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Host islands within the California Northern Channel Islands create fine-scale genetic structure in two sympatric species of the symbiotic ectomycorrhizal fungus *Rhizopogon*

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Abstract

We have examined fine-scale genetic structure of the symbiotic ectomycorrhizal fungi *Rhizopogon occidentalis* and *R. vulgaris* on two of the California Channel Islands using five and six microsatellite loci, respectively. Both *Rhizopogon* species are sympatric on Santa Cruz and Santa Rosa Islands and are ectomycorrhizal with bishop pine (*Pinus muricata*) on both islands or Santa Rosa Island Torrey pine (*P. torreyana* ssp. *insularis*) on Santa Rosa. The combination of disjunct pine host distributions and geographic barriers within and among the islands have created highly structured *Rhizopogon* populations over very short distances (8.5 km on Santa Cruz Island; F_{ST} = 0.258, F_{ST} = 0.056, *R. occidentalis* and *R. vulgaris*, respectively). Both species show similar patterns of genetic differentiation as a result of limited dispersal between host populations as revealed by a significant isolation by distance relationship (r = 0.69, P < 0.04; r = 0.93, P < 0.001, *R. occidentalis* and *R. vulgaris*, respectively) and Bayesian clustering analyses, and is most likely a function of the small foraging range of the few mammals that disperse *Rhizopogon* on these islands and the enormous spore bank characteristic of *Rhizopogon* species.

Keywords: animal dispersal, genetic distance, hypogeous fungi, microsatellite loci, phylogeography, spore banks

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Introduction

Islands are considered to be natural laboratories for evolution (Darwin 1859; Mayr 1963). Founding events are typically believed to involve few individuals or propagules (Mayr 1942; Whittaker 1998) and thus random genetic drift and selection may result in rapid evolution (Barton 1998). Insular populations are likely to have less genetic diversity within populations and greater genetic divergence between populations (Nei *et al*. 1975; Chakraborty & Nei 1977; reviewed by Frankham 1997).

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Ectomycorrhizal fungi form obligate, symbiotic interactions with roots of many forest trees. This mutualism involves the exchange of mineral nutrients that the fungi obtain from the soil for photosynthetically fixed carbon, produced by the plants (Smith & Read 1997). The obligate nature of the interaction means that ectomycorrhizal fungi are restricted to habitats that contain appropriate host plants. When host populations are discrete and isolated, such 'host islands' have the potential to affect the structure of fungal populations, particularly in cases where dispersal of the fungus is relatively limited and thus functions as a mosaic of isolated subpopulations. Furthermore, isolated host islands within real islands provide an opportunity to examine the factors causing geographic variation compared to strictly inter-island or island–mainland studies (Thorpe & Malhotra 1998).

Rhizopogon species (Basidiomycota, Boletales) might be expected to be particularly prone to the host island effect

because they tend to have narrow host ranges and their spores are animal dispersed. *Rhizopogon* is symbiotic almost exclusively with members of the Pinaceae and species are often restricted to *Pinus* or *Pseudotsuga* species (Molina *et al*. 1999). In many ecosystems this means that individual *Rhizopogon* species are effectively restricted to a single host species. *Rhizopogon* produces meiotic spores in below-ground, 'hypogeous' sporocarps, called false-truffles. These sporocarps are dispersed primarily by small rodents, and less frequently by larger mammals (Maser *et al*. 1978; Currah *et al*. 2000; Ashkannejhad & Horton 2006). The dispersal of *Rhizopogon* is limited by the foraging range of mammal vectors compared to above-ground 'epigeous' mushroom-forming fungi that have the potential to have windborne spore dispersal.

Local populations of *Rhizopogon* are likely to be large and persistent through time because they form extensive soil spore banks, analogous to seed banks in plants. Spores are deposited in soil when *Rhizopogon* false-truffles that are not consumed by mammals deliquesce in place or when dispersed spores are deposited by mammal fecal pellets (Miller *et al*. 1994). Within pine forests these spore banks are so dense that even when soils are diluted 50 to 100-fold into sterile soil more that half of the test seedlings planted will still be colonized by *Rhizopogon* species (Taylor & Bruns 1999; Kjøller & Bruns 2003; Rusca *et al*. 2006). We now estimate that this translates to at least 3000 *Rhizopogon* spore/mL of soil for an average pine forest in California (TD Bruns, unpublished). The longevity of these spore banks is not known, but some observations suggest that they may last at least for decades (Izzo *et al*. 2005). Thus, we would anticipate that *Rhizopogon* spore banks have increased over time, and that rare migration events would likely be undetected without the selective sweep of advantageous alleles from migrant individuals and hitchhiking by neutral microsatellite loci.

In the present study our primary goal was to examine the fine-scale population structure, based on microsatellite loci, of *Rhizopogon occidentalis* and *R. vulgaris*, two pine-associated *Rhizopogon* species, in a setting where populations appeared to be separated into localized host islands on the scale of a few kilometers. We hypothesized that we would find significant genetic structure in such a setting, due to the dispersal, host and population biology of *Rhizopogon* discussed above. We were able to use two *Rhizopogon* species as independent replicates because both occur together in our study area and seem to have similar dispersal and host-dependency.

Santa Cruz and Santa Rosa Islands are the only two California Northern Channel Islands where pine occurs today. *R. occidentalis* and *R. vulgaris* occur on these islands and are restricted to a single host, *Pinus muricata* (bishop pine), on Santa Cruz Island (SCI) and two hosts, *P. muricata* and *P. torreyana* ssp. *insularis* (Santa Rosa Island Torrey pine), on Santa Rosa Island (SRI). Pine populations are few, small and disjunct on the islands, and are separated by 5 km or 8.5–18.5 km of nonpine habitat on SRI and SCI, respectively. On SCI, a valley that separates two mountain ranges with elevation up to 753 m provides geographic barriers between pine populations.

Our secondary goal was to see if the observed population structure of these *Rhizopogon* species correlated with the geographic partitioning among the two islands. Fluctuating sea levels during the last glaciation (> 13 000 years ago) led to periods in which the four Northern Channel Islands were a contiguous land mass forming one large island called Santarosae (Vedder & Howell 1980). There is no evidence that a land bridge connected any of these islands to the mainland, although during the Pleistocene the oceanic levels were much lower and at various times only a 7 km distance separated the eastern end of Santarosae (closer to SCI) to the mainland at Ventura, California, compared to the current distance of 20 km (Junger & Johnson 1980). Thus, long-distance dispersal would be necessary for the initial establishment of *Rhizopogon* on the islands.

Colonization of the islands may have been difficult, as evidenced by the relatively small number of mammals that inhabit them (Schoenherr *et al*. 1999). Only three or four native, endemic mammals occur on both islands that could disperse *Rhizopogon* spores. On Santa Rosa, the native mammals are the Santa Rosa island deer mouse (*Peromyscus maniculatus santarosae*), the Santa Rosa island fox (*Urocyon littoralis santarosae*) and the island spotted skunk (*Spilogale gracilis amphialus*). In addition to the island spotted skunk, the Santa Cruz endemic version of the deer mouse and island fox also occur. A fourth endemic mammal is the Santa Cruz island harvest mouse (*Reithrondtomys megalotis santacruzae*). *Rhizopogon* colonization of the islands is similarly sparse. We found only these two *Rhizopogon* species on the islands, while most pine forests in California have five to eight species of *Rhizopogon* (Kjøller & Bruns 2003; Rusca *et al*. 2006). In addition, members of *Rhizopogon* subgenus *Amylopogon* are usually dominant species in mature California pine forests (Kjøller & Bruns 2003; Rusca *et al*. 2006; Grubisha unpublished) but are entirely absent from both islands. *R. occidentalis* and *R. vulgaris* are common throughout the state, but tend to be found in early pine forest succession or disturbed settings.

Materials and methods

Study area

SCI and SRI differ by size and distance from the mainland (Fig. 1). SCI is larger than SRI (249 km^2 and 217 km^2, respectively), and it lies 30 km southeast of the coastal city of Ventura, while SRI is 9 km west of SCI and 44 km from the mainland (Schoenherr *et al*. 1999). The highest points

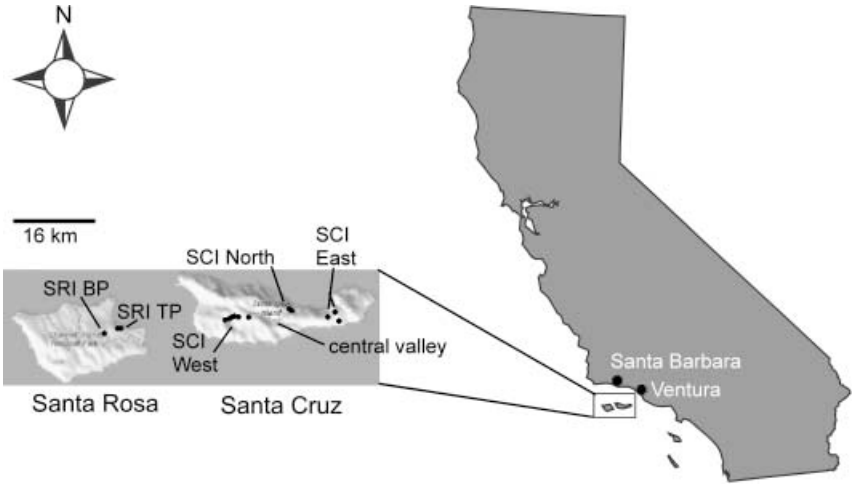

Fig. 1 Location of Santa Cruz Island and Santa Rosa Island of the California Northern Channel Islands. Population location labels are the same as in Table 1. Black circles represent approximate sampling locations for sporocarps and inoculum soil for spore bank bioassays.

are 753 m and 484 m on SCI and SRI, respectively. Both islands have a Mediterranean climate characterized by cool, wet winters and warm, dry summers.

The small number of pine populations on SCI and SRI made it possible to sample all of them, so that we could obtain a relatively complete picture of population structure of *Rhizopogon* on the islands (Fig. 1). On SCI, bishop pine was located in three well-separated regions in the east, north and west of the island (Fig. 1). The eastern and northern pine were separated from each other by non-pine rolling hills, and the western pine was on ridge tops and was separated from these by the dry central valley. The GPS location for *Rhizopogon* populations was calculated by averaging the coordinates from the two most distant *Rhizopogon* sample points in each pine island. This GPS point was then used to calculate the distance between any two populations (Table 3). The range of sample points for these populations are as follows: SCI East (34 01.00′N, 119 36.27′W–34 00.11′N, 119 37.39′W–33 59.76′N, 119 36.33′W); SCI North (34 01.70′N, 119 41.56′W–34 01.82′N, 119 42.16′W); SCI West (34 00.77′N, 119 47.65′W –34 00.48′N, 119 48.70′W–33 59.96′N, 119 49.29′W–34 01.04′N, 119 49.88′W). On SRI, the two pine stands are very small. The bishop pine stand (SRI BP 33 58.92′N, 120 04.39′W) is located along a small portion of Black Mountain, about 5 km inland from the coastal Santa Rosa Island Torrey pine stand (SRI TP 33 58.94′N, 120 01.39′W). Bishop pine trees are few and scattered, while the Torrey pines form a small, dense stand of trees.

Sample collection

We sampled *Rhizopogon* species as sporocarps and as spores from the soil spore bank. Sporocarps are produced below-ground, primarily during the rainy season, and we found them by raking the soil under pines. *Rhizopogon occidentalis* sporocarps were collected from all pine populations on both islands while *R. vulgaris* sporocarps were only found on SCI. We sampled sporocarps on SCI in March and April 2001 and January 2002. On SRI, sporocarps were collected in March 2001. On SRI, sporocarps were collected from both the bishop pine and Torrey pine stands. Sporocarps were collected, processed and dried as previously described (Grubisha *et al*. 2005b).

Sporocarps were collected from under individual trees that were each separated by a minimum of 10 m at all sites and up to 1000 m in SCI West. This was done to prevent re-sampling the same vegetative mycelium. This distance is greater than the size of most individual genets for the *Rhizopogon* species previously sampled by Kretzer *et al*. (2005) and most other ectomycorrhizal fungi sampled to date. Large genets (> 10 m), as measured by the maximum distance between two sporocarps (Dmax), have been found in a few ectomycorrhizal species, e.g. *Suillus* spp. (Dahlberg & Stenlid 1994; Bonello *et al*. 1998; Hirose *et al*. 2004) and *Xerocomus* spp. (Fiore-Donno & Martin 2001). However, more studies have found Dmax < 10 m for most genets in species such as *Amanita fracheti*, *Lactarius xanthogalactus* and *Russula cremoricolor* (Redecker *et al*. 2001), *Hebeloma cylindrosporum* (Gryta *et al*. 1997), *Laccaria amythestina* (Gherbi *et al*. 1999; Fiore-Donno & Martin 2001), *Russula brevipes* (Bergemann & Miller 2002) and *Tricholoma* spp. (Gryta *et al*. 2006; Lian *et al*. 2006). In addition, a high proportion of the sampled area was in discontinuous pine stands where vegetative growth of *Rhizopogon* was highly unlikely.

Rhizopogon spores are abundant in soil (Kjøller & Bruns 2003) and can be sampled at any time of year, by simply planting *Pinus muricata* seeds in soil collected from island sites under greenhouse conditions, and then collecting the distinctive colonized roots (i.e. mycorrhizae) of *Rhizopogon*. Soil was collected from SCI in July 2000 and in March 2001 from SRI. Twenty soil samples were collected with a hand trowel at 1-m intervals along a transect and stored in plastic zip-lock freezer bags. All sampling points were kept as

unique points for the entire process; soil samples were not pooled at any time. The number of transects per pine population varied depending on the size of the population. On SCI, soil was collected from two transects in the east region, two from the north region and five from the west region. On SRI, soil was collected from two transects from the bishop pine population and two from the Torrey pine population. Processing of island soil, set-up of bioassays and conditions for the growth of seedlings followed the procedure described by Kjøller & Bruns (2003) with few exceptions. Inoculum soil was sieved in the laboratory through a 1.0-mm sieve and dried at room temperature in paper bags for two weeks. In this study, growth medium consisted of a 50:50 mixture of autoclaved sand:soil. Approximately 2 mL of inoculum soil was added to 80 mL of growth media and this was applied to each pot (RLC-4 Super 'Stubby' Cell Cone-tainer™, Stuewe & Sons Inc). Three replicate assays from each meter point were set up for a total of 60 bioassays/transect. Twenty negative controls/transect were set up by the same procedure minus the inoculum soil. This gave a total of 780 bioasssays with inoculum soil and 260 negative controls for both islands combined. Seeds were sterilized, thoroughly rinsed and sown in the pots immediately.

Bioassay harvest and processing

Rhizopogon ectomycorrhizal roots are easy to distinguish by the white colouration often with pink or orange stains, conspicuous rhizomorphs of similar colour, and a densely coralloid branching pattern. *Rhizopogon* mycorrhizas from SCI bioassays were freeze-dried and stored at room temperature until DNA extraction. The ectomycorrhizal fungi were isolated into pure cultures from mycorrhizae following Kjøller & Bruns (2003).

DNA isolation and species confirmation

Fungal tissue was homogenized in a bead beater. DNA was extracted from tissue by incubation in 2X CTAB buffer (100 mM Tris pH 8-9, 1.4 M NaCl, 20 mM EDTA, 2% cetyltrimethylammonium bromide) at 65 C for 15–60 min followed by chloroform extraction. Genomic DNA was further purified using a GENECLEAN II Kit (Q-BIOgene). Alternatively, genomic DNA was extracted by using a Qiagen DNeasy Tissue Kit (Qiagen), following manufacturer's instructions. To confirm species identifications, we polymerase chain reaction (PCR)-amplified the nuclear ribosomal internal transcribed spacer region (ITS) with the fungal specific primer ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990). The PCR-amplicons were digested with the restriction enzyme *Hha*I (New England Biolabs, Inc), an enzyme that produces distinctive band patterns from ITS sequences that separated all the known *Rhizopogon* species on the islands. Restriction fragment length polymorphism (RFLP) patterns were compared and representative samples were sequenced with the primers ITS1F and ITS4. Sequencing and editing of sequences was previously described (Grubisha *et al*. 2005a). Sequences were compared to each other and against a database of *Rhizopogon* ITS sequences previously compiled (Grubisha *et al*. 2002).

Sporocarp and spore bank samples

To determine if spore bank and sporocarp samples are drawn from the same underlying population, we tested the null hypothesis of no difference between allele frequencies between spore bank and sporocarp samples within the same sampling region using exact tests of nondifferentiation between population pairs using ARLEQUIN (Schneider *et al*. 2000). We also measured the strength of the correlation of allele frequencies between sporocarp and spore bank samples by computing the Spearman rank correlation coefficient using SPSS 10.0. For *R. occidentalis*, samples from SCI East and SCI North and for *R. vulgaris*, SCI North and SCI West were used. These locations were selected because population sizes derived from sporocarp and spore bank at these sites were relatively large for both species.

Microsatellite isolation and genotyping

Isolation of microsatellite loci was achieved using an enrichment protocol for $(CAC)_n$ repeats that was previously described (Grubisha *et al*. 2005a). Microsatellite enriched libraries for both species were constructed partially or entirely from isolates collected on SCI. In fact, one library for each species was constructed from mycelium grown from sporocarps collected from SCI West, LCG331 and LCG343, while a third library was also constructed from a *R. vulgaris* collection from northern California (Grubisha *et al*. 2005a). Primer development, screening and genotyping techniques were previously described (Grubisha *et al*. 2005a). For this study, we used five and six microsatellite loci for *R. occidentalis* and *R. vulgaris*, respectively (Grubisha *et al*. 2005a; Table S1, Supplementary material).

Genetic data analysis

Allele frequencies and observed (H_O) and expected (H_E) heterozygosities were calculated per locus and population using MICROSATELLITE-ANALYSER (MSA) 3.12 (Dieringer & Schlötterer 2002). Linkage disequilibrium (LD) across all pairs of loci and departures from Hardy–Weinberg equilibrium (HWE) for each locus within each population were tested by estimating exact *P*-values using Markov chain Monte Carlo parameters as implemented in the web-based version of GENEPOP version 3.4 (Guo & Thompson 1992; Raymond & Rousset 1995). Sequential Bonferroni

correction for multiple significance tests was used to calculate critical significance values (initial $\alpha = 0.05$; Rice 1989). Allelic richness was computed using FSAT (Goudet 2001) which corrects for differences in sample sizes. To determine the probability that two individuals taken at random within a population have different genotypes, gene diversity was estimated by:

$$\frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right) \quad \text{(eqn 1)}$$

where n = the number of gene copies in the sample, k is the number of haplotypes and p_i is the sample frequency of the i-th haplotype in ARLEQUIN (Schneider *et al*. 2000).

In Ascomycetes that have dispersed mitotic spores, fungal genotypic data sets are corrected for multiple sampling of the same genetic individual (Chen & McDonald 1996; Milgroom 1996). However, *Rhizopogon* has no mitotic spore states and our sampling scheme for sporocarps was designed to minimize the chances of re-sampling the same vegetative mycelium. The only populations that recovered a high frequency of genetically identical isolates were those in which allelic diversity was low, and the probability of recovering identical genotypes due to chance was high (e.g. *R. occidentalis*, SCI West and SRI). The fact that identical genotypes were recovered in both spore bank and sporocarps and between populations on both islands confirms that these were not re-sampled vegetative clones, but rather the most probable genotypes within genetically depauperate populations.

To assess whether gene flow among populations on and between islands was an important evolutionary force, isolation-by-distance analysis (Slatkin 1993) was performed using Isolation By Distance Web Service (IBDWS; Bohonak 2002; Jensen *et al*. 2005). This program (IBD; Bohonak 2002) uses reduced major-axis regression to calculate the slope and intercept of the isolation-by-distance relationship and assesses significance using a Mantel test (Mantel 1967); and significance was tested by 30 000 permutations. Genetic distance was estimated as pairwise linearized F_{ST} (Rousset 1997) for all population pairs and plotted against the log-transformed geographic distance, that was estimated as the linear distance between population pairs (Table 3). Average measures of latitude and longitude were calculated from the endpoints of each sampled population.

Pairwise population genetic distance was compared to examine genetic differentiation among populations. F_{ST} estimates, following Weir & Cockerham (1984), significance levels and strict Bonferroni correction for significance levels for multiple tests were calculated in MSA 3.12 (Dieringer & Schlötterer 2002). Significance levels for F_{ST} estimates were tested by 10 000 permutations of genotypes among samples.

The distribution of genetic variation was assessed in a hierarchical framework using analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) as implemented in ARLEQUIN (Schneider *et al*. 2000). To further examine the effect of geographic barriers on the level of genetic differentiation, total variance of allele frequency data (F_{ST}) was partitioned into covariance components due to differences among islands, among populations within islands and within populations for *R. occidentalis*, and among populations within SCI and within populations for *R. vulgaris*.

Population structure was further assessed by assigning individuals to K populations that are characterized by a set of allele frequencies at each locus that are not constrained by sampling information using the program STRUCTURE version 2 (Pritchard *et al*. 2000). This program uses a model-based Bayesian clustering approach to assign multilocus genotypes to populations while minimizing linkage disequilibrium and deviations from HWE. Markov Chain Monte Carlo simulations were run for up to $K = 10$ clusters for both species. For each simulation, an initial burnin length of 5×10^5 was followed by 10^6 iterations. The Markov Chain was replicated independently three times for each K to ensure consistency of the estimate of ln $\Pr(X/K)$ between runs. Individuals would be assigned to more than one cluster if admixture was inferred from the multilocus genotype profile. The most likely estimated number of K clusters is based on the highest log-likelihood of the data. Analysis parameters were set so that sampling location data was not included (USEPOPINFO = 0), using the admixture model (NOADMIX = 0). Results of individuals' membership proportions for each cluster were graphically displayed using the program DISTRUCT (Rosenberg 2002).

Results

Samples

Reasonable size populations ($n > 30$) were retrieved from all sites for *Rhizopogon occidentalis*, and all but one site for *R. vulgaris* (SRI, $n = 6$). Table 1 summarizes the number of samples by species, sample type and population. Few sporocarp collections were made on SRI, probably due to the time the sampling was conducted (Grubisha *et al*. 2005b). Only seven *R. occidentalis* sporocarps were collected on SRI whereas 109 were sampled on SCI. We collected 88 *R. vulgaris* sporocarps from SCI. *R. occidentalis* was abundant in the bioassays from both islands, although the number per site varied (Table 1). *R. vulgaris* was found in the bioassays on SCI at all sites, but at a lower frequency than *R. occidentalis* especially in the SCI East population (Table 1). On SRI, *R. vulgaris* was recovered from only six bioassay seedlings. Due to an insufficient sample size, *R. vulgaris* samples from SRI were not included in the analyses.

Table 1 Population location by island and site, and descriptive statistics for *Rhizopogon occidentalis* and *R. vulgaris* populations

Island/population	*n*	Richness	Loci	H_O	H_E	Genotype diversity
R. occidentalis						
Santa Cruz Island (SCI)						
SCI East	56 (26, 30)	2.4	0.6	0.24	0.26	0.83 ± 0.02
SCI North	37 (11, 26)	2.8	0.6	0.18	0.25	0.87 ± 0.03
SCI West	52 (52, 0)	1.5	0.4	0.06	0.07	0.31 ± 0.05
Santa Rosa Island (SRI)						
SRI (TP & BP)	64 (5, 59)	1.0	0.0	NA	NA	0.00 ± 0.00
R. vulgaris						
Santa Cruz Island						
SCI East	30 (25, 5)	3.8	1.0	0.41	0.40	0.97 ± 0.01
SCI North	48 (30, 18)	3.9	1.0	0.47	0.46	0.98 ± 0.00
SCI West	46 (26, 20)	3.1	1.0	0.31	0.42	0.96 ± 0.01
Santa Rosa Island						
SRI	6 (0, 6)*					

n = sample size (sporocarps, spore bank); Richness, mean number of alleles per population corrected for differences in sample size; Loci = proportion of polymorphic loci; H_O, observed heterozygosity; H_E, expected heterozygosity and both values are averaged over all loci, including homozygous loci; Genotype diversity is the probability that two individuals taken at random in the same population have different genotypes. **R. vulgaris* spore bank samples from SRI were not included in analyses due to such a small sample size.

Microsatellite variation

Most populations were polymorphic across microsatellite loci (Table S1, Supplementary material). The primary exceptions include the *R. occidentalis* from SCI West and SRI populations. *R. occidentalis* also exhibited lower genetic diversity than *R. vulgaris* in terms of number of variable loci. Two loci of *R. occidentalis* were monomorphic across all six populations. The number of alleles for the remaining three loci ranged from four to eight. After a sequential Bonferroni correction for multiple comparisons, two loci showed significant levels of heterozygote deficiency in *R. occidentalis*: Roc27.11 and Roc27.56 in the SCI East population, and locus Roc27.56 in the SCI North population. No significant linkage disequilibrium was detected in *R. occidentalis* ($P > 0.05$). The *R. occidentalis* population on SRI had no polymorphic loci and estimates of LD should be interpreted with caution.

All loci were polymorphic in *R. vulgaris* populations (Table S1, Supplementary material). The number of alleles ranged from two to seven. After employing Bonferroni correction, two loci showed significant heterozygote deficiency (Rvu20.46 and Rvu21.83, both in SCI West). No significant linkage disequilibrium was detected in *R. vulgaris* ($P > 0.05$).

Private alleles were found in both species, but none were found in the SCI West population for either species (Table S1, Supplementary material). For *R. occidentalis*, SCI East and SCI North had four unique alleles each. In *R. vulgaris*, SCI East had one and SCI North had three unique alleles, and all occurred at a low frequency.

Table 2 Location and sample size (*n*) of sporocarps and spore bank samples and comparison of significance of difference in allele frequencies using the Spearman rank correlation coefficient test and the exact test of differentiation; SCI, Santa Cruz Island; SRI, Santa Rosa Island

Location	*n** sporocarps	*n** spore bank	Spearman rank correlation coefficient†	Exact *P*-value for non differentiation‡
Rhizopogon occidentalis				
SCI North	11	26	0.89	0.48 ± 0.03
SCI East	26	30	0.89	0.34 ± 0.02
R. vulgaris				
SCI North	30	18	0.96	1.00 ± 0.00
SCI West	26	20	0.93	0.49 ± 0.05

*Sample size of sporocarps or spore bank; †$P < 0.01$; ‡Exact *P*-value for nondifferentiation between population pairs computed from Markov chain of 10 000 steps.

Sporocarp and spore bank comparisons

Spore bank and sporocarps for both species were drawn from the same underlying population. No significant differentiation between spore bank and sporocarps samples was detected using the exact test for both species (Table 2). There was a high correlation between sporocarp and spore bank allele frequencies based on Spearman rank correlation coefficient in all four populations tested ($P < 0.01$).

Table 3 Geographic distances (above diagonal) in km and pairwise estimates of genetic differentiation (F_{ST}) for all population pairs (below diagonal). All pairwise population comparisons were significant. A, *Rhizopogon occidentalis;* B, *R. vulgaris*; SCI, Santa Cruz Island; SRI, Santa Rosa Island

A

	SCI East	SCI North	SCI West	SRI
SCI East	0	8.5	18.5	40.5
SCI North	0.258	0	11	33.0
SCI West	0.316	0.241	0	22.0
SRI	0.482	0.359	0.165	0

B

	SCI East	SCI North	SCI West
SCI East	0	8.5	18.5
SCI North	0.055	0	11.0
SCI West	0.249	0.195	0

Genetic divergence among populations

Genetic differentiation among populations was assessed by pairwise population F_{ST} estimates (Table 3). Both species showed significant among-population differentiation between the SCI West population from the SCI East and SCI North populations (Table 3). Strong population differentiation was found between both SCI East and SCI North populations for *R. occidentalis* ($F_{ST} = 0.258$, $P < 0.001$); whereas moderate but significant differentiation was found among populations for *R. vulgaris* (SCI East and SCI North $F_{ST} = 0.054$, $P < 0.001$). All pairwise comparisons were significant before and after Bonferroni correction (initial $\alpha = 0.05$).

Genetic differentiation between all population pairs increased significantly with increasing geographic distance for *R. occidentalis* and *R. vulgaris* ($r = 0.69$, $P < 0.04$; $r = 0.93$, $P < 0.001$, respectively; Fig. 2), indicating that there is not an equilibrium between gene flow and an accumulation of private alleles through processes of stochastic drift. This pattern is expected for organisms that have low levels of dispersal.

Most of the genetic variation was found within populations (Table 4). The amount of genetic variation partitioned between islands for *R. occidentalis* was not significant (–7.40%, $P = 0.5029$; Table 4). However, a significant amount of genetic variation was detected among populations within SCI for both species (38.48%, $P \geq 0.0001$; 17.60%, $P \geq 0.0001$, *R. occidentalis* and *R. vulgaris*, respectively).

We used the program STRUCTURE to determine the number of populations that best described the distribution of the data into *K* clusters and examine how individual multilocus genotype profiles were assigned to predefined populations (Fig. 3). For *R. occidentalis*, $K = 4$ clusters had the highest estimated probability of the data (ln = –602) when posterior probabilities for the three runs for each value of *K* were averaged. The SRI and majority of SCI West were equally split across the same two clusters. The lack of genetic diversity in SCI West and SRI populations may have made it difficult for an accurate estimate of clusters to be determined. For *R. vulgaris*, $K = 3$ had the highest probability (ln = –1239). When $K = 2$, the average log likelihood of Pr(X/*K*) was –1243, suggesting that with this number of loci it may be difficult to determine whether two or three clusters is most appropriate for explaining the data (Pritchard *et al*. 2000). Individuals' membership proportion into each cluster is presented in Fig. 3 based on *K* inferred clusters.

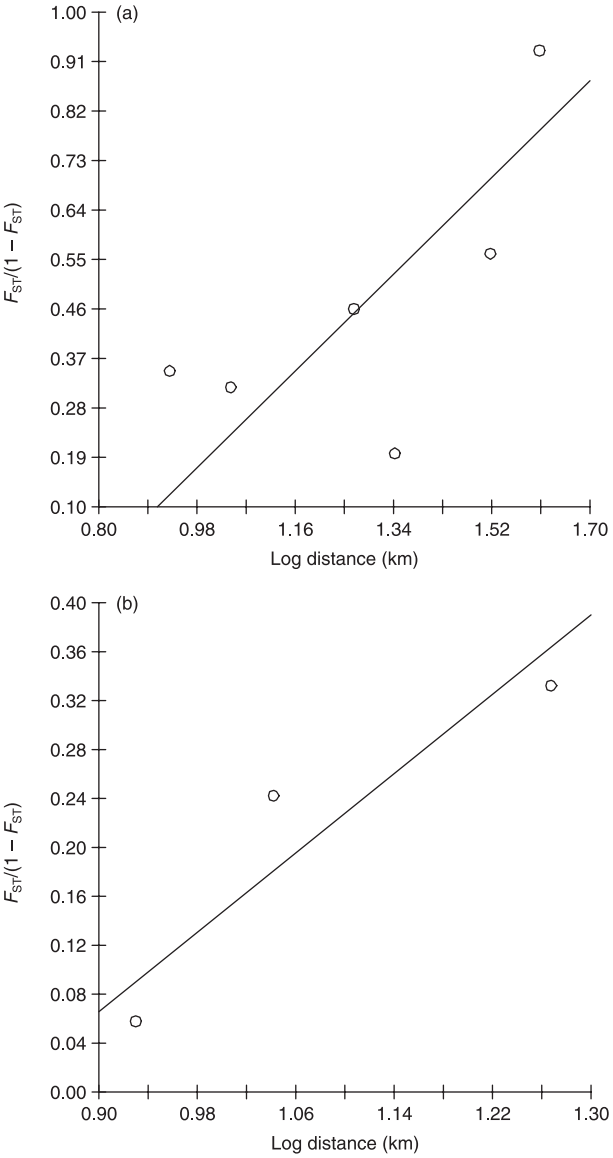

Fig. 2 Genetic distance ($F_{ST}/1 - F_{ST}$) plotted against log-transformed geographic distance (km) for all pairwise population comparisons. a, *Rhizopogon occidentalis*; b, *R. vulgaris*.

Table 4 Hierarchical analysis of molecular variance (AMOVA) results for *Rhizopogon occidentalis* and *R. vulgaris*

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
R. occidentalis					
Among islands	1	15.89	−0.04	−7.40	0.5029
Among populations within islands	2	35.50	0.18	38.48	0.0001
Within populations	414	135.61	0.32	68.92	0.0001
R. vulgaris					
Among populations within SCI	2	47.43	0.28	17.60	0.0001
Within populations	245	317.05	1.29	82.40	

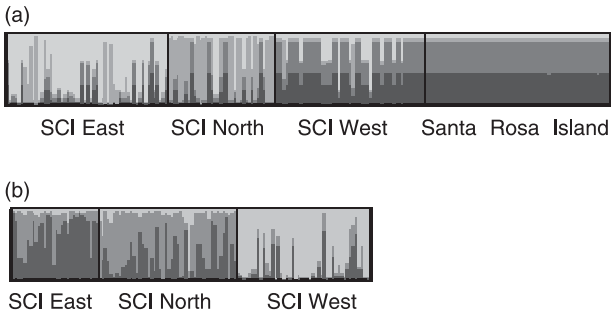

Fig. 3 Histograms of STRUCTURE assignment tests. Each vertical bar represents an individual and its assignment proportion into one of *K* clusters. More than one colour per individual indicates admixture. Population labels are those indicated in Table 1. a, *Rhizopogon occidentalis*; *K* = 4; b, *R. vulgaris*, *K* = 3.

Discussion

The high levels of genetic differentiation seen among populations that are separated by only a few kilometers demonstrates that both *Rhizopogon occidentalis* and *R. vulgaris* can be limited by dispersal on a relatively fine spatial scale (F_{ST} = 0.054, $P < 0.001$, F_{ST} = 0.258, $P < 0.001$, *R. vulgaris* and *R. occidentalis*, respectively). In addition, there was a linear relationship between genetic distance and the log of geographic distance suggesting that dispersal between host populations is affected by increasing distance between populations. Given the disjunct distribution of pine host islands, the paucity of mammal vectors that disperse *Rhizopogon* sporocarps on these islands, and the spore bank characteristic of *Rhizopogon*, this pattern was expected. Nevertheless, this is the highest level of among-population structure reported in any fungus at this relatively fine spatial scale, and it adds strong support to the growing body of evidence that at least some microbe populations form a genetic mosaic of isolated subpopulations across fine geographic scales (Martiny *et al*. 2006).

Comparisons with other studies

There have been few other studies of population structure in ectomycorrhizal fungi, but these have either been focused on broader geographic scales, or if they sampled finer scales, little genetic differentiation was found. The European black truffle (*Tuber melanosporum*), a hypogeous, animal-dispersed fungus, was found to have a high level of differentiation (F_{ST} = 0.20) among populations in France, Italy and Spain where postglacial range expansion of the host trees from refugia in Italy was hypothesized to be a major influence on the present truffle population structure (Murat *et al*. 2004). Strong patterns of genetic differentiation were found among populations of *Russula brevipes*, an epigeous, wind-dispersed fungus, between Colorado and California in the western United States, but no genetic structure was found at smaller scales (0.2–1.0 km) when discontinuous host populations were sampled within the California Sierra Nevada region (Bergemann & Miller 2002; Bergemann *et al*. 2006). Similarly, the epigeous fungus, *Suillus grevillei* did not show among-population differentiation (F_{ST} = 0.02) between populations separated by 0.7 km in Japan (Zhou *et al*. 2001).

Kretzer *et al*. (2005) studied populations of two Douglas-fir (*Pseudotsuga menziesii*) associated *Rhizopogon* species separated by 1–5.5 km in continuous Douglas-fir forest, and found small but significant amounts of genetic structure in *R. vesiculosus* at distances < 6 km between populations (Φ = 0.078, 5 km; Φ = 0.066, 5.5 km) that they attributed to local adaptation or small sample size. This study, which is the most readily comparable to ours, differs in two important ways. First, host populations were part of larger contiguous tracts of Douglas-fir forests with no obvious barriers to gene flow; and second, there were several other mammals with greater foraging ranges coexisting in this continental setting, such as the Northern flying squirrel and deer (Maser *et al*. 1978; Currah *et al*. 2000; Ashkannejhad & Horton 2006), compared to the mammal vectors on the two Channel Islands investigated here.

The islands have two non-native dispersal agents that probably disperse *Rhizopogon* spores. The exotic mule deer (*Odocileus hemionus*) was introduced to SRI within the past 100 years (Schoenherr *et al*. 1999) and could easily vector *Rhizopogon* between the island's two pine populations (Ashkannejhad & Horton 2006). Feral pigs (*Sus scrofa*) have existed on Santa Cruz for ~150 years (Junak *et al*. 1995). These mammals are potential vectors for dispersing *Rhizopogon* spp.; however, the home ranges for these animals is quite small and was estimated at 2.0–4.0 km^2 for boars and 0.8–1.5 km^2 for sows (Sterner 1990), thus frequent cross-island dispersal by pigs is unlikely. Sterner (1990) reported that boars occasionally were found outside of their range with the extreme being 9 km out, and that pigs were found to prefer canyon bottoms to ridge tops (where the pine are found). It is possible that low levels of gene flow may occur among populations due to rare dispersal events by feral pigs, but it is insufficient to counter the effects of genetic drift that is most likely affecting *Rhizopogon* in the disjunct pine populations on the Channel Islands.

Geographic barriers

The phylogeographic patterning of the *R. occidentalis* and *R. vulgaris* on the islands is similar and seems to correlate with the geography and geological history of the islands. The strongest pattern in both species is the genetic differentiation between eastern/northern and western populations on SCI (Fig. 3, Table 3). These results demonstrate that the hot, dry central valley is a potent barrier to mammal dispersal, and suggests pines have not been continuously distributed across this divide for a long time, if ever. Interestingly, the central valley on SCI is also a barrier to gene flow of native fox populations, which have separate monophyletic lineages for the western and northern/eastern regions based on microsatellite genetic distances (see Fig. 1 in Goldstein *et al*. 1999). Studies of the other island mammals have focused on colonization events and distribution of genetic variation among the Channel Islands but not within islands (Gill 1980; Ashley & Wills 1987; Wayne *et al*. 1991). To our knowledge, there has not been examination of among- or within-island population structure in the Northern Channel Islands spotted skunk, the Santa Cruz island harvest mouse, or the host bishop pine trees.

The ocean was also a clear barrier to gene flow (Table 3, Fig. 3a). However, the pattern of genetic similarity observed in the SCI West and SRI *R. occidentalis* populations may reflect the fact that the islands were joined near these populations approximately 13 000 years ago (Vedder & Howell 1980) and that they may have a shared ancestry. The weaker genetic differentiation between SRI and SCI West populations of *R. occidentalis* may also suggest that the 9 km span of ocean that separates the islands and the 20 km that separate the pine stands has not been as strong of a barrier to dispersal as the central valley on SCI, but current gene flow among islands seems unlikely given the dispersal by small mammals on these islands.

Differences between the two species

Although both *Rhizopogon* species show similar geographic patterns, they differ in the amount of genetic variation observed (Table 1). *R. occidentalis* had more monomorphic loci and lower allelic richness than *R. vulgaris*. *R. vulgaris* showed a lower level of genetic differentiation (F_{ST}) between the SCI North and SCI East populations, whereas these populations were highly differentiated in *R. occidentalis*. If this was due to increased dispersal between these populations *R. occidentalis* should be equally affected since there is no evidence of foraging preference by the mammals. In addition, the genetic diversity within the SCI West and SRI populations of *R. occidentalis* was strikingly lower than all other populations (Table 1). The reasons for these differences between species remain unclear but are likely to be related to either different historical patterns of colonization or unknown differences in the biology of the two species. Differentiating between these two options will require more knowledge of their biology and dispersal vectors and could benefit from additional genetic markers.

Spore bank sampling

Bioassays proved to be an efficient way of obtaining *Rhizopogon* samples for estimating population structure when the *Rhizopogon* species under study were abundant in the spore bank. These two species are the only *Rhizopogon* species found on these islands and both were retrieved in large enough numbers from bioassays to make a considerable contribution to the population sample number. In fact on SRI, significantly more samples of *R. occidentalis* were obtained from the bioassays than were collected as sporocarps and thus sampling from the spore bank was absolutely essential for this study. Furthermore, *R. vulgaris* samples were only detected on SRI by sampling the spore bank. We observed that *R. vulgaris* exists on SRI but at an extremely low frequency that may indicate a recent introduction, or that it is competitively excluded by other species on SRI.

Allele frequencies of spore bank isolates were not different from the fruiting populations at the same sites (Table 2). This would be expected if the same underlying population (spore bank and sporocarp) is sampled, since the spores in the soil are deposited from the fruiting population. Sampling artefacts may create a biased representation of allele frequencies especially for sampling hypogeous fungi. For example, there is a potential for 'hot-spots' of spore density where sporocarps have deliquested in place (Miller *et al*. 1994). If soil samples were pooled for a population a particularly dense spore contribution from a single sporocarp

could bias the allele frequencies. Since our samples were collected as discrete points along transects and not pooled, this potential artefact was avoided.

Effects of spore bank size on population structure — an overwhelming effect of founding populations?

Our results show that the limited opportunities for dispersal between isolated host islands are probably a major factor in observed population structure. We hypothesize that the size of the spore bank populations is an additional factor that may accentuate this structure in two ways. First, the large size of established spore banks should buffer genetic change. For example, rare migration between established populations would be difficult to detect, because in lieu of positive selection of advantageous alleles and hitchhiking by neutral microsatellite loci, the immigrant alleles would be swamped by the overwhelming size of the resident population. This same effect should hold for novel alleles generated within existing populations by mutation. Second, when new populations are founded, they may expand rapidly from a small genetic base until a large spore bank is established. Random genetic drift would be a strong influence when populations are small, and less so as the population increases to a sufficiently large size. Decreased genetic variation within newly founded insular populations and increased genetic divergence among populations is expected since meiotic spores will be produced from individuals within the resident population (Nei *et al*. 1975; Chakraborty & Nei 1977). If these scenarios are correct, it would mean that founding effects associated with small population size predominate over mutation and drift in older, larger populations as determinates of population structure in *Rhizopogon*. This model would predict that the differences we see between *R. occidentalis* and *R. vulgaris* are likely due to differences in founding events.

Conclusions

Earlier work that examined fine-scale genetic structure in ectomycorrhizal fungi showed little or no genetic differentiation among populations. In this study, we found high levels of genetic structure in *Rhizopogon occidentalis* and *R. vulgaris* populations on the Northern Channel Islands that were separated by as little as 8.5 km. This high degree of genetic differentiation among populations is attributed to isolation among *Rhizopogon* populations as a consequence of the host pine islands within Santa Cruz Island, geographic barriers to dispersal, a limited number of mammal dispersers on Santa Cruz Island, and the large *Rhizopogon* spore bank. Differences seen between *R. occidentalis* and *R. vulgaris* in the level of genetic variation and levels of genetic differentiation between SCI North and SCI East populations may be explained by historical events such as different colonization events or demographic factors; these cannot be addressed here and will be pursued further in a future study.

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC3264/MEC3264sm.htm

Table S1 Observed allele frequencies at microsatellite loci for: A, Five loci for *Rhizopogon occidentalis*; B, Six loci for *R. vulgaris*.

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This work was completed as part of the PhD research of Lisa Grubisha. She studied geographic and genetic structure of mainland and island populations of the ectomycorrhizal fungus *Rhizopogon*. Sarah Bergemann is interested in evolutionary ecology of fungi. Her current research focuses on landscape-level patterns and processes and fine-scale genetic structure of ectomycorrhizal fungi. Tom Bruns is broadly interested in fungal ecology and evolution and is particularly interested in spore bank dynamics of *Rhizopogon*.
