

Within-population genetic structure differs between two sympatric sister-species of ectomycorrhizal fungi, *Rhizopogon vinicolor* and *R. vesiculosus*

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Abstract: Using spatial autocorrelation analysis, we examined the within-population genetic structure of *Rhizopogon vinicolor* and *R. vesiculosus*, two hypogeous ectomycorrhizal (EM) species that are sympatric sister taxa known to differ in their clonal structure. We collected 121 sporocarps and 482 tuberculate EM of both species from a 20 ha forest stand dominated by Douglas-fir (*Pseudotsuga menziesii*). Field collections were identified to species with restriction fragment length polymorphism analysis of the nuclear ribosomal internal transcribed spacer. Five and six microsatellite markers were used to characterize the genetic diversity of EM and sporocarp samples from *R. vesiculosus* and *R. vinicolor* respectively. After correcting for genet structure, spatial autocorrelation analyses of the EM samples were used to test the null hypothesis that multilocus genotypes characterized from each species were randomly distributed within the study area. We detected positive and statistically significant fine-scale genetic structure up to 120 m within the *R. vesiculosus* sample. In contrast, no spatial genetic structure was evident for *R. vinicolor*, indicating that the genotypes characterized for this species were randomly distributed throughout the study area. Differences in statistical power or the nuclear count of basidiospores are unlikely agents of the genetic patterns observed. Our results suggest that differences in reproductive output or competitive ability may act individually or in combination to create clusters of similar genotypes for *R. vesiculosus* throughout the study area.

Key words: ectomycorrhizal, fungi, genet, genetic structure, *Rhizopogon*, spatial autocorrelation

INTRODUCTION

Ectomycorrhizal (EM) fungi form obligate mutualisms with the roots of many seed plants and comprise a critical component of terrestrial ecosystems (Smith and Read 2008). Although for processes such as carbon flow (Höberg et al. 2001) and nutrient cycling (Read and Perez-Moreno 2003) the ecological roles of EM fungi are increasingly well documented, research into the population dynamics of EM species is comparatively limited (Bruns and Kennedy 2009). This is due in part to the difficulties inherent in collecting population-level samples for organisms that are both highly diverse and distributed belowground. Continued study of population dynamics however will greatly increase our understanding of how EM species both contribute to and are affected by forest ecosystem processes (Douhan et al. 2011).

Both extrinsic habitat features and intrinsic life history characteristics may contribute to genetic structure (or lack thereof) within and among populations (Slatkin and Barton 1989). Several recent studies have examined how extrinsic features such as distance, geographic barriers or host species may structure genetic variability among populations of EM fungi (Douhan et al. 2011). While these studies are restricted to a handful of EM species, the available data suggest that both distance and geographic barriers can play significant roles in structuring genetic variation among populations of both wind and animal dispersed species (Kretzer et al. 2005, Grubisha et al. 2007, Carriconde et al. 2008, Amend et al. 2010) while host species may be less important (Roy et al. 2008). There is also evidence that populations of some wind-dispersed, generalist EM species are highly connected over long distances. For example, Roy et al. (2008) found that distances up to 450 km between populations did not pose a significant barrier to gene flow for *Laccaria amethystina*.

The structuring of genetic variation within a population of EM fungi may depend on a number of intrinsic or extrinsic factors, including the persistence and growth of existing fungal individuals (genets), the recruitment of new genets into the population, the size of the population, the mating system and its inherent level of inbreeding and any

Submitted 19 Jul 2012; accepted for publication 26 Dec 2012.

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selective forces acting on the population. In the past two decades genetic studies have revealed high variability in clonality and recruitment patterns of EM fungi (Douhan et al. 2011). Some species produce genets more than 10 m diam (Dahlberg and Stenlid 1994, Bonello et al. 1998, Sawyer et al. 1999, Fiore-Donno and Martin 2001, Hirose et al. 2004, Kretzer et al. 2005) whereas others form comparatively smaller genets (Fiore-Donno and Martin 2001, Redecker et al. 2001, Kretzer et al. 2005, Gryta et al. 2006, Lian et al. 2006, Carriconde et al. 2008), some less than 1 m² (e.g. Gherbi et al. 1999). Data from genet studies often have been used to draw inferences about the population biology of EM species (Douhan et al. 2011). Species that form larger genets may be strong competitors that are long-lived and spread via both spore dispersal and abundant vegetative growth (clonal propagation; Dahlberg and Stenlid 1990, Bonello et al. 1998, Redecker et al. 2001). In contrast, species that form small genets may have high reproductive potentials, short persistence and spread predominantly through spore dispersal (sexual reproduction; Gherbi et al. 1999, Fiore-Donno and Martin 2001, Carriconde et al. 2008). These descriptive characterizations, however, may oversimplify the innate complexity in many EM life histories. Populations of EM fungi can contain mixtures of both small, potentially short-lived genets and large, persistent genets depending on the disturbance history of the site (Gryta et al. 1997, Gryta et al. 2000, Guidot et al. 2002) or recruitment patterns of new genets (Amend et al. 2009). On the other hand, EM species that form small genets may be slow growing, as opposed to short-lived, due to unfavorable abiotic conditions or interactions with other soil organisms (Zhou et al. 2001, Selosse 2003, Douhan et al. 2011).

Characterizing how variation in clonality and recruitment relate to genetic structure within populations of EM fungi may let ecologists use existing information from genet studies to develop predictions about how some life history characteristics (e.g. genet structure) may influence the population dynamics of EM fungi. Toward this end, comparing the within-population structure of sister species that are sympatric in distribution and associated with a single EM host controls a number of potentially confounding factors (e.g. phylogeny, disturbance history of the site, host tree physiology) and allows for stronger inference about how life history may influence genetic structure within populations of EM fungi. *Rhizopogon vinicolor* and *R. vesiculosus* are sister species in the Boletales (Basidiomycota; Kretzer et al. 2003) that provide a useful system for the study of how life history might correlate with differences in population structure. Both species form EM exclusively with Douglas-fir (*Pseudotsuga menziesii*) and

neither are known to form any structures, such as mitotic spores, that facilitate asexual dispersal (Molina et al. 1999, Kretzer et al. 2003). In comparing the genetic structure among sympatric populations of *R. vinicolor* and *R. vesiculosus* in Oregon, Kretzer et al. (2004, 2005) demonstrated that *R. vesiculosus* genets frequently are larger than 10 m diam while *R. vinicolor* genets are generally smaller. Similar results were found by Beiler et al. (2010) at an independent study site near Kamloops, Canada.

It is possible that the difference in genet size documented for *R. vinicolor* and *R. vesiculosus* may correlate with the development of population genetic structure at larger spatial extents within populations. *Rhizopogon* spores can accumulate in forest soils (Kjøller and Bruns 2003, Grubisha et al. 2007) and may remain viable for extended periods (Bruns et al. 2008). Genets that encompass larger spatial extents (i.e. *R. vesiculosus*) may create patches of high spore density if these genets are longer-lived or produce more sporocarps over the lifespan of a genet, compared to *R. vinicolor*. Grubisha et al. (2007) proposed that spore banks accumulated by *Rhizopogon* species might influence the relationship between genetic and geographic distance because patches of high spore density could effectively swamp out any influence of spores arriving from other patches or populations. The primary goal of our study was to use spatial autocorrelation analysis to investigate patterns of within-population genetic structure in sympatric and continuously distributed populations of *R. vinicolor* and *R. vesiculosus*. Individual-based statistical approaches, such as spatial autocorrelation analysis (Smouse and Peakall 1999, Epperson 2003, Peakall and Smouse 2006), can provide significant insight into how genetic variability is structured within naturally occurring populations of EM fungi (e.g. Zhou et al. 2001, Liang et al. 2004, Kretzer et al. 2005, Dunham et al. 2006, Carriconde et al. 2008, Amend et al. 2009). Using spatial autocorrelation analysis, we evaluate alternate hypotheses of panmixia and localized inbreeding as explanations for the patterns in genetic distance observed between *R. vinicolor* and *R. vesiculosus* genets separated by 5–400 m. Based on the genet size differences and potential differences in spore banks described above, we predicted that the genetic variability within the *R. vesiculosus* population would be structured over shorter distances compared to *R. vinicolor*.

MATERIALS AND METHODS

Study area.—We collected sporocarps and EM of *R. vinicolor* and *R. vesiculosus* from a 20 ha (0.45 × 0.48 km) study plot located on the north slope of Mary's Peak in the

Oregon Coast Range (44°32.1'N, 123°32.1'W). The area is dominated by 40–80 y old Douglas-fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*), with vine maple (*Acer circinatum*), western sword fern (*Polystichum munitum*), Oregon grape (*Mahonia nervosa*) and salal (*Gaultheria shallon*) as prominent components of the understory. In mid-Jun to mid-Jul 2004, we installed a sampling grid comprising 27 individual transects, 4.2 km long. Individual transects were arrayed in either north-south or east-west directions such that many transects intersected at right angles. Parallel transects were separated by a minimum of 10 m and the relative location of each transect was checked at several points with a measuring tape, compass, and a handheld GPS device. The 20 ha plot was centered on a 50 × 100 m study plot sampled by Kretzer et al. (2005; plot identified as MP3 in this study) during an investigation of genet and population genetic structure in *R. vinicolor* and *R. vesiculosus*.

Field sampling.—An advantage of working with *R. vinicolor* and *R. vesiculosus* is that both species form large, distinctive EM with a tuberculate morphology (Zak 1971, Massicotte et al. 1992). Tuberculate EM grow up to several centimeters in diameter and comprise dense clusters of root tips surrounded by a rind of hyphae. The EM of *R. vinicolor* and *R. vesiculosus* are more common, less seasonal, and hence easier to sample than sporocarps and likely provide better representation of the genetic diversity within a study location compared to sporocarps. It is possible, however, that both species also form EM that are not clustered (i.e. non-tuberculate) at least as precursors to the tuberculate form and these simpler EM would be missed by our sampling. We are not aware of any information indicating that the two species form tuberculate EM at different rates or ratios (non-tuberculate/tuberculate). In sampling only tuberculate EM we assume that these structures represent the genetic diversity in each population as well as if our samples included both forms of EM.

The size and distinctive morphology of the EM allow for sampling via raking. The area within one meter of transects was systematically searched for tuberculate EM by raking upper organic soil layers and decomposing wood. *R. vesiculosus* forms larger genets (>10 m in width) more frequently than *R. vinicolor* (Kretzer et al. 2005, Beiler et al. 2010). To balance the number of discrete genets sampled for each species we maintained a minimum distance of 5 m between any tuberculate EM collected along individual transects. The spatial coordinates for each tuberculate EM collection were recorded to the nearest 0.1 m. Subtle morphological differences can be used to differentiate tuberculate EM of *R. vesiculosus* and *R. vinicolor* in the field (Kretzer et al. 2003). When we found both tuberculate EM morphological types in the same location, we collected one EM of each type and assigned identical spatial coordinates to both collections. All collections were cleaned of excess soil and frozen (–20 C) the day they were collected and freeze dried within a week of collection.

In Jun and Jul 2009 we sampled *Rhizopogon* sporocarps from a one-hectare subplot of the study area. Within this subplot we systematically searched for sporocarps by raking

and sorting the surface organic soil and top 8 cm of mineral soil within 10 × 10 × 100 m belt transects centered on individual transects sampled as part of this study in 2004 and by Kretzer et al. (2005; plot identified as MP3). As with EM collections, we recorded the spatial coordinates of each sporocarp collection to the nearest 0.1 m. Sporocarps were measured and stored on ice immediately after collection and dried at 35 C within 24 h. Before drying, we divided each sporocarp along its length with a clean scalpel and excised two small tissue samples from the spore-bearing tissue. One tissue sample was immediately subjected to DNA extraction, while the other was pressed between clean glass microscope slides to preserve spores for analysis by fluorescence microscopy. We determined the size of sporocarps with the volumetric formula $(4/3)\pi abc$ (where a, b and c are the radii of the sporocarp).

Molecular typing.—We carried out DNA extractions, PCR amplification of the nuclear ribosomal internal transcribed spacer (ITS) and microsatellite loci, and scoring of restriction fragments and microsatellite alleles using methods detailed in Kretzer et al. (2000, 2003, 2004). Briefly, we confirmed the species identity of all tuberculate EM samples by amplifying the ITS using the fungal specific primer ITS1f (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and restricting the unpurified PCR product with *Aha*I. We scored restriction enzyme digests directly from agarose gels (1% SeaKem GTG; 2% NuSieve GTG, Cambrex Bio Science, Rockland, Maine) visualized with ethidium bromide staining. We used the methods and microsatellite markers developed by Kretzer et al. (2000, 2004) to genotype all *R. vinicolor* EM and sporocarps with six microsatellite loci (*Rv*15, *Rv*46, *Rv*53, *Rve*2.77, *Rve*3.21, *Rve*1.34.). We genotyped *R. vesiculosus* EM with five microsatellite loci (*Rv*02, *Rve*1.21, *Rve*1.34, *Rve*2.44, *Rve*2.14) and *R. vesiculosus* sporocarps with these five loci along with an additional sixth microsatellite locus (*Rve* 2.10).

Genet assignments.—Clonal (vegetative) growth, somatic mutation and errors in microsatellite scoring can confound interpretation of spatial patterns in genetic variability and must be accounted for before drawing inferences about genetic structure. Kretzer et al. (2005) found that the *Rhizopogon*-specific microsatellite loci used here do not fully resolve all genets. To address these issues, we used both genetic and spatial criteria to assign multilocus genotypes to independent fungal genets. We identified potential genets of both *R. vinicolor* and *R. vesiculosus* by using the FIND CLONES option in GenAIEx (6.1; Peakall and Smouse 2006) which performs a search for groups of samples with identical multilocus genotypes, calculates the maximum linear distances between sets of samples with identical multilocus genotypes and calculates the probability of encountering each multilocus genotype more than once (Pse; Peakall and Smouse 2006). The Pse statistic is calculated with the frequencies of the alleles that make up the multilocus genotype and a model that assumes random mating and sexual reproduction within each population (Peakall and Smouse 2006). After this analysis, we culled each sample set (i.e. *R. vinicolor* and *R. vesiculosus*) such that any two samples with the same multilocus genotypes

were (i) separated by at least 30 m and (ii) possessed only genotypes that had a high probability ($P_{se} > 0.7$) of arising more than once in a sexually reproducing population. The 30 m distance criterion exceeds the maximum width previously observed for *Rhizopogon* genets in the study area (Kretzer et al. 2004, 2005). The P_{se} cut off was selected by examining the frequency distribution of P_{se} values within each population. Only samples that fit these criteria were retained in subsequent spatial genetic analyses. We thought, however, that both the distance and P_{se} criteria were somewhat arbitrary. As a conservative measure, we also performed spatial autocorrelation analyses on sample sets where each unique genotype was represented by only one randomly selected sample.

Either somatic mutation or errors in scoring microsatellite alleles can produce situations where individual samples that might otherwise be recognized as repeated samples from one fungal genet are instead found to possess highly similar, but not identical, genotypes. Because such samples will likely occur in proximity, this may yield misleading results in spatial analyses of genetic data. If all multilocus genotypes are the result of sexual reproduction, a frequency histogram of genetic distances should produce a unimodal and normal distribution. On the other hand, a bimodal distribution with one peak centered over small genetic distances could indicate the occurrence of somatic mutation or errors in the scoring of microsatellite alleles. We examined frequency histograms of pairwise genetic distances for each species (as in Arnaud-Haond et al. 2007, Amend et al. 2009) to ensure that all unique multilocus genotypes included in spatial genetic analyses arose from sexual reproduction and not from somatic mutation or errors in scoring microsatellite alleles. Spatial autocorrelation analyses were conducted on sample sets for which the frequency histograms of pairwise genetic distances contained only one unimodal peak.

Spatial genetic analysis.—To examine the spatial distribution of genotypes for both *R. vinicolor* and *R. vesiculosus* we used the program GenALEX (6.1; Peakall and Smouse 2006). This program employs multivariate analysis methods to calculate a correlation coefficient (r ; range -1 to $+1$) between genetic and geographic distances for all pairs of individuals within user-specified distance classes. The starting point for this analysis is a pair of genetic and geographic distance matrices calculated for all possible pairwise comparisons in the pool of samples. We used the genotypic distance option to calculate linear genetic distances between all possible pairs of collections (Peakall and Smouse 2006). Genetic distance calculations combine information from all alleles and loci thus reducing allele to allele noise and enhancing the signal from existing spatial patterns. We examined signal contributions from individual microsatellite markers to ensure that any significant results were not driven by one or two loci. We selected distance classes that produced an even number of pairwise comparisons across all classes. To increase pattern resolution we selected the smallest distances classes that allowed sufficient sample size and statistical power (at least 500 pairwise comparisons) for calculating the r statistic.

For each distance class statistical significance of r calculated from the data was determined by comparison to a randomized distribution of r values created via 1000 permutations that swapped genetic data across spatial locations. The resulting randomized distribution represents the expected behavior of the correlations statistic (given the data) under the null hypothesis that genotypes are distributed randomly across the study area. Randomized r values were sorted and the 25th and 975th values used to define upper and lower 95% confidence intervals (Smouse and Peakall 1999). Bootstrap errors for r within each distance class also were used as a more conservative significance test of the autocorrelation statistic (Peakall et al. 2003). In combination, r values were considered statistically significant when they exceed the 95% confidence interval around the null hypothesis of zero and their 95% bootstrap interval did not contain zero. We estimated the extent of genetic autocorrelation in *R. vesiculosus* and *R. vinicolor* by repeatedly testing for statistical significance of r in the shortest distance class in analyses that varied distance class size 40–400 m by 40 m intervals (Peakall et al. 2003, Double et al. 2005). In each analysis our primary interest was the detection of positive autocorrelation within the shortest distance classes. Because of this, we report one-tailed tests for positive spatial structure (Smouse and Peakall 1999, Peakall et al. 2003) with an alpha of 0.05 used in hypothesis testing. Adjustments for multiple tests (i.e. Bonferroni adjustments) were not made to the alpha value because we were testing an explicit, a priori hypothesis that involved evaluating p -values for only the shortest distance classes.

Fluorescence microscopy.—We examined basidiospores from six randomly chosen sporocarps of each species (*R. vinicolor* and *R. vesiculosus*) for nuclear count using 4'-6-diamidino-6-phenylindole (DAPI, Anaspec Inc. San Jose, California) staining procedures to determine the potential for secondary homothallism in each species. Each sporocarp examined was fully mature (dark, soft spore bearing tissue at time of collection) and was selected from a distinct genet as determined by microsatellite multilocus genotype analyses. We carried out DAPI preparations following the methods of Coleman et al. (1981) and Horton (2006). We scraped dried spores and tissue from microscope slides prepared from field collections and placed them into microcentrifuge tubes containing 500 μ L 70% ethanol. Each sample was vortexed, centrifuged, allowed to stand 1–2 h at room temperature and then centrifuged at 12000 g for 1 min to pellet spore material. After removing the residual ethanol a sample of each spore pellet was placed onto a fresh microscope slide and allowed to air dry. Dried samples were flooded with McIlvaine's solution, pH 4.4, allowed to stand for 5 min and blotted dry. A single drop of DAPI stain solution (5 μ g/mL) was applied to each sample, which was covered with a clean cover slip and sealed with clear nail polish. We allowed stain preparations to set at 25 C in a dark container 1–3 h before viewing at 1000 \times with a Leica DMRB fluorescence microscope. We determined the nuclear count of 200 basidiospores from each sporocarp.

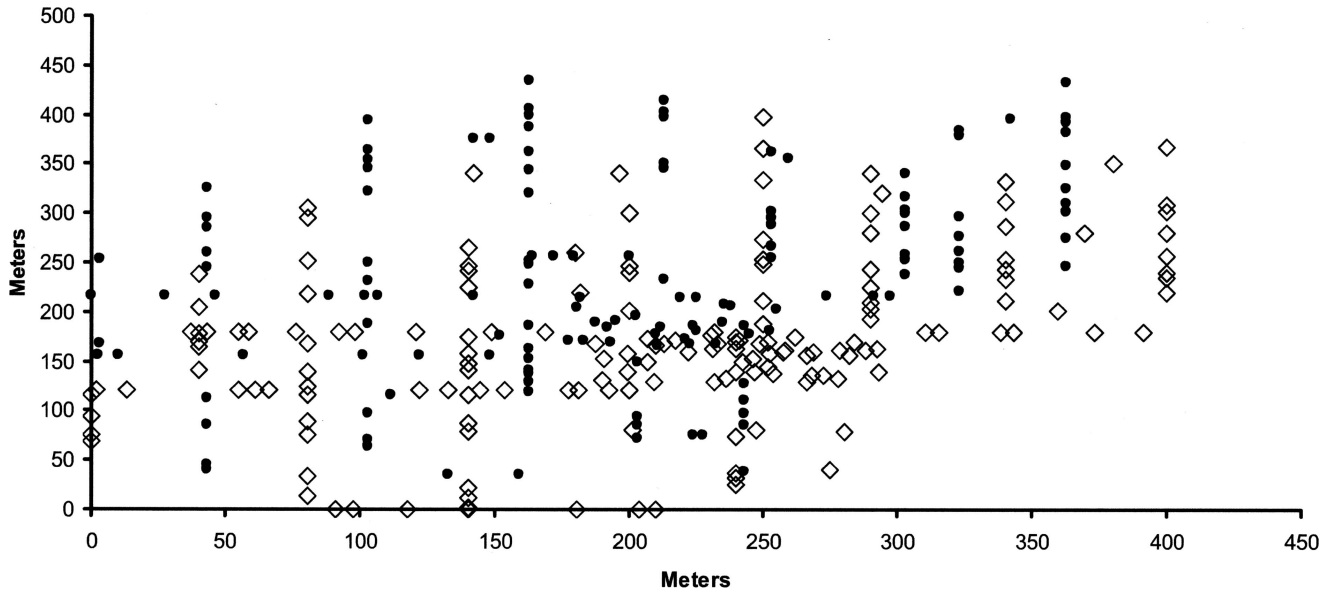


FIG. 1. Distribution of *R. vinicolor* and *R. vesiculosus* EM collections included in spatial autocorrelation analyses. Unfilled diamonds represent the locations of 169 individual roots colonized by *R. vesiculosus*; filled circles represent the locations of 142 individual roots colonized by *R. vinicolor*. Spatial positions of ectomycorrhizae are represented to the nearest 0.1 m.

RESULTS

Genet assignment.—In 2004 we collected 482 tuberculate mycorrhizae from the 20 ha plot with 195 and 287 collections confirmed as *R. vinicolor* and *R. vesiculosus* respectively by ITS-RFLP analysis. Of these collections, 154 *R. vinicolor* samples and 247 *R. vesiculosus* samples yielded high-quality PCR amplifications for all microsatellite loci and were included in subsequent genet assignments. Microsatellite screening produced 142 and 129 unique multilocus genotypes from the 154 *R. vinicolor* and 247 *R. vesiculosus* collections respectively. Culling samples using spatial and genetic criteria reduced the samples from 154 to 142 for *R. vinicolor* collections and from 247 to 169 for *R. vesiculosus* collections (FIG. 1). We analyzed the fine-scale genetic structure for each culled sample set. Each of the 142 *R. vinicolor* samples included in subsequent spatial autocorrelation analyses possessed a unique multilocus genotype. For *R. vesiculosus* we ran analyses using two sample sets. The first *R. vesiculosus* sample set included 169 samples represented by 129 unique genotypes; the second sample set included 129 samples, each possessing a unique multilocus genotype.

In 2009 we collected 121 sporocarps, 61 *R. vesiculosus* and 60 *R. vinicolor*. RFLP identification of sporocarps was confirmed by incorporation of ITS sequence data from three sporocarps representative of each restriction pattern into a parsimony based phylogenetic analysis using the dataset and methods of Kretzer et al. (2003). The spatial distributions of *R.*

vesiculosus and *R. vinicolor* sporocarps were similar across the one hectare plot (FIG. 2). Multilocus microsatellite genotypes were successfully determined for 57 *R. vesiculosus* sporocarps and 54 *R. vinicolor* sporocarps. Microsatellite screening produced 26 unique *R. vesiculosus* genotypes and 38 unique *R. vinicolor* genotypes of which 11 and 26 respectively were represented by a single sporocarp collection. Culling both samples using the same spatial and genetic criteria as for the EM samples yielded 27 unique *R. vesiculosus* genets and 38 unique *R. vinicolor* genets. Genotypes from *R. vesiculosus* were resampled more frequently than those of *R. vinicolor* (i.e. a genotype was represented by more than one sporocarp; FIG. 3). The *R. vesiculosus* sporocarps collected were larger (2.58 cm^3) on average than those of *R. vinicolor* (1.69 cm^3 ; two-tailed *t*-test, $P = 0.001$, 95% CI: 0.36, 1.42).

Variability in microsatellite loci.—In the 142 EM collections from *R. vinicolor*, microsatellite loci had 2–9 alleles with expected heterozygosities of 0.35–0.76. The number of effective alleles (the reciprocal of homozygosity; Hartl and Clark 1989) averaged across all loci was 2.30 ± 0.45 and the mean expected heterozygosity was 0.50 ± 0.07 . In the 169 EM collections from *R. vesiculosus*, microsatellite loci had 3–9 alleles with expected heterozygosities of 0.32–0.60. The number of effective alleles averaged across all loci was 2.10 ± 0.18 and the mean expected heterozygosity was 0.50 ± 0.05 . These statistics were similar in the *R. vesiculosus* sample culled to include

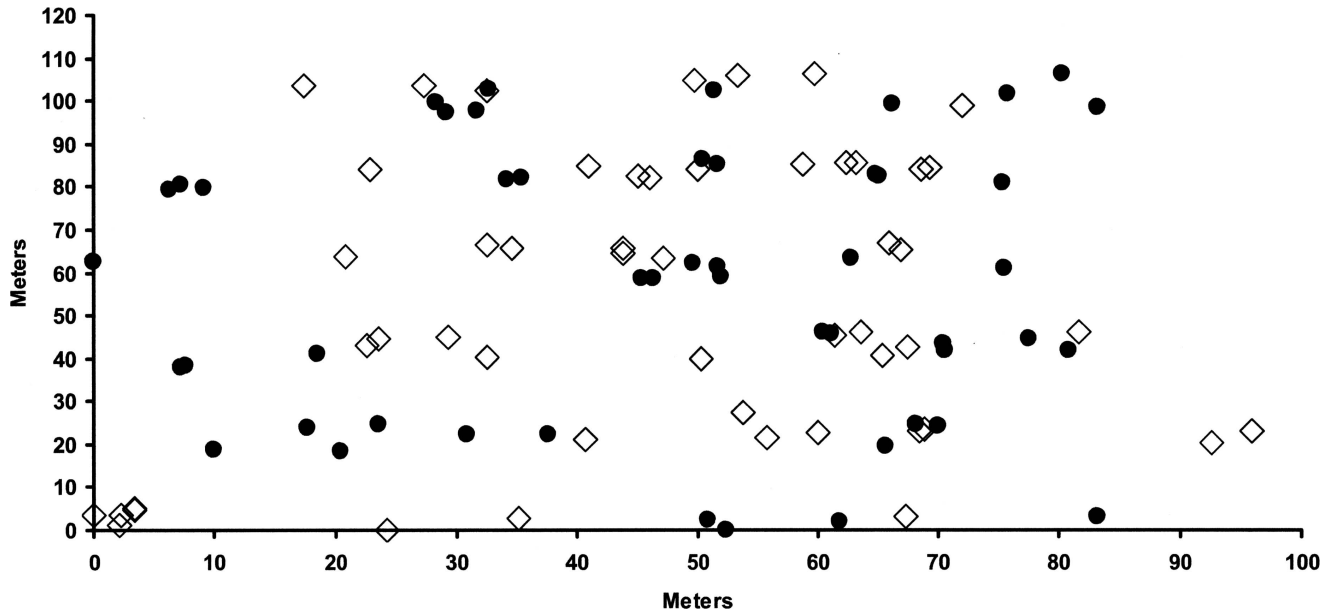


FIG. 2. Distribution of 61 *R. vesiculosus* and 60 *R. vinicolor* sporocarps collected from a 1 ha subplot of the study site in 2009. Sampling occurred within 10-m-wide N/S belt transects separated by 10 m. Filled circles indicate the locations of *R. vinicolor* sporocarps, and unfilled diamonds represent the locations of *R. vesiculosus* sporocarps. Spatial positions of sporocarps are represented to the nearest 0.1 m.

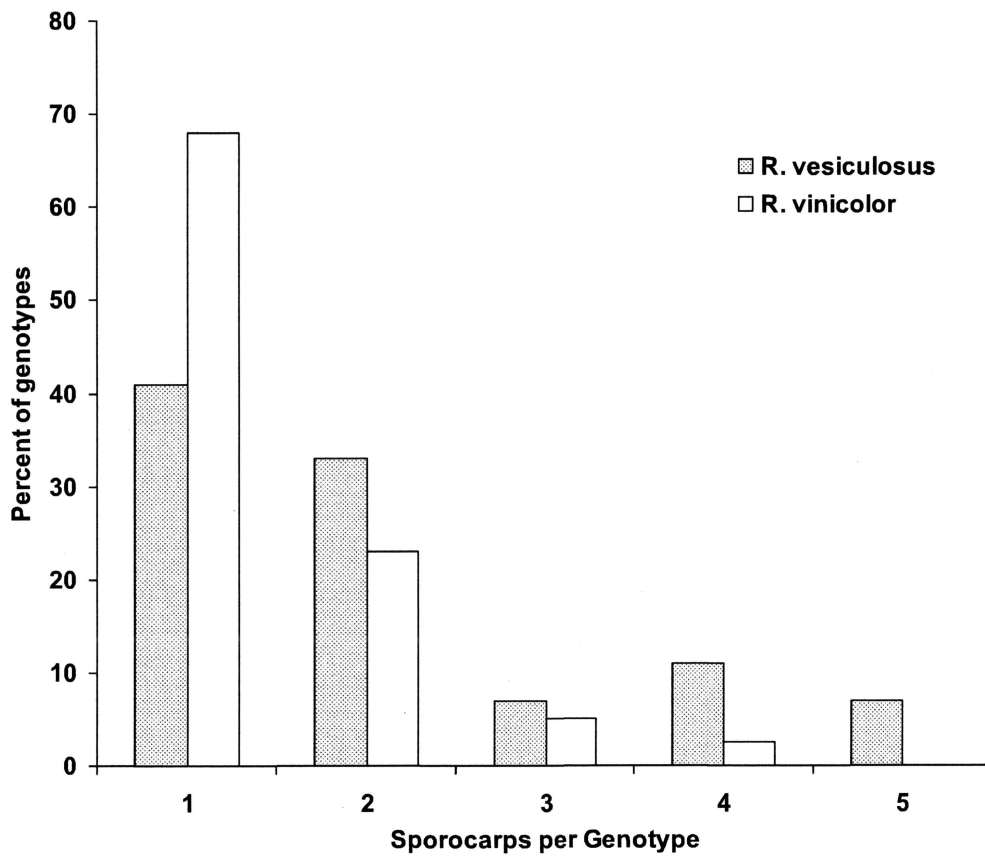


FIG. 3. Frequency distribution of sporocarp number per genotype across all multilocus genotypes identified in microsatellite analyses. Microsatellite screening identified 26 *R. vesiculosus* genotypes and 38 *R. vinicolor* genotypes.

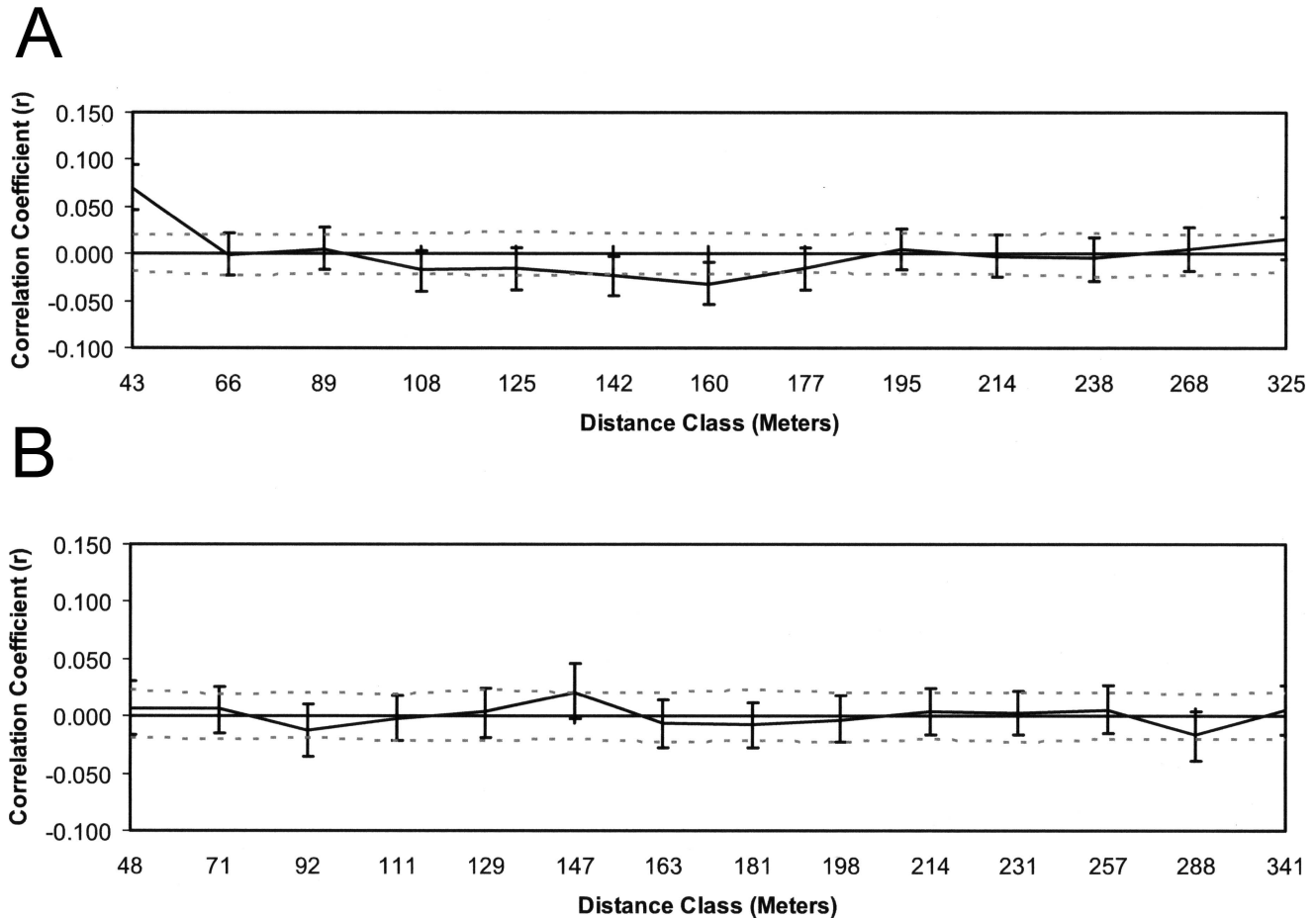


FIG. 4. Correlograms showing the mean spatial autocorrelation values for *R. vesiculosus* (A) and *R. vinicolor* (B). Sample sizes for each analysis were similar (*R. vesiculosus* $n = 169$, *R. vinicolor* $n = 142$). Brackets represent the 95% confidence interval around the r -value (a correlation coefficient between genetic and geographic distances) calculated for each distance classes. Dashed lines represent the 95% confidence interval around the null hypothesis of no spatial structure ($r = 0$). Confidence intervals around an r -value (brackets) that do not fall within the confidence interval around $r = 0$ (dashed lines) indicate statistically significant and non-random genetic signal. For *R. vesiculosus* the r -value in the first distance class is statistically significant ($r = 0.07$, $P < 0.001$); the spatial signal is random in all distance classes for *R. vinicolor*.

only a single collection per unique multilocus genotype ($n = 129$).

Spatial genetic structure.—Three patterns commonly observed in autocorrelation statistics include (i) random fluctuations about zero, indicating that genotypes are randomly distributed, (ii) a stabilizing profile where significant autocorrelation is observed in the shortest distance classes and the autocorrelation statistic fluctuates around zero in the longer distance classes, and (iii) a long-distance cline with significant positive autocorrelation in the shortest distances classes that declines into significant negative autocorrelation in the longer distance classes (Sokal et al. 1997, Diniz-Filho and Telles 2002). For *R. vesiculosus* EM the correlation between geographic and multilocus genetic distance was positive and statistically significant in the shortest

distance class (0–43 m, $r = 0.07$, $P = 0.001$, FIG. 4a) and fit a stabilizing profile in longer distance classes. The results for the *R. vesiculosus* EM sample where each unique genotype was represented by only one randomly selected sample were similar (0–38 m, $r = 0.05$, $P = 0.002$, analyses not shown). For *R. vinicolor* EM the spatial signal did not depart from the null hypothesis of randomly distributed genotypes in any distance class (FIG. 4b). Analyses of individual microsatellite loci showed that all loci consistently mirrored the combined analysis for each species.

The radius of genetically homogenous patches as determined from the x-axis intercept (Epperson 1993, Epperson et al. 1999) was 65 m for *R. vesiculosus* (FIG. 4a). Sequential tests for significantly positive spatial structure in the shortest distance class demonstrated that this x-axis intercept underestimates the

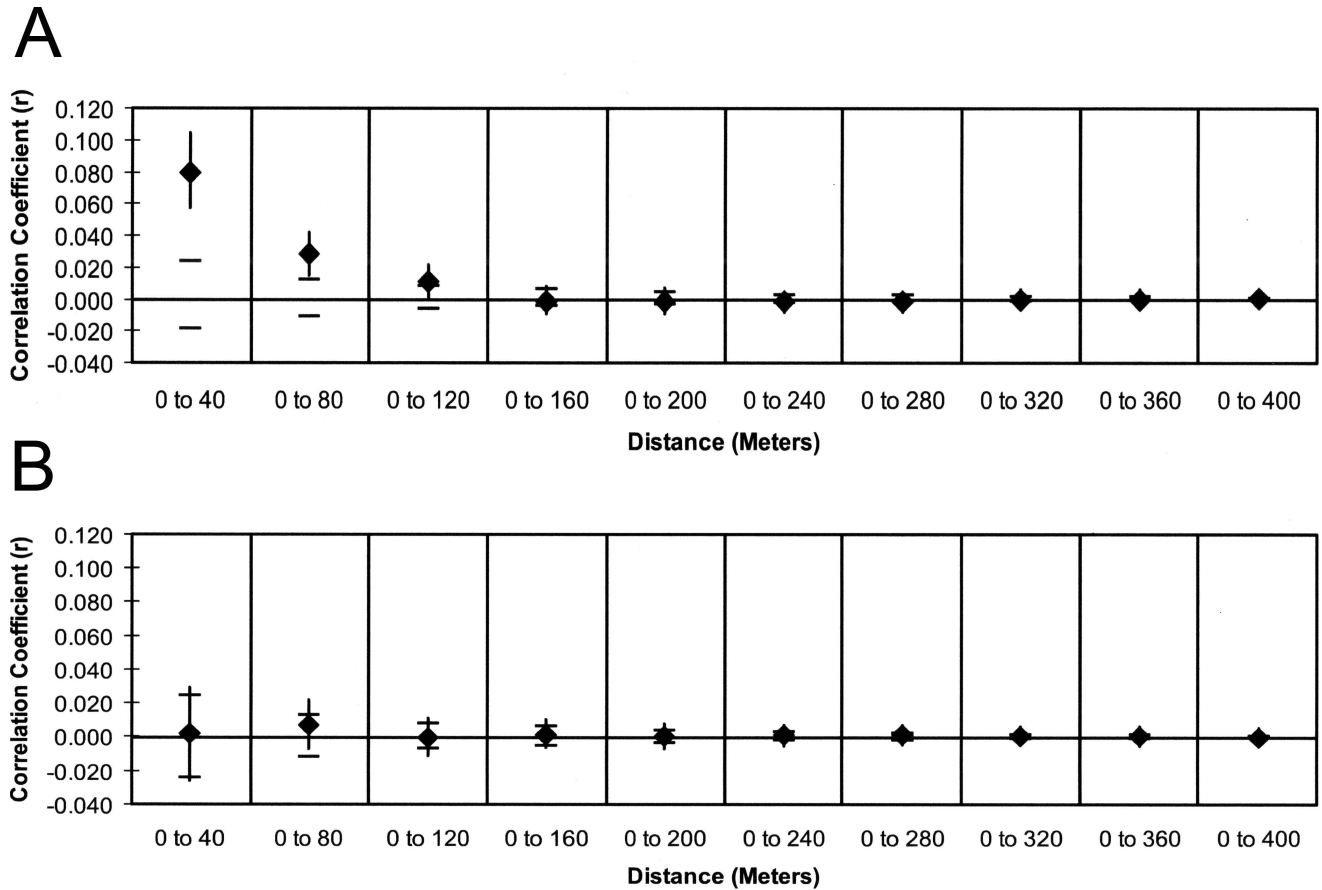


FIG. 5. A plot of the spatial autocorrelation values for the shortest (first) distances class from analyses that sequentially increase distance class size 40–400 m for *R. vesiculosus* (A) and *R. vinicolor* (B). Black diamonds represent the estimated r -value (a correlation coefficient between genetic and geographic distances) for each analysis. Vertical lines passing through each diamond represent the 95% confidence interval around each r -value estimate calculated with 1000 bootstrap replicates. Black dashes represent the 95% confidence interval around the null hypothesis of no spatial structure ($r = 0$) for each distance class. The extent of non-random spatial signal extends to the distance class for which the confidence interval around r (vertical line) intersects zero. For *R. vesiculosus* this is the 0–120 m distance class; the spatial signal is random in all distance classes for *R. vinicolor*.

true extent of spatial structure for this species (FIG. 5a; Peakall et al. 2003, Double et al. 2005). As distance class size is increased 40–400 m, the magnitude of r steadily declines. Bootstrap errors around r overlap zero when distance class size reaches 120 m. Further analysis of larger distance classes to the maximum extent possible for the data show r values converging on zero. Therefore, the distance at which two genetically similar genets of *R. vesiculosus* are equally likely to be in the same patch or different patches within this 20 ha study area is 65–120 m (Sokal and Oden 1978, Sokal and Wartenberg 1983, Epperson 1995). As expected, bootstrap errors and r overlap zero in the first distance class (0–40 m) analyzed for *R. vinicolor* (FIG. 5b).

Nuclear status of basidiospores.—We determined the nuclear count of 1200 basidiospores (200 spores \times 6

collections) for both *R. vesiculosus* and *R. vinicolor* to determine the relative potential of these species to produce secondary homothallic basidiospores. Of the spores counted from each species, an average of 0.5% of *R. vesiculosus* and 0.9% of *R. vinicolor* spores were binucleate.

DISCUSSION

Studying closely related and sympatric species allows for comparative analysis of how differences in life history can influence spatial genetic structure within populations. In this study we examined the genetic structure within sympatric populations of EM sister taxa that differ substantially in genet size (Kretzer et al. 2005, Beiler et al. 2010). Our goal was to use this comparative approach to examine how variation in genet size may contribute to the development of fine-

scale genetic structure. We found significant and positive autocorrelation of genotypes within *R. vesiculosus* and no evidence for similar patterns in *R. vinicolor* within the single 20 ha site that we sampled. The strikingly different patterns of fine-scale structure suggest that additional aspects of reproductive biology (e.g. inbreeding or dispersal) may be affected by patterns of clonality in *R. vesiculosus* and *R. vinicolor* in this location. Our results represent the first demonstration that sympatric, closely related EM taxa with similar host and habitat associations can show dramatically different patterns of genetic structure at fine spatial scales.

The positive spatial autocorrelation detected in *R. vesiculosus* closely resembles a stabilizing profile (FIG. 4a) indicating that genetic variability is structured in repeated patches of closely related genotypes throughout the study area. The sequential distance class analysis (FIG. 5a) indicates that the patch size for *R. vesiculosus* extends up to 120 m. In other words, genotypes within 120 m of each other tend to have higher genetic similarity than would be expected if genotypes were distributed randomly throughout the study area. Such clustering of similar genotypes may result from either restricted gene flow (Peakall et al. 2003), high variance in reproductive output among individuals in a breeding population (Double et al. 2005) or some combination of these two factors. Although the general interpretation of our results is relatively straightforward, identifying the mechanisms that underlie the observed differences requires consideration of several potential processes that might lead to the spatial clustering of similar genotypes in *R. vesiculosus* but not *R. vinicolor*. It is possible that more than one of the following processes operate within this system.

Differences in the power to detect fine-scale genetic structure.—In spatial autocorrelation analyses statistical power is a function of the number of individuals and the number of patches sampled as well as the level of polymorphism across all genetic loci scored (Epperson et al. 1999, Smouse and Peakall 1999). Based on these measures, it is likely that the statistical power was higher in the *R. vinicolor* analysis compared to the *R. vesiculosus* analysis. *R. vinicolor* samples were genotyped at six (as opposed to five) microsatellite loci that exhibited slightly higher numbers of effective alleles and mean heterozygosity. The numbers of samples analyzed for the two species were similar (142 EM for *R. vinicolor*, 169 or 129 EM for *R. vesiculosus*) and the EM of both taxa were similarly distributed across the study area (FIG. 1). Because we were able to detect genetic structure in *R. vesiculosus* it is logical to assume we could have

detected genetic structure with the *R. vinicolor* analysis were it present. In fact, the lower statistical power in the *R. vesiculosus* analysis actually might have hampered our ability to detect highly similar genotypes that are the product of localized selfing. If this is the case, then our estimate of the strength of the genetic structure for *R. vesiculosus* may be conservative.

The distance classes set for each autocorrelation analysis (FIGS. 4, 5) were selected to maximize statistical power for calculating the *r* statistic within the smallest possible distance classes. The number of pairwise comparisons (minimum of 500) within each distance class was similar in both analyses. It is possible that fine scale structure exists in *R. vinicolor* at spatial scales within the 0–40 m distance class, but either the power of this analysis or the field sampling design for tuberculate EM was insufficient to detect this structure. Kretzer et al. (2005) used spatial autocorrelation analysis to demonstrate significant differences in genet size between *R. vinicolor* and *R. vesiculosus*. The sample design used by Kretzer et al. (2005) involved more intensive collecting over a 50 × 100 m plot. While this allowed for the analysis of genetic structure within very small distance classes, up to 80–100 m, no fine scale genetic structure was detected once each genet was represented by only a single sample. Given this, we think it is unlikely any unresolved fine scale genetic structure exists in *R. vinicolor* within the 0–40 m distance class in our analysis.

Differences in dispersal.—It has been shown that small rodents (e.g. Bruns et al. 1989, Molina et al. 1999) and occasionally larger mammals such as deer (Ashkannejhad and Horton 2006) disperse the meiotic spores of hypogeous EM fungi such as *R. vinicolor* and *R. vesiculosus*. The most likely dispersers of these fungi in western forests dominated by Douglas-fir are the northern flying squirrel (*Glaucomys sabrinus*) with a home range of 3.4–4.9 ha (Witt 1992), Townsend's chipmunk (*Tamias townsendii*) with a home range of 0.6–0.96 ha (Trombulak 1985) and the western red-backed vole (*Myodes californicus*) with a home range of 0.06–0.34 ha (Tallmon and Mills 1994). The plot sampled in this study was roughly 20 ha (450 × 500 m) and has the potential to contain several territories for any of these small mammal species. *Rhizopogon* spores frequently are found in the scats of small mammals collected in western Oregon forests (e.g. Jacobs and Luoma 2008). In such dietary studies, however, spores typically are identified to genus only. It is possible that *R. vesiculosus* and *R. vinicolor* have different nutritional qualities or olfactory characteristics that affect the rate at which sporocarps are sought out or

detected by small mammals. However we are not aware of any research that has documented such differences (or lack thereof) in these sister taxa. As a result, we cannot currently propose or exclude differences in dispersal as a possible explanation for the genetic patterns observed.

Differences in the mating system.—Although EM fungi possess mating-type genes that typically function to reduce inbreeding, compatible basidiospores from the same genet can germinate and fuse to form a dikaryotic mycelium. The mating systems of *R. vesiculosus* and *R. vinicolor* have never been investigated, but *R. rubescens* (synonym *R. roseolus*) possesses a bipolar mating system (Kawai et al. 2008) and the mating system is known to be bipolar in several species of *Suillus*, which are closely related to *Rhizopogon* (Fries and Neumann 1990, Fries and Sun 1992). Under a bipolar mating system, each spore from a given sporocarp would be sexually compatible with 50% of the spores from the same sporocarp. Heterozygote deficiency, which would be expected under high rates of selfing, was not observed in either species in this study or by Kretzer et al. (2005), and there is no evidence to suggest that these sister taxa would have mating systems that differ from each other.

Another possible form of selfing is through secondary homothallism, in which heterokaryotic, binucleate spores are formed. This has been shown to occur in *Suillus pungens* (Bonello et al. 1998). We do not know whether the binucleate spores observed in this study were homo- or heterokaryotic, but even if they were heterokaryotic, they were produced in similar proportions in both species.

Differences in patterns of genet establishment.—It is possible that competitive interactions between *R. vinicolor* and *R. vesiculosus* play a role in structuring the genetic patterns observed for these two species within this site. Spore dispersal represents only the potential for gene flow but must be followed by successful germination, dikaryon formation and root colonization before a new genet is actually established. Several recent experiments involving *Rhizopogon* species have demonstrated that the mode and timing of EM root tip colonization (i.e. priority effects) can determine the outcome of competition between EM species (Kennedy et al. 2007a, b, 2009; Kennedy 2010). Kennedy et al. (2009) reported that in manipulative experiments the outcome of competitive interactions among *Rhizopogon* species may be dependent on which species occupies a higher proportion of the root system. A genet that is extensively connected to host roots may have greater access to carbon resources compared to germinating spores, newly formed dikaryons or smaller genets. *R.*

vesiculosus is known to form larger genets and may be able to occupy a larger proportion of the host root system (Beiler et al. 2010). Under such conditions, *R. vesiculosus* may constrain where genets of *R. vinicolor* can establish and grow and through this interaction, influence the genetic structure of *R. vinicolor*. If this were the case one could predict that in stands where *R. vesiculosus* is absent, the spatial distribution of *R. vinicolor* genotypes may differ from what we detected in this study.

Differences in the potential for inbreeding.—Several characteristics of the genus *Rhizopogon* and of *R. vesiculosus* in particular could facilitate the development of inbred patches within our study area. *Rhizopogon* spores can be abundant in forest soils (Kjøller and Bruns 2003, Grubisha et al. 2007) and also have the potential to remain viable for extended periods, possibly decades (Bruns et al. 2008). Hot spots of spore density may dominate soil in localized areas when sporocarps that are not consumed by small mammals deliquesce in place (Miller et al. 1994). Grubisha et al. (2007) demonstrated that local *Rhizopogon* spore banks in the California Channel Islands could not be genetically differentiated from the sporocarp population in the same site. They hypothesized that local spore banks might enhance the level of genetic structure created by isolation by distance because alleles possessed by incoming migrants would have little effect on local allele frequencies in the large resident spore population. It is possible that a similar phenomenon may occur over much finer spatial scales within our study area.

When new *Rhizopogon* genets become established their sporocarps can contribute to a localized spore bank that continues to develop over the life span of that genet and may persist beyond its demise. For *R. vesiculosus*, the potential to form genets that span more than 10 m could facilitate the development of a spore bank from a single genotype over a relatively large area, particularly if larger genets truly are longer lived. This effect could be amplified for *R. vesiculosus*, given its tendency to produce larger sporocarps that may contain more spores than the smaller sporocarps of *R. vinicolor*. These spores have a 50% chance of selfing if the mating system is bipolar. In areas dominated by a spore bank produced by a single genotype, incoming spores will be more likely to mate with the locally dominant genotype or its progeny, resulting in localized patches of closely related genets. Dispersal consequently may not be sufficient to completely disrupt localized patches of inbreeding. Genets of *R. vinicolor* also may be able to form local spore banks with similar properties. However, the small size and possibly short persistence genets as well

as production of fewer, smaller sporocarps may not allow spore banks to develop to the extent possible for *R. vesiculosus*. For *R. vinicolor* dispersal may be sufficient to disrupt any fine-scale structure that might form as the result of spores accumulating in the soil.

The data presented here support the hypothesis that differences in genet size may contribute to patterns of genetic structure within populations of EM fungi. With the experimental design and marker system used we have eliminated many confounding variables that could cloud our interpretation of the different genetic structures observed for *R. vinicolor* and *R. vesiculosus*. The species are sister taxa that are sympatric in distribution under a single host species. With the exception of the variable of interest (genet structure), both species are similarly distributed throughout the study area and genetic markers for each species are similar in their power to define genets and to detect genetic spatial structure.

It is not clear whether the size differences between genets of *R. vesiculosus* and *R. vinicolor* can be attributed to differences in genet persistence times, mycelial growth rates or some other ecological or genetic factor. The presence of similar patterns in relative genet size in a different location (Beiler et al. 2010) supports the hypothesis that the differences at least in may be determined by genetics. Regardless of the exact mechanism behind the observed differences in genet size, we have identified several phenomena that may act individually or in combination to create clusters of similar genotypes for *R. vesiculosus* throughout the study area. Based on our current understanding of the population biology of *Rhizopogon* species, the best supported explanation for the genetic patterns we observed is that the larger genets formed by *R. vesiculosus* dominate local spore banks resulting in localized patches of closely related genets even in the presence of substantial sporocarp dispersal. However we have proposed additional viable hypotheses and further study is required to eliminate these as possible mechanisms.

Our results have important implications for the design of population genetic studies in other EM species. Understanding the spatial scales at which populations of EM fungi are likely to differentiate and the factors that shape genetic structuring within and among populations is crucial for predicting how changes in forest ecosystem structure or process may affect EM species. The contrasting patterns of fine-scale genetic structure between *R. vinicolor* and *R. vesiculosus* highlight the importance of considering intrinsic influences on population structure, such as life history variability, in addition to extrinsic landscape features.

ACKNOWLEDGMENTS

The authors thank Jason Dunham for many useful discussions regarding sampling design, Michelle Gerdes, Alicia Leytem, Tri Tran, Ryan Woolverton, Mary Beth Latvis and Rita Dunham for field and laboratory assistance. This manuscript greatly benefited from comments made by Susan Kephart, Tom Bruns and four other anonymous reviewers. Our research was financially supported by NSF grant DEB-0137531 to Annette Kretzer.

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