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The theory of island biogeography applies to ectomycorrhizal fungi in subalpine tree "islands" at a fine scale

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Citation: Glassman, S. I., K. C. Lubetkin, J. A. Chung, and T. D. Bruns. 2017. The theory of island biogeography applies to ectomycorrhizal fungi in subalpine tree "islands" at a fine scale. Ecosphere 8(2):e01677. 10.1002/ecs2.1677

Abstract. The theory of island biogeography, which predicts that species richness is a function of island size and distance from the mainland, is well tested with macro-fauna and flora. Yet, in many ways, microbes are more appropriate for testing this and other ecological theories due to their small size and short generation times that translate to an ease of replication. We used a natural experimental system of isolated "host islands" to test the generality of the theory of island biogeography. Specifically, we tested whether ectomycorrhizal fungal (EMF) richness increased with tree size and decreased with distance from forest in a subalpine basin in Yosemite National Park for two congeneric pine species, *Pinus albicaulis* and *Pinus contorta*. We determined EMF richness with next-generation sequencing, measured the size and age of each tree island (n = 40), and calculated geographic distances from each tree to the nearest forest edge. We found that EMF richness increased with island size (as measured by tree volume) and tree age for both pine species and decreased with distance from forest edge for *P. albicaulis*. Thus, we show the applicability of the theory to microbial symbionts in harsh, dry, and likely non-equilibrium systems. In addition, we found that despite the fact that our tree islands had a mean age of 65 yr, a pioneer community of EMF dominated. We interpret this as evidence that water stress interacts with succession to create a sustained period of early-stage fungi even in mature trees.

Key words: distance decay; ectomycorrhizal (ECM) fungi; Illumina MiSeq; island biogeography; next generation sequencing (NGS); *Pinus albicaulis; Pinus contorta;* taxa–area relationship; Yosemite National Park.

Received 12 December 2016; accepted 19 December 2016. Corresponding Editor: Debra P. C. Peters.
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INTRODUCTION

Islands are attractive model systems to study the basic processes of ecology and evolution due to their simplified and easily replicated nature. In 1963, MacArthur and Wilson originally recognized the utility of island biotas as providing discrete, manageable, and replicated microcosms of biological communities (MacArthur and Wilson 1963, Warren et al. 2015). The theory of island biogeography, which predicts that species richness is island size and distance from the mainland (MacArthur and Wilson 1967), is well tested with macro-fauna and flora. Early on, it was recognized that host plants could function as islands as well, in the context of plant-associated insects (Janzen 1968). Due to recent advances in next-generation sequencing techniques, ecologists can now apply these principles to microorganisms, which lend themselves well to ecological studies because their habitats are easily replicated in a small geographic space. In recent years, we have made advances in the application of theory to microbial ecology (Prosser et al. 2007), and the theory of island biogeography has been applied to archaea (Whitaker et al. 2003), bacteria (Bell et al.

2005), unicellular fungi (Belisle et al. 2012), and ectomycorrhizal fungi (EMF) (Peay et al. 2007, 2010).

Ectomycorrhizal fungi are associated primarily with woody plants where they provide increased access to soil nutrients in addition to many other benefits in exchange for photosynthetically derived carbon (Smith and Read 2008). Their obligate associations with members of the Pinaceae and Fagales make them a dominant symbiosis in boreal and temperate forests (Smith and Read 2008). Despite dispersing astronomical numbers of spores (Dahlberg and Stenlid 1994, Peay et al. 2012) via air or through animal vectors (Maser et al. 1978, Lilleskov and Bruns 2005, Ashkannejhad and Horton 2006), EMF can experience either "fundamental" or "realized" dispersal limitation for a number of reasons. EMF can experience "fundamental" dispersal limitation by processes that prevent propagules from reaching appropriate habitat: The vast majority of spores never make it more than 0.5 m from the mushroom (Li 2005). EMF can also experience "realized" dispersal limitation, in which spores may reach an appropriate habitat, but fail to establish before they perish because uncolonized roots are rare and difficult to access. For example, germinating spores experience intense competition for roots (Kennedy et al. 2007), and strong priority effects can inhibit establishment (Kennedy and Bruns 2005).

Ectomycorrhizal fungi that inhabit tree roots thus offer unique opportunities for the application of the principles of island biogeography. Similar to many insect associates of host plants (Janzen 1968), EMF are obligate symbionts of trees. When trees are isolated in a matrix of non-EMF hosts, trees can be viewed as islands (Peay et al. 2007, 2010). Isolated trees can thus represent well-replicated habitat islands, among which the host-associated fungi disperse via wind or animal dispersal agents.

Island biogeography theory has previously been tested in EMF using isolated "tree islands" in a wetter, sea-level, coastal pine forest in Point Reyes National seashore (Peay et al. 2007, 2010). Using 10-yr-old EMF tree patches ranging in size from <10 to >10,000 m^2, it was shown that EMF richness increases with tree island size (Peay et al. 2007). In isolated trees with a mean age of 45 yr that ranged from 1 m to >1 km from the nearest forest edge, it was found the EMF richness significantly decreased with increasing distance to the forest "mainland" (Peay et al. 2010).

However, there are many reasons why island biogeography theory might not apply within some environmental conditions, and why ecological outcomes could differ when measured at finer scales (Martiny et al. 2011). For instance, EMF richness tends to decrease with increasing altitude (Bahram et al. 2012). Thus, it is reasonable to expect that processes affecting EMF richness in low vs. high altitudes might differ. Furthermore, precipitation as rain is necessary for mushrooms that disperse spores via wind to form, and thus, drier, colder habitats would create a shorter, less predictable fruiting period that could translate into more intense dispersal limitation. Moreover, species of EMF that are dispersed primarily by animal vectors would be affected by presence and availability of dispersal agents. Thus, testing these principles at a finer scale and in a high-elevation, dry system that might be dominated by EMF with different dispersal modes could lead to very different outcomes than those that were previously tested in a wetter, sea-level, coastal pine forest (Peay et al. 2010).

Here, we utilize a natural experimental system of isolated "tree islands" in a subalpine basin in Yosemite National Park to test the generality of the theory of island biogeography, which states that richness is a function of island size and distance from mainland. This system contains an "archipelago" of isolated congeneric pine tree "islands" established at varying distances from each other and the forest edge. Two *Pinus* congeners are the only ectomycorrhizal hosts in the basin, and individual tree "islands" of the two species are interspersed within the basin (Fig. 1). This system allowed us to test the generality of island biogeography and to use isolated tree islands to test for the effects of the size, age, and degree of isolation of tree host on EMF species richness in a harsher, drier, colder, subalpine system than has been tested previously. Furthermore, we test whether the theory of island biogeography is still applicable when tested at a fine scale (<1 km), and in a non-traditional island system where there are multiple possible nearby "mainland" forest patches. We identified EMF from soil using Illumina MiSeq sequencing of internal transcribed spacer (ITS) amplicons, used distance from nearest forest edge as the proxy of

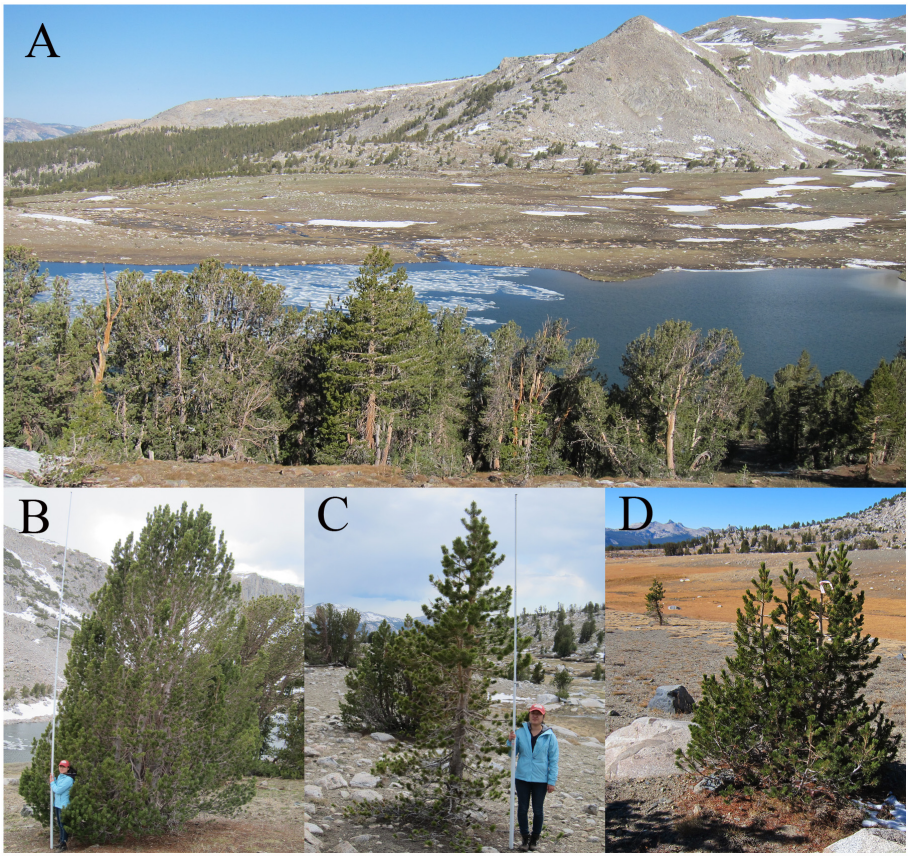


Fig. 1. Tree islands in the Gaylor Lake Basin. (A) View looking north across Gaylor Lake into basin from the forested ridge with individual trees barely visible; individual sampled tree islands showing a range of size, isolation, and species. Examples of some of the isolated tree "islands" with a range of isolation shown in panels B–D. (B) *Pinus albicaulis* (PA32), (C) *Pinus contorta* (PC8), and (D) *P. albicaulis* (PA30). Photographs of all individual trees located in Appendix S1: Fig. S1. Judy Chung for scale.

continental source pool, and volume of photosynthetic tissue of tree as size of islands, to test whether EMF richness decreases with distance from the forest edge and increases with tree size and/or age.

Materials and Methods

Description of tree islands

Gaylor Lake Basin (37°54′47.4″ N; 119°16′ 07.8″ W), a subalpine basin located near Tioga Pass in Yosemite National Park, California, was selected because it offers a very simple "island" system of isolated congeneric ectomycorrhizal host trees that have established at various distance from each other and the forest edge. Individuals of *Pinus albicaulis* and *Pinus contorta* are the only trees present; they occur in the same areas, are isolated from the forest, and experience identical soil, elevation, and climate (Fig. 1). The trees exist in a matrix of non-ectomycorrhizal grasses and sedges dominated by the shorthair sedge *Carex exserta* (Cole et al. 2004). This is an arid, high-elevation habitat, where precipitation falls primarily as snow (Callaway et al. 2002). The elevation of the selected trees in Gaylor Lake Basin is on average 3176 m ranging from 3147 to 3200 m.

Tree size measurements

A total of 40 trees (20 of each tree species) were selected for this study. We estimated tree photosynthetic volume of each tree in the following

way. Tree and canopy heights were measured with a telescoping height pole (Fig. 1), canopy width was measured in two directions with a meter tape, and radius was calculated by halving the averaged diameter of the canopy. As most trees were shrubby in stature, volume was estimated for each tree as either a cone or a cylinder using the averaged radius and measured height. All islands were single trees, but in the case of multiple main stems per tree, canopy volumes of each stem were estimated as above and summed.

Tree age measurements

In October 2014, ages were estimated for the 40 trees based on a combination of node counts and tree ring analysis. Previous studies have shown that in this region, *P. albicaulis* (Millar et al. 2004) and *P. contorta* var. *murrayana* (K. C. Lubetkin, A. L. Westerling, and L. M. Kueppers, *unpublished manuscript*), are uni-nodal, producing a single whorl of branches each year. Node counts were thus used to estimate tree ages for all trees <2 m tall. Stem cores were taken from trees larger than 0.1 m dbh, at either 0.5 m or 1.0 m height, depending on the geometry of the tree. All cores were visually cross-dated with a dissecting microscope and measured to 0.01-mm resolution using a Velmex sliding stage apparatus. COFECHA was then used for further cross-dating (Holmes 1983). Years to the cross-dated ring age were added to account for the age of the tree at core height based on previous age–height relationships, determined from separate node counts of *P. contorta* and *P. albicaulis* (Kueppers et al., *unpublished manuscript*).

Distance calculations

The location of each tree was determined in the field with a GPS unit with an accuracy of 3 m to be used for distance calculations. Each tree was fairly isolated from its neighbors and from the forest edge (Fig. 1). However, since every tree in the basin was not sampled, an isolation index was estimated by counting every stem of each tree species located within a 10 m radius circle of the tree. On average, EMF-dominant taxa show patchiness at a scale of <3 m (Lilleskov et al. 2004). From this, we infer that average genet size of EMF must be within 10 m of the focal tree. We thus recorded the number of stems belonging to each species within the 10 m radius circle of the focal tree and used the sum of all stems for the isolation index. Images of all 40 trees used in this study show the variation in size, shape, and degree of isolation of each host "island" (Appendix S1: Fig. S1).

This is not a traditional island system with a single forest "continent." There are multiple forests surrounding the basin, as can be seen in Fig. 1. We define the surrounding forests as the "mainland." Similar principles have previously been used when applying the theory of island biogeography in other non-traditional island systems, such as patches of serpentine outcrops, where a single "mainland" source population does not exist (Harrison et al. 2000, Harrison and Inouye 2002). Although there are hundreds of possible definitions for the term "forest" (Lund 2002), we took the international Kyoto Protocol definition as 10–30% minimum crown cover (Lund 2002) and we took the median value of 20% as our definition. We accounted for distance from the mainland by measuring the distance from the nearest forest edge, which we measured in GIS. Forest polygons were drawn by hand using Google Earth imagery in GIS using the add polygon tool by visually estimating >20% forest cover (Appendix S1: Fig. S2). Topographic distance from the nearest edge, accounting for Gaylor Lake as a hard barrier, was calculated for each tree using the "gdistance" (van Etten 2015) and "rgeos" (Bivand and Rundel 2014) packages in R. The topographic distances were estimated as the shortest overland distances between tree and forest edge based on topographic relief using a digital elevation model with 1/3 arc-second (~10 m) resolution from the National Elevation Dataset (ned.usgs.gov).

Soil sampling and DNA extractions

The fungal community associated with each tree was assessed by taking four soil samples per each of 40 trees twice, once in October 2012, and again in June 2013, approximately 3 weeks after snowmelt. On each date, we took four (~250 mL) soil samples from each tree, one sample in each cardinal direction. In each direction, a sample could be one of four distances from the tree (adjacent to tree trunk, half distance to drip line, 3/4 distance to drip line, and drip line), with the distance chosen using a random number generator. The June 2013 samples were pooled by tree, whereas the October 2012 samples were not. All soils were stored on ice or at 4°C for 2 d until homogenized and sieved in a 2-mm sieve, and 0.25 g of soil was

weighed and dispensed into tubes from the MoBio Power Soil DNA Extraction Kit (Carlsbad, California, USA), and DNA was extracted within the week. For the soils collected in October 2012, each of the four samples per tree was extracted separately for a total of 160 DNA extractions, while an aggregated soil sample for each tree was extracted from the soil collected in June 2013 for a total of 40 DNA extractions. Prior to amplification, a pooled sample from the October 2012 DNA extractions was created for each tree island by combining 10 μL DNA from each of the four DNA extractions per tree. Negative controls of empty MoBio Power soil tubes were also extracted, and gels were examined for PCR products.

Amplification, Illumina sequencing, and bioinformatics

Soil samples were PCR-amplified using Illumina sequencing primers designed by Smith and Peay (2014). These primers target the ITS1 spacer, a part of the ITS region that serves as the universal DNA barcode for fungi (Schoch et al. 2012). PCR mixtures for amplification contained 0.130 μL of HotStar Taq Plus (5 units/μL) DNA polymerase (Qiagen, Valencia, California, USA), 2.5 μL of 10× PCR buffer supplied by the manufacturer, 0.50 μL 10× each dNTPs (200 μmol/L), 0.50 μL each of 10 μmol/L forward and reverse primers, 1 μL of DNA template (diluted to 1:20 to overcome inhibitors), and water up to 25 μL. PCR conditions were as follows: denaturation at 95°C for 5 min; 29–34 amplification cycles of 30 s at 94°C, 30 s at 51°C, 1 min at 72°C, followed by a 10-min final extension at 72°C. Triplicate amplifications for each barcoded sample were pooled, cleaned with AMPure magnetic beads (Beckman Coulter Inc., Brea, California, USA), and quantified and quality-checked as previously described (Glassman et al. 2015). Samples were sequenced in an Illumina (Illumina, Inc., San Diego, California, USA) MiSeq PE 2x250 run at the DNA Technologies Core, UC Davis Genome Center Davis, California, United States, in August 2013. Bioinformatics processing is as described in Glassman et al. (2016). Briefly, trimmed, paired, high-quality sequences were grouped into operational taxonomic units (OTUs) based on 97% similarity in UPARSE (Edgar 2013), and taxonomy assignments were made in QIIME using BLAST based on the UNITE database (Koljalg et al. 2013).

Ectomycorrhizal fungi were separated from the OTU table based on genera determined to be ectomycorrhizal by Tedersoo et al. (2010) following the established methods from previous studies (Glassman et al. 2015). OTUs belonging to questionable genera, including *Ramaria, Entoloma, Lyophyllum, Sistorema, Sebacina,* among others, in which only part of the lineages are known to be ectomycorrhizal, were examined more closely via BLAST and included only if we could conclusively identify them as EM fungi (Tedersoo et al. 2010). OTUs in genera considered dark septate endophytes (DSE), such as *Acephala, Meliniomyces, Cadophora, Phialocephala,* were also examined more closely. DSE are a miscellaneous group of ascomycetous anamorphic fungi that colonize root tissues intracellularly and intercellularly (Jumpponen 2001). While they are consistently and ubiquitously found in studies of ectomycorrhizal tree roots, they are not consistently considered mycorrhizal, and their exact biology remains unclear (Rinaldi et al. 2008, Tedersoo et al. 2010). We examined OTUs belonging to these genera more closely via BLAST and retained only the OTUs that we could conclusively identify as EMF such as *Cadophora finlandica* and *Meliniomyces bicolor* (Tedersoo et al. 2010). The specific QIIME command used to filter out putative EMF OTUs can be found in Appendix S1: Methods S1, and the QIIME commands used to filter out OTUs that we conclusively identified as non-mycorrhizal can be found in Appendix S1: Methods S2. Sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under accession number SRP079403.

Statistical analysis

We tested the predictions of island biogeography of a positive exponential relationship between species richness and island size, and a negative exponential relationship between species richness and distance from forest edge with linear regression. Because island biogeography predicts an exponential relationship between richness, size, and distance from forest edge, we employed a log10 transformation to those variables. We combined OTUs from the June and October sampling dates to acquire a single richness value per tree.

There is no clear best estimator of species richness (Brose et al. 2003). Thus, we ran our analyses with four estimates of fungal richness in an effort

to ensure that our results were robust to method of richness estimation. We calculated the observed species number (S obs) per sample, and estimated richness using the Fisher's alpha, chao1, and ACE (Abundance-based Coverage Estimator) diversity indices using the diversity, fisher.alpha, and estimate R functions in vegan (Oksanen et al. 2012). To accurately compare richness among samples with uneven sequencing depth, OTU tables were rarefied to an equal number of sequences per sample. To ensure that our analyses were robust to various rarefaction levels, we ran all analyses on the EMF OTU table not rarefied, rarefied to an even sequencing depth (284 sequences/sample) but retaining all samples ($n = 40$), rarefied to remove the bottom 10% quantile (647 sequences/sample, $n = 36$), and bottom 15% quantile (1066 sequences/sample, $n = 34$) of samples with low sequencing yields. In this paper, all results are presented with EMF rarefied to 647 sequences per sample, with richness measured as observed species number (S obs) as the response variable; the results of all other analyses are included in the supplements.

We used volume of tree photosynthetic tissue (m^3) as a proxy for island size. Distance of each tree from the nearest forest edge was used as a proxy for distance from mainland (Peay et al. 2010). We used linear regression model to test whether distance from nearest forest edge and island size predicted EMF richness. Since two species of trees were included as tree hosts, we also tested the principles of island biogeography in each tree species separately and with both trees together. Since not every tree was equally isolated, we tested whether including the extra explanatory variable of isolation significantly improved model fit to predict EMF richness with an ANOVA of full vs reduced model. In addition to the multivariate model, we ran univariate regressions of EMF richness against tree size, age, and distance from forest edge to visualize the results. All analyses were conducted in R version 3.0.2 (R Core Team 2013).

Results

Tree information

The trees averaged 65 ± 5.2 yr old (mean ± SE). *Pinus contorta* trees (71 ± 6.9) and *P. albicaulis* trees (59 ± 7.68) did not significantly differ in age (t test, $t = -1.1761$, $P = 0.24$; Appendix S1: Fig. S3). *Pinus contorta* trees measured approximately 10.75 ± 2.86 m^3 vs. 13.56 ± 3.4 for *P. albicaulis* and did not significantly differ in size (t test, $t = 0.6311$, $P = 0.53$; Appendix S1: Fig. S4). Trees ranged from 308 to 837 m from the nearest forest edge (Appendix S1: Fig. S5). *Pinus contorta* trees measured approximately 630 ± 2.76 m from nearest forest edge vs. 629 ± 2.72 for *P. albicaulis*, and the host trees did not significantly differ in distance to the nearest forest edge (Appendix S1: Fig. S6). The vast majority of sampled trees had fewer than five stems (of either *Pinus* species) within 10 m of them (29 of 40), and nearly 50% were devoid of neighboring trees within 10 m (18 of 40; Appendix S1: Fig. S7). For both species combined, tree age and tree size were significantly correlated with each other (Pearson's $r = 0.33$, $P = 0.036$; Appendix S1: Fig. S8A). Examined separately, tree age and tree size were significantly correlated with each other for *P. albicaulis* (Pearson's $r = 0.45$, $P = 0.049$; Appendix S1: Fig. S8B), but not for *P. contorta* (Pearson's $r = 0.25$, $P = 0.30$; Appendix S1: Fig. S8C). For both species combined, tree size and distance from forest edge were not significantly correlated (Pearson's $r = 0.25$, $P = 0.12$; Appendix S1: Fig. S9A). Examined separately, tree size and distance from forest edge were not correlated for *P. albicaulis* (Pearson's $r = 0.09$, $P = 0.71$; Appendix S1: Fig. S9B), but were strongly correlated for *P. contorta* (Pearson's $r = 0.44$, $P = 0.05$; Appendix S1: Fig. S9C). However, this relationship appeared to be driven by the largest tree (PC10), and when removed, the correlation between distance from forest edge and size for *P. contorta* was reduced (Pearson's $r = 0.35$, $P = 0.14$; Appendix S1: Fig. S9D). Location, age, size, isolation, and distance for all 40 trees are summarized in Appendix S1: Table S1.

Richness information

After extensive quality control, OTUs that we could confidently identify as EMF were selected. These consisted of 238,895 sequences constituting 96 OTUs. Of these, 68 OTUs occurred under *P. albicaulis* and 62 OTUs under *P. contorta* trees. We present the results as observed species number (S obs) with EMF OTU table rarefied to 647 sequences per sample, which yielded 76 OTUs (51 OTUs under *P. albicaulis* and 54 OTUs under *P. contorta*). EMF richness averaged 10.15

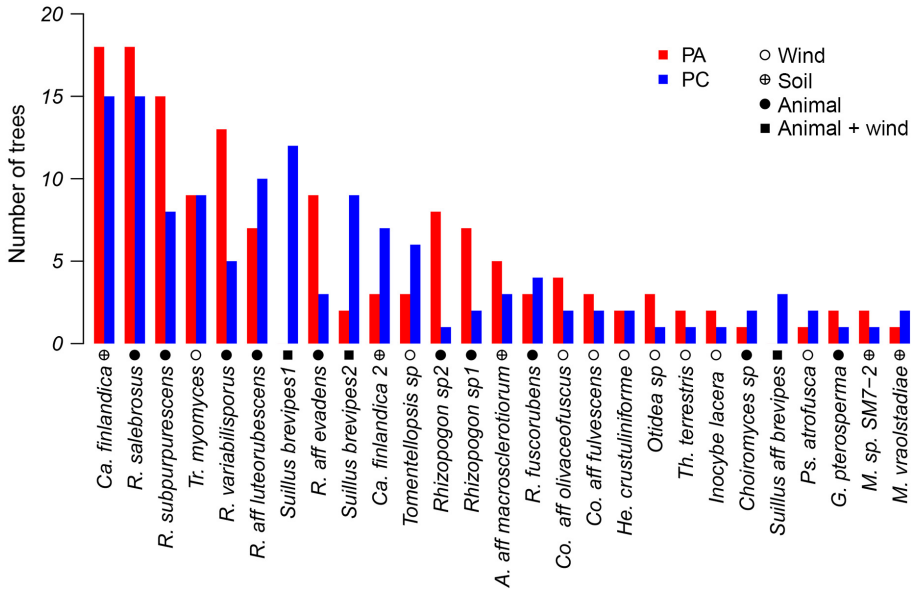


Fig. 2. Rank–abundance curve of frequency of ectomycorrhizal fungal operational taxonomic units occurring on more than two trees (top 27 most frequent) of either *Pinus albicaulis* (PA; $n = 20$) or *Pinus contorta* (PC; $n = 16$). Symbols represent primary dispersal modes of taxa. Genus abbreviations: Ca. = *Cadophora*; R = *Rhizopogon*; Tr. = *Tricholoma*; A = *Acephala*; Co = *Cortinarius*; He = *Hebeloma*; Th = *Thelephora*; Ps = *Pseudotomentella*; G = *Gautieria*; M = *Meliniomyces*.

OTUs ± 3.27 SD per *P. albicaulis* tree and 10.71 OTUs ± 4.97 SD per *P. contorta* tree and did not differ significantly between tree hosts (*t* test; $t = -1.025$, $P = 0.3157$). EMF richness did not differ between tree hosts regardless of rarefaction level (Appendix S1: Fig. S10). However, EMF community composition did significantly differ between trees (S. I. Glassman et al., *unpublished manuscript*). Observed richness and Fisher's alpha at the four rarefaction levels for every tree are summarized in Appendix S1: Table S2A, and the chao1 and ACE richness estimates at the four rarefaction levels for every tree are summarized in Appendix S1: Table S2B. The mean and standard deviation of EMF richness per tree species are summarized in Appendix S1: Table S3. The richness estimated as observed species number (S obs) and ACE were highly correlated with each other (Appendix S1: Fig. S11) as were the chao1 and ACE indices (Appendix S1: Fig. S12), regardless of rarefaction level employed. The three rarefaction levels employed neared saturation and yielded similar richness results (Appendix S1: Fig. S13).

Five of the 10 most frequent EMF OTUs belonged to the genus *Rhizopogon*, with the rest belonging to the genera *Cadophora, Tricholoma,* and *Suillus* (Fig. 2; Appendix S1: Table S4). The primary dispersal mode of the top 27 most frequent EMF OTUs (present on more than two trees) is denoted in the rank–abundance curve (Fig. 2). Seven of the top 10 most frequent EMF OTUs are either solely vectored by mammals (e.g., *Rhizopogon;* Frank et al. 2009) or can be effectively vectored by mammals (e.g., *Suillus;* Ashkannejhad and Horton 2006). Of the 76 EMF OTUs (Appendix S1: Fig. S14), the frequency of EMF OTUs on trees belonging to *P. contorta* vs. *P. albicaulis* was strongly correlated with each other (Pearson's $r = 0.72$, $P < 0.001$; Appendix S1: Fig. S15). The primary exception is *Suillus brevipes,* which prefers *P. contorta* (Appendix S1: Fig. S15). We previously sampled a nearby *P. albicaulis* forest upslope of the basin (Talbot et al. 2014) and identified 49 EMF OTUs using 454 pyrosequencing. Of those, only two of the top 10 most frequent OTUs belonged to the genus *Rhizopogon*, with the rest belonging to the genera *Cortinarius, Cenococcum, Russula, Piloderma, Meliniomyces, Pseudotomentella* (Appendix S1: Fig. S16). A second nearby mixed *P. albicaulis* and *P. contorta* forest, which we

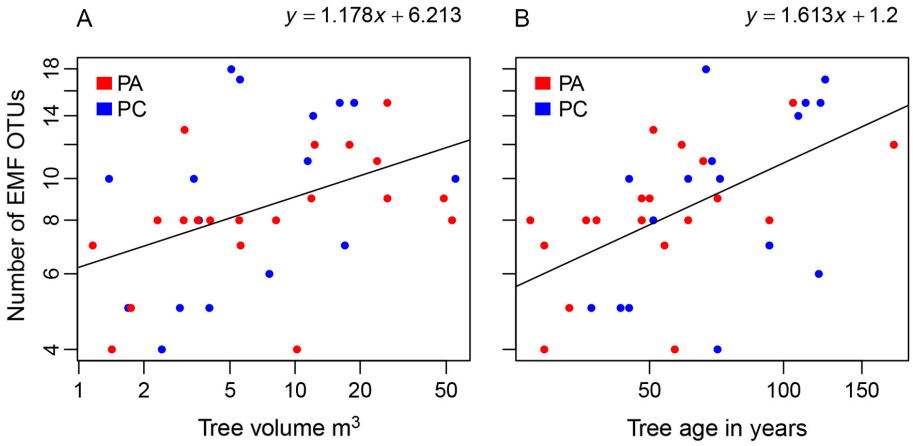


Fig. 3. Ectomycorrhizal fungal richness vs. tree (A) island size (measured as volume m^3) and (B) island age (in years). All scales are logarithmic. *Pinus albicaulis* (PA) colored in red and *Pinus contorta* (PC) trees colored in blue.

had also previously sampled (Talbot et al. 2014), showed similar results (Appendix S1: Fig. S17). Only two of the top 10 most frequent OTUs were *Rhizopogon*, and the rest were in the genera *Cortinarius, Tricholoma, Meliniomyces, Russula, Amanita,* and *Cadophora* (Appendix S1: Fig. S17).

Testing the predictions of island biogeography on EMF richness

For both trees combined, the number of EMF OTUs significantly increased with both size ($F_{1,34}$ = 7.661; adj. R^2 = 0.16, P < 0.01; Fig. 3A) and age of trees ($F_{1,34}$ = 13.68; adj. R^2 = 0.27, P < 0.01; Fig. 3B), but did not decrease with distance from forest edge ($F_{1,34}$ = 0.2789; adj. R^2 = 0.27, P = 0.6). For *P. albicaulis*, where size and distance were not correlated (Appendix S1: Fig. S9B), the linear model with log EMF richness as the response variable and with log tree size and log distance from forest edge as explanatory variables indicated that the theory of island biogeography successfully predicts EMF richness in this system ($F_{2,17}$ = 5.891, adj. R^2 = 0.34, P < 0.05; Table 1). Richness significantly increased with tree size (t = 2.513, P < 0.01) and decreased with distance from forest edge (t = −2.322, P < 0.05; Table 1). These results were largely robust to rarefaction level and richness index employed, with an adjusted R^2 ranging from 0.18 to 0.52 (Appendix S1: Table S5). Adding in the isolation metric as an additional explanatory variable did not significantly increase the fit of the model. Univariate regressions corroborated results from the multivariate island biogeography model; *P. albicaulis* EMF richness significantly increased with island size ($F_{1,18}$ = 5.136; adj. R^2 = 0.18, P < 0.05; Fig. 4A) and significantly decreased with distance from forest edge ($F_{1,18}$ = 4.221; adj. R^2 = 0.15, P < 0.055; Fig. 4B). These results were largely robust to rarefaction level and richness index employed; EMF richness nearly always increased with *P. albicaulis* size and age, and decreased with distance from forest edge (Appendix S1: Table S6).

Because size and distance were confounded for *P. contorta* (Appendix S1: Fig. S9C), the explanatory variables cancel each other out in the multivariate model and island biogeography theory cannot be applied to those trees ($F_{2,13}$ = 1.559; adj. R^2 = 0.07, P = 0.25). Removing the outlier (the largest and farthest tree; PC10) decreased the correlation between tree size

Table 1. The results of the linear regression model testing the effect of log size of island as measured by tree volume (m^3) and log distance (measured in m from nearest forest edge) on log richness of ectomycorrhizal fungal for *Pinus albicaulis* ($F_{2,17}$ = 5.891; mult. R^2 = 0.41; adj. R^2 = 0.34, P < 0.05).

Predictors	Estimate	SE	*T*	*P*
Intercept	5.60308	1.63418	3.429	0.0032**
log Tree size	0.14036	0.05586	2.513	0.0224*
log Distance	−0.58955	0.25387	−2.322	0.0329*

$^{**}P \leq 0.01$; $^{*}P \leq 0.05$.

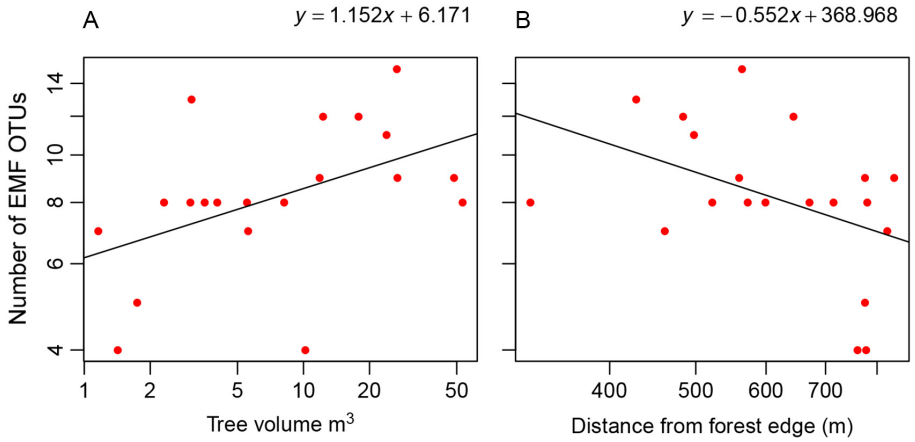


Fig. 4. Ectomycorrhizal fungal richness vs. (A) island size (measured as volume m^3) and (B) distance from nearest forest edge (in m) for *Pinus albicaulis* trees. All scales are logarithmic.

and distance (Appendix S1: Fig. S9D), but did not improve the model fit ($F_{2,12} = 1.964$; adj. $R^2 = 0.12$, $P = 0.18$). However, model fit was improved for *P. contorta* EMF richness based on the OTU table rarefied to 284 sequences per sample, which retained four more *P. contorta* trees than the OTU table rarefied to 647 sequences per sample ($F_{2,16} = 3.423$; adj. $R^2 = 0.21$, $P = 0.058$).

Discussion

We have shown that even in an extremely stressful, subalpine, non-traditional island system, and at spatial scales below 1 km, the theory of island biogeography largely applies (Table 1; Fig. 4). Regardless of tree species, EMF richness always increased with island size and island age (Fig. 3). While one of the tree species proved problematic due to a confounding of size and distance, the signal for *P. albicaulis*, in which size and distance were not at all correlated (Appendix S1: Fig. S9B), was unambiguous (Table 1). In spite of the fact that trees were not completely isolated from a single distinct source, EMF richness decreases with distance from forest edge (Fig. 4B). Without invoking any biology, we were able to attribute approximately one-third to one half of the variance in EMF richness simply to size and distance (Table 1; Appendix S1: Table S5).

With advances in sequencing technologies, there is a growing literature corroborating that theory developed for macro-organisms applies to microorganisms. For example, studies have shown that dispersal limitation affects archaea in isolated geothermal hot springs (Whitaker et al. 2003), and floral nectar yeast assemblages in individual *Mimulus* flowers (Belisle et al. 2012). Dispersal limitation, which predicts that the number of species will decrease with increasing distance from a source pool, has been more widely tested in microorganisms (Martiny et al. 2006) than the taxa–area relationship, which predicts that the number of species will increase with increasing size of area available to them (Green and Bohannan 2006). In one application of the taxa–area relationship to microorganisms, water-filled treeholes were used to show that larger islands (represented by water volume) house more bacteria taxa (Bell et al. 2005).

In this study, we show that both the taxa–area relationship and dispersal limitation apply to EMF in a high-elevation system. These results show a very similar pattern to what has been found in a wetter, coastal pine forest located hundreds of kilometers from Gaylor Lake Basin (Peay et al. 2007, 2010). The aforementioned studies involved a single species of pine tree growing in isolation, *Pinus muricata*, and a single, obvious, isolated forest edge (Peay et al. 2010). We have now increased the generality of this pattern by showing that the theory of island biogeography can also apply in harsher, drier systems, with less precipitation as rain, and where trees are not isolated from a single obvious forest edge. We have also shown that for two more pine species, *P. albicaulis* and *P. contorta*, EMF richness unambiguously increases with tree

size (Fig. 3A). We have also added novel information that EMF richness increases with tree age (Fig. 3B).

Despite the similarities in overall patterns, there are some striking differences in the EMF species composition of the trees in the current study system vs. those in the previously studied wetter coastal pine tree islands (Peay et al. 2007, 2010), and we interpret these differences to be a result of the reduced precipitation as rain in Gaylor Lake Basin. It is striking that seven of the 10 most frequent EMF OTUs on the tree islands in this study are mammal-dispersed (Fig. 2). For example, the most common genus in these subalpine tree islands is *Rhizopogon*, which produces a belowground, truffle-like fruitbody that is dispersed by small mammals (Maser and Maser 1988). Its fruiting habit is thought to be an advantage when precipitation is limiting (Bruns et al. 1989). Furthermore, *Tricholoma myomyces* and *S. brevipes* are the only common EMF taxa that are dispersed primarily by wind (Fig. 2), and *S. brevipes* can also be effectively dispersed by mammals such as deer (Ashkannejhad and Horton 2006). In contrast, in the wetter coastal pine forest, species of *Suillus*, *Rhizopogon,* and *Tricholoma* were frequent on small, isolated patches of 10-yr-old trees (Peay et al. 2007), while the EMF composition of 45-yr-old tree islands was much more diverse and included many mature forest EMF genera that produce aboveground mushrooms, such as *Amanita*, *Cortinarius*, *Lactarius*, *Russula,* and *Tomentella* (Peay et al. 2010). Thus, the EMF composition of the tree islands in Gaylor Lake Basin, despite their advanced age (up to 177 yr with a mean of 65 yr), is similar to that of 10-yr-old tree islands in a wetter, coastal habitat. We hypothesize that this is due to the fact that Gaylor Lake Basin is at high elevation and under water stress, limiting fruiting of aboveground mushrooms to rarer occurrences than in the wetter, coastal habitat.

It also appears that dispersal ability interacts with the ability of certain spore bank fungi to sit and wait long periods of time for a tree seedling to establish in this system. Five of the 10 most frequent OTUs belonged to the genus *Rhizopogon* (Fig. 2). In addition to dispersal by mycophagy (Frank et al. 2009), *Rhizopogon* species are also known to have a large capacity for dormancy and longevity in the soil (Bruns et al. 2009), which allows them to maintain a large, viable spore bank (Glassman et al. 2015). Mycophagous small mammals, including the yellow-bellied marmot (*Marmota flaviventris*), alpine chipmunk (*Tamais alpinus*), Belding's ground squirrel (*Spermophilus beldingi*), and pika (*Ochotona princeps*), are prevalent at this elevation in Yosemite National Park (Moritz et al. 2008) and were sighted during our fieldwork trips to Gaylor Lake Basin. They all can eat *Rhizopogon* and defecate spores throughout the basin. The abundance of relatively large rodent dispersal agents in this subalpine site differs from the coastal pines (Peay et al. 2010). In the coast, the primary rodent dispersers are smaller animals that move shorter distances, such as the mice species *Peromyscus maniculatus* and *Reithrodontomys megalotis* that typically travel <100 m (T. D. Bruns, *unpublished data*). The frequency of *Rhizopogon* sequences seen in our study is likely due to the frequency of animal dispersal and the persistence of these spores, and it indirectly shows that belowground fruiting is common in this subalpine setting. Whether the DNA sequences we are detecting are derived from spores in the soil, or mycelium, is not clear, but in either case it is evident that belowground fruiting and mammal dispersal is an exceptionally successful strategy in this setting.

Despite a nearby source pool of pine trees containing many later-stage EMF species (Appendix S1: Figs. S16, S17), and despite their advanced age, the isolated tree islands in Gaylor Lake Basin are dominated primarily by early-stage highly ruderal pioneer species. The *P. albicaulis* forest on the ridge directly above the basin to the east that we had previously sampled (Talbot et al. 2014) was dominated by later-stage species of fungi such as *Cortinarius*, *Cenococcum*, *Russula*, and *Piloderma*, in addition to *Rhizopogon* (Appendix S1: Fig. S16), as was the nearby mixed *P. albicaulis* and *P. contorta* forest (Appendix S1: Fig. S17). In particular, *Cortinarius* and *Russula* are considered "late-stage" fungi (Last et al. 1987), and species of *Russula* are typically dominant in mature pine forests in California (Horton and Bruns 2001). While Appendix S1: Figs. S16, S17 are certainly not a comprehensive assessment of the total diversity of all EMF source pools in the area, they are two examples of the diversity available in a nearby forest "mainland." Even though the source pool for these EMF species is within 1 km, establishment of these fungi on the isolated trees is quite rare. For example, *Piloderma* was not present on any of

the tree islands, *Russula* was only present on two of the tree islands, *Amanita* was only present on three of the tree islands, and species of *Cortinarius* and *Cenococcum* were rare (Appendix S1: Table S3, Fig. S14). We know that this is not just a single tree effect, or an age effect, because single trees averaging 45 yr in age in the wetter coastal pine forest studied by Peay et al. (2010) were dominated by what we would consider mature forest fungi in that area such as *Tomentella, Russula, Amanita,* and *Cortinarius* (Last et al. 1987, Taylor and Bruns 1999), even though the distances to source forest were slightly greater than in this subalpine basin. Thus, in this high-elevation system, it appears that water stress interacts with dispersal to yield mature tree islands that remain colonized primarily by pioneer EMF. In addition, we provide further evidence that fungal richness decreases with altitude (Bahram et al. 2012). The Jack2 estimates of EMF richness for the 45-yr-old isolated tree islands in the coastal, sea-level forest (Peay et al. 2010) ranged from 7 to 42 species (median = 16; SD = 9). In contrast, our high-elevation, 65-yr-old isolated tree islands ranged from 4 to 18 observed EMF species per tree (median = 8; SD = 3.7), and the chao1 estimates were not much higher (range of 4–27; median = 9.25; SD = 5.6).

It is possible that the patterns seen here of mature trees that are primarily colonized by pioneer EMF species could be driven largely by strong priority effects. For example, if upon initial germination and growth, the trees were first colonized by strong dispersers such as *Rhizopogon* and *Suillus*, then strong priority effects could have allowed these early colonists to resist secondary colonization by "later-successional" fungi. However, if this scenario is correct, it still must be driven by the subalpine environment since the younger 45-yr-old tree islands of the wetter, coastal pine forest were frequently colonized by late-stage successional fungi such as *Amanita* and *Russula* (Peay et al. 2010), and only the smallest, youngest, 10-yr-old trees were dominated by *Rhizopogon* and *Suillus* (Peay et al. 2007). Thus, strong priority effects did not allow for the protracted domination of early colonists of isolated trees in a less harsh environment even with a shorter time span. Thus, the observed patterns are more consistent with the idea that wind dispersal of spores is highly limited in this subalpine environment.

We conclude by noting that a recent review of the theory of island biogeography stated that the availability of genetic tools is key to provide a wider coverage across taxa and individuals (Warren et al. 2015). By using such tools, we now add to the growing body of knowledge that the theory is even more general than had previously been demonstrated. Equally important in the more specific realm of individual organisms, we show that composition of EMF species present in this subalpine tree island system is enriched for ruderal and animal-dispersed species relative to a coastal pine system. This difference in species composition is likely driven by the difference in environment, and yet the general tenets of island biogeography are still strongly predictive.

Acknowledgments

Thanks to L. Boddy, A. Carver, L. Forero, A. DiRocco, and N. Nguyen for help with fieldwork. Thanks to K. Peay and D. Smith for providing primers, molecular protocols, and bioinformatics assistance; L. Ponisio and I. Wang for providing analytical advice; and J. Battles and E. Simms for discussions and providing comments on the manuscript. We also thank R. Lankau and an anonymous reviewer for helpful comments on the manuscript. Thanks to Yosemite National Park for allowing us to sample, to the Battles laboratory for use of cross-validation equipment, and to the University of Arizona Laboratory of Tree Ring Research for curating the tree cores. S.I.G was supported by the NSF Graduate Research Fellowship Program. This project was funded by the NSF Doctoral Dissertation Improvement Grant to S.I.G. and T.D.B. (Award: 1403215) and an NSF Dimensions of Biodiversity Grant to TDB (Award 1046115).

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Supporting Information

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