A continental view of pine-associated ectomycorrhizal fungal spore banks: a quiescent functional guild with a strong biogeographic pattern

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Summary

• Ecologists have long acknowledged the importance of seed banks; yet, despite the fact that many plants rely on mycorrhizal fungi for survival and growth, the structure of ectomycorrhizal (ECM) fungal spore banks remains poorly understood. The primary goal of this study was to assess the geographic structure in pine-associated ECM fungal spore banks across the North American continent.

• Soils were collected from 19 plots in forests across North America. Fresh soils were pyrosequenced for fungal internal transcribed spacer (ITS) amplicons. Adjacent soil cores were dried and bioassayed with pine seedlings, and colonized roots were pyrosequenced to detect resistant propagules of ECM fungi.

• The results showed that ECM spore banks correlated strongly with biogeographic location, but not with the identity of congeneric plant hosts. Minimal community overlap was found between resident ECM fungi vs those in spore banks, and spore bank assemblages were relatively simple and dominated by *Rhizopogon*, *Wilcoxina*, *Cenococcum*, *Thelephora*, *Tuber*, *Laccaria* and *Suillus*.

• Similar to plant seed banks, ECM fungal spore banks are, in general, depauperate, and represent a small and rare subset of the mature forest soil fungal community. Yet, they may be extremely important in fungal colonization after large-scale disturbances such as clear cuts and forest fires.

Introduction

Understanding the processes that maintain species richness is a central goal in ecology. Alternative strategies for colonization and reproductive success, such as trade-offs between colonization and competition (Tilman, 1994), or dispersal and longevity (Ehrlen & van Groenendael, 1998), can contribute to species diversity by partitioning niche space. These functional differences between species can interact with disturbance regimes to increase species richness (Bohn *et al.*, 2014). Some plant species capitalize on disturbance by forming seed banks that remain dormant in the soil for long periods of time. These seeds then germinate following disturbances, allowing the plants to thrive in the temporary absence of competitively dominant species. Seed banks have been studied extensively and much is known about the identity of the species present, specific cues that trigger germination, negative correlations between seed size and longevity, and other associated functional traits (Thompson, 1987; Thompson *et al.*, 1993).

Analogous to soil seed banks, fungal spore banks are reservoirs of dormant fungal propagules (Bonito *et al.*, 2012), but much less

is known about them compared with seed banks. Also referred to as resistant propagule communities because they include both spores and sclerotia (Baar *et al.*, 1999; Taylor & Bruns, 1999; Buscardo *et al.*, 2010; Hoeksema *et al.*, 2012), we prefer the term spore bank because it draws immediate comparisons with seed banks where the parallels are obvious and empirical and the theoretical literature is better developed. Spore banks are functionally important biotic components of terrestrial ecosystems (Bonito *et al.*, 2012), and may remain quiescent in the soil for decades or longer (Bruns *et al.*, 2009). With advances in sequencing technologies, there has also been an increased interest in understanding the role of dormancy and longevity as mechanisms for niche partitioning and species coexistence in bacteria, which has been referred to by microbial ecologists as the microbial seed bank (Lennon & Jones, 2011; Caporaso *et al.*, 2012).

As many dominant tree species are reliant on ectomycorrhizal (ECM) fungal symbionts for growth and survival, the ability of ECM fungi to form spore banks is likely to be of importance for ecosystem recovery following disturbance, and there is evidence that some ECM fungi from spore banks behave in this way.

However, these studies have been geographically limited. Although some studies on spore banks have been conducted outside of these regions (Brundrett *et al.*, 1996; Cowden & Peterson, 2013), the vast majority have focused on western North America, especially California, Oregon and British Columbia (Baar *et al.*, 1999; Hagerman *et al.*, 1999; Taylor & Bruns, 1999; Jones *et al.*, 2003; Kjøller & Bruns, 2003; Bruns *et al.*, 2009). For instance, it is known that western *Pinus* and *Pseudotsuga* spore banks are dominated by *Rhizopogon* species (Taylor & Bruns, 1999; Kjøller & Bruns, 2003; Rusca *et al.*, 2006), but does this pattern hold true when sampling is conducted at a broader scale? Furthermore, some species (particularly *Rhizopogon* and *Suillus* spp.) are host specific, at least at the level of host genera (Massicotte *et al.*, 1994). However, the extent to which host specificity shapes spore bank composition across large geographic regions remains unknown.

The primary goal of this study was to achieve a comprehensive understanding of ECM fungal spore banks associated with pine-dominated forests in North America, and to determine the major ecological factors shaping these spore bank communities. Previous studies have shown that the diversity of ECM fungi in the spore bank is low relative to ECM fungi active in the mature forest (Kipfer *et al.*, 2011; Cowden & Peterson, 2013), and the species dominating spore banks are rare or absent from mature forest soil fungal communities (Taylor & Bruns, 1999). Both of these patterns are consistent with the plant seed bank literature (Hopfensperger, 2007). Although these studies provide critical baseline information on the relationship between ECM fungal spore banks and the mature forest soil community, methods for microbial identification have improved exponentially in recent years and now allow us to conduct studies at larger scales. By conducting a study of ECM fungal spore banks at the continental scale from the same sample locations in which ECM fungal communities in mature forest soil have been analyzed previously (Talbot *et al.*, 2014), we have the opportunity to determine whether ECM fungal spore bank patterns seen at local and regional scales are repeatable and general, and we can thereby increase the knowledge of ECM spore banks to be on a par with plant seed banks.

In this paper, we address the following four hypotheses: (H1) ECM fungal spore banks will be geographically patterned at local, regional and continental scales; (H2) identity of the bioassay host will affect the alpha and beta diversity of recovered taxa; (H3) ECM fungal spore banks will be dominated by only a few genera; (H4) ECM fungal taxa in spore banks will be rare or absent from mature forest soil ECM fungal communities. In addition to investigating whether there is geographic patterning in these spore banks, we also ask whether this pattern correlates more strongly with geographic distance, host, climatic or edaphic factors.

To test these hypotheses, our study of ECM fungal spore banks was performed in conjunction with a large-scale continental study of fungal diversity associated with pine forests across North America (Talbot *et al.*, 2013, 2014). This study involved sampling pine forest soils across North America, using a replicated nested sampling design, which allowed for detection of geographic patterning at multiple scales. Here, we report the findings from the first 19 plots spanning boreal, western and south-eastern forests in North America. All of these plots were previously analyzed by Talbot *et al.* (2014) for soil fungi and mature ECM communities. Here, we analyze the spore bank community from the same locations and sample points. We define the spore bank community as those ECM fungi recovered in a bioassay of pine seedlings planted in soil that had been air dried to kill the active hyphae, but retain spores and sclerotia (Taylor & Bruns, 1999; Bonito *et al.*, 2012). We used next-generation sequencing (NGS) to identify the ECM fungal species formed on the bioassay seedlings of the native and a common pine species host. We then compared the results of the spore banks with those of the total soil fungal community assessed through NGS of the fresh soil, which includes both hyphae and spores. This sampling design allowed us a unique opportunity to compare the way in which the mature forest soil fungal communities and ECM fungal spore bank guilds are patterned at local, regional and continental scales.

Materials and Methods

Soil sampling

We used a hierarchical sampling design that allowed comparisons of multiple samples within a plot and across the continent. Plots were chosen with the help of local experts to find mature stands with monodominance of a single targeted species in the Pinaceae (Talbot *et al.*, 2014). Sampling was carried out in 2011 and 2012 near the period of peak plant biomass for each region, and a total of 19 plots from across six states were sampled for spore bank fungi, including Alaska, California, Florida, Minnesota, Mississippi and North Carolina (Supporting Information Methods S1). Soil cores were collected from the corners of nested squares (5×5 m^2, 10×10 m^2, 20×20 m^2) within a 40×40-m^2 grid for a total of 13 samples per plot (Methods S2). At each sampling location, a soil core 14 cm deep and 7 cm in diameter (*c.* 40 cm^3) was collected for analysis of the mature forest fungal community as described in Talbot *et al.* (2014), and three additional soil cores were collected for spore bank bioassays. Soil cores collected for bioassays were sieved through 2-mm mesh to remove roots and rocks and homogenized by hand before drying. Soil cores were air dried in paper bags for several weeks to months to kill active vegetative fungal hyphae before assaying for resistant propagules (Taylor & Bruns, 1999; Bonito *et al.*, 2012). Before each bioassay, soil moisture was measured on a subset of all soils to confirm negligible water content (Methods S3).

Glasshouse bioassays

Pine seedlings have been successfully used to bioassay for ECM fungal spore banks from air-dried soils for nearly two decades (Taylor & Bruns, 1999; Izzo *et al.*, 2006; Bonito *et al.*, 2012; Hoeksema *et al.*, 2012), and glasshouse studies have shown good concordance with *in situ* colonization after disturbance in the field (Baar *et al.*, 1999). To recover as much biodiversity as

possible and to account for potential host specificity differences, all soils collected from each sampling location within a plot were grown with both a common host, *Pinus muricata*, and the native pine host associated with the plot (Methods S1). All seedlings were grown in a common ambient temperature glasshouse at the University of California, Berkeley, CA, USA. *P. muricata* seeds were collected from Point Reyes National Seashore; *Pinus contorta*, *Pinus monticola*, *Pinus taeda*, *P. ponderosa*, *Pinus banksiana* and *Picea glauca* seeds were purchased from Sheffield's Seed Co. Inc. (Locke, NY, USA). *Pinus monticola* was used as a surrogate for the endangered *P. albicaulis*; both are members of the subgenus *Strobus*. Purchased seeds were stratified according to the vendor's instructions, and *P. muricata* seeds were soaked in water for 48 h before surface sterilization (Rusca *et al.*, 2006). Three to five replicates of each host species were planted in the dried soil from each of 13 sampling locations within a plot to hedge for mortality and to increase chances of recovering the most fungal diversity. An uninoculated control was also added to each plot to control for airborne fungal spores in the glasshouse. For the control treatment, both *P. muricata* and native plants were grown in twice-autoclaved soils (250°C for 1 h each) from Point Reyes National Seashore, known to be lacking in ECM fungal spores (Bruns *et al.*, 2009). Pine seedlings were planted in 50-ml Cone-tainers (Super 'Stubby' Cell Cone-tainer; Stuewe & Sons Inc., Tangent, OR, USA) using a 1 : 1 ratio of dried native soil and autoclaved coarse yellow sand to improve drainage. Plants were watered every other day and grown in the glasshouse without fertilizer for *c.* 6 months before harvesting. Treatments were randomized among trays on initial planting with trays randomized every other week. In total, 1806 seedlings were planted (19 plots × 13 samples (+ aerial control) × 2 tree hosts (common and native) × 3–5 replicates per tree). Plants were harvested by rinsing the soils from the roots under tap water, and collecting ECM root tips with sterilized forceps under the dissecting microscope. The roots of the control plants were inspected under the dissecting microscope to confirm noncolonization.

Molecular identification of species

Approximately six root tip clusters representing different ECM morphotypes were harvested from each plant, frozen at −80°C and lyophilized. Root tips from the replicates of each host plant per sampling location were pooled for DNA extraction following a modified version (see Methods S4) of the Qiagen (Valencia, CA, USA) DNAeasy Blood and Tissue Kit.

We PCR amplified the internal transcribed spacer (ITS). Forward primers comprised the 454 Fusion Primer A-adaptor, a specific multiplex identifier (MID) barcode and the ITS1F primer (Gardes & Bruns, 1993), whereas the reverse primer was composed of the B-adaptor and ITS4 primer (White *et al.*, 1990). Pyrosequencing PCR mixtures contained 0.25 μl of HotStartTaq polymerase (Qiagen), 2.50 μl of 10× PCR buffer (Qiagen), 2.50 μl of 10× each deoxynucleoside triphosphate (dNTP) (200 μM), 0.20 μl of 50 μM reverse primer, 1 μl of 10 μM forward primer, 2 μl of DNA template (some samples diluted 1 : 10 to overcome inhibitors) and water up to 25 μl. Thermocycler

conditions were the same as described previously (Adams *et al.*, 2013). Triplicate PCRs per barcode were pooled and cleaned using AMPure magnetic beads (Beckman Coulter Inc., Brea, CA, USA), quantified fluorescently with the Qubit dsDNA HS kit (Life Technologies Inc., Gaithersburg, MD, USA) and pooled at equimolar concentration. Libraries were quality checked for concentration and amplicon size using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) at the Functional Genomics Laboratory, University of California, Berkeley, CA, USA. Pyrosequencing was performed on the Roche/454 Genome Sequencer FLX+ at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, IL, USA. Sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under accession number SRP047454.

Pyrosequencing data were processed using the QIIME pipeline versions 1.60–1.80 (Caporaso *et al.*, 2010). Initial sequence processing and sample assignment were performed using the split_libraries.py command with a minimum/maximum sequence length cut-off of 200/800 bp, maximum primer mismatch of 0 and minimum quality score of 25. Sequences were denoised in separate batches using the flowgram clustering algorithm (Reeder & Knight, 2010). We extracted the ITS1 subregion from the nuclear ITS sequences using the perl script FungalITSextrator.pl in an effort to reduce chimera formation by removing the highly conserved 5.8S region (Nilsson *et al.*, 2010). We then used USEARCH (Edgar, 2010) as implemented in the QIIME pipeline to remove the remaining chimeric sequences employing the UCHIME algorithm (Edgar *et al.*, 2011) and referenced against the UNITE database (Koljalg *et al.*, 2005). Sequences were clustered *de novo* into operational taxonomic units (OTUs) to 95% similarity, and singletons were removed. Taxonomy was assigned by searching representative sequences from each OTU against the UNITE database (Koljalg *et al.*, 2005) accessed on 19 December 2013 with the QIIME assign_taxonomy.py script. We then built an OTU table, removed the samples of uninoculated controls and filtered for ECM fungal or putative ECM taxa based on current knowledge of the metabolic lifestyle of the BLAST matches for each individual taxon (Tedersoo *et al.*, 2010a). For the QIIME script used to filter for ECM fungal taxa, see Methods S5, and for the taxa added on to filter for putative ECM fungal taxa, see Methods S6. After filtering for ECM fungal taxa, the OTU table was rarefied to 100 sequences per sample to enable comparisons across samples. Unless otherwise stated, all analyses and results are based on prefiltering for ECM fungal taxa and rarefying to 100 sequences per sample.

Soil chemistry and climate

Each soil horizon was analyzed for total carbon, total nitrogen, pH and percentage of soil moisture before drying for bioassays as described previously (Talbot *et al.*, 2014). Climate data for each sample were obtained using the WorldClim global climate dataset (Hijmans *et al.*, 2005) as described previously (Talbot *et al.*, 2014).

Data analysis and statistics

To evaluate the role of different spatial and environmental factors in determining the structure of fungal communities, we collapsed environmental variables into vectors using principal components analysis (PCA) as described previously (Talbot *et al.*, 2014). Community dissimilarity matrices were created in QIIME and exported to R version 3.0.2 (R Core Team, 2013) for statistical analyses. We graphed OTU accumulation curves of ECM fungi and all fungal OTUs per sample against sequences per sample in QIIME (Fig. S1), and found that all samples had been sequenced to saturation for ECM OTUs. We also graphed spore bank ECM OTU accumulation curves per plot for richness against the number of samples in R (Fig. S2) to determine whether sampling was adequate to fully describe the fungal assemblages associated with each plot; all of them were nearly saturated. Thus, we used the observed OTU number to estimate the alpha richness for each sample. We conducted all beta diversity analyses using both the Sorensen–Dice and Jaccard indices as the binary (presence/absence)-based metrics, and the Bray–Curtis and Morisita–Horn indices as the abundance-based metrics. Community analyses were conducted using the vegan (Oksanen *et al.*, 2012) and ecodist (Goslee & Urban, 2007) packages in R. Mantel tests implemented in vegan were used to directly compare geographic distances with community composition differences. Because our study spanned from 5 m to nearly 6000 km, spatial data were log-transformed. Mantel correlograms, as implemented in vegan, were used to determine at what spatial scale distance decay occurs (Legendre & Fortin, 1989). The effect of plant host on beta diversity and community composition was analyzed with ADONIS, a nonparametric version of permutational multivariate analysis of variance (Anderson, 2001), and the effect of plant host on alpha richness was analyzed using ANOVA and generalized linear models. The most frequent taxa in each site were identified by creating rank abundance curves in R based on presence/absence in the 13 sampling points per plot.

To disentangle the relative importance of various spatial and environmental factors controlling fungal community composition in spore bank communities across samples, we used multiple regression on matrices (MRM) (Lichstein, 2007) as implemented in the ecodist package of R. Permutation tests were conducted with spatial distance (log-transformed meters), soil chemistry PC1 or PC2, or climate principal component axes as independent variables, and with Jaccard community dissimilarity among samples as the dependent variable. For comparative analyses among samples, samples were filtered for ECM fungal taxa only and rarefied to 100 ITS reads per sample ($n = 315$). To determine the relative importance of geography and local environmental factors in structuring communities, we then conducted multiple regression using MRM. To reduce the effect of spurious relationships between variables, we ran the MRM test, removed nonsignificant variables and then ran the test again (Martiny *et al.*, 2011). We report the model results from the second run. Patterns of community dissimilarity among plots were visualized with nonmetric multidimensional scaling (NMDS) as implemented in the MASS package (Venables & Ripley, 2002) in R. To visualize

the role of geography in structuring spore bank ECM fungal communities, color was assigned to each sample point based on a location in North America following a modified version of this approach (Kreft & Jetz, 2010), using the same color codes as described previously (Talbot *et al.*, 2014). All analyses were repeated with the four beta diversity metrics and several rarefaction levels (50, 100 or 200 sequences per sample). In cases in which data did not conform to assumptions of normality and homogeneity of variance, values were log-transformed before analysis. All statistical tests were considered to be significant at $P < 0.05$.

Comparison of spore bank and soil fungi

Soil fungal sequences were determined as described previously (Talbot *et al.*, 2014). To compare the soil fungal sequences with the spore bank fungi assayed from the same sampling locations, all raw fasta files from the soil fungi and from the spore bank fungi were concatenated and OTUs were picked together using the bioinformatics pipeline described above. All samples were rarefied to 100 sequences per sample so that comparisons could be made at the same sequencing depth, and ADONIS was used to compare the community composition of soil vs spore bank at the same locations. Correlation tests with the Pearson product moment correlation coefficient were performed to determine the relationship between the abundant ECM fungal OTUs present in the soil vs the spore bank. We determined the abundance of an OTU as the frequency across soil samples ($n = 342$) and across spore bank samples ($n = 312$), removed the rare OTUs by including only the OTUs that were present in at least 10% of the soil samples ($n = 34$), or the OTUs that were present in at least 10% of the spore bank samples ($n = 31$), and included only the OTUs that were present in both the soil and the spore bank. We then plotted the frequency across samples of each OTU in the spore bank vs the soil community, and performed a correlation test to determine the relationship between the frequency of the abundant ECM fungal OTUs in the soil vs spore bank.

Results

Taxonomic richness and community composition of ECM fungal spore bank

Our initial dataset included a total of 940 592 raw sequence reads. After initial quality filtering, in which sequences were trimmed to 200–800 bp and had to meet a mean minimum quality score of 25, there were 571 052 sequences (570 744 sequences after ITS1 was extracted) for downstream analysis (see Table S1 for complete 454 pyrosequencing library statistics). A total of 655 fungal OTUs were identified from a total of 393 samples across 19 plots. Of these 655 fungal OTUs, 176 could be identified as putative ECM fungal OTUs, and 137 of these could confidently and conservatively be identified as ECM fungal OTUs. The overall mean per sample ECM fungal OTU richness values $\pm$ SE for each spore bank sample were 3.95 ± 0.11 and 4.5 ± 0.21 for the mature forest soil fungi. Table S2(a) lists per

sample means for all 19 plots for spore bank vs mature forest soil ECM fungal communities. Per sample spore bank ECM richness was lowest in Alaska and highest in Mississippi and North Carolina (ANOVA: $F_{7,308} = 6.472$, $P < 0.0001$; Fig. 1; Table S2b). The total ECM fungal richness per plot was much higher in the soil than in the spore bank. It ranged from 42 to 107 OTUs in the mature forest soil (72.1 ± 16.5) vs 13 to 34 OTUs in the spore bank (23.4 ± 5.4) (Table S3).

Effect of bioassay host on OTU recovery

There was no significant effect of bioassay host on per sample ECM fungal richness. In all cases, *P. muricata* recovered as many if not more OTUs per sample than the native host (Fig. 2), and there was no significant main effect of bioassay host on ECM fungal richness. In general, *P. muricata* recovered communities statistically similar in composition to the native host grown at each sampling location (Table S4). *P. muricata* recovered similar communities to *P. taeda* and *P. contorta*, but recovered different communities from *P. banksiana* (ADONIS: $R^2 = 0.2$, $P < 0.01$; Table S4), which is probably because *P. banksiana* recovered fewer OTUs on average per sample than did *P. muricata*. *P. muricata* recovered statistically similar communities to *P. ponderosa* using abundance-based beta diversity metrics; using binary beta diversity metrics, *P. ponderosa* recovered slightly different communities, but only *c.* 6% of the variance was explained by tree host (Table S4). The native hosts *P. monticola* and *P. glauca* had insufficient survival to detect significant differences. The limited survival may be a result of the fact that *P. monticola* and *P. glauca* are montane and boreal species, respectively, and the ambient temperature in the glasshouses in California was too high for them.

Biogeography and distance decay at a continental scale

A significant effect of distance decay on ECM fungal diversity was observed (Fig. 3a). There was a significant spatial autocorrelation of fungal communities, with Mantel *r* ranging from 0.33 to 0.51 and $P < 0.01$. This pattern was robust to beta diversity metric and rarefaction level (Table S5), and to removal of the Alaska plots, which have a *Picea* rather than a *Pinus* host (Fig. S3). Communities were more similar than expected by chance within 500 km, and were more dissimilar than expected by chance at spatial scales ranging from 500 to 6000 km (Fig. 3b). NMDS ordination showed that fungal community composition was correlated strongly with geographic region within North America (Fig. 4).

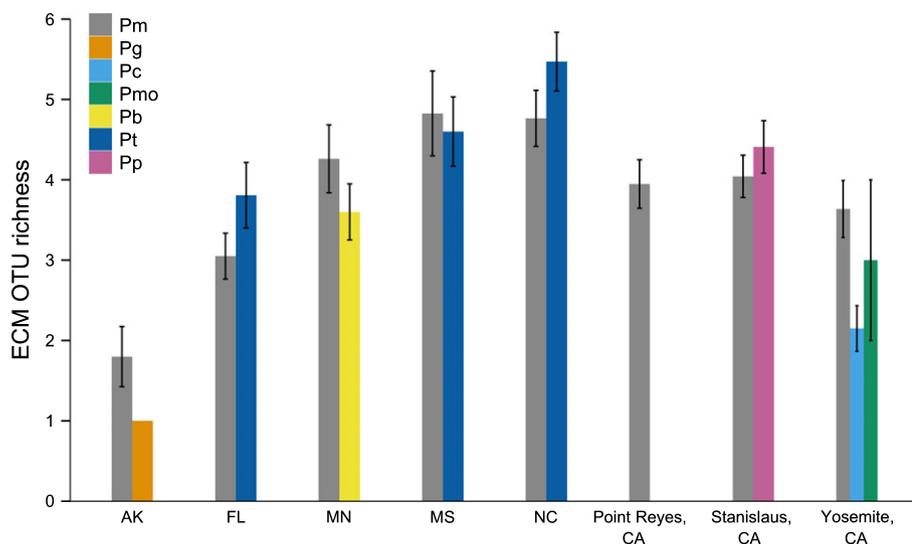## Relative importance of environmental vs spatial factors

Climate, host plant identity and local environment varied across our sites (Methods S1). However, these factors played a small role in structuring spore bank ECM fungal communities relative to spatial distance, which had an R^2 value that was 35 times larger than the next-nearest parameter (Table 1).

## Dominant taxa in spore banks across North America

Overall, we recovered ECM fungal taxa from 18 genera and 10 families. The top 10 most frequent ECM fungal spore bank OTUs across the 19 plots belonged to *Rhizopogon*, *Wilcoxina*, *Cenococcum*, *Thelephora*, *Tuber* and *Laccaria*. With the exception of *Laccaria*, which is known to be an early successional mycorrhizal partner common in disturbed settings (Danielson, 1984; Last *et al.*, 1987), all of the most frequent taxa form hypogeous fruiting bodies (*Rhizopogon*, *Tuber*) or sporulate in the soil either by forming resupinate crusts or producing asexual spores directly on hyphae (*Wilcoxina*, *Cenococcum*, *Thelephora*). The top five most frequent ECM fungal OTUs from spore banks across the continent are summarized in Table 2. Briefly, the western and southeastern parts of the USA were dominated by species of *Rhizopogon*, which dropped out in more northern regions. Minnesota was dominated by *Laccaria* and *Suillus*, and Alaska was dominated by *Wilcoxina* and *Sphaerosporella*, two genera in the
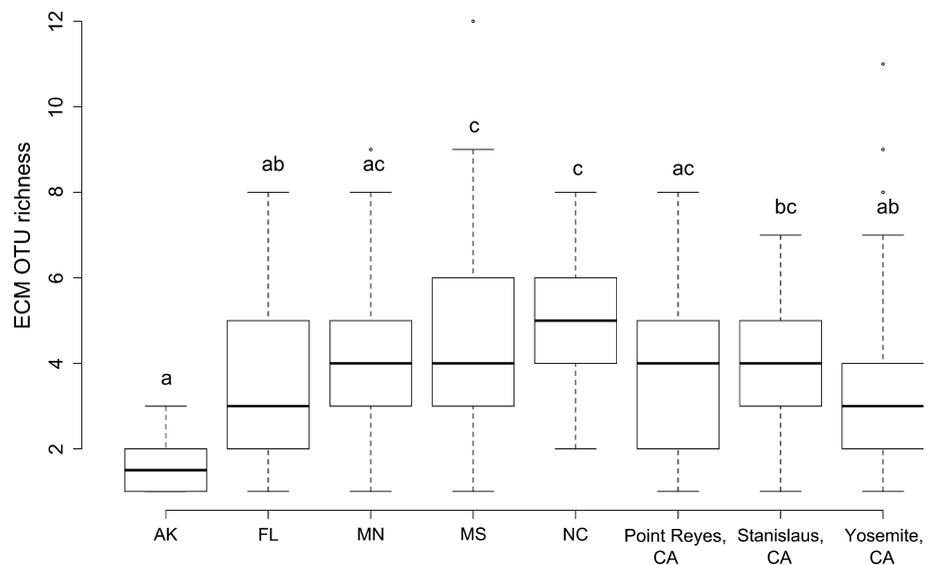
Fig. 1 Median richness of ectomycorrhizal (ECM) fungal operational taxonomic units (OTUs) per spore bank sample by sampling location. Lowercase letters represent Tukey honestly significant differences (HSDs) at $P < 0.05$. AK, Alaska; FL, Florida; MN, Minnesota; MS, Mississippi; NC, North Carolina; Point Reyes National Seashore, Stanislaus National Forest and Yosemite National Park are all sampling locations within California (CA). Whiskers represent interquartile range (IQR) × 1.5.
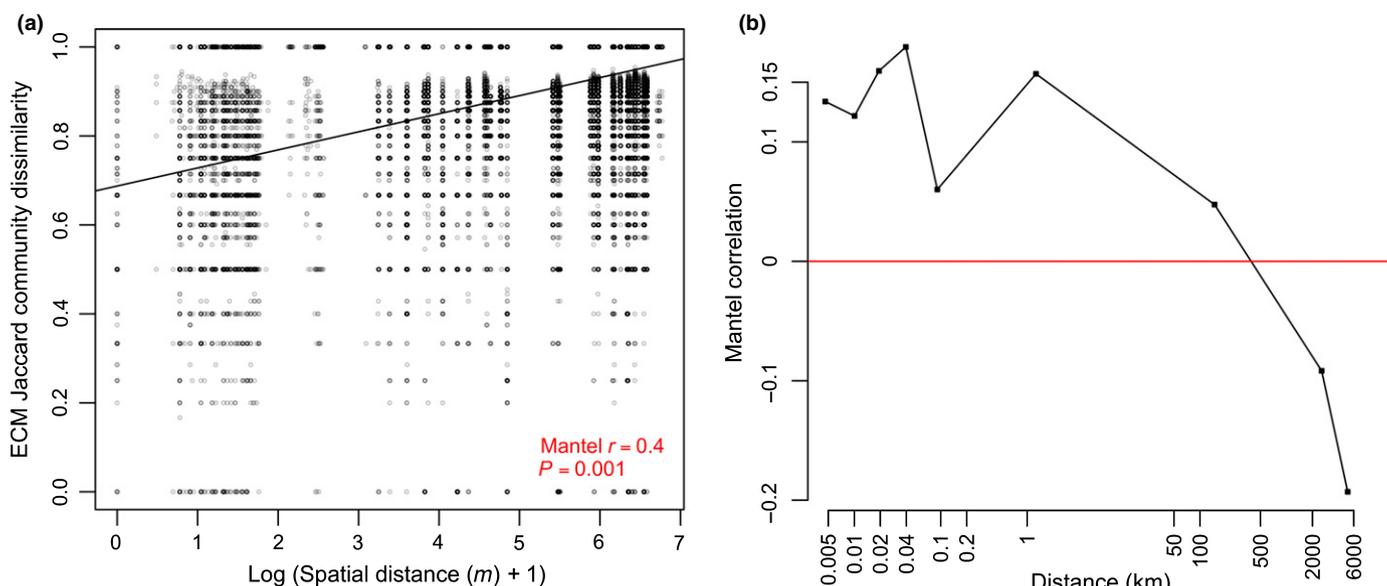
Fig. 3 (a) Mantel test of ectomycorrhizal (ECM) fungal Jaccard community dissimilarity against log-transformed spatial distance (m). There is a significant effect of distance decay across the continent (Mantel $r = 0.4$, $P < 0.001$). Points are transparent and so darker circles indicate multiple points on top of each other. (b) Mantel correlogram shows that samples are more similar than expected by chance within 500 km, and more dissimilar than expected by chance at scales above 500 km. Solid black squares indicate significance at $P < 0.05$.

Pyronemataceae, and the resupinates *Piloderma* and *Thelephora*. Rank abundance curves by both sample frequency and read abundance for each sampling location are shown in Figs S4–S12.

Comparison with mature forest soil taxa

The spore bank ECM fungal community was much less species rich and significantly different from the mature forest soil ECM fungal community at every sampling location (Table S6). Within region, soil and spore bank communities were least different at Point Reyes National Seashore (ADONIS: $R^2 = 0.13$; $P < 0.01$) and most different in North Carolina (ADONIS: $R^2 = 0.26$; $P < 0.01$; Table S6). Although our a priori expectation was that

the spore bank would be a small subset of the mature forest soil fungal community, in all cases over half of the spore bank taxa were never detected in the mature forest soil community (Fig. 5). Indeed, there was a strong negative correlation between the frequency of the overlapping abundant spore bank vs abundant soil ECM fungal OTUs (Fig. 6). The negative correlation was strengthened by the addition of putative ECM taxa to the analyses (Pearson's $r = -0.83$, $P < 0.001$).

In most locations, the most frequent ECM fungal taxa detected in the soil vs the spore bank did not overlap (Table S7). The notable exceptions included *Rhizopogon salebrosus* in Point Reyes National Seashore, an uncultured *Cenococcum* species in Florida and Mississippi, and *Wilcoxina* and *Piloderma* in Alaska.
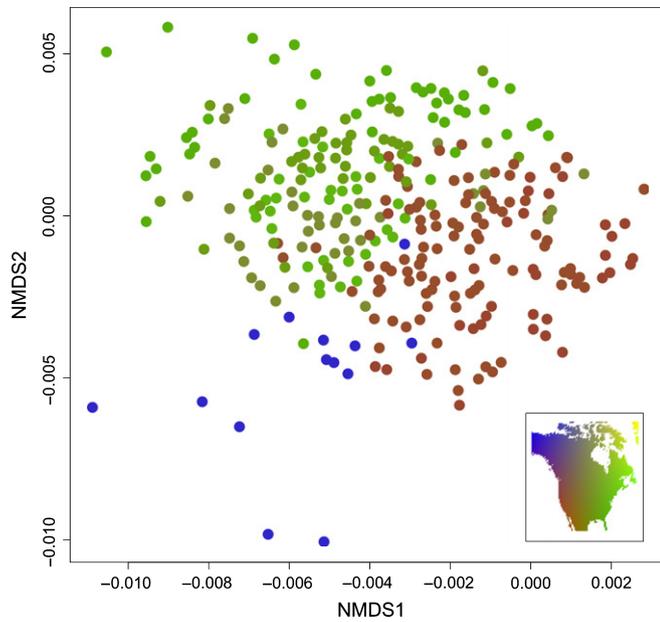
Fig. 4 Ordination of spore bank ectomycorrhizal (ECM) fungal communities. Samples were prefiltered for ECM and putative ECM fungal taxa and rarefied to 200 sequences per sample. Samples are colored by geographic location in North America (inset). NMDS, nonmetric multidimensional scaling.

Table 1 Multiple regression on matrices (MRM) statistics predicting ectomycorrhizal fungal community composition

Explanatory factor	Fungal community composition MRM R^2
Single factor	
Spatial distance (m)	0.16***
Climate PC1	0.036***
Climate PC2	0.067***
Climate PC3	0.0414***
Soil chemistry PC1	0.0015**
Soil chemistry PC2	0.0082***
Multiple regression	0.17***
Spatial distance (m)	0.12***
Climate PC1	
Climate PC2	
Climate PC3	0.0034***
Soil chemistry PC1	0.00074*
Soil chemistry PC2	0.0014**

Single factor statistics are generated from single regression analyses (for climate, soil chemistry, spatial distance) or MRM analysis (for community composition and spatial distance) using single factors. To reduce the effect of spurious relationships between variables, we ran the MRM test, removed nonsignificant variables and then reran the test again. The results of the second run are shown. In MRM analysis, partial R^2 for individual factors represents the unique fraction explained. Asterisks represent significance of regression (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

Discussion

Our results largely support our initial hypotheses, and extend what was a regional pattern to a basic understanding of ECM fungal spore banks at a continental scale. We found that ECM fungal spore banks are geographically patterned (H1); that the

identity of the bioassay host did not significantly affect the alpha and beta diversity of the recovered taxa, at least within congeneric host comparisons (H2); that ECM fungal spore banks were composed of a limited set of genera (H3); and that ECM fungal taxa in spore banks were rare or absent from mature forest soil ECM fungal communities (H4), with a few exceptions, such as *Cenococcum*, *R. salebrosus*, some *Thelephoraceae*, and *Piloderma*.

Geographic differentiation appears to be a general feature of fungal communities. Our results add further support to patterns seen in the mature forest soil fungal communities sampled at the same locations (Talbot *et al.*, 2014), and a recent meta-analysis of NGS data (Meiser *et al.*, 2014). This geographic signal is evident in ECM fungal spore banks as a significant distance decay of community dissimilarity spanning the meter to continental scale (Fig. 3a), and is consistent with a strong role for dispersal limitation as a driver of fungal community turnover (Peay *et al.*, 2012; Talbot *et al.*, 2014). Geographic endemism has long been known as a key feature of plants and animals, and our results now add to the growing evidence that fungi also have discrete ranges (Taylor *et al.*, 2006; Amend *et al.*, 2010; Sato *et al.*, 2012; Talbot *et al.*, 2014). The geographic pattern observed in North American ECM fungal spore banks (Fig. 4) broadly parallels the biogeographic provinces previously described for North American plants (Kreft & Jetz, 2010).

At finer spatial scales, it is interesting that the ECM fungal richness in the spore bank and the mature soil scale very differently. At the level of individual soil cores, both measures show limited richness (3.95 ± 0.11 for the spore bank vs 4.5 ± 0.21 for the soil community) but, when scaled up to the plot level (40×40 m^2), the estimates of richness are roughly three-fold different, with an average plot richness $\pm$ SE of 72.1 ± 16.5 in the soil vs 23.4 ± 5.4 in the spore bank (Table S3). These results show that species richness in the spore bank scales up more slowly with distance at the local scale than does the mature forest community. We speculate that this difference is caused by spore longevity (Bruns *et al.*, 2009) which helps to homogenize spore dispersal at this scale. By contrast, the forest fungal soil community, which is likely dominated by active mycelium, may be more sensitive to environmental heterogeneity and competitive interactions at the plot level. Our analysis may underestimate the scale of this effect, because the sequencing depth was sufficient to fully capture and saturate the spore bank, but not the soil fungal richness (Fig. S1), and because each spore bank sample represents a much larger volume of soil than each fresh soil sample.

Host specificity is known to be an important niche axis for structuring the function and diversity of ECM fungal communities (Dickie, 2007; Ishida *et al.*, 2007; Tedersoo *et al.*, 2010c). In this study, however, we did not find host specificity to be a major driver of the observed patterns. Our study was limited to host comparisons within the genus *Pinus*, and our results may be limited to the seedling stage of these hosts. Therefore, host effect may have been stronger if a taxonomically broader set of plants had been used as bioassays. At least some of the dominant ECM fungal species recovered in our studies are known to be restricted to the genus *Pinus* (e.g. *Suillus* and *Rhizopogon* spp.). However, many of these pine species tend to form a near monoculture

Table 2 Top five most frequent ectomycorrhizal (ECM) fungal operational taxonomic units (OTUs) in spore banks across North America

Location	Assigned ID	Morphology	% Samples	% Sequences
Alaska	*Sphaerosporella1*	Soil sporulator	68	20.5
Alaska	*Wilcoxina1*	Soil sporulator	58	39.1
Alaska	*Wilcoxina2*	Soil sporulator	35	14.5
Alaska	*Piloderma1*	Crust	28	2.0
Alaska	*Thelephora terrestris*	Crust	18	3.3
Florida	*Rhizopogon2*	Truffle	51	10.8
Florida	*Cenococcum geophilum1*	Soil sporulator	31	5.6
Florida	*Thelephora1*	Crust	29	3.0
Florida	*Tomentella2*	Crust	27	9.3
Florida	*Tomentella1*	Crust	24	12.8
Minnesota	*Laccaria1*	Mushroom	54	17.2
Minnesota	*Suillus brevipes*	Mushroom	44	16.3
Minnesota	*Suillus*	Mushroom	44	8.7
Minnesota	*Rhizopogon1*	Truffle	44	1.3
Minnesota	*Tuber1*	Truffle	28	15.9
Mississippi	*Rhizopogon1*	Truffle	84	21.3
Mississippi	*Cenococcum geophilum1*	Soil sporulator	47	4.4
Mississippi	*Cenococcum geophilum3*	Soil sporulator	42	4.3
Mississippi	*Rhizopogon2*	Truffle	32	5.1
Mississippi	*Chloridium virescens*	Soil sporulator	29	0.7
North Carolina	*Rhizopogon1*	Truffle	94	7.8
North Carolina	*Tuber2*	Truffle	79	9.1
North Carolina	*Tuber shearii*	Truffle	77	18.9
North Carolina	*Tuber separans*	Truffle	51	3.9
North Carolina	*Cenococcum geophilum2*	Soil sporulator	40	1.5
Point Reyes	*Rhizopogon occidentalis1*	Truffle	82	0.5
Point Reyes	*Rhizopogon salebrosus*	Truffle	69	10.3
Point Reyes	*Rhizopogon fuscorubens2*	Truffle	62	0.4
Point Reyes	*Wilcoxina mikolae*	Soil sporulator	59	36.7
Point Reyes	*Thelephora albomarginata*	Crust	51	20.0
Stanislaus	*Rhizopogon arctostaphyli*	Truffle	100	28.8
Stanislaus	*Rhizopogon variabilisporus*	Truffle	53	5.6
Stanislaus	*Rhizopogon salebrosus*	Truffle	47	2.3
Stanislaus	*Rhizopogon1*	Soil sporulator	45	5.5
Stanislaus	*Wilcoxina3*	Soil sporulator	43	4.1
Yosemite lodgepole pine	*Wilcoxina mikolae*	Soil sporulator	72	75.1
Yosemite lodgepole pine	*Rhizopogon fuscorubens3*	Truffle	46	0.9
Yosemite lodgepole pine	*Rhizopogon pseudoroseolus2*	Truffle	40	6.2
Yosemite lodgepole pine	*Rhizopogon occidentalis2*	Truffle	34	0.5
Yosemite lodgepole pine	*Rhizopogon pseudoroseolus1*	Truffle	22	0.2
Yosemite white pine	*Rhizopogon fuscorubens1*	Truffle	72	2.7
Yosemite white pine	*Rhizopogon occidentalis2*	Truffle	48	1.8
Yosemite white pine	*Rhizopogon pseudoroseolus2*	Truffle	41	10.2
Yosemite white pine	*Wilcoxina mikolae*	Soil sporulator	38	21.5
Yosemite white pine	*Rhizopogon pseudoroseolus1*	Truffle	34	0.7

Stanislaus, Point Reyes and Yosemite are all sampling locations within California, and Yosemite was separated because it is the only location in which we sampled in sites with distantly related hosts within the genus *Pinus*. Alaska includes plots AK1 and AK2, Florida includes plots FL1 and FL2, Minnesota includes plots MN1 and MN2, Mississippi includes plots MS1 and MS2, North Carolina includes plots NC1 and NC2, and Point Reyes includes plots PR1, PR2 and PR3. Stanislaus includes plots CA1 and CA2, Yosemite white pine includes plots CA3 and CA6, and Yosemite lodgepole pine includes plots CA4 and CA5.

following disturbance, and so the effect of host diversity in the field may be similarly limited.

Pinus muricata was an effective bioassay host with respect to the detection of regional patterns. It yielded fungal communities similar to those of the native host (Table S4), and was the best host to maximize OTU recovery and biodiversity (Fig. 2). We know from previous studies that *P. muricata* is a highly receptive host, recovering as many or more taxa than native hosts for *Rhizopogon* species (Rusca *et al.*, 2006). We now know that this is

a much more general pattern. This property may be because *P. muricata* grows well in glasshouses in our climate, thus guaranteeing sufficient survival from all treatments. The fact that it is a ruderal, serotinous-coned species that colonizes rapidly after stand-replacing fire events may also be important, because, under such disturbed settings, broad receptivity to ECM fungi probably provides an important advantage for colonization and survival.

ECM fungal spore banks, in general, appeared to be enriched for nonaerially dispersed species. Unlike the mature forest ECM

Fig. 5 Venn diagrams comparing ectomycorrhizal fungal operational taxonomic units (OTUs) in the mature forest soil (dark gray) relative to ectomycorrhizal fungal OTUs in the spore bank (white) for all the sampling locations. Sequences not rarefied. AK, Alaska; FL, Florida; MN, Minnesota; MS, Mississippi; NC, North Carolina; Point Reyes National Seashore, Stanislaus National Forest and Yosemite National Park are all sampling locations within California (CA).
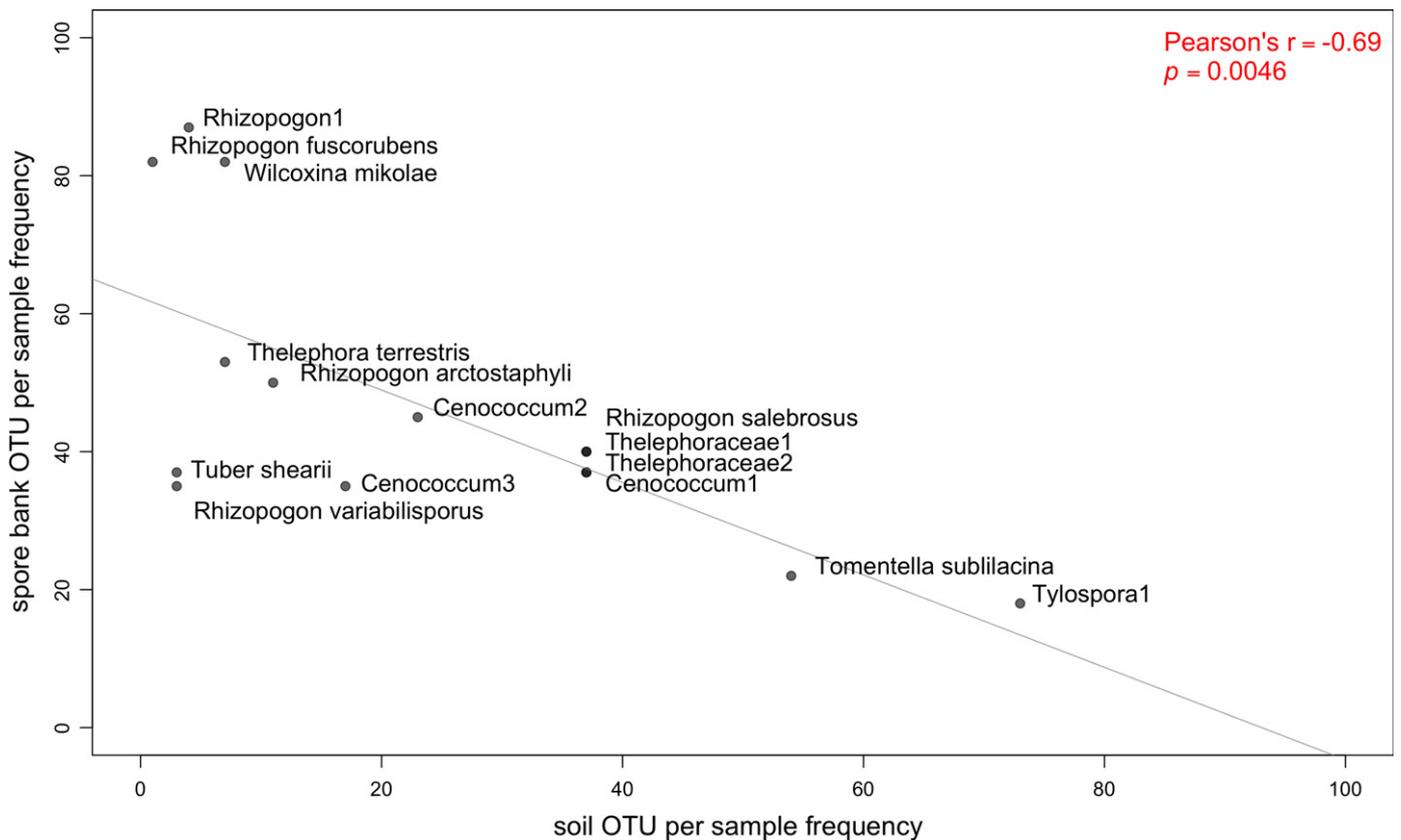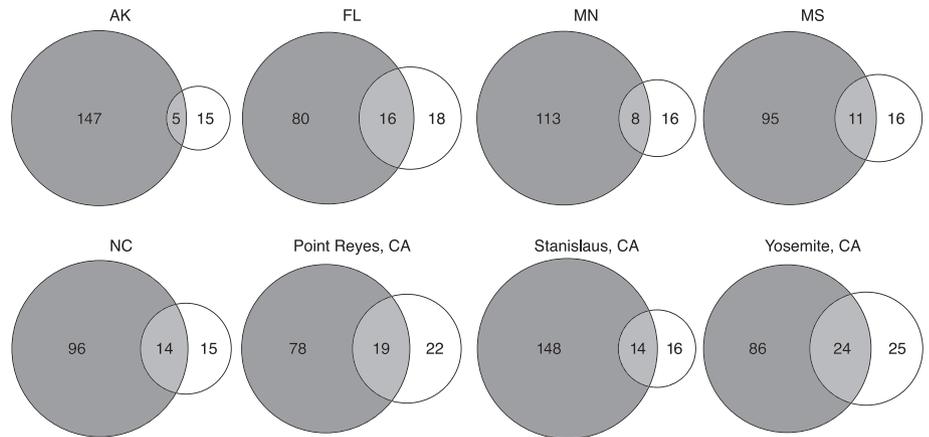
Fig. 6 Correlation test of frequency of overlapping ectomycorrhizal fungal operational taxonomic units (OTUs) present in spore bank and mature forest soil samples. Comparison is restricted to those present in at least 10% of either sample type. There is a significant negative correlation between the frequency of abundant OTUs in the spore bank vs soil (Pearson's $r = -0.69$; $P < 0.01$). Points are transparent so darker circles indicate multiple points on top of each other.

fungal community, the spore bank was dominated by a variety of hypogeous fungi (e.g. *Rhizopogon*, *Tuber*), fungi that sporulate close to or on the soil as crusts (*Thelephora*, *Tomentella*, *Piloderma*) or fungi that sporulate mitotically within the soil (*Wilcoxina*, *Cenoccocum*). Spores and resistant propagules of these fungi may be deposited in the soil at locally higher concentrations as a result of *in situ* deposition deeper into the soil profile (Miller *et al.*, 1994). Moreover, fungi dispersed by mycophagy may be preadapted for persistence (Frank *et al.*, 2009), as they must

retain their viability after passage through the microbially active digestive tracts of animals (Bonito *et al.*, 2012).

Minnesota ECM fungal spore banks were exceptional in that the most frequent taxa were wind-dispersed epigeous mushrooms, such as *Laccaria* spp. and *Suillus brevipes*, rather than primarily hypogeously deposited species. This pattern may be typical of *P. banksiana* forests in other parts of the boreal zone. *Suillus brevipes* was one of the most frequent colonizers of *P. banksiana* roots in 6-yr-old pine stands after a forest fire,

appearing in nearly 80% of the seedlings (Visser, 1995). *Laccaria*, however, was not detected as mycorrhizae in these stands (Visser, 1995), but was a frequent fruiter in disturbed *P. banksiana* areas in more southern sites in Canada (Danielson, 1984). The prevalence of epigeous wind-dispersed mushrooms in the Minnesota spore banks is perhaps a result of the fact that Minnesota is wetter and thus has more reliable fruiting of mushrooms and fewer hypogeous fungi, which are thought to be adapted to water scarcity (Bruns *et al.*, 1989). Buscardo *et al.* (2010) found *Laccaria*, *Lactarius* and *Scleroderma* only in spore banks from unburned forests or forests with long fire return intervals, and so these genera could be indicative of less fire-prone sites.

Another exceptional finding was the high frequency of *Tuber* in the spore banks of the two North Carolina plots. Although *Tuber* species have certainly been recovered in spore banks in previous pine seedling bioassays, they were always minor components of the community (Baar *et al.*, 1999; Taylor & Bruns, 1999). In one study, *T. candidum* was a frequent colonizer of roots of oak bioassay seedlings, where *Quercus garryana* was planted 5 m beyond the forest edge in Oregon (Frank *et al.*, 2009). However, an experiment that intentionally assayed for *Tuber* by collecting soils from beneath *Tuber*-colonized pecan trees found only limited *Tuber* colonization within a spore bank community dominated by other Pezizalean taxa (Bonito *et al.*, 2012).

Similar to previous studies (Taylor & Bruns, 1999), we found minimal overlap between ECM fungi in spore banks vs the mature forest soil (Fig. 5). Indeed, we found a strong negative correlation between the frequency of the abundant soil and spore bank ECM fungal taxa (Fig. 6). Our results parallel those of the plant seed bank literature, where a review of 108 articles found that plant seed banks always have fewer species than the aboveground vegetation, and that the similarity between seed banks and standing vegetation is low in forest systems (Hopfensperger, 2007). The ECM fungal taxa that were found to be common in both the soil and spore bank were *Cenococcum*, *R. salebrosus* and species in the Thelephoraceae (Fig. 6). This result is similar to findings in previous studies (Taylor & Bruns, 1999; Kipfer *et al.*, 2011; Cowden & Peterson, 2013).

We expected the spore bank to be a small subset of the soil ECM fungal community but, surprisingly, in all cases, over half of the spore bank fungi were never detected from sequencing of the soil (Fig. 5). This may be a result of sequencing depth limitations for the soil fungal community, of the limited amount of soil extracted for direct sequencing, or perhaps because the DNA extraction does not efficiently break up some fungal spores. In any case, it is clear that the bioassays used in this study are effective at selecting a subset of rare or persistent propagules in the soil community. Although it is likely that some part of the spore bank remains undetected by these methods, evidence from the field suggests that *in situ* colonization after a massive disturbance, such as forest fire (Baar *et al.*, 1999) or seedling colonization following trenching (Fleming, 1983), agrees well with results from glasshouse bioassays. Results from the study of ECM seedling colonization in windthrows are also consistent with the model that this 'reactive' part of the spore bank is the functional part in disturbance settings (Cowden & Peterson, 2013).

NGS techniques have rapidly expanded our ability to detect novel microbial diversity, but we continue to have problems with OTU inflation. As in other studies of pyrosequencing data (Adams *et al.*, 2013), we found that some of the 'distinct' OTUs were given identical taxon assignments from BLAST matches. Although this could indicate true variation in taxa, it could also be a result of a limitation in the analysis software to accurately pool taxa (Reeder & Knight, 2009). We took steps to reduce OTU inflation caused by PCR and sequencing analysis error, such as denoising the raw sequences (Reeder & Knight, 2010; Adams *et al.*, 2013), removing singletons (Tedersoo *et al.*, 2010b), extracting ITS1 in order to reduce chimeras (Nilsson *et al.*, 2010) and picking OTUs at 95% similarity. We used multiple rarefaction levels and analyzed data with several binary- and abundance-based beta diversity metrics in order to avoid the vagaries of these assumptions. In almost all cases (Tables S4–S6), we found the same qualitative and quantitative results.

From our results and knowledge gained from the previous literature, we can conclude that ECM fungal spore bank communities of pines are, in general, depauperate (Fig. 1), and represent a small, rare subset of the mature forest soil community (Fig. 5). Yet, they may be important in dictating which fungi will colonize after large-scale stand-replacing disturbances, such as clear cuts and forest fires. Judging from plant ecology, one would predict a reduction in competitive and stress-tolerant c- and s-selected species and an increase in r-selected species following disturbance (Grime, 1977). In ECM fungi, there is evidence that spore bank species are an important guild of r-selected fungal partners in regenerating stands (Last *et al.*, 1987). We suggest that it is a general successional pattern that the first ECM fungal colonizers after a severe disturbance will be those that have persisted over time in the spore bank, followed by r-selected fungi that do not persist well through time, but are capable of rapid colonization by aerial dispersal, and, finally, by more c- and s-selected species. The distinction between the spore bank and wind-dispersed ruderals seems to be clear in most, but not all, regions sampled here (e.g. Minnesota sites), and with a broader sample it will be interesting to determine whether climate and disturbance regime are predictors of this distinction.

A successional trend from high to low host specificity in dominant ECM fungal genera was pointed out by Dickie *et al.* (2013) and was hypothesized previously by Bruns *et al.* (2002). Assuming that the spore bank represents an early successional guild in waiting, this specificity pattern is also evident in the current study. The highly pine-specific *Rhizopogon* and *Suillus* species were dominants in most spore banks, whereas the mature forest communities at the same locations were more often dominated by host-generalist taxa, such as *Amanita*, *Cortinarius*, *Thelephora* and *Tomentella* (Table S7). Dickie *et al.* (2013) also suggested that genera commonly reported in mature ecosystems, but notably absent from seedlings and spore banks, such as *Amanita*, *Boletus* and *Russula*, may have higher carbon requirements relative to early successional species, and thus depend both on soil

development (Dickie *et al.*, 2009) and the presence of mature trees to be successful (Fleming, 1984).

Despite the vast increase in scale from previous studies, 19 plots is still not a lot when considering the massive scale of the North American continent. Given the knowledge of limited host effect and of the scale of spatial structure (Fig. 3), we are in the process of scaling up our experimental design by removing the native host and within plot replicates, allowing us to sample 49 more plots. With these new data, we plan to formulate a predictive framework for which fungal traits correspond with persistence in the spore bank.

Conclusions

With this study, we found remarkably generalizable results that are concordant with the previous literature, but also greatly expand on current knowledge. We found that ECM fungal spore banks in pine forests contain a limited set of fungal genera – predictable across the continental scale, yet geographically patterned at regional scales. This geographic pattern, and the underlying endemism that drives it, is itself an emerging generalization for fungal communities (Amend *et al.*, 2010; Meiser *et al.*, 2014; Talbot *et al.*, 2014). ECM fungi in the spore bank are a functionally important guild that are often the first colonizers of seedlings after disturbance (Baar *et al.*, 1999; Jones *et al.*, 2003; Buscardo *et al.*, 2010), and we show strong evidence that different pine species can access the same dominant components of this guild independent of geographic or host provenance.

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Supporting Information

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

Fig. S1 QIIME rarefaction curves.

Fig. S2 Species accumulation curves for ectomycorrhizal (ECM) fungal taxa in spore banks across North America.

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Fig. S3 Mantel test for the effect of spatial distance on ectomycorrhizal fungal community dissimilarity with Alaska removed.

Fig. S4 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in Alaska spore bank samples.

Fig. S5 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in California spore bank samples: Stanislaus National Forest.

Fig. S6 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in California spore bank samples: Yosemite National Park *Pinus contorta*.

Fig. S7 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in California spore bank samples: Yosemite National Park *Pinus monticola*.

Fig. S8 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in California spore bank samples: Point Reyes National Seashore.

Fig. S9 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in Florida spore bank samples.

Fig. S10 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in Mississippi spore bank samples.

Fig. S11 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in North Carolina spore bank samples.

Fig. S12 Rank abundance curves for ectomycorrhizal fungal operational taxonomic units (OTUs) in Minnesota spore bank samples.

Table S1 Four hundred and fifty-four Pyrosequencing library statistics

Table S2 Per sample alpha richness of ectomycorrhizal (ECM) fungi, where sequences were prefiltered for ECM fungal taxa and then rarefied to 100 sequences per sample

Table S3 Total alpha richness per plot of ectomycorrhizal (ECM) fungi, where sequences were prefiltered for ECM fungal taxa and then rarefied to 100 sequences per sample

Table S4 Effect of bioassay host on beta diversity of ectomycorrhizal fungal operational taxonomic units (OTUs) recovered

Table S5 Table of Mantel *r* statistics and *P* values across all fungi, rarefactions and beta diversity metrics used

Table S6 Beta diversity comparisons for ectomycorrhizal fungal communities in the mature forest community vs the spore bank in the same locations

Table S7 Taxonomy information on most frequent taxa for ectomycorrhizal fungal communities in spore bank vs mature forest soil for every sample location

Methods S1 Plot characteristics at each sampling site.

Methods S2 Plot sampling design.

Methods S3 Soil moisture of a subset of samples before bioassays.

Methods S4 Description of DNA extraction of ectomycorrhizal fungal root tips.

Methods S5 Script to filter ectomycorrhizal fungal taxa (conservative list) from operational taxonomic unit (OTU) table in QIIME.

Methods S6 Taxon names that were added to a previous list to filter possible ectomycorrhizal fungal taxa from operational taxonomic unit (OTU) table in QIIME.

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