immigration is indeed because of dispersal limitation, and that reduced immigration rates leads to lower ectomycorrhizal colonization and diversity on more isolated host plants. While it is often assumed that dispersal limitation drives the isolation effect in island biogeography (Lomolino *et al.* 2006), most studies do not measure the dispersal or colonization process directly (Diamond 1972; Lomolino 1984; Cody 2006). Our finding that regional or landscape context has important effects on community structure appears to be in contrast to other recent studies indicating that local factors are the primary determinants of microbial community structure (Fierer & Jackson 2006; Bell 2010; Queloz *et al.* 2011). Direct measurements of dispersal in other microbial communities may resolve these differences as high rates of dispersal increase the importance of local interactions in determining community structure (Tilman *et al.* 1994; Mouquet & Loreau 2003). However, high rates of dispersal may lead to low local diversity (Mouquet & Loreau 2003), a finding not consistent with most studies of fungal or bacterial communities (O'Brien *et al.* 2005; Fierer *et al.* 2007; Buée *et al.* 2009).

Dispersal can have both predictable and historical effects on the species composition of ecological communities. While passive dispersal of spores or seeds is

Table 2 Summary of ectomycorrhizal spore community observed via 454 pyrosequencing of spore traps. The table summarizes results by Family or Order and shows the number of sequences assigned, the number of species from each group observed by spore, and the number of species from that group that were also observed on seedlings. Genera in bold are those observed to colonize seedlings

Family	Genera	No. reads (%)	Spore richness	Seedling richness (% colonized)
Suillaceae	***Suillus***	8305 (71.11)	7	5 (57.9)
Cantherellales	*Clavulina*, *Hydnum*	949 (8.12)	10	0 (0)
Thelephoraceae	*Pseudotomentella*, *Sarcodon*, ***Thelephora***, ***Tomentella***, *Tomentellopsis*	728 (6.23)	27	3 (68.68)
Cortinariaceae	*Cortinarius*	440 (3.77)	40	0
Rhizopogonaceae	***Rhizopogon***	473 (4.05)	5	2 (2.37)
Hymenogastraceae	***Hebeloma***	240 (2.05)	6	2 (1.05)
Tricholomataceae	*Tricholoma*	207 (1.77)	18	0 (0)
Inocybeaceae	*Inocybe*	91 (0.78)	19	0 (0)
Amanitaceae	*Amanita*	66 (0.56)	6	0 (0)
Russulaceae	*Lactarius*, *Russula*	63 (0.54)	14	0 (0)
Boletaceae	*Boletus*, ***Paxillus***, *Pisolithus*, *Xerocomus*	54 (0.46)	8	1 (0.53)
Hydnangaceae	***Laccaria***	37 (0.32)	2	1 (1.31)
Atheliaceae	*Athelia*, *Tylospora*	26 (0.22)	4	0 (0)
Gomphaceae	*Ramaria*	17 (0.15)	2	0 (0)
Total	24	11 678	166	14

stochastic in nature, strong differences between species in dispersal ability can lead to statistically predictable outcomes in community composition in some situations (Tilman 1994; Nathan & Muller-Landau 2000; Levin *et al.* 2003). In contrast, because early-arriving species have a competitive advantage and exert strong effects on later-arriving species (Kennedy & Bruns 2005; Peay *et al.* 2012), community composition can also depend on historical variation in arrival order (Drake 1991; Fukami 2004; Dickie *et al.* 2012). The identity of ectomycorrhizal spores and overall species richness levels in this study corresponded well with those observed in fruit body and molecular surveys of roots in both young (10 years) and old (*c.* 50 years) *P. muricata* forests, suggesting that we have performed a fairly thorough job of sampling the pool of species dispersing from *P. muricata* patches at PRNS. These results demonstrate that at PRNS, differences in dispersal ability lead to highly predictable species composition at the early stages of community assembly (Baar *et al.* 1999; Peay *et al.* 2007). The most widespread taxa we found colonizing 10-year-old *P. muricata* islands (Peay *et al.* 2007) were the most prolific colonizers and dispersers, measured both by qPCR and 454 pyrosequencing, in this study (Fig. 3, Table 2). While 454 pyrosequencing reads cannot be used in a strict quantitative sense (Amend *et al.* 2010b), the read abundance for taxa such as, *Suillus*, *Thelephora*, *Hebeloma*, *Laccaria* and *Tricholoma*, matches well with the patterns of abundance on 10-year-old tree islands (Peay *et al.* 2007). The only major exception is taxa with animal dispersed spores, such as *Rhizopogon*, which were intentionally minimized in the current study. However, we have also found that the predictability of community assembly based on dispersal ability erodes over longer timescales as a large pool of competitively superior species with weak dispersal abilities replaces the small pool of dispersal specialists (Peay *et al.* 2010a). Our study of 50-year-old tree islands (Peay *et al.* 2010a) showed a similar negative relationship between isolation and ectomycorrhizal species richness; however, there was little overlap with the early successional species pool on seedlings in this study or on 10-year tree islands (Peay *et al.* 2007). In addition, species composition on older tree islands was not predictable based on island isolation or environmental conditions. Based on these two previous studies and the current study, it appears that as ectomycorrhizal community assembly progresses, the predictable effects of the dispersal ability of early colonizers are outweighed by the historical effects of stochasticity in the later phases of colonization.

There is increasing evidence for a diversity of dispersal-related functional traits that could drive colonization differences across ectomycorrhizal taxa. Studies have demonstrated the ecological importance of spore dormancy (Bruns *et al.* 2009), stress tolerance (Peay *et al.* 2009) and differences in germination patterns (Nara 2009). In this study, we show quantitative differences in spore production within ruderal taxa that are common on seedlings and in early successional settings.

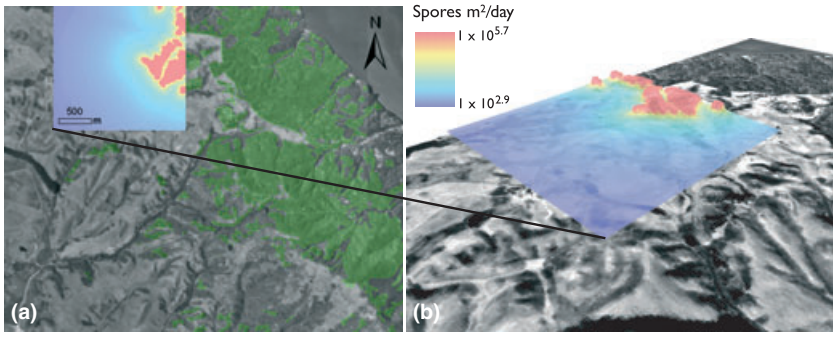

Fig. 6 Spatially explicit sporescape for *Suillus pungens*. Figure shows estimated spore deposition of *S. pungens* per m^2 per day across a 4-km^2 mixed landscape of *Pinus muricata* forest and coastal scrub. (a) Two-dimensional topographic map showing spore deposition and landscape layout. Green indicates mapped patches of *P. muricata*, and spore loads increase from cool to warm colours. (b) Three-dimensional rendering of spore deposition demonstrates the exponential decrease of spore loads moving away from the forest edge (*z*-scale = 30). Average spore load per km^2 across the modelled landscape is 8×10^{12} spores.

We found that three common early successional taxa varied in total spore production and long-distance colonization patterns (*S. pungens* > *T. terrestris* > *T. sublilacina*, Fig. 3). Our results thus suggest that even within good dispersers, there are gradations in dispersal ability. In addition to total fruit body production, differences in fruiting body construction and spore size have also been shown to affect dispersal distances (Galante *et al.* 2011). While it was not possible to account for quantitatively in this study, fruit body height and size do match the observed patterns of long-distance dispersal (e.g. *S. pungens* > *T. terrestris* > *T. sublilacina*). Thus, fruit body construction, spore receptivity, spore production and spore viability and are key functional traits that will be useful to measure to predict community assembly trajectories of ectomycorrhizal fungi.

Differences between species in dispersal ability may be driven in part by a trade-off between dispersal ability and competitive ability. It is well established at PRNS that reproductive output (e.g. fruit body production) is *not* correlated with fungal abundance (Gardes & Bruns 1996; Peay *et al.* 2007), that is, some species appear to invest more in fruiting than others. For other taxonomic groups, there is good evidence that differences in dispersal investment may come at a trade-off with competitive ability (Tilman 1994; Cadotte 2007). We see some support for this in our data. Colonization of *S. pungens* in the field is well below the predicted potential from single-species spore inoculations (Figs 3 and 4) in the areas closest to the forest where its spores overlap with those of *T. terrestris* and *T. sublilacina*. This pattern could be caused by competitive interactions, where established *T. terrestris* and *T. sublilacina* on seedling roots suppress colonization by *S. pungens*. While we do not have direct evidence for these competitive interactions, *S. pungens* has been shown to be a competitive inferior in a separate test of the competition–colonization hypothesis (Kennedy *et al.* 2011) and *T. sublilacina* has been shown to be competitively superior to other early successional species (Lilleskov & Bruns 2003). Given the preponderance of evidence, it seems likely that competition–colonization trade-offs are important in these fungal communities.

Many late successional ectomycorrhizal taxa have spores that do not germinate readily or colonize seedlings (Miller *et al.* 1993; Nara 2009). This difference in spore behaviour has been proposed as an explanation for ectomycorrhizal successional patterns. Species with nonresponsive spores have been shown to be less abundant colonizers in early successional settings at this study site (Peay *et al.* 2007) and in other settings (Last *et al.* 1987; Deacon & Fleming 1992; Nara 2009). Based on our 454 data, some late successional taxa such as *Amanita*, *Russula* and the *Boletaceae*, simply seem to be poor dispersers overall (<1% of 454 read abundance). However, there are some taxonomic groups that were somewhat abundant in 454 but not present on bait seedlings (*Tricholoma*, *Cortinarius*, *Clavulina*), possibly suggesting differences in spore behaviour on young seedlings (Table 2, Fig. 5b). Understanding what controls variation in spore behaviour would represent major progress in ectomycorrhizal ecology.

The prevalence of dispersal limitation in this system has important implications for plant communities. First, it suggests that symbiont availability will vary in quantity and identity across a landscape and that establishing hosts do not have unlimited variety of ectomycorrhizal fungi to choose from. Second, it suggests that symbiont availability may limit host migration rates, in particular by making it less likely that long-distance dispersal events will lead to successful establishment. Availability of N-fixing bacterial symbionts has already been suggested to limit range expansion in some leguminous plants (Stanton-Geddes & Anderson 2011). Our data

indicate that for ectomycorrhizal host plants, this may occur at the scale of 10^3 to 10^2 m, depending on the identity of ectomycorrhizal species in the community. *Suillus* species are know to be specific to the Pinaceae and appear to be the major long-distance colonizers in other systems, for example pine invasions in Argentina (Nunez *et al.* 2009). Studies of *Quercus* (Dickie & Reich 2005) show ectomycorrhizal limitation over much shorter spatial scales. It is important to note that our study intentionally excluded pre-existing soil-borne inoculum (i.e. the spore bank), which is an important component of colonization in this and other systems (Baar *et al.* 1999). Identity of species in and size of the spore bank should also be considered when assessing the effects of symbiont availability on plant communities.

Dispersal takes place across multiple spatiotemporal scales and orders of biological organization. At the ecological scale, short-term variation in the quantity and quality of dispersal may strongly influence the patterns of community assembly, while at the evolutionary timescale relatively infrequent long-distance dispersal events may be sufficient to homogenize biogeographic patterns. There is some evidence for this from ammonia-oxidizing bacteria (Martiny *et al.* 2011), which seem to show evidence of local scale dispersal limitation but appear to share a global taxonomic pool (although this may be affected by the taxonomic resolution inferred from bacterial 16S ribosomal sequences). Population genetic studies have shown large panmictic populations for the ectomycorrhizal fungus *Laccaria amethystina* within Europe, but significant genetic differentiation between European and Japanese populations (Vincenot *et al.* 2012). In addition, a number of long-distance dispersal events have been documented for fungi (Brown & Hovmoller 2002; Geml *et al.* 2011). On the other hand, fungal communities overall appear to harbour few cosmopolitan species (Amend *et al.* 2010a) and fungi investigated in new biomes, such as neotropical (Smith *et al.* in press) and paleotropical rainforests (Peay *et al.* 2010b) appear to be composed primarily of endemic taxa. In addition, the order in which species disperse to a site has been demonstrated repeatedly to have strong effects on fungal community assembly (Kennedy *et al.* 2009; Fukami *et al.* 2010; Dickie *et al.* 2012; Peay *et al.* 2012). Based on these reports, it appears that fungal communities are strongly influenced by dispersal limitation at both ecological and evolutionary scales.

Conclusions

Dispersal plays a major role in most ecological theories. However, actual measurements of dispersal in real ecological systems are hard to come by. In this study, we provide an experimental field test demonstrating that dispersal limitation at the landscape scale reduces immigration rates of ectomycorrhizal fungi on host seedlings. In doing so, we provide empirical support for a key mechanism in island biogeography theory and confirm the importance of higher-scale processes in understanding local community dynamics of microbes. Despite some debate over the macroecology of cryptic organisms, such as bacteria and fungi, the application of molecular tools has the potential to make microbial field systems, a model for understanding the role of dispersal in ecological theory.

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Data accessibility

Root tip DNA sequences: GenBank accession numbers JN858071:JN858083

Site data, ECM colonization data, spore dispersal data and NGS sequence data available at DRYAD entry doi:10.5061/dryad.b70pn