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LETTER

Greater local diversity under older species pools may arise from enhanced competitive equivalence

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Abstract

Ecological communities typically contain more species when located within geologically older regions. This pattern is traditionally attributed to the long-term accumulation of species in the regional species pool, with local species interactions playing a minor role. We provide evidence suggesting a more important role of local species interactions than generally assumed. We assembled 320 communities of root-associated fungi under 80 species pools, varying species pool richness and the mean age of the sites from which the fungi were collected across a 4-myr soil chronosequence. We found that local diversity increased more with increasing species pool richness when species were from older sites. We also found that older species pools had lower functional and phylogenetic diversity, indicating greater competitive equivalence among species. Our results suggest that older regions have higher local richness not simply because older pools are more speciose but also because species have evolved traits that allow them to locally co-occur.

Keywords

biodiversity, chronosequence, coexistence, community assembly, functional diversity, regional species pool.

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INTRODUCTION

Processes occurring across large geographical scales, such as vicariance and allopatric speciation, can dictate local patterns of species diversity (Terborgh and Faaborg, 1980; Ricklefs, 1987; Cornell and Lawton, 1992; Hillebrand and Blenckner, 2002). For example, geologically older regions and historically more abundant habitats tend to be associated with greater local diversity (Pärtel, 2002; Fine and Ree, 2006; Zobel *et al*., 2011). This relationship between the age of a region and the level of local diversity has traditionally been attributed to the accumulation of regional species richness over time (Zobel *et al*., 2011; Lososová et al. 2015). However, in addition to having greater richness, species pools in older regions may also include taxa that have evolved traits that facilitate local coexistence, enhancing local diversity (Aarssen, 1989; Zobel, 1992). Such traits may evolve as a result of coevolution among species in the same regional species pool (Rabosky, 2009; Cornell, 2013; Cornell and Harrison, 2014; Price et al. 2014; Brodie, 2019; Close et al. 2019), but this possibility remains to be fully investigated.

If competitive interactions favoured co-occurrence of species with greater trait differences in the past, niche filling through diversification and immigration could increase functional diversity in the regional species pool (Purschke et al. 2013; Lososová et al. 2015; Gillespie, 2016; Spasojevic *et al*., 2018). Greater functional diversity in the species pool could then contribute to greater local diversity by reducing trait overlap among members of the species pool. Alternatively, if the outcomes of local species interactions were primarily determined by a competitive trait hierarchy, convergence on traits that equalise species' intrinsic fitness differences could ensue (Mayfield and Levine, 2010; Kunstler *et al*., 2012). This convergence could also promote local diversity because reduced fitness differences would allow locally rare species to persist (Herben and Goldberg, 2014). However, these hypotheses that invoke traits that govern how species locally interact remain largely untested.

Determining whether the composition of older species pools contributes to greater local diversity is challenging because it requires comparisons that account for differences in species pool richness and other potentially confounding factors, such as differences in regional species abundance distributions (Fox and Srivastava, 2006), local extinction and colonisation dynamics (He *et al*., 2005), and the rate of local disturbance (Caswell and Cohen, 1993). To overcome this challenge, we conducted an experiment using species pools comprised of fungi collected across a 4-myr soil chronosequence in Hawaii (Vitousek, 2004). We focused on fungi associated with the roots of a native understory plant, *Vaccinium calycinum*, which hosts a phylogenetically and functionally diverse community of root-associated symbionts, predominately ericoid mycorrhizal fungi (Leopold, 2016). This study system is uniquely suited for testing hypotheses linking regional species pools to local community because the host plant is relatively slow growing and many of the root-associated fungal symbionts can be isolated and grown in pure culture. In addition, *V. calycinum* is the only common host to ericoid mycorrhizal fungi across the chronosequence, which is dominated by a single canopy tree species and a common suite of understorey

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plants (Kitayama and Mueller-Dombois, 1995), limiting overall biotic variation. As a result of these features, experimental assembly of fungal communities on *V. calycinum* seedlings in laboratory microcosms serves as a tractable proxy for the natural process of fungal community assembly.

The primary goal of this experiment was to test the hypothesis that the accumulation of fungal symbiont diversity within a host plant will saturate with increasing species pool richness, but that greater local diversity will be realised when the region contributing to the species pool is older. For this purpose, we assembled local communities of root-associated fungi using experimental species pools that varied in species richness and the geological age of the sites contributing to each species pool. In addition, we quantified functional and phylogenetic relationships among species to test the hypothesis that species pools from older ecosystems would have greater functional diversity, which should facilitate local species coexistence.

MATERIALS AND METHODS

Overview and experimental design

We isolated fungi from the roots of an ericaceous plant, *V. calycinum*, collected from five sites across a 4.1-million-year soil chronosequence in Hawaii, known as the Long Substrate Age Gradient (LSAG) (Vitousek, 2004). We then assembled experimental species pools of 2–30 fungi, manipulating geologic age by systematically varying the mean of the $\log_{10}$-transformed ages of the sites where individual species were collected. We also manipulated the variance of the $\log_{10}$-transformed site ages to account for the potential confounding effects of edaphic variation among sites on species pool functional diversity and assembly outcomes. We used site age-variance as a proxy for edaphic variation because the LSAG chronosequence represents a progression of long-term soil development, resulting in greater differences in soil properties (e.g. physical structure, nutrient availability, etc.) with increasing differences in site age (Vitousek, 2004). A total of 80 experimental species pools (including one control treatment with no fungi) were inoculated onto sterile *V. calycinum* seedlings in individual microcosms, each replicated four times (320 total microcosms), and community assembly outcomes were assessed using Illumina metabarcoding after 5 months.

Fungal culture collection

At each LSAG site, we sampled fungi from the roots of 12 randomly selected *V. calycinum* plants. A portion of the root system of each plant and the adhering soil (*c.* $25 \times 25 \times 10$ cm) was excavated with a hand trowel and transported immediately to the University of Hawaii, Hilo campus, where samples were refrigerated (4 °C) and processed within 24 h. Fine terminal roots were manually separated from the soil and rinsed in tap water to remove all visible soil particles and four segments (*c.* 2 cm) were haphazardly selected and pooled for each plant. Pooled root samples were surface-sterilised by sequential vortexing for 1 min in sterile water, 70% EtOH and 50% household bleach (4.5% available chlorine), followed by three rinses in sterile water. From each surface-sterilised root fragment, two segments (*c.* 2 mm) were excised and aseptically transferred to separate petri dishes containing modified Melin-Norkrans (MMN) agar, amended with the antibiotics gentamycin (15 mg l^{-1}), streptomycin (15 mg l^{-1}) and tetracycline hydrochloride (12 mg l^{-1}). For each pair of excised root segments, one was placed on media that also contained benomyl (4 mg l^{-1}), a fungicide that suppresses many ascomycetous fungi, facilitating the isolation of basidiomycetous fungi from roots (Vohník *et al.*, 2012; Bruzone *et al.*, 2015). Plates were monitored regularly, and hyphae growing from root segments were immediately transferred to new MMN plates and maintained by serial transfer every 4–8 weeks, depending on growth rate.

Identifying fungal isolates

One representative of each fungal morphotype from each site was selected for possible use in the microcosm study and identified using molecular methods. Fungal DNA was extracted by scraping hyphae from the surface of a colonised agar plate and lysing cells with Extract-N-Amp Plant (Sigma-Aldrich) extraction and neutralisation solutions. The complete nrDNA ITS gene and partial large subunit (28S) gene were PCR amplified using the primer pairs ITS1f-ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993) and LROR-LR5 (Vilgalys and Hester, 1990). PCR products were sequenced using Sanger sequencing (Beckman Coulter Genomics, Danvers, MA, USA) and sequences were deposited in GenBank (MT321740–-MT321793). Taxonomy was assigned by manually searching the full ITS sequence and the complete ITS and partial LSU sequences against the UNITE species hypothesis database v8.2 (Kõljalg et al. 2013; Abarenkov et al. 2020).

We tentatively identified the fungal isolates used in the microcosm experiment as belonging to a range of ascomycetous taxa commonly associated with ericaceous plants, predominantly in the order Helotiales (Fig. S1). Many of the closest matching sequences in the UNITE database originated from the roots of other ericaceous species and were taxonomically affiliated with ericoid mycorrhizal fungi, including *Hyaloscypha bicolor* (syn. *Meliniomyces bicolor*), *Hyaloscypha hepaticicola* (syn. *Rhizoscyphus ericae*) *and Oidiodendron maius* and poorly classified species within the Helotiales. Other taxa had affinities to groups of common endophytic fungi associated with ericoid roots, including *Chaetosphaeria*, *Cladophialophora Phialocephala* and *Rhizoderma veluwensis*. There is a considerable taxonomic overlap between these two functional groups and the interactions between our isolates and the host plants are not well documented (Lukešová *et al.*, 2015; Leopold, 2016). However, given the aggressive surface sterilisation protocol used and the taxonomic affiliations, we expect all taxa to be either ericoid mycorrhizal fungi or non-mycorrhizal endophytes with intimate associated with *Vaccinium calycinum*.

Functional/phylogenetic characterisation of fungi

Two complementary approaches were used to characterise the functional similarity among fungal isolates, direct measurements of functional traits and estimation of phylogenetic

relationships. For functional traits, we quantified the relative abilities of each isolate to grow on 95 different carbon substrates using phenotypic microarrays (Biolog, FF microplate). Hyphal suspensions were prepared for inoculating microarrays by blending hyphae scraped from the surface of an agar plate in 60 ml sterile water. The hyphal suspension was then centrifuged at 1500 x g for 5 min, decanted and resuspended in a sterile aqueous solution of 0.25% Phytagel and 0.03% Tween 40 to achieve a total volume of *ca*. 50 ml and an optical density of 0.06 at 590 nm. Three replicate plates were inoculated with 100 µl of this solution, sealed with Parafilm and incubated at room temperature for 6 weeks. Fungal growth was monitored by reading optical density at 740 nm using a microplate reader (Spectramax 190, Molecular Devices), and measurements were collected at 1, 3 and 6 days, and weekly thereafter. Substrate use profiles for each species were generated by fitting a loess smoother to the optical density values in each well at each time point and integrating the area under the curve as a cumulative measure of substrate use. Values for the no-substrate control well were subtracted from all other wells to account for growth not attributable to the available carbon substrates.

To estimate phylogenetic relationships (Fig. S1), we sequenced a 740-bp region of the nrDNA large ribosomal subunit (28S), which allows better resolution of phylogenetic relationships in ascomycetous fungi than the ITS region (Liu *et al*., 2012). Sequences were aligned on the T-Coffee web server (Di Tommaso et al. 2011) and alignment columns were weighted using transitive consistency scoring (Chang *et al*., 2014) before phylogenetic tree construction using maximum likelihood and nonparametric, approximate likelihood-ratio tests of branch support with PhyML (Guindon *et al*., 2010).

Fungal species pools

Experimental species pools were constructed using a subset of the total culture library that excluded replicate isolates from the same site with identical nrDNA sequences. Initial Sanger sequencing revealed four Basidiomycete taxa, which we excluded from the assembly experiment to avoid excessive influence of phylogenetic outliers in our analyses. Preliminary tests also revealed four pathogenic isolates (all *Cryptosporiopsis* spp.) that rapidly killed seedlings *in vitro*; these isolates were also excluded. The phylogenetic distribution of the remaining isolates was not correlated with site age ($K = 8.41 \times 10^{-7}$; $P = 0.12$), and all 54 were used in the construction of the experimental species pools.

To ensure that individual isolates could be identified in mixed communities, isolates with > 97% similarity in their ITS2 nrDNA gene sequences were never included in the same species pool. The compositions of species pools were selected using a stratified random sampling of the possible parameter space defined by the species pool richness (2–30 species) and the range of possible values of mean $\log_{10}$-transformed site age and variance of $\log_{10}$-transformed site age (age-variance), given the available species (Fig. 1). Briefly, 20 million random possible pools were generated and the parameter space was divided into 79 equal area bins, from which one species pool was randomly selected. This approach assured that more extreme parameter values would be sampled and allowed us to disentangle the effects of pool age from age-variance.

Microcosms

Microcosms were assembled in 2.5 cm × 15 cm cylindrical glass culture tubes. Each tube received 3 g (dry) of a mixture of sphagnum peat, fine vermiculite and perlite (1:5:1) and 7 ml of a low-carbon mineral nutrient solution, following (Grelet *et al*., 2009), containing 0.600 mM NH_4NO_3, 0.599 mM $(NH_4)2HPO_4$, 0.662 mM KH_2PO_4, 0.170 mM $MgSO_4.7H_2O$, 0.300 mM NaH_2PO_4, 0.080 mM K_2SO_4, 0.034 mM FeNaEDTA, 0.006 mM $ZnSO_4.7H_2O$, 3.69 µM $MnSO_4.H_2O$, 0.04 µM $Na_2MoO_4.2H_2O$, 9.55µM H_3BO_3, 1 µM $CuSO_4.5H_2O$ and 100 mg l^{-1} glucose. Culture tubes were fitted with polypropylene closures, modified to include 3 2-mm-diameter vent holes which were covered with two layers of micro-pore tape to allow gas exchange. Assembled microcosms were autoclaved twice for 30 minutes at 121 °C with a 24-hour interval.

Seedlings

V. calycinum fruits were collected at the 300-year LSAG site and were cleaned, dried and refrigerated before use (Zee et al. 2008). Seeds were surface-sterilised by gentle vortexing in a 10% solution of H2O2 for 5 min, rinsing in sterile water and then germinated in petri plates on 0.8% water agar in a lighted growth chamber. Individual 6-week-old seedlings (*c*. 1-cm tall) were aseptically transferred into the sterile microcosms, which were sealed with Parafilm. Microcosms were then placed in a lighted growth chamber providing 150 µmol m^{-1} s^{-1} of photosynthetically active radiation with lighting cycle of 16-h light (25 °C) and 8-h dark (20 °C). The positions of the microcosms within the growth chamber were randomised once a week throughout the experiment and seedlings were allowed to acclimate after being transferred to the microcosms for 3 weeks before fungi were added.

Fungal inoculation

Fungal inocula consisted of hyphal suspensions prepared in individual sterilised jars fitted with a stainless steel blender assembly and filled with 80 ml distilled water. Each sterile jar received four agar plugs (1 cm) taken from the growing edge of a 4-week-old fungal culture, and was blended at high speed for 60 seconds. Inoculum for each species pool was then prepared by combining 150 µl of the hyphal suspension of individual isolates and adding sterile water for a total of 5 ml. Microcosms were inoculated under a sterile air-flow hood and received 1 ml of a mixed-species inoculum (or sterile water) which was applied at the base of the seedling.

Microcosm harvest

Microcosms were harvested 5 months after the introduction of the fungal species pools. Roots were separated from shoots and were gently cleaned of growth media using flame sterilised forceps and briefly dried on sterile filter paper. The fresh weight of roots and shoots was recorded. Shoots were dried

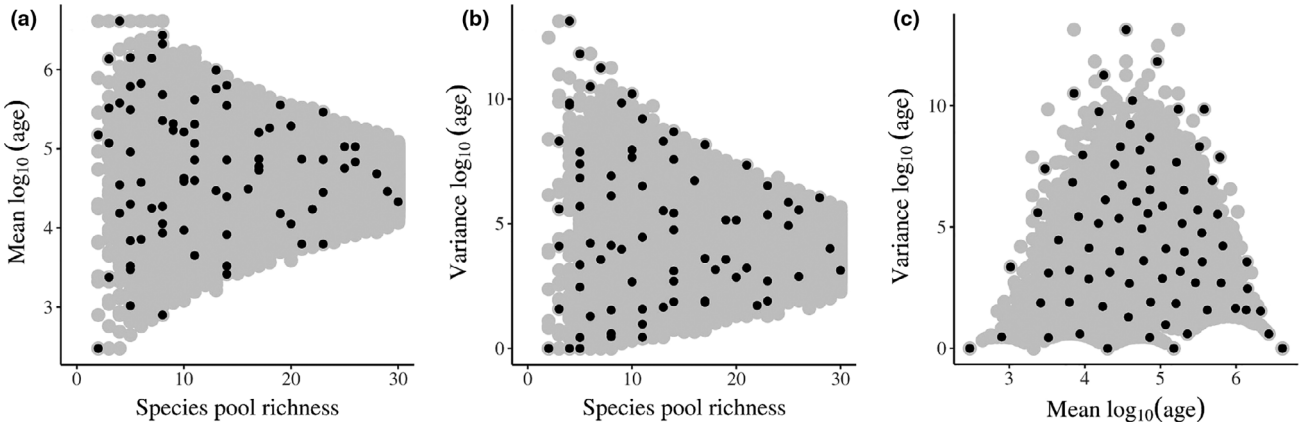

Figure 1 Graphical summary of experimental pool design, showing the pairwise relationships between species pool richness (a and b), and the mean (a and c) and variance (b and c) of the $\log_{10}$-transformed ages of the chronosequence sites included. Light grey points indicate the parameter values for 20 million randomly generated possible species pools and black points show the values for the 79 species pools used in the experiment.

at 60 °C and analysed for C and N concentration using an elemental analyzer (Carlo Erba NA 1500). After weighing, roots were transferred to sterile 2-ml screw cap tubes and stored at −80°C. We did not find any evidence for an effect of species pool composition on seedling biomass or leaf chemistry and present a graphical summary of these results as supplemental material (Fig. S2).

Fungal species composition

Fungal species composition at the end of the experiment was determined using Illumina metabarcoding, targeting the ITS2 region of the nrDNA gene. This region was identified by Sanger sequencing as being variable enough to discriminate between isolates while having minimal length variation to minimise amplicon sequencing bias (Lindahl *et al.*, 2013). In addition to the microcosm samples, we included samples that consisted of DNA from pure cultures of each fungal isolate to verify the Sanger sequencing of the target barcoding region.

DNA was extracted from the entire root system of each seedling using bead beating and the Nucleomag 96 Plant Kit (Macherey-Nagel) on a KingFisher Flex (ThermoFisher) automated magnetic particle processor. A dual indexed, two-stage fusion-PCR procedure was used for Illumina library preparation, following the primer design of (Toju *et al.*, 2016). We modified the stage-one primers to include the gene primers 58A1F (Martin and Rygiewicz, 2005) and ITS4. Because these primers amplify both fungal and host ITS2 nrDNA, we designed an amplification-blocking oligo (Vestheim and Jarman, 2008) that overlapped with 2-bp at the 3' end of the forward primer, extended into a host-specific sequence, and terminated with a 3' C3 spacer to block elongation during PCR (5'-GTA GCG AAA TGC GAT ACT TGG-3SpC3-3'). Mismatches between the blocking oligo and fungal DNA were consistent across all species, limiting the probability of introducing amplification bias. Stage-one PCRs (25 μl) were carried out using MyTaq Hot-Start Red Mix (Bioline), 10 μM primers, 100 μM blocking oligo, 1 μl template DNA and a thermocycler programme consisting of an initial cycle of 95°C (3 min), 32 cycles of 95°C (20 s), 60°C (10 s) and 72°C (10 s), followed by a final elongation cycle at 72 °C (5 min). Stage-two PCRs (25 μl) used 2 μl of the stage-one PCR product as template, 10 μM primers and a thermocycler programme consisting of an initial cycle of 95°C (3 min), 6 cycles of 95°C (20 s), 55°C (10 s) and 72 °C (10 s), followed by a final elongation cycle at 72°C (5 min). Final PCR products were cleaned and normalised to 2.5 ng μl^{-1} using Just-a-Plate, 96-well normalisation and purification plates (Charm Biotech), then pooled and sent to the Stanford Functional Genomics Facility for 250-bp paired-end sequencing on the Illumina MiSeq platform. The resulting raw data were publicly archived in the NCBI Sequence Read Archive (BioProject PRJNA613615).

Bioinformatics

Bioinformatic processing of the Illumina sequence data involved first removing gene primers and length heterogeneity spacers with cutadapt v1.18 (Martin, 2011). Initial quality assessment indicated poor quality for the reverse reads which were not used in subsequent analyses. The forward reads were cropped to 200 bp, filtered at a maximum expected error rate of 2 bp, and denoised with the R-package DADA2 (Callahan *et al.*, 2016) to identify unique amplicon sequence variants. Priors, consisting of the expected amplicons for each sample, were provided to facilitate accurate denoising. Because the initial denoising did not fully resolve all single species control samples to a single cluster, amplicon sequence variants with a Hamming distance < 4 bp within each sample were collapsed. Unexpected reads in microcosm samples were predominantly host DNA or likely contaminants from the sample processing and library preparation procedure, which were present in very low abundance and often also identified in the no-DNA negative control samples. These reads were filtered from the data before analysis. Read counts for each denoised amplicon in each sample were merged into a species-by-sample matrix for analyses, discarding samples with low sequencing depth (<5000 reads). After bioinformatic processing of the metabarcoding data, 286 samples remained, leaving three or four replicates of 75 unique species pools.

Statistical analyses

To quantify local diversity, we used multiple rarefaction of the community sequencing data, sampling without replacement to the minimum sequencing depth across all samples (5736 sequences), and calculating the mean species richness over 1000 iterations. To determine whether the relationship between local diversity and species pool richness was linear or curvilinear (i.e. saturating), we used linear mixed models in the R-package nlme (Pinheiro *et al.*, 2017), including species pool composition as a random effect. To account for heteroscedasticity, we allowed the model variance to increase with increasing species pool richness. Support for a curvilinear model was tested by comparing models with and without a polynomial term using a likelihood-ratio test.

We used two approaches to test whether the relationship between species pool richness and local diversity was affected by species pool age. First, we used polynomial mixed models, as above, testing for a significant interaction between species pool richness and age. To account for a possible effect of site heterogeneity, we also included age-variance and the interaction between age-variance and species pool richness. In the second approach, we fit a generalised nonlinear least squares model with nlme, using a Michaelis–Mentin function, which takes the form,

$$\alpha = (\alpha_{max} * \gamma)/(\alpha_k + \gamma).$$

where α is the local diversity and γ is the species pool richness. The estimated parameters, α_{max} and α_k, represent the asymptotic value of α and the value of γ at which half of the asymptotic value of α is reached, respectively. As with the polynomial mixed-model approach, we allowed the model variance to increase with species pool richness. We then modelled the standardised (Pearson) residuals of the nonlinear model as a function of species pool richness and age to test whether the residuals varied systematically with species pool age. Specifically, we fit a linear mixed model with species pool composition as a random effect and interactions between species pool richness and both species pool age and age-variance as fixed effects. We then used likelihood ratio tests to simplify the model, removing both the interaction between pool richness and age-variance ($\chi^2_{(1)} = 0.061$, $P = 0.81$) and the direct effect of age-variance ($\chi^2_{(1)} = 0.16$, $P = 0.68$). We then refit the final model using restricted maximum likelihood estimation to test the significance of the remaining fixed effects. Additionally, for both modelling approaches, we tested whether the results were sensitive to the inclusion of species pool phylogenetic diversity (mean pairwise phylogenetic distance), which is necessarily correlated with species pool richness and possibly with species pool age (see below). Conclusions from the polynomial mixed-model approach and analysis of the generalised nonlinear model residuals were identical. We present the results of the generalised nonlinear model here because the functional form more closely approximates the expected saturating relationship between regional richness and local diversity.

To test the relationship between phylogenetic or functional diversity and species pool composition, we first quantified the mean pairwise phylogenetic and functional distances among isolates for each species pool. Phylogenetic distances were square root transformed to account for nonlinear scaling between evolutionary and functional distances (Letten and Cornwell, 2015). Carbon substrate use data were reduced to the first 10 principle components with PCA (accounting for *c.* 75% of total variation) to collapse highly correlated substrates. Euclidean distance of the PCA coordinates was then used to calculate mean pairwise functional distances among isolates for each species pool. Because patterns were broadly similar for phylogenetic and functional diversity, we combined these metrics with equal weight, following the method of Cadotte *et al.* (2013), resulting in a single measure of functional and phylogenetic diversity, FPdist. We then used multiple linear regression to test the hypothesis that mean pairwise FPdist increases with species pool age, accounting for both species pool richness and site heterogeneity (age-variance).

To test whether changes in phylogenetic and functional diversity during community assembly (i.e. increased clustering or dispersion) were related to species pool age we calculated the standardised effect sizes for the mean pairwise FPdist, using the R-package picante (Kembel *et al.*, 2010). We weighted species abundances using Hellinger-transformed community sequencing data and used a null model where taxa names were permuted 999 times on the distance matrix. The standardised effect sizes were modelled as a response using a linear mixed-effects model, with species pool richness, age and age-variance as fixed effects and species pool composition as a random effect.

RESULTS

Fungal communities within *V. calycinum* seedlings accumulated diversity at a decreasing rate with increasing species pool richness ($\chi^2_{(1)} = 10.0$, $P = 0.002$; Fig. 2; Table S1). As hypothesised, the relationship between pool richness and realised local diversity varied with species pool age, with older pools showing increasingly higher diversity as regional species richness increased ($t_{1,74} = 2.61$, $P = 0.011$; Fig. 2; Table S1). This result was not sensitive to the inclusion of species pool phylogenetic diversity, which did not account for additional variation in realised local diversity ($t_{1,73} = -0.20$, $P = 0.984$).

We also found that the functional and phylogenetic diversity of the species pools decreased with increasing pool age ($t_{1,74} = -4.92$, $P < 0.001$; Fig. 3; Table S2). Moreover, communities assembled with species pools from younger sites tended to become phylogenetically more dispersed during community assembly than communities associated with species pools from older sites, which tended to become phylogenetically more clustered ($t_{1,70} = -4.24$, $P = 0.001$; Fig. 4; Table S3).

DISCUSSION

Taken together, our results indicate that local diversity increases with the geological age of a region not just as a result of increased regional species richness but also because the regional species pool contains species that are more likely to co-occur in local communities. Although the mechanisms responsible for fine-scale species co-occurrence in our

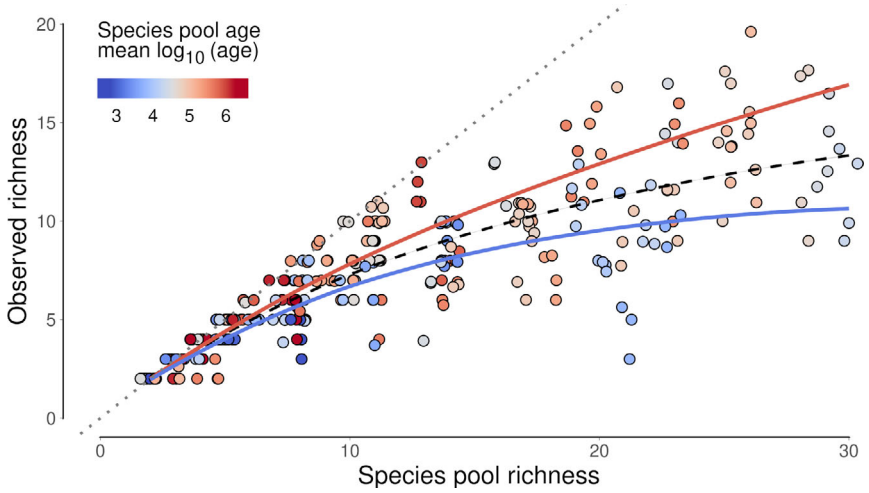

Figure 2 The relationship between species pool richness and local diversity (observed richness) in experimental microcosms, showing the theoretical maximum local diversity (dotted grey line) and a nonlinear saturating model (a Michaelis–Menten functional form) fit to the data (dashed black line). Point colours indicate the mean of the $\log_{10}$-transformed age of the chronosequence sites represented in each species pool. The blue and red lines show the results of our analysis of the residuals from the nonlinear model, indicating the predicted mean relationship between species pool richness and observed richness for young and old species pools, respectively, using the 0.05 and 0.95 quantiles of the range of species pool age used in the experiment.

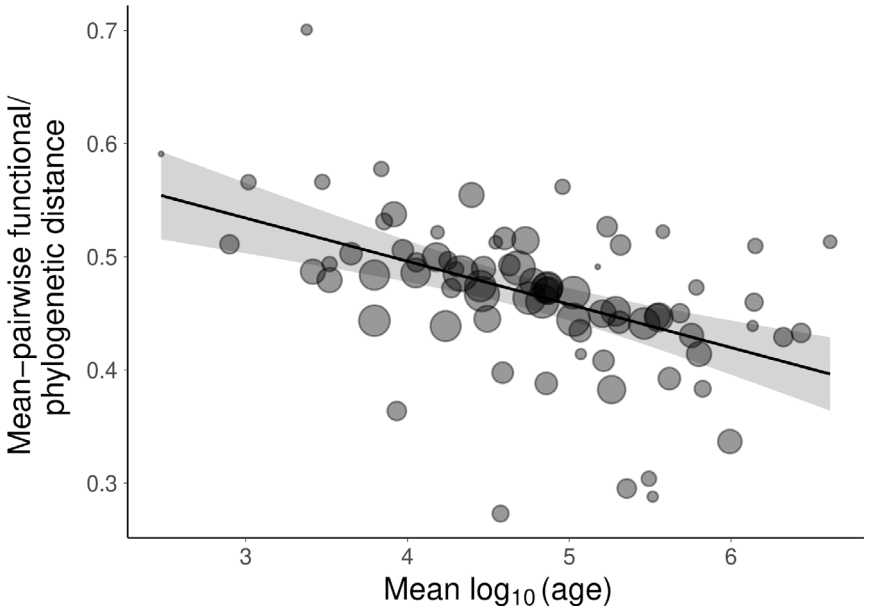

Figure 3 The relationship between the combined functional and phylogenetic diversity of the experimental species pools and the mean of the log10-transformed ages of the sites where the constituent species originated. Point size is proportional to species pool richness.

microcosms are unknown, our results are consistent with evidence from molecular profiling of fungi in field-collected *V. calycinum* roots, which showed that the diversity and evenness of the fungal symbiont community associated with an individual plant increased with ecosystem age (Leopold et al. 2020).

The finding that older species pools had lower functional and phylogenetic diversity (Fig. 3) suggests that increased coexistence of species from older sites may be the result of convergence on similar ecological strategies (i.e. equalising fitness differences), rather than niche differentiation (Chesson, 2000; Mayfield and Levine, 2010). We do not know which traits determine the outcome of species interactions in this system, but both evolutionary and ecological mechanisms could contribute to trait convergence in older ecosystems. For example, a competitive trait hierarchy may lead to evolutionary convergence on similar root colonisation strategies over geological time (TerHorst *et al.*, 2010; Kunstler *et al.*, 2012; Tobias *et al.*, 2013). It is also possible that changing conditions with long-term soil development led to trait convergence only in older sites. In plants, progressive phosphorus limitation with increasing soil age can result in a shift of species coexistence mechanisms from niche differentiation to trait convergence (Mason *et al.*, 2012). Similar trends may exist in root-associated fungi so that they coexist through niche

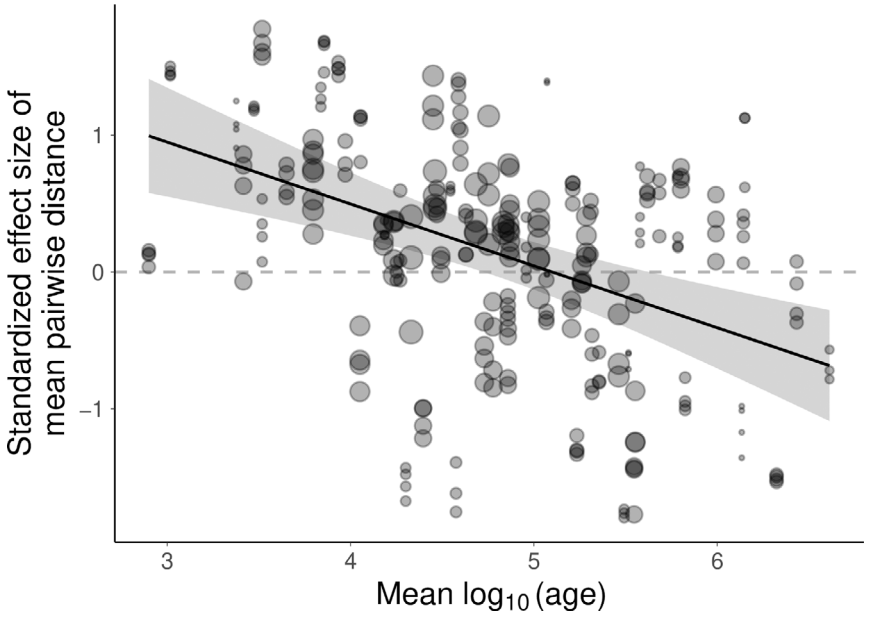

Figure 4 Effect of species pool age on the standardised effects size of mean pairwise functional and phylogenetic distance, showing that assembled communities had higher dispersion than the species pool if the pool was from young sites, whereas assembled communities had less dispersion than the species pool if the pool was from old sites. Point size is proportional to species pool richness.

differentiation in younger soils, but converge on a common resource-retentive strategy with declining phosphorus availability (Lambers *et al*., 2008), resulting in coexistence through functional convergence and reduced fitness differences.

We cannot entirely rule out niche partitioning due to environmental heterogeneity as a mechanism facilitating diversity in the natural fungal communities we sampled for this experiment. For example, the accumulation of complex organic layers and recalcitrant nutrient pools during pedogenesis could promote diversity in ericaceous root-associated fungi through niche partitioning along nutrient resource axes (Wurzburger *et al*., 2012). This fine-grained resource heterogeneity in older soils may facilitate coexistence among species in the regional species pool more than the broad scale environmental heterogeneity present across the chronosequence (Tamme *et al*., 2010). However, soil nutrient resource partitioning is unlikely to explain the patterns we observed in the experimental microcosms, where a single growth media was used and nutrients were supplied in mineral form.

We did not detect a significant signal of clustering or dispersion in the phylogenetic or functional structure of the assembled communities. This lack of pattern could be due to the relatively small number of species used in our experiment and the biases introduced by our focus on ascomycetous fungi that could be easily cultured (Allen *et al*., 2003). In addition, spatial resource partitioning within roots between mycorrhizal fungi and non-mycorrhizal endophytes may not result in a strong phylogenetic signal due to the phylogenetic overlap among these functional groups (Lukešová *et al*., 2015; Leopold, 2016). Nonetheless, we did find that species pools from younger sites tended to increase in functional and phylogenetic dispersion during assembly more than species pools from old sites (Fig. 4). This result is consistent with a shift towards equalising fitness differences, rather than niche differentiation, as the primary mechanism of species coexistence (Mason *et al*., 2012).

The results of our experiment must be considered in the context of two important caveats concerning our experimental approach. First, our study uses the geologic age of a site as a proxy for species pool age. Because the individual chronosequence sites are not completely isolated from one another, geographically, the species pools may not be entirely independent, and site age may not always reflect the amount of shared co-evolutionary history between pairs of co-occurring taxa. Nonetheless, fungi associated with *V. calycinum* roots are likely to be highly dispersal limited because few ericaceous host plants are present in montane Hawaiian forests and most ericoid fungi do not effectively disperse over long distances (Hutton *et al*., 1997). This assumption is further supported by long-term (> 30 years) nutrient-addition experiments conducted at the youngest and oldest sites, which resulted in relatively subtle changes in fungal species composition of *V. calycinum* roots, compared to the species turnover observed across the chronosequence (Leopold et al. 2020). Furthermore, the conditions present in younger soils persist for only a short time, relative to the conditions present in older soils, limiting the opportunities for groups of co-occurring taxa to collectively disperse across the archipelago.

The second limitation of our experimental design concerns the partial overlap in the composition of species pool treatments, particularly with increasing species pool richness. This raises the possibility that our results primarily reflect the competitive abilities of the particular isolates used, as opposed to a more general pattern. However, post-hoc analysis of each isolate's ability to persist in the local community, and of their final relative abundance, suggests that there was no significant bias in the competitive abilities of the fungi we obtained from the different chronosequence sites, and that our main findings

are not simply the result of the inclusion of highly influential outlier taxa from certain sites (Fig. S3). Nonetheless, future studies should seek to replicate the findings of our highly simplified and controlled experimental system using larger collections of taxa, possibly from more geographically isolated locations.

Greater local diversity in geologically older habitats has been thought to reflect increased richness of the regional species pool (Zobel *et al*., 2011; Lososová et al. 2015). The novel finding of our study is that older species pools can also consist of species that are more likely to coexist. Our results are consistent with an increasing role of equalising fitness differences in older species pools and a shift from niche partitioning in younger ecosystems to convergence on similar ecological strategies in older ecosystems, possibly in response to progressive phosphorus limitation. Increased local diversity as a result of strengthened species coexistence mechanisms might be a general feature of species pools in geologically older regions.

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AUTHORSHIP

DRL and TF conceived of and designed the experiment. DRL conducted the experiment and performed the analyses with guidance from TF. DRL wrote the initial manuscript draft and both authors contributed to revisions.

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OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at: https://doi.org/10.5281/zenodo.4136633.

DATA AVAILABILITY STATEMENT

Raw sequence data have been deposited in the NCBI SRA (BioProject PRJNA613615). All code and additional sample meta data needed to reproduce the analyses have been publicly archived (https://doi.org/10.5281/zenodo.4136633).

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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