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Geographic divergence in a species-rich symbiosis: interactions between Monterey pines and ectomycorrhizal fungi

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Abstract. A key problem in evolutionary biology is to understand how multispecific networks are reshaped by evolutionary and coevolutionary processes as they spread across contrasting environments. To address this problem, we need studies that explicitly evaluate the multispecific guild structure of coevolutionary processes and some of their key outcomes such as local adaptation. We evaluated geographic variation in interactions between most extant native populations of Monterey pine (*Pinus radiata*) and the associated resistant-propagule community (RPC) of ectomycorrhizal (EM) fungi, using a reciprocal cross-inoculation experiment with all factorial combinations of plant genotypes and soils with fungal guilds from each population. Our results suggest that the pine populations have diverged in community composition of their RPC fungi, and have also diverged genetically in several traits related to interactions of seedlings with particular EM fungi, growth, and biomass allocation. Patterns of genetic variation among pine populations for compatibility with EM fungi differed for the three dominant species of EM fungi, suggesting that Monterey pines can evolve differently in their compatibility with different symbiont species.

Key words: coevolution; ectomycorrhizal fungi; geographic divergence; Monterey pine; multispecific mutualism; Pinus radiata.

Introduction

Natural selection on species varies among environments because gene expression and the fitness of genotypes varies among environments, creating a genotype by environment interaction for fitness (Williams 1966, Endler 1986). The result, if selection is strong enough to counter gene flow, is the local adaptation of populations (Hereford 2009) and, sometimes, ecological speciation (Schluter 2009). This process can occur even more rapidly when the main agent of selection is another species, because the species impose reciprocal selection on each other, which often varies among environments (genotype by genotype by environment, or G × G × E, interactions; Thompson 2005). The result is a geographic mosaic of coevolution due to three factors: (1) geographic variation in the structure of coevolutionary selection, e.g., ranging from mutualism to parasitism (selection mosaics, or G × G × E interactions); (2) geographic variation in the intensity of coevolutionary selection, resulting in coevolutionary hotspots and coldspots; and (3) trait remixing resulting from gene flow, random genetic drift, and metapopulation dynamics (Thompson 1994, 2005). The mosaics are likely to increase in complexity as pairwise interactions diversify into multispecific interactions. A key problem in evolutionary biology is therefore to understand how multispecific networks are reshaped by evolutionary and coevolutionary processes as they spread across contrasting environments.

Although multiple studies have now shown how coevolution between two focal species may vary with the presence of a third species (G × G × G interactions; e.g., Thompson and Cunningham 2002, Siepielski and Benkman 2007, Thompson et al. 2010), a further challenge is to understand how the outcomes of coevolution vary among different members of diverse interacting guilds (i.e., groups of species with similar roles in communities). If we are to understand how coevolution proceeds within multispecific assemblages, we need to assess the extent to which all species in a guild evolve in the same manner in response to evolution in another guild, and which guilds are most likely to drive the structure of selection within complex assemblages. Recent theoretical studies have suggested that coevolution within mutualistic assemblages is driven especially by supergeneralists interacting with species across guilds (Guimaraes et al. 2011). We therefore need

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empirical studies that assess not only geographic differences in coevolution driven by other species, but also the multispecific guild structure of coevolution and its key outcomes such as local adaptation and varying degrees of specialization.

We addressed this problem by experimentally manipulating the ectomycorrhizal (EM) interaction between most native populations of Monterey pine (*Pinus radiata* D. Don) and members of the diverse guild of fungal species that colonize the roots. Post-Pleistocene native populations of Monterey pine are restricted to a small set of geographically separated sites along the west coast of California (USA) and two islands off Baja California (Mexico) (Axelrod 1980), and therefore provide an opportunity to study how geographically isolated diverse interactions evolve amid limited gene flow among populations. We focused on the guild of ectomycorrhizal fungal species that typically colonize pine roots via resistant propagules in the soil such as spores and sclerotia, rather than via mycelial growth from other colonized roots. In pine-dominated ecosystems on the West Coast of North America, members of the resistant propagule community (RPC) of ectomycorrhizal fungi are particularly important colonists of pine seedlings and saplings in early secondary succession, after stand-replacing disturbances or during stand expansion into adjacent habitat (Taylor and Bruns 1999, Ashkannejhad and Horton 2006). Bishop pine (*Pinus muricata* D. Don), a sister species to Monterey pine in northern and central California, has been shown to have a relatively low-diversity EM fungal community overall (Horton and Bruns 2001), with an RPC fungal guild dominated by members of the genera *Rhizopogon*, *Tomentella*, *Tuber*, and *Cenococcum* (Gardes and Bruns 1996, Horton and Bruns 1998, Baar et al. 1999, Taylor and Bruns 1999, Grogan et al. 2000).

In previous studies, we found that one of the RPC fungi, *Rhizopogon occidentalis*, varies considerably in its ecological interactions with Monterey pine, bishop pine, and shore pine (*Pinus contorta* var. *contorta* Dougl. ex Loud.), a more northerly coastal species. Using cross-inoculation experiments with multiple genotypes of fungi and pines, we found that within populations, fungal and pine seedling performance frequently varied with fungal genotype, pine genotype, and abiotic factors, suggesting the potential for ongoing coevolution ($G \times G$ interactions) mediated by selection mosaics ($G \times G \times E$ interactions) among populations (Piculell et al. 2008, Hoeksema et al. 2009). We also found evidence for local adaptation of fungal populations to nearby pine populations at a large geographic scale, suggesting pines are exerting geographically variable selection on fungal populations (Hoeksema and Thompson 2007). Nevertheless, much of the variation among populations in pine seedling traits was independent of *R. occidentalis* genotype and vice versa and was correlated with latitude, pointing to the importance of abiotic selection on plant and fungal traits (Hoeksema and Thompson 2007).

Rhizopogon occidentalis, however, is only one species in this complex mycorrhizal assemblage. Here, we expand this analysis by evaluating geographic variation in the interaction between disjunct native Monterey pine populations in coastal California and insular Mexico and the associated RPC fungi. Using a reciprocal cross-inoculation experiment with all factorial combinations of plant genotypes and fungal guilds from each population, we aimed to answer two questions:

1) Has the species composition of the RPC fungi diverged among populations of Monterey pine?
2) Have populations of pines and fungi diverged genetically in their compatibility or traits, and are patterns of divergence consistent across different members of the fungal guild?

Methods

We used a factorial experiment in the laboratory to assess the main and interactive effects of soils (containing resistant propagules of mycorrhizal fungi) and Monterey pine genotypes on plant performance traits and the composition of ectomycorrhizal fungi colonizing pine roots. We sampled soils and open-pollinated families from five native stands of Monterey pine, and combined them in all 25 possible combinations in a growth chamber pot experiment. We measured plant performance traits for 28 weeks and used molecular methods to identify the mycorrhizal fungi that colonized the roots of the experimental pine seedlings. We used root colonization on these experimental seedlings planted into field soils as a "bioassay" of the resistant propagule community (RPC) of ectomycorrhizal fungi. This procedure has been shown to provide a relatively accurate assessment of the RPC of ectomycorrhizal fungi that are ecologically important during early succession in coastal pine forests (e.g., Baar et al. 1999, Taylor and Bruns 1999). Moreover, we posited that variation among seedling genotypes in observed relative abundances of fungal taxa in such a bioassay would provide an estimate of plant genetic variation for compatibility with those fungal taxa.

Collection and preparation of soil and seeds

Soil and seeds were collected from stands in two native Monterey pine populations in mainland California (Año Nuevo, 37°3′46.8″ N, 122°14′16.8″ W; Cambria, 35°32′6.0″ N, 121°4′48.0″ W) and three stands in insular Mexico (Guadalupe Island, 29°9′36.0″ N, 118°17′60.0″ W; northern Cedros Island, 28°20′48.0″ N, 115°13′24.0″ W; southern Cedros Island, 28°10′45.9″ N, 115°12′39.0″ W). Although the latter two stands are usually considered to be part of the same population (Cedros Island), they are separated by ~20 km, and one of our goals was to explore variation between these isolated stands within the Cedros Island. Hereafter, we

refer to the five sampled stands, including the two on Cedros Island, as five "populations." No sampling was conducted in the pine population at Monterey, California, in an effort to reduce the complexity of the experiment. Monterey pine was the dominant ectomycorrhizal tree species at all five sampling sites, although occasional Douglas-firs (*Pseudotsuga menziesii*) were observed at Año Nuevo, and occasional coast live oaks (*Quercus agrifolia*) were observed at both Cambria and Año Nuevo. In addition, knobcone pine (*P. attenuata*) is known to occur in the Año Nuevo population and to hybridize with Monterey pine, but no knobcone pines were observed in the vicinity of our specific sampling site. On average from 1950 to 2000, the Cedros Island sites were the driest and warmest compared to the other sites (~105 mm annual precipitation, ~18°C annual mean temperature), Año Nuevo was the wettest and coolest (841 mm, ~13°C), with Cambria and Guadalupe Island intermediate but closer to Año Nuevo in temperature and closer to Cedros Island in precipitation (Supplement). Additional characteristics of each sampling site, including soil characteristics and bioclimatic variables often used in ecological niche modeling (Hijmans et al. 2005), can be found in the Supplement. As variation in environmental characteristics occurs within each population, it should be noted that these characteristics are representative of our particular sampling sites. Collections were made from the Mexican populations (under permit from the Mexican government) during an expedition on 18–26 May 2006, and from the California sites in August, 2006 (see Appendix B for photos of the Mexican sites).

At each of the two California sites, soil was obtained by collecting 20 cores, each 10 cm in diameter and 20 cm deep (excluding litter), spaced 30 m apart along two randomly placed 300-m transects through the closed-canopy pine forest. On the Mexican islands, the soils were thin and rocky, and were not conducive to removal of cylindrical soil cores. Consequently, equivalent volumes of soil were removed using a hand trowel. In addition, because tree density at the Mexican sites was either low (Guadalupe Island) or irregular and clumped (Cedros Island), at those sites we collected soil samples within 10 m of specific individual trees. At all five sites, we collected ~20 pine cones from each of 10 different trees. At the California sites, those trees were chosen as the nearest cone-bearing trees adjacent to every second soil sampling point. In the Mexican populations, the 10 trees were chosen haphazardly at equally spaced intervals across the populations. Soils and seeds were imported to the United States under USDA APHIS permits P526P-06-02719 and 37-86851, respectively.

Cones were immersed in hot water and dried to extract seeds, and samples of 100 seeds from each cone were weighed, so that seed mass could be used as a covariate in analyses to control for potential maternal effects on seedling growth traits. Soils were dry at the time of collection, and were stored dry until use in experiments, at which time the multiple soil samples from each site were bulked (separately for each site), homogenized, and sifted through a 5-mm sieve. A subset of the soil was subjected to gamma irradiation (Sterigenics, Hayward, California, USA) to reduce densities of mycorrhizal fungal propagules and other microbes for use in sterile controls. Subsamples of the soil from each of the five populations were passed through a 2-mm sieve and submitted to the University of California, Davis Analytical Laboratory for determination of chemical and physical characteristics including extractable nutrients, cation exchange capacity, percentage organic matter, percentage organic carbon, and texture. Seeds were surface sterilized with 10% sodium hypochlorite and cold stratified at 4°C for four weeks.

Growth chamber experiment

Experimental units were established by filling cylindrical pots (6 cm diameter by 15 cm deep) with a 3:1 mixture of field-collected soil and sterile sand (to improve drainage), and planting three seeds from the appropriate tree (i.e., open-pollinated, or OP, seed family) into each pot. After seed germination, each pot was thinned to contain only one seedling. We created different treatments by pairing soil from each of the five populations with seedlings grown from seeds collected from three different OP familes at each of the five populations, resulting in 75 different (soil × population × OP family) combinations. Each combination was replicated five times, for a total of 375 pots (hereafter referred to as the "main experiment"). In addition, three gamma-irradiated replicates of each OP family with each soil were established for an additional 225 pots, to determine plant growth in soils with reduced microbial densities. Beginning in February 2007, seedlings were grown in a controlled-environment room at the University of California, Santa Cruz, on a 14:10 h day : night cycle (20:10°C day : night temperature, 225 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of light at plant height) and watered to capacity twice weekly with deionized water. Pots were set in racks with 6.5 cm between pots, with pot locations assigned randomly, and locations were re-randomized once every 4 weeks during the experiment. A 5-mm layer of sterile sand was added to the surface of the soil in each pot to minimize splashing of soil and microbes among pots.

Seedling height was measured after 12 weeks of growth and at the end of the experiment by measuring needle-bearing stem length, which we have found in previous work to be tightly correlated with total biomass for Monterey pine seedlings grown in the laboratory. After 28 weeks, the experiment was harvested. Fourteen plants in the main experiment did not survive to harvest and were not included in any analyses. Soil was washed from the roots of surviving seedlings on a 2-mm sieve, and 5–10 root tips colonized by ectomycorrhizal fungi (as indicated by a lack of root hairs) were randomly selected from each root system under a dissecting microscope by choosing the colonized root tips nearest

to sampling points on a regular grid. Uncolonized root tips were rarely observed. Collected colonized root tips were lyophilized to preserve DNA for molecular identification of fungi. Ninety percent of seedlings growing in the gamma-irradiated soils were observed to be colonized by ectomycorrhizal fungi, and this contamination did not differ among treatments; but contamination only occurred on feeder roots in the bottom 1 cm of each pot, suggesting that it took place via spores carried in water that splashed into the drainage holes on the bottom of these pots. Therefore, when sampling ectomycorrhizal root tips on the main experiment, we did not sample from any roots growing in the bottom 5 cm of the pots. Representative root tips from contaminant ectomycorrhizal root tips were also saved for molecular identification. Root length of each plant was estimated using the gridline intercept method (Newman 1966). Aboveground (shoot) and belowground (root) biomass of seedlings were separated, oven-dried for 48 h at 60°C, and weighed. Four plant performance variables were calculated and used for analysis: relative growth rate of height over the final 16 weeks (RGR, $cm \cdot cm^{-1} \cdot d^{-1}$), final total biomass (MASS, g), final ratio of root to shoot biomass (RSR), and final specific root length (SRL, m/g of root).

Molecular identification of ectomycorrhizal fungi

We extracted DNA from each ectomycorrhizal root tip, used PCR with fungal-specific primers to amplify DNA from the nuclear ITS genomic region of the ectomycorrhizal fungal symbionts, and used Sanger sequencing and matching with public databases to classify similar fungal sequences into operational taxonomic units (OTUs, hereafter "species"). Methods were similar to recent published community studies of ectomycorrhizal fungi (e.g., Izzo et al. 2006), and details can be found in Appendix A.

Data analysis

Species richness of the RPC ectomycorrhizal fungi.—All observed species (including "singlets," i.e., species observed only once) were included in analyses to estimate species richness and diversity in each of the five soils studied. We generated estimates of fungal species richness in each soil (across all tree genotypes) by conducting separate analyses (using EstimateS 8.2 software; Colwell 2009) for each of the five soils across all of the replicate seedlings grown in each of those soils. In each of these five separate analyses, the Mao Tau rarefaction function was used to create sample-based species accumulation curves, rescaled as a function of the number of ectomycorrhizal root tips identified (as recommended by Gotelli and Colwell 2001). Because none of the species accumulation curves reached an asymptote, we estimated asymptotic or total species richness in each analysis by calculating a nonparametric total richness estimator, ICE (the incidence-based coverage estimator), and by functional extrapolation of the Mao Tau rarefaction curve using the Michaelis-Menten function (Colwell 2009). For all analyses, parameters and measures of their uncertainty were estimated from 50 randomizations of sample order.

Variation in composition of the dominant RPC ectomycorrhizal fungi.—We tested for variation in composition of the RPC ectomycorrhizal fungal guild due to the main and interactive effects of soils and pine genotypes by conducting a permutation-based multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis dissimilarity of species relative abundances among samples. These analyses excluded species that occurred on fewer than 5% of seedlings, as these rare taxa are expected to contribute little to multivariate patterns of community composition (McCune and Grace 2002). An initial full model for the PERMANOVA included soil, pine population, and their interactions as fixed effects, and included plant family (nested within plant population) and the interaction of plant family with soil as random factors. In initial analyses, the factors other than soil and plant population had negative estimated variance components and were eliminated from the final analysis. P values were calculated from 10 000 permutations, and significance of all statistical tests was assessed using $\alpha = 0.05$. Multivariate analyses were conducted using PRIMER version 6 (PRIMER-E, Plymouth, UK).

We explored the importance of each of the three dominant species for the results of the PERMANOVA analyses by testing for potential local adaptation of fungal species to particular pine populations using separate univariate mixed ANOVAs (in SAS PROC MIXED version 9.2; SAS Institute, Cary, North Carolina, USA) for each of the three dominant species. Each univariate analysis on a particular species used only data from soils in which that species occurred in the data set. Hence, if a fungal species was never detected in a particular soil, then all replicates for that soil were excluded from univariate analyses of that species. In these analyses, the response variables analyzed were the relative abundances of the species being analyzed, and the statistical models had the same fixed effects as the full model for the PERMANOVA analyses (soil, pine population, and their interactions). The corresponding random effects were included only when their variance components were estimated to be greater than zero. We tested for patterns of local adaptation of fungal species to pine populations by following any significant soil by pine population interactions with planned contrasts comparing the relative abundance of a particular species in sympatric combinations (i.e., from the same population) of soils plus tree genotypes vs. allopatric combinations (i.e., from different populations). Because residuals in these analyses were all highly non-normal, P values were obtained with randomization tests (10 000 iterations) using a macro wrapper in SAS PROC MIXED.

Variation in plant growth traits.—Univariate mixed ANOVA models identical to those used for the

univariate analyses of dominant fungal species were used to test for variation in plant performance in the main experiment due to the main and interactive effects of soils and pine genotypes on each plant performance variable. Average seed mass for each open-pollinated family was explored as a covariate to account for potential effects of maternal environment on seedling traits, but results of analyses were not qualitatively different, so seed mass was not used as a factor in the final analyses presented. When soil by pine population interactions were significant, we also tested the overall contrast between sympatric and allopatric combinations of soils and tree populations. However, for some measures of plant performance, their qualitative relationship with plant relative fitness may be context dependent; thus, overall differences in such variables between sympatric and allopatric combinations of soils and plant populations may not be indicative of local adaptation. For example, higher RGR may be favored in some environments and lower RGR may be favored in others; thus, finding consistently higher RGR in sympatric combinations of soils and plant populations would not necessarily indicate that plants are locally adapted to soils. Due to the observation of some contaminant ectomycorrhizal fungi on the lower 1 cm of roots of seedlings in the gamma-irradiated soils, comparisons of seedling growth between the main experiment and the gamma-irradiated soils would be difficult to interpret, i.e., they would not represent clear comparisons of plant growth in sterilized and non-sterilized soils. Thus, we do not present analyses of plant performance in the gamma-irradiated soils.

Results

Question 1: Has the species composition of the RPC fungi diverged among populations of Monterey pine?

Richness and diversity of the RPC ectomycorrhizal fungi.—Sequencing of the ectomycorrhizal fungi identified a rich mycorrhizal assemblage of 35 operational taxonomic units (species) among the 789 samples, including 2% (17 sequences) that occurred only once in the experiment (Appendix C: Table C1). The high fungal diversity found in these samples did not exhaust the full diversity of fungal species associated with the pines at any of these sites, after analyzing more than 40 seedlings and 100 ectomycorrhizal root tips from each soil. Asymptotic species richness of the RPC ectomycorrhizal fungi was estimated to be more than twofold higher in Guadalupe Island soil (ICE = 26.8 ± 3.9 taxa, mean ± SD) than in Cambria soil (ICE = 11.8 ± 6.5), with intermediate levels of richness in the other three soils (Cedros Island South, 12.3 ± 2.6; Año Nuevo, 14.3 ± 3.1; Cedros Island North, 16.1 ± 5.7), although uncertainty around these richness estimates was substantial, with Cambria and Guadalupe Island exhibiting overlapping 95% confidence intervals on Mao Tau rarefaction curves (Appendix C: Fig. C1).

Variation in composition of the dominant RPC ectomycorrhizal fungi.—Fungal diversity showed a strongly exponential distribution of abundance among the samples, suggesting that the assemblage was dominated by a few fungal species (Appendix C: Fig. C2). Three dominant species (Wilcoxina1, Pyronemataceae1, and *Rhizopogon roseolus*) occurred each on at least 5% of experimental seedlings, and thus were used in multivariate analyses. Among these species, Pyronemataceae1 and *Rhizopogon roseolus* occurred in all five soils (Wilcoxina1 was never found in Cambria soil) and all three dominants occurred on all five plant populations of genotypes (Appendix C: Table C1), but their relative abundances differed significantly among soils (pseudo-$F_{4,251} = 85.2$, $P = 0.0001$; PERMANOVA) and among populations of tree genotypes (pseudo-$F_{4,251} = 2.3$, $P = 0.0238$; PERMANOVA). The sharpest break in composition among soils was between Año Nuevo and the other four sites (Fig. 1a). Wilcoxina1 dominated the roots of plants growing in Año Nuevo soil, but was a minor component or did not appear in the others (ANOVA, $F_{3,185} = 139.8$, $P < 0.0001$). In contrast, Pyronemataceae1 dominated the other four soils (ANOVA, $F_{4,225} = 94.3$, $P < 0.0001$), and *Rhizopogon roseolus* was the second most abundant taxon in each soil except Cedros Island South (ANOVA, $F_{4,49} = 2.02$, $P = 0.078$).

The only contaminants found in the gamma-irradiated soils were *Rhizopogon occidentalis* gr and Pyronemataceae1, both of which were also found in the main experiment. This fact, combined with the observation that contaminants only colonized the bottom 1 cm of feeder roots in pots with gamma-irradiated soils, suggests that contaminants were not from airborne propagules and did not influence the RPC fungal community data in the main experiment (collected only from the upper 10 cm of those pots).

Question 2: Have populations of pines and fungi diverged genetically in their compatibility or traits, and are patterns of divergence consistent across different members of the fungal guild?

Mycorrhizal compatibility traits of pine seedlings.—Comparisons of relative abundances of the dominant fungal species on seedlings suggest that the five plant populations have diverged genetically for their relative compatibility with two of the dominant species, Wilcoxina1 (univariate ANOVA, $F_{4,185} = 2.8$, $P = 0.025$) and *Rhizopogon roseolus* (univariate ANOVA, $F_{4,49} = 2.6$, $P = 0.0315$). Specifically, Wilcoxina1 and *Rhizopogon roseolus* were both moderately abundant on the California pine genotypes, but on the Mexican pine genotypes Wilcoxina1 was more common and *Rhizopogon roseolus* was only rarely encountered (Fig. 1b). Pyronemataceae1 was similarly abundant across all five populations of tree genotypes (univariate ANOVA, $F_{4,10} = 0.68$, $P = 0.56$, average relative abundance = 0.68 ± 0.04, mean ± SE). We found no evidence that any of the

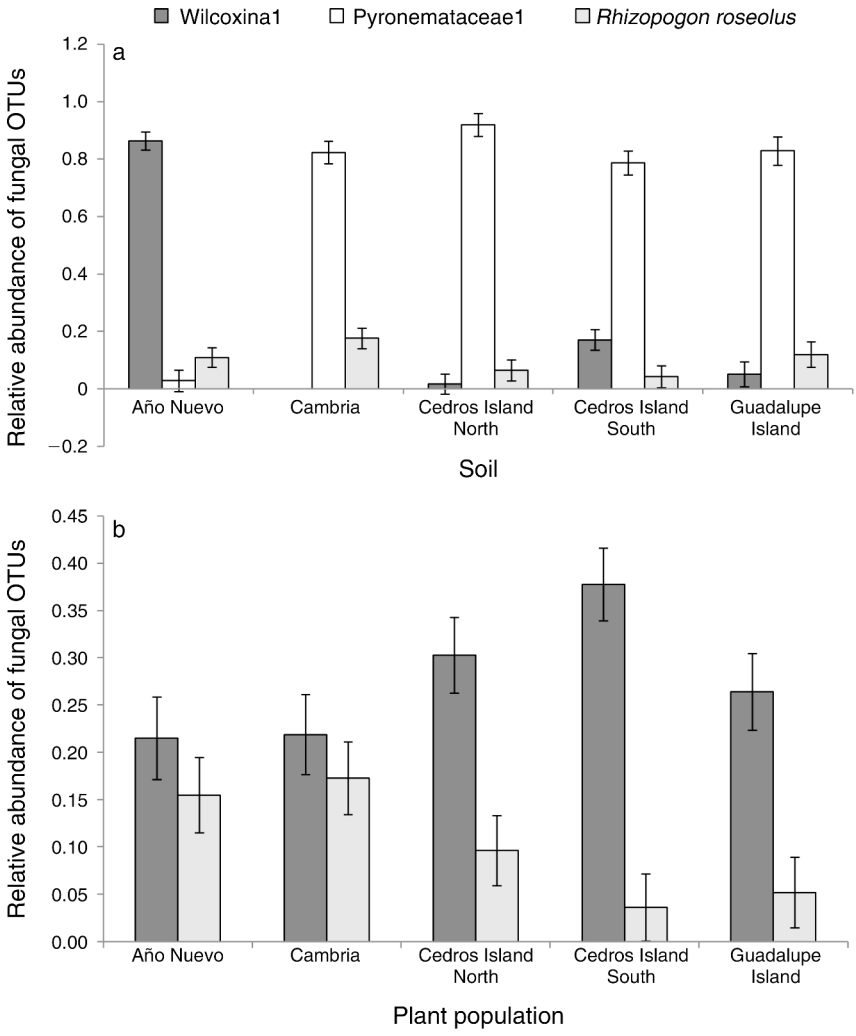

FIG. 1. Variation in relative abundance (proportion of root tips occupied per seedling, mean ± SE) of three dominant ectomycorrhizal fungal species (a) among the five soils studied (each sampled across all five genetic populations of plants), and (b) among the five Monterey pine (*Pinus radiata*) populations studied (each sampled across all five soils) in mainland California, USA, and Guadalupe and Cedros Islands, Mexico. OTUs are operational taxonomic units. Pyronemataceae1 [not shown in panel (b)] was equally abundant across all five pine populations of genotypes (mean relative abundance = 0.68 ± 0.04, mean ± SE). Wilcoxina1: soil, $F_{3,185} = 139.8$, $P < 0.0001$; plant, $F_{4,185} = 2.8$, $P = 0.025$; soil × plant, $F_{12,185} = 0.82$, $P = 0.64$. *Rhizopogon roseolus* gr: soil, $F_{4,49} = 2.02$, $P = 0.078$; plant, $F_{4,49} = 2.6$, $P = 0.032$; soil × plant, $F_{16,49} = 0.85$, $P = 0.55$. Pyronemataceae1: soil, $F_{4,225} = 94.3$, $P < 0.0001$; plant, $F_{4,10} = 0.68$, $P = 0.56$; soil × plant, $F_{16,225} = 0.73$, $P = 0.77$.

three dominant species was locally adapted (or maladapted) to particular plant populations.

Variation in plant growth traits.—Several seedling growth traits have also diverged among the five native populations of *Pinus radiata*. Soils and plant genotypes interacted to control three plant performance traits: RGR, MASS, and SRL (Fig. 2). Genetic differences among plant populations in RGR were strongest in Guadalupe Island soils in which Año Nuevo and Cedros Island North genotypes grew the fastest, and were weakest in Cedros Island South soils in which RGR was equally low among all plant genotypes (Fig. 2a). RGR was significantly lower in sympatric combinations of soils and plant genotypes, mainly driven by the Cambria, Cedros Island North, and Guadalupe soils in which sympatric genotypes exhibited low RGRs relative to other genotypes in those soils.

Patterns of genetic variation among plant populations in seedling MASS differed among soils (Fig. 2b). This pattern was largely driven by Año Nuevo genotypes, which had relatively high mass in California soils and relatively low mass in Mexican island soils. Overall, the other four populations of genotypes exhibited relatively consistent high (Cambria and Guadalupe Islands) or low (both Cedros Island populations) MASS, and MASS was typically lower in the Mexican soils than the California soils. Patterns of genetic variation in SRL also varied among soils, and SRL was lower (i.e., roots

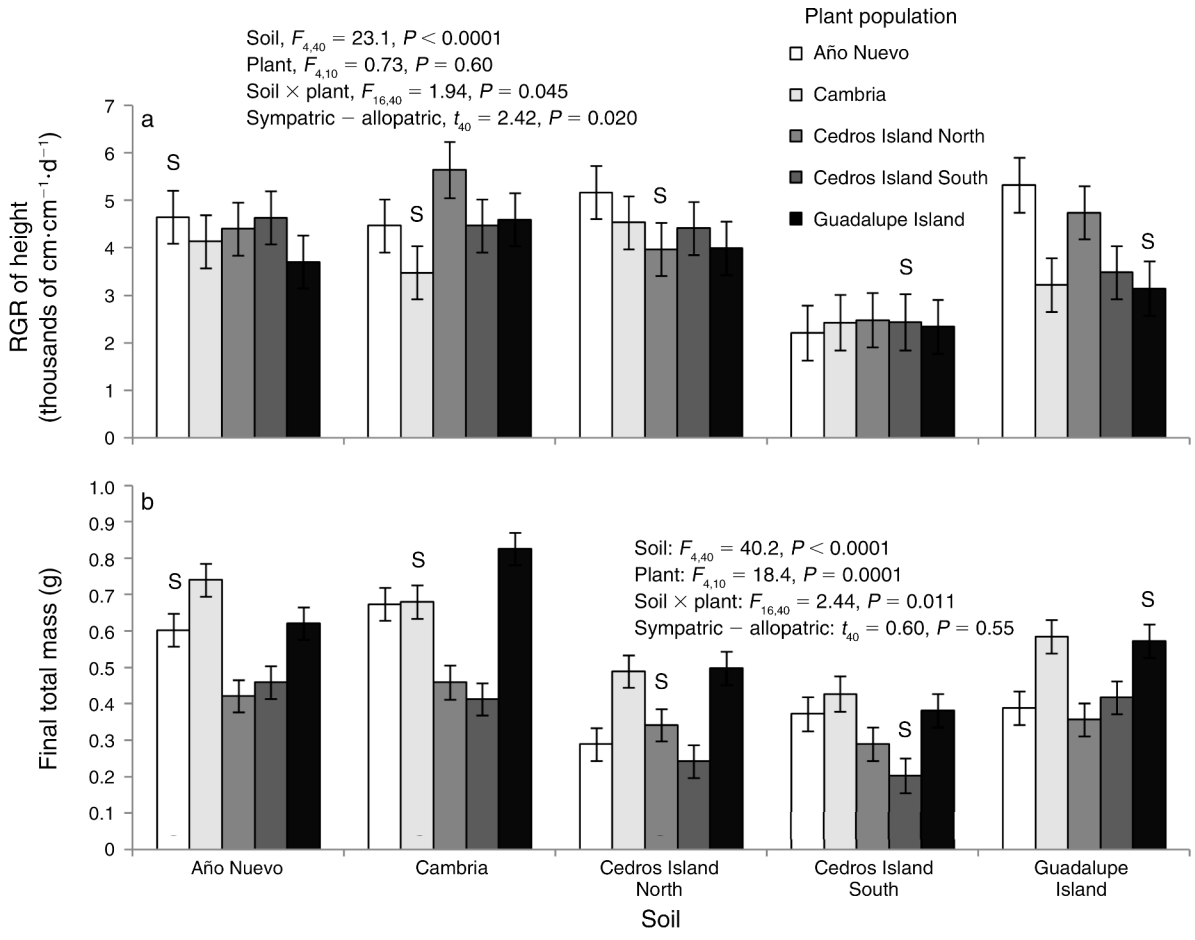

FIG. 2. Variation in *Pinus radiata* seedling growth traits among soils and plant genotypes from each of five populations: (a) relative growth rate (RGR) of height during the final 16 weeks of the experiment, (b) final total biomass, (c) specific root length, and (d) final root : shoot biomass ratio. All data are presented as mean ± SE. In panels (a)–(c), sympatric combinations of plant genotypes and soils are marked with an uppercase "S." Note that panels (c) and (d) are on the following page.

were coarser) in sympatric combinations of soils and plant genotypes (Fig. 2c). Overall, SRL was lowest in Año Nuevo soils and highest in Cedros Island North soils. Final root : shoot ratio of biomass varied significantly among plant genotype populations and also among soils (Fig. 2d). Variation among genotypes was greater than variation among soils, with Cedros Island genotypes exhibiting the lowest root : shoot ratios.

DISCUSSION

The results presented here suggest that the five studied populations of Monterey pine have diverged in the community composition of their associated resistant-propagule community (RPC) of ectomycorrhizal (EM) fungi, and have also diverged genetically in several traits related to interactions of seedlings with EM fungi, growth, and biomass allocation. Patterns of genetic variation among pine populations differed among three dominant species of EM fungi (*Rhizopogon roseolus*, Wilcoxina1, and Pyronemataceae1), suggesting that Monterey pines can independently evolve in their compatibility with different ectomycorrhizal symbiont species.

Geographic variation among native Monterey pine populations in guild composition of RPC ectomycorrhizal fungi

Climatic variation and biogeographic processes such as dispersal and extinction frequently cause geographic variation in community composition and environmental variables (Lomolino et al. 2010). Such geographic divergence in biotic and abiotic context can drive divergent direct selection on species traits (G × E interactions), resulting in local adaptation of a single species (Williams 1966, Endler 1986), or can modify interactions between species, resulting in a geographic mosaic of coevolutionary selection (G × G × E interactions) that drives divergence in traits governing those interactions (Thompson 1994, 2005). We sought to characterize geographic variation among native

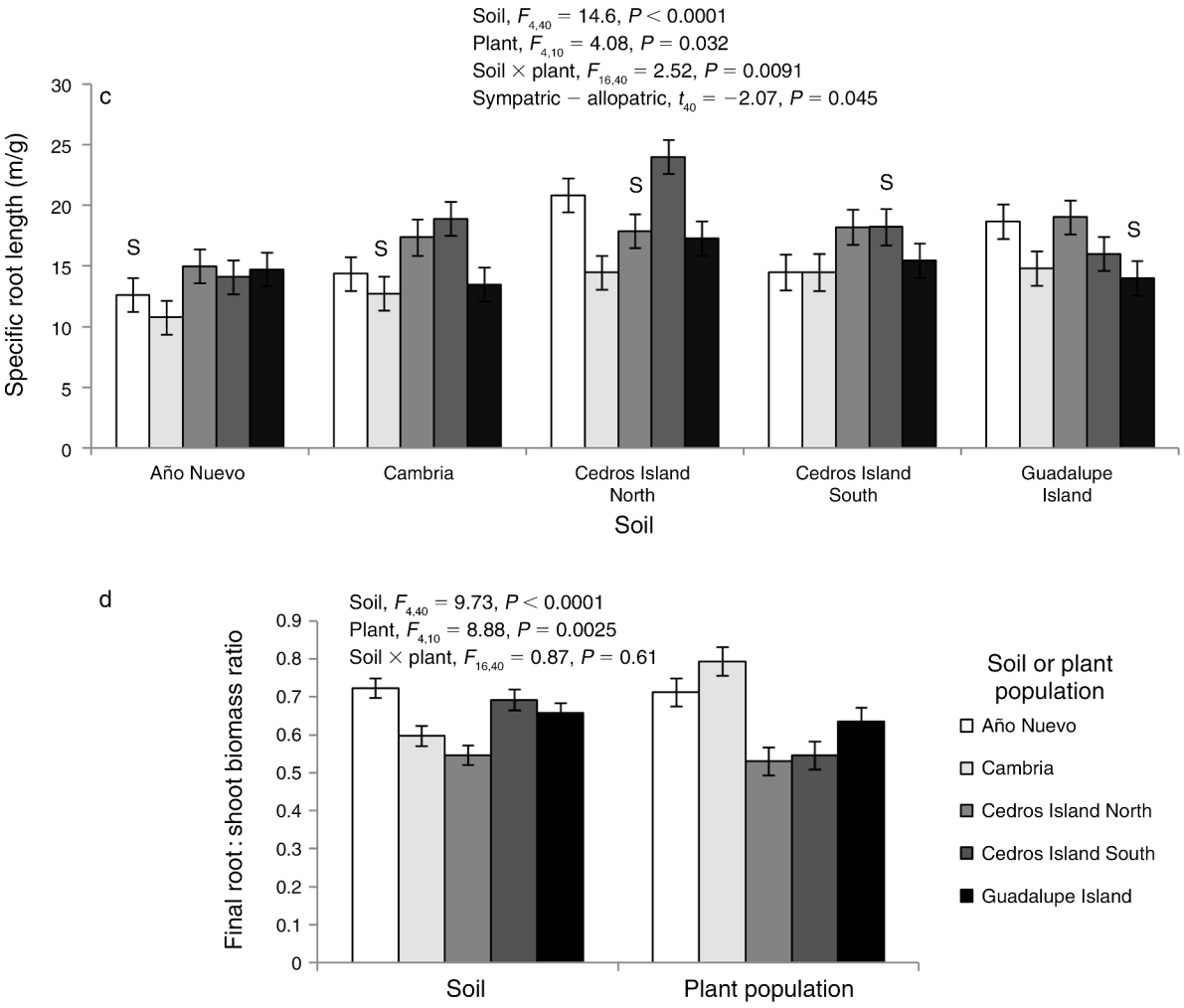

FIG. 2. Continued.

Monterey pine populations in one component of community composition—the RPC ectomycorrhizal fungi, in order to understand the potential for such biotic variation to drive trait divergence among populations of pines and their ectomycorrhizal fungi. Our results suggest that both diversity and composition of the RPC ectomycorrhizal fungi vary among the five studied soils, with the Año Nuevo soil exhibiting contrasting relative abundances of the dominant fungal taxa (Fig. 1a). Given that different EM fungal taxa can have dramatically different ecological effects on their host plants (Jones and Smith 2004), and that plant growth and productivity often varies with the diversity of mycorrhizal fungal taxa (Hoeksema et al. 2010), these results suggest the potential for divergent selection from variable RPC ectomycorrhizal fungi on Monterey pine traits among populations, and/or geographic variation in coevolutionary dynamics of particular ectomycorrhizal interactions. Any divergent selection likely depends on the degree to which the dominant RPC fungal taxa (*Rhizopogon roseolus*, Wilcoxina1, and Pyronemataceae1) differ significantly in key traits affecting Monterey pine fitness or affecting each other's interactions with Monterey pine, and whether the low-diversity community of RPC fungi found in Cambria soils has a different suite of average traits that affect pines, compared to the other communities.

Studies in other ectomycorrhizal systems have also typically found significant variation in EM fungal community composition among geographically isolated populations of the same host plant species (e.g., Kjoller and Bruns 2003, Walker et al. 2005, Rusca et al. 2006, Moser et al. 2009), perhaps in part due to limited dispersal among populations. Rusca et al. (2006) and Kjoller and Bruns (2003) used bioassay methods similar to those used in this study, but focused on variation in relative abundances of *Rhizopogon* species in the RPC ectomycorrhizal fungal guilds associated with pines in California. They studied a series of geographically isolated populations of bishop pine (*Pinus muricata*),

which is a sister species of Monterey pine and ecologically similar to it (Axelrod 1980), along a latitudinal gradient overlapping in the north with the one examined in this study. From their site in Monterey, California, which is located geographically between our Año Nuevo and Cambria sites, they retrieved several *Rhizopogon* species, the most abundant of which were *R. occidentalis* and a member of the *Amylopogon* subgenus (clade I); the soils from their Santa Barbara site (~120 km southeast of our Cambria site) were dominated by *R. brunsii* (called Amylopogon clade II at the time; Grubisha et al. 2005). In contrast, *R. roseolus*, which was detected by Kjoller and Bruns on the coast only at Point Reyes (~125 km north-northwest of our Año Nuevo site) but was frequently detected in pine-dominated sites in the Sierra Nevada mountains (Izzo et al. 2006, Rusca et al. 2006), was the most frequent *Rhizopogon* species in all of our soils. *R. brunsii* and *R. occidentalis* were detected only in small numbers in our study (Appendix C: Fig. C2, Table C1), although our observation of their occurrence in soils from Cedros Island significantly extends the southern limit of the known range of these widespread taxa. Our root tip sampling method (collecting tips nearest to points on a fixed grid) may have underestimated relative abundances of taxa such as *Rhizopogon* with clumped mycorrhizal morphology, and a better understanding of the biogeography of *Rhizopogon* species among our study sites would be facilitated by further studies designed to enhance detection of *Rhizopogon*, including significant dilution of soils for bioassays in a neutral soil medium (as in Kjoller and Bruns 2003), which could also help remove potentially confounding effects of differences among soils in physicochemical factors and other microbes. In general, deeper sampling within the soils from each of our sites would likely reveal additional rare species at each site, and molecular studies of available propagules in the soil would help paint a more complete picture of these communities.

In comparison to other laboratory bioassay studies of RPC ectomycorrhizal fungi, taxonomic composition and diversity in our five Monterey pine soils were most similar to those associated with bristlecone pine (*Pinus longaeva*) in the White Mountains of California (Bidartondo et al. 2001). In both the bristlecone and the Monterey pine study systems, which occur in relatively arid climates, the guilds of RPC fungi were dominated by three taxa: an unidentified species in the Pyronemataceae (different species in the two studies), a *Wilcoxina* species (both very similar to *W. rehmii*), and a *Rhizopogon* species in the subgenus *Roseoli* (Grubisha et al. 2002). The unidentified Pyronemataceous species was the most abundant in the bristlecone pine soils and in soils from all of our sites except the least arid one, Año Nuevo (Fig. 1a). Moreover, the observed species richness in each of our five soils was relatively low (Appendix C: Fig. C1), comparable to that found by Bidartondo et al. (2001), and lower than has been typically found in studies of bishop pine soils farther north along the California coast (e.g., Taylor and Bruns 1999). Perhaps this guild structure of the RPC ectomycorrhizal fungi will prove to be typical of pine-dominated habitats in arid climates. Future analyses of the RPC fungal guild structure in this system will be enhanced by consideration of phylogeographic structure of the fungal taxa, as the fungal species identified here likely contain multiple phylogenetic lineages.

Geographic divergence in growth and mycorrhizal traits of Monterey pine

The five studied populations of Monterey pine have diverged genetically in most of the traits we examined, including growth and biomass allocation traits (Fig. 2) and mycorrhizal traits, i.e., compatibility with particular taxa of ectomycorrhizal fungi (Fig. 1b). Moreover, the results for mycorrhizal traits demonstrate that patterns of variation in Monterey pine compatibility with its ectomycorrhizal symbionts differ among fungal taxa, i.e., mycorrhizal colonization of Monterey pine seedlings depends on a $G_P \times G_F$ interaction, where G_F represents different fungal taxa. Specifically, the Mexican pine populations have evolved higher compatibility with Wilcoxina1 and lower compatibility with *Rhizopogon roseolus*, compared to California populations, and the populations have not diverged with respect to compatibility with Pyronemataceae1 (Fig. 1b). In a prior study, Hoeksema and Thompson (2007) found yet a different pattern of divergence among the Año Nuevo, Guadalupe Island, and Cedros Island South populations for their compatibility with another RPC ectomycorrhizal fungus, *Rhizopogon occidentalis*. Specifically, the Cedros Island South population of pines (see Plate 1) seems to have evolved significantly lower compatibility with *R. occidentalis*, compared to the Año Nuevo and Guadalupe Island populations (as well as the Monterey, California population, which was not included in the current study).

The conclusion that Monterey pines can evolve unique patterns of compatibility with different ectomycorrhizal symbiont species has significant implications for how diverse mutualisms may coevolve, or for how diverse guilds of species may exert selection on a focal species (if the interaction is not coevolving). Different fungal taxa, rather than exerting conflicting selection on the same plant trait (e.g., overall plant compatibility with mycorrhizal fungi), could have selective effects on different plant traits, such as specific signaling and recognition loci (Hoeksema 2010). A high degree of specificity in such traits could allow coevolutionary cycling to drive fluctuating polymorphisms at corresponding recognition loci in plants and fungi, as has been found for the major histocompatibility complex (MHC) loci in vertebrates (Hughes and Nei 1988) and self-incompatibility loci in fungi (May and Matzke 1995). Selection should strongly favor recognition by plants of more beneficial vs. less beneficial taxa of

PLATE 1. Groves of *Pinus radiata* in the Cedros Island South population (Mexico). The Cedros Island *Pinus radiata* habitats are significantly drier than Guadalupe Island and sites in California, receiving just over 100 mm of precipitation each year on average. Photo credit: J. D. Hoeksema.

ectomycorrhizal fungi, and subsequent exclusion or rejection of the less beneficial taxa (Kiers and van der Heijden 2006, Bever et al. 2009). In turn, less beneficial fungal taxa would be under selection to avoid recognition, which would drive further reciprocal evolution in the recognition genes of the plant.

Divergence among Monterey pine populations in plant growth and allocation traits was also substantial, but differences among genotypes also interacted with soils for all traits but root : shoot (Fig. 2), and geographic patterns of seedling growth traits were more correlated than were the mycorrhizal traits. Overall, Cedros Island plants allocated relatively less biomass to roots and made finer roots (i.e., higher SRL) compared to the other three populations, although in some soils the Año Nuevo genotypes also exhibited relatively fine roots. Similarly, mass was typically lowest for the Cedros Island genotypes, but genetic variation in RGR during the final 16 weeks depended heavily on soils. These overall patterns are qualitatively similar to those found in a previous study of variation in seedling growth traits among native Monterey pine populations (Hoeksema and Thompson 2007). Although greater relative allocation to aboveground biomass is often interpreted as a response to light limitation in plants (Tilman 1988), in native Monterey pine populations fog moisture is also (in addition to light) an important "aboveground" resource (Rogers et al. 2005), and lower root : shoot ratios of Cedros Island seedlings may reflect adaptation for capturing fog moisture on needles in that extremely arid climate. The observations that RGR and SRL were both lower in sympatric combinations of pine genotypes and soils compared to allopatric combinations suggests the possibility of overall local adaptation or maladaptation of those traits to one or more soil characteristics, e.g., chemical properties or composition of pathogenic or ectomycorrhizal fungi. However, this idea depends on the assumption that plant fitness has the same relationship with those two plant traits in all five populations, which may not be the case. For example, higher seedling RGR may be less advantageous for survival and reproduction in a drier climate than in a wetter climate. It should be kept in mind that our estimates of plant traits and fungal abundances would likely vary across alternative light, moisture, and temperature regimes compared to the conditions used in our experiment. In addition, the presence of contaminant ectomycorrhizal fungi on the lower 1 cm of feeder roots of some plants in our experiment may have reduced the influence of different soils on plant trait estimates.

CONCLUSIONS

The emerging story on evolution of interactions between pines and their associated resistant-propagule community of ectomycorrhizal fungi is that they are highly variable geographically; community composition of the RPC fungi, soil and climatic factors, and compatibility of pine seedlings with particular RPC taxa have all diverged among communities. Moreover, pine compatibility with particular RPC fungi appears to evolve as a set of relatively independent traits. The challenge now is to develop clear tests of hypotheses

on the specific selective sources and other evolutionary forces that may be driving these divergences. In particular, if pine populations are diverging independently in their compatibility with different ectomycorrhizal symbiont taxa, are fungal traits acting as sources of selection driving this divergence? If so, what are the fungal traits in question and is their selection on plant traits modulated by environmental factors? Answering these questions will lend insight into how coevolution proceeds in widespread species-rich interactions.

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Literature Cited

Ashkannejhad, S., and T. R. Horton. 2006. Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving *Pinus contorta*, suilloid fungi and deer. New Phytologist 169:345–354.

Axelrod, D. I. 1980. History of the maritime closed-cone pines, Alta and Baja California. University of California Press, Berkeley, California, USA.

Baar, J., T. R. Horton, A. M. Kretzer, and T. D. Bruns. 1999. Mycorrhizal colonization of *Pinus muricata* from resistant propagules after a stand-replacing wildfire. New Phytologist 143:409–418.

Bever, J. D., S. C. Richardson, B. M. Lawrence, J. Holmes, and M. Watson. 2009. Preferential allocation to beneficial symbiont with spatial structure maintains mycorrhizal mutualism. Ecology Letters 12:13–21.

Bidartondo, M. I., J. Baar, and T. D. Bruns. 2001. Low ectomycorrhizal inoculum potential and diversity from soils in and near ancient forests of bristlecone pine (*Pinus longaeva*). Canadian Journal of Botany 79:293–299.

Colwell, R. K. 2009. EstimateS: statistical estimation of species richness and shared species from samples. Version 8.2. User's guide and application. http://purl.oclc.org/estimates

Endler, J. A. 1986. Natural selection in the wild. Princeton University Press, Princeton, New Jersey, USA.

Gardes, M., and T. D. Bruns. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Canadian Journal of Botany 74:1572–1583.

Gotelli, N. J., and R. K. Colwell. 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters 4:379–391.

Grogan, P., J. Baar, and T. D. Bruns. 2000. Below-ground ectomycorrhizal community structure in a recently burned bishop pine forest. Journal of Ecology 88:1051–1062.

Grubisha, L. C., J. M. Trappe, A. R. Beyerle, and D. Wheeler. 2005. NATS truffle and truffle-like fungi 12: *Rhizopogon ater* sp nov and *R. brunsii* sp nov (Rhizopogonaceae, Basidiomycota). Mycotaxon 93:345–353.

Grubisha, L. C., J. M. Trappe, R. Molina, and J. W. Spatafora. 2002. Biology of the ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of ITS sequences. Mycologia 94:607–619.

Guimaraes, P. R., P. Jordano, and J. N. Thompson. 2011. Evolution and coevolution within mutualistic networks. Ecology Letters 14:877–885.

Hereford, J. 2009. A quantitative survey of local adaptation and fitness trade-offs. American Naturalist 173:579–588.

Hijmans, R. J., S. E. Cameron, J. L. Parra, P. G. Jones, and A. Jarvis. 2005. Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology 25:1965–1978.

Hoeksema, J. D. 2010. Tansley review. Ongoing coevolution in mycorrhizal interactions. New Phytologist 187:286–300.

Hoeksema, J. D., et al. 2010. A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. Ecology Letters 13:394–407.

Hoeksema, J. D., B. Piculell, and J. N. Thompson. 2009. Within-population genetic variability in mycorrhizal interactions. Communicative and Integrative Biology 2:110–112.

Hoeksema, J. D., and J. N. Thompson. 2007. Geographic structure in a widespread plant–mycorrhizal interaction: pines and false truffles. Journal of Evolutionary Biology 20:1148–1163.

Horton, T. R., and T. D. Bruns. 1998. Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). New Phytologist 139:331–339.

Horton, T. R., and T. D. Bruns. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black box. Molecular Ecology 10:1855–1871.

Hughes, A. L., and M. Nei. 1988. Pattern of nucleotide substitution at major histocompatibility complex class-I loci reveals overdominant selection. Nature 335:167–170.

Izzo, A., D. T. Nguyen, and T. D. Bruns. 2006. Spatial structure and richness of ectomycorrhizal fungi colonizing bioassay seedlings from resistant propagules in a Sierra Nevada forest: comparisons using two hosts that exhibit different seedling establishment patterns. Mycologia 98:374–383.

Jones, M. D., and S. E. Smith. 2004. Exploring functional definitions of mycorrhizas: Are mycorrhizas always mutualisms? Canadian Journal of Botany 82:1089–1109.

Kiers, E. T., and M. G. A. van der Heijden. 2006. Mutualistic stability in the arbuscular mycorrhizal symbiosis: exploring hypotheses of evolutionary cooperation. Ecology 87:1627–1636.

Kjoller, R., and T. D. Bruns. 2003. *Rhizopogon* spore bank communities within and among California pine forests. Mycologia 95:603–613.

Lomolino, M. V., B. R. Riddle, R. J. Whittaker, and J. H. Brown. 2010. Biogeography. Fourth edition. Sinauer Associates, Sunderland, Massachusetts, USA.

May, G., and E. Matzke. 1995. Recombination and variation at the A mating-type of *Coprinus cinereus*. Molecular Biology and Evolution 12:794–802.

McCune, B., and J. B. Grace. 2002. Analysis of ecological communities. MjM Software Design, Gleneden Beach, Oregon, USA.

Moser, A. M., J. L. Frank, J. A. D'Allura, and D. Southworth. 2009. Ectomycorrhizal communities of *Quercus garryana* are

similar on serpentine and nonserpentine soils. Plant and Soil 315:185–194.

Newman, E. J. 1966. A method of estimating the total root length of a root in a sample. Journal of Applied Ecology 3:139–145.

Piculell, B. J., J. D. Hoeksema, and J. N. Thompson. 2008. Interactions of biotic and abiotic environmental factors in an ectomycorrhizal symbiosis, and the potential for selection mosaics. BMC Biology 6:23.

Rogers, D. L., J. J. V. Hernandez, A. C. Matheson, and J. J. G. Santos. 2005. Conserving the pines of Guadalupe and Cedros Islands, Mexico: an international collaboration. Pages 31–53 *in* A. Romero and S. West, editors. Environmental issues in Latin America and the Caribbean. Springer-Verlag, Berlin, Germany.

Rusca, T. A., P. G. Kennedy, and T. D. Bruns. 2006. The effect of different pine hosts on the sampling of *Rhizopogon* spore banks in five eastern Sierra Nevada forests. New Phytologist 170:551–560.

Schluter, D. 2009. Evidence for ecological speciation and its alternative. Science 323:737–741.

Siepielski, A. M., and C. W. Benkman. 2007. Convergent patterns in the selection mosaic for two North American bird-dispersed pines. Ecological Monographs 77:203–220.

Taylor, D. L., and T. D. Bruns. 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Molecular Ecology 8:1837–1850.

Thompson, J. N. 1994. The coevolutionary process. University of Chicago Press, Chicago, Illinois, USA.

Thompson, J. N. 2005. The geographic mosaic of coevolution. University of Chicago Press, Chicago, Illinois, USA.

Thompson, J. N., and B. M. Cunningham. 2002. Geographic structure and dynamics of coevolutionary selection. Nature 417:735–738.

Thompson, J. N., A. L. Laine, and J. F. Thompson. 2010. Retention of mutualism in a geographically diverging interaction. Ecology Letters 13:1368–1377.

Tilman, D. 1988. Plant strategies and the dynamics and structure of plant communities. Princeton University Press, Princeton, New Jersey, USA.

Walker, J. F., O. K. Miller, and J. L. Horton. 2005. Hyper-diversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. Molecular Ecology 14:829–838.

Williams, G. C. 1966. Adaptation and natural selection. Princeton University Press, Princeton, New Jersey, USA.

Supplemental Material

Appendix A

Details of methods used for molecular identification of ectomycorrhizal fungi (*Ecological Archives* E093-212-A1).

Appendix B

Photographs of native *Pinus radiata* populations on Guadalupe and Cedros Islands, which are located in the Pacific Ocean off the west coast of Baja California, Mexico (*Ecological Archives* E093-212-A2).

Appendix C

Figures showing a sample-based species accumulation curve for ectomycorrhizal fungal OTUs in each of the five soils and the frequency of ectomycorrhizal fungal OTUs on seedlings across the entire study, and a table showing the frequencies of each fungal OTU in each of the five soils along with Genbank best matches and accession numbers for each fungal OTU (*Ecological Archives* E093-212-A3).

Supplement

Soil and climate data for each of the five sampled native populations of *Pinus radiata* (*Ecological Archives* E093-212-S1).