

# Patterns of vegetative growth and gene flow in *Rhizopogon vinicolor* and *R. vesiculosus* (Boletales, Basidiomycota)

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## Abstract

We have collected sporocarps and tuberculate ectomycorrhizae of both *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* from three 50 × 100 m plots located at Mary's Peak in the Oregon Coast Range (USA); linear map distances between plots ranged from c. 1 km to c. 5.5 km. Six and seven previously developed microsatellite markers were used to map the approximate size and distribution of *R. vinicolor* and *R. vesiculosus* genets, respectively. Genetic structure within plots was analysed using spatial autocorrelation analyses. No significant clustering of similar genotypes was detected in either species when redundant samples from the same genets were culled from the data sets. In contrast, strong clustering was detected in *R. vesiculosus* when all samples were analysed, but not in *R. vinicolor*. These results demonstrate that isolation by distance does not occur in either species at the intraplot sampling scale and that clonal propagation (vegetative growth) is significantly more prevalent in *R. vesiculosus* than in *R. vinicolor*. Significant genetic differentiation was detected between some of the plots and appeared greater in the more clonal species *R. vesiculosus* with  $\Phi_{ST}$  values ranging from 0.010 to 0.078\*\*\* than in *R. vinicolor* with  $\Phi_{ST}$  values ranging from -0.002 to 0.022\*\* (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). When tested against the null hypothesis of no relationship between individuals, parentage analysis detected seven likely parent/offspring pairs in *R. vinicolor* and four in *R. vesiculosus* ( $\alpha = 0.001$ ). Of these 11 possible parent/offspring pairs, only two *R. vinicolor* pairs were still supported as parent/offspring when tested against the alternative hypothesis of being full siblings ( $\alpha = 0.05$ ). In the latter two cases, parent and offspring were located at approximately 45 m and 28 m from each other. Challenges to parentage analysis in ectomycorrhizal fungi are discussed.

**Keywords:** false-truffles, genetic structure, microsatellite markers, mycophagy, spore dispersal, tuberculate ectomycorrhizae

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## Introduction

Genetic studies of ectomycorrhizal fungal populations have so far been primarily concerned with characterizing the size and distribution of genets. Genets (clones) are generally identified as 'repeatedly sampled, multilocus genotypes that are unlikely to arise by chance in sexual reproduction' (Anderson & Kohn 1998). Because ectomycorrhizal fungi do not generally produce asexual

(mitotic) spores, genets are propagated entirely by vegetative, hyphal growth and thus form fairly coherent localized units. The traditional approach to delineating genet size has been to sample macroscopic structures (typically sporocarps) and use either mycelial interactions (somatic incompatibility) or molecular markers for genet differentiation; the distance between the outermost samples of a genet is used as an estimate of genet size (see references in Kretzer *et al.* 2004). However, because the sampled structures are not necessarily located at the periphery of an actual genet, this approach likely underestimates genet size. Despite these and other limitations, valuable

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comparative data have been gained. Genets of ectomycorrhizal fungi have been found to range in size from less than 1 m<sup>2</sup> (e.g. Gherbi *et al.* 1999) to 300 m<sup>2</sup> or more (e.g. Bonello *et al.* 1998). In *Suillus bovinus* and *Suillus variegatus*, genet size increases with forest age presumably due to continued vegetative growth (Dahlberg & Stenlid 1994; Dahlberg 1997). In contrast, *Laccaria amethystina* forms small and apparently short-lived genets in a 150-year-old forest (Gherbi *et al.* 1999). Ectomycorrhizal fungi such as *S. bovinus* and *S. variegatus* on the one hand and *L. amethystina* on the other seem to represent two extremes in a spectrum of life history traits that are dominated either by long-lived mycelia and abundant vegetative growth (clonal propagation) or by short-lived mycelia and frequent spore establishment (sexual reproduction). Both strategies have sometimes been likened to K- and r-selection strategies, respectively (Deacon & Fleming 1992). It has initially been assumed that the former strategists should be more prevalent with mature trees in stable environments, while the latter should be more prevalent with young trees and in early successional settings, but recent work has shown that a number of ectomycorrhizal fungi form small genets in fairly mature (Redecker *et al.* 2001) to very mature forests (Gherbi *et al.* 1999; Fiore-Donno & Martin 2001); it is possible that these fungi take advantage of microdisturbances for spore establishment.

The genetic structure of ectomycorrhizal fungal populations is additionally shaped by short- and long-distance gene flow, which is a function of spore dispersal followed by successful establishment. Two common spore-dispersal strategies are either wind dispersal or animal dispersal (mycophagy). While many fungi may use a mix of both dispersal strategies, the general belief is that wind dispersal dominates in fungi with epigeous (mushroom-like) sporocarps and animal-dispersal dominates in fungi with hypogeous (truffle-like) sporocarps (e.g. Bruns *et al.* 1989). Gene flow cannot be measured directly in ectomycorrhizal fungi, and indirect, genetic methods are required, but literature in this area continues to be sparse. Both Zhou *et al.* (2001) and Liang *et al.* (2004) found some fine-scale clustering of similar genotypes in *Suillus grevillei* and *Russula vinosa*, respectively. Beyond that, Zhou *et al.* (2001) found little genetic differentiation ( $F_{ST} = 0.024$ ) between two populations of *Suillus grevillei* that were separated by 700 m. At larger spatial scales, Bergemann & Miller (2002) found strong genetic differentiation ( $F_{ST} = 0.434$ ) between populations of *Russula brevipes* from California and Wyoming, but based on the almost complete absence of shared alleles, these populations may actually represent distinct species. Similarly, LoBuglio & Taylor (2002) detected strong differentiation ( $F_{ST} = 0.256$ ) between populations of *Cenococcum geophilum* from New York State and Alberta, as did Jany *et al.* (2002) along a 250 km transect across northeastern France. *Cenococcum geophilum* is, however, an

unusual ectomycorrhizal fungus in that it is not known to form any type of spores and is thought to disperse exclusively via sclerotia. Recently, Murat *et al.* (2004) detected a strong subdivision ( $F_{ST} = 0.20$ ) between populations of *Tuber melanosporum* from France, northern Italy and north-eastern Spain; population structure within France strongly mirrored known routes of postglacial recolonization for ectomycorrhizal host tree species. Also, Grubisha *et al.* (private communication) recently found that mainland and island populations of *Rhizopogon vulgaris* from California are heavily structured with pairwise  $F_{ST}$  values up to 0.45 (Grubisha *et al.*, personal communication). Given the overall sparseness of literature on ectomycorrhizal fungi, relevant information may also be gained from work on other nonmitosporic forest fungi, mostly wood decay and saprotrophic fungi. Most of these studies have been done at intracontinental scales and have revealed very little (e.g. Saville *et al.* 1996; Högberg *et al.* 1999; Kausrud & Schumacher 2002) to moderate (Högberg & Stenlid 1999) interpopulation differentiation.

We are interested in the population genetics of two ectomycorrhizal sister species, *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* (Boletales, Basidiomycota). Both species form hypogeous sporocarps, and their spores are thought to be dispersed primarily by small mammal mycophagy. *Rhizopogon vinicolor* is well known as a predominant ectomycorrhizal fungus on Douglas-fir (*Pseudotsuga menziesii*) roots (Molina *et al.* 1999) and forms distinctive, large, tuberculate ectomycorrhizae (Zak 1971; Massicotte *et al.* 1992). Recently, however, we reported that its sister species, *R. vesiculosus* (*sensu* Kretzer *et al.* 2003), is commonly mistaken for *R. vinicolor* and also forms tuberculate ectomycorrhizae on Douglas-fir. Tuberculate ectomycorrhizae are relatively easy to sample because of their size (up to several centimetres across) and distinctive morphology and they provide ample material for genetic analysis; they are also more common and less seasonal than the associated sporocarps. *Rhizopogon vinicolor* and *R. vesiculosus* thus constitute ideal model systems to (i) study population genetics of ectomycorrhizal fungi with hypogeous sporocarps and to (ii) improve fine-scale sampling by including ectomycorrhizae in addition to sporocarp collections. Finally, previous studies on the phylogeny of the genus *Rhizopogon* (Grubisha *et al.* 2001, 2002; Kretzer *et al.* 2003) put comparative analyses of genetic structure into a broader phylogenetic context.

We have recently developed moderately polymorphic microsatellite markers for both *R. vinicolor* and *R. vesiculosus* and have demonstrated their utility in differentiating genets from both sporocarp and ectomycorrhizal samples (Kretzer *et al.* 2000, 2004). In short it was found that 11 previously mapped genets had multilocus genotypes with very low frequencies under Hardy–Weinberg expectations ( $\leq 4 \times 10^{-3}$ ) making it unlikely for two samples to possess

identical multilocus genotypes by chance rather than by clonal descent. Furthermore it was found that when the number of genets resolved was plotted over the number of loci analysed, it only increased up to the fifth locus in both species. Adding a sixth or seventh locus did not increase the number of genets resolved in either species (Kretzer *et al.* 2004). Here we use previously developed microsatellite markers to analyse genetic structure in both *R. vinicolor* and *R. vesiculosus*. We use spatial autocorrelation analysis for comparative analysis of genet size, and results demonstrate that clonal reproduction (hyphal growth) is much more prevalent in *R. vesiculosus* than in *R. vinicolor*. We were further able to detect small but significant differentiation between plots at 5–5.5 km scales. Finally we conduct parentage analysis, which to our knowledge has not been done in ectomycorrhizal fungi before. Limitations of parentage analysis for ectomycorrhizal fungi are discussed.

## Materials and methods

### Plots and Sampling

Three 50 × 100 m plots were established on the slopes of Mary's Peak in the Oregon Coast Range (USA) and designated as plots MP1, MP3, and MP4. Plots MP1 and MP3 are located on the north side of the peak at approximately 44°31.8'N, 123°32.9'W and 44°32.1'N, 123°32.1'W, respectively; MP4 is located on the south side of the peak at approximately 44°28.6'N, 123°29.8'W. Because of the canopy cover over the plots, GPS readings are affected with an approximately ± 0.1' error. Plots are within 40- to 80-year-old second-growth forests; MP1 is dominated by Douglas-fir (*Pseudotsuga menziesii*), western hemlock (*Tsuga heterophylla*) and western red cedar (*Thuja plicata*), while the overstory in MP3 and MP4 is mostly dominated by Douglas-fir with some western hemlock present. According to the pollen record, Douglas-fir which is the only known host for both *Rhizopogon vinicolor* and *Rhizopogon vesiculosus* does not appear to have been common in the Pacific Northwest until about 10 000 BP (Whitlock 1992). Pre-logging, wildfire and wind were the major natural disturbances in the forests of the Pacific Northwest with infrequent catastrophic fires occurring at intervals of several hundred years (Franklin 1988; Agee 1993).

Plots were sampled between mid-May and mid-July 2000 as follows. In each plot, eleven 50 m transects were run in parallel spaced 10 m apart. In western Oregon, tuberculate ectomycorrhizae of Douglas-fir have been reported to be particularly common in coarse woody debris (Zak 1971). Hence at locations with coarse woody debris within 5 m distance of a transect, the litter was removed with a rake from an approximately 0.25 m<sup>2</sup> large area, and roots were examined for the presence of tuberculate ectomycorrhizae. When tuberculate ectomycorrhizae were found, the centre

of the raked area was mapped relative to the closest transect at a resolution of 0.5 m. Tuberculate ectomycorrhizae were removed, cleaned and freeze dried within a week from sampling. Because both *R. vinicolor* and *R. vesiculosus sensu* Kretzer *et al.* (2003) are spring fruiters, the previously described sampling strategy did also yield a few sporocarp collections, which were treated the same as tuberculate ectomycorrhizal samples. If multiple collections were made within the same c. 0.25 m<sup>2</sup> sampling area, only one representative was subjected to genetic analyses, except when morphological examinations suggested the presence of both *R. vinicolor* and *R. vesiculosus*; in the latter case, one representative per species was subjected to genetic analysis. Finally, samples from a previous study that had been retrieved from within the MP1 plot were also included in this study (Kretzer *et al.* 2004).

### Molecular typing

Although subtle morphological differences exist between tuberculate ectomycorrhizae and sporocarps of *R. vinicolor* and *R. vesiculosus* (see Kretzer *et al.* 2003), species identification of all samples was confirmed using restriction fragment length polymorphisms of the internal transcribe spacer region (ITS-RFLPs) as described in Kretzer *et al.* (2003, 2004). *Rhizopogon vinicolor* samples were subsequently genotyped at microsatellite loci *Rv15*, *Rv46*, *Rv53*, *Rve1.34*, *Rve2.77*, *Rve3.21*, and *R. vesiculosus* samples were genotyped at loci *Rv02*, *Rve1.21*, *Rve1.34*, *Rve2.10*, *Rve2.14*, *Rve2.44*, *Rve2.74*; development of these markers and genotyping techniques including PCR primer sequences have been described in Kretzer *et al.* (2000, 2004).

### Statistical analyses

Spatial autocorrelation analysis based on the multivariate approach of Smouse & Peakall (1999) was completed using GENALEX version 5.1 (available at <http://www.anu.edu.au/BoZo/GenALEX/>). This approach is suitable for the simultaneous analysis of multiallelic, multilocus data, and thus reduces allele to allele noise and enhances the signal from existing spatial patterns. Linear genetic and geographical distances were calculated for all pairwise sample comparisons, and values were binned into user-defined geographical distance classes. Geographic distance classes were chosen to yield even numbers of comparisons across distance classes. For each distance class, a correlation coefficient (*r*; range -1 to +1) between genetic and geographical distances was calculated. Statistical significance of *r* was determined using 999 permutations that randomized samples across distance classes and created a null distribution of *r* values for each distance class. The null distributions of *r* values were used to define 95% confidence intervals. Observed *r* values above the