Microsatellite markers reveal the below ground distribution of genets in two species of *Rhizopogon* forming tuberculate ectomycorrhizas on Douglas fir

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Summary

• We have developed microsatellite markers for two sister species of *Rhizopogon*, *R. vesiculosus* and *R. vinicolor* (Boletales, Basidiomycota), and used selected markers to investigate genet size and distribution from ectomycorrhizal samples. Both species form ectomycorrhizas with tuberculate morphology on Douglas-fir (*Pseudotsuga menziesii*).

• Tuberculate ectomycorrhizas were sampled and mapped in two 10 x 10 m core plots located at Mary's Peak in the Oregon Coast Range and at Mill Creek in the Oregon Cascade Mountains, USA; additional samples were obtained from a larger area surrounding the Mary's Peak core plot.

• Gene diversities at the newly described microsatellite loci ranged from 0.00 to 0.68 in *R. vesiculosus,* and from 0.00 to 0.43 in *R. vinicolor.* Both taxa appeared to be in Hardy-Weinberg and linkage equilibrium. The largest distance observed between tuberculate ectomycorrhizas of the same genet was 13.4 m for *R. vesiculosus*, but only 2 m for *R. vinicolor.*

• This is to our knowledge the first study to differentiate fungal genets from ectomycorrhizas with great confidence using multiple codominant markers.

Key words: *Rhizopogon*, tuberculate, ectomycorrhizas, fungal genets, microsatellite markers.

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Introduction

A central problem to population genetic work in most fungal systems including ectomycorrhizal systems is that the mycelium resides in the soil (or substrate) and that the extensions and distribution of individual genets cannot easily be determined (for review see McDonald, 1997; Anderson & Kohn, 1998). Fungal 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. A general approach to estimate the distribution and size of individual genets in ectomycorrhizal fungi has been to map and collect sporocarps appearing in a study area over a given amount of time and to use mycelial interactions or molecular markers to type them as belonging to either the same genet or different genets. Markers used include vegetative incompatibility (Baar *et al.*, 1994; Dahlberg & Stenlid, 1994; Dahlberg, 1997), randomly amplified polymorphic DNA (RAPDs; Jacobson *et al.*, 1993), mating types (De la Bastide *et al.*, 1994), restriction fragment length polymorphisms (RFLPs; Gryta *et al.*, 1997), inter-simple sequence repeats (ISSRs; Anderson *et al.*, 1998; Gherbi *et al.*, 1999; Sawyer *et al.*, 1999; Zhou *et al.*, 1999), single-strand conformational polymorphisms (SSCPs) of anonymous RAPD-bands (Bonello *et al.*, 1998), amplified fragment length polymorphism (AFLPs; Redecker *et al.*, 2001), and more recently also microsatellite markers (Zhou *et al.*, 2001; Bergemann & Miller, 2002; Dunham *et al.*, 2003).

Results from recent work on the community ecology of ectomycorrhizal fungi has shown that there is generally a poor correspondence between species that produce the dominant above ground sporocarps and those that appear dominant on the roots (for review see Horton & Bruns, 2001). This disconnect between above and below ground community pictures has raised the question if a similar disconnect exists at the population level mid has prompted population genetic investigations to also move below ground. Guidot *et al.,* (2001) collected both sporocarps and ectomycorrhizas of *Hebeloma cylindrosporum* and used RFLPs of the ribosomal intergenic spacer to demonstrate a close correspondence between above- and below-ground distribution of genets, but additional genets were commonly detected as ectomycorrhizas that were not observed fruiting in the plots over the sampling period. Using a finer sampling scale and one microsatellite marker, Zhou *et al*., (2001) determined that sporocarp formation in *Suillus grevillei* correlated with formation of extra-radical mycelia and ectomycorrhizas of the same genet, but that number of sporocarps was not a good predictor of the extension of the subterranean parts of the genet, nor were sporocarps necessarily centered over the subterranean parts of the genet. This might explain why Gardes & Bruns (1996) found ectomycorrhizas of *Suillus pungens* to be rare in soil samples taken directly underneath *S. pungens* sporocarps.

We are interested in both small and large scale population structure of ectomycorrhizal fungi, and have chosen to work with two sister species, *Rhizopogon vesiculasus* and *Rhizopogon vinicolor sensu* Kretzer *et al*., (2003). 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 roots (Molina *et al*., 1999) and forms distinctive, large, tuberculate ectomycorrhizas (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 ectomycorrhizas on Douglas-fir. Tuberculate ectomycorrhizas of Douglas-fir are abundant in the Pacific North-west and can easily be sampled owing to their large size and distinctive morphology. In fact, tuberculate ectomycorrhizas are easier to sample than hypogeous sporocarps which are less common and more seasonal in occurrence. In addition, work quoted in the previous paragraph suggests that ectomycorrhizas are a more accurate sampling unit than sporocarps for determining the dimensions of the vegetative thallus of an ectomycorrhizal fungus. To our knowledge, only two other fungi with hypogeous sporocarps are currently being studied at the population genetic level. One is *Rhizopogon occidentalis*, but results are still preliminary (L. Grubisha, pers. comm.). The other is *Tuber melanosporum* (Pezizales, Ascomycota) that has been shown to have very low genetic diversity probably because of severe bottlenecks during glaciation (Bertault *et al*., 2001).

Microsatellite markers (also known as simple sequence repeat markers) are an excellent choice for use with ectomycorrhizal samples because they are highly variable, codominant markers that do not require cultivation and work with relatively small amounts of sample material. Most importantly, through the use of locus-specific polymerase chain reaction (PCR) primers, they allow the analysis of specific fungal loci from a mix of plant and fungal DNA present in ectomycorrhizal samples. We have previously reported the development of six microsatellite markers for *R. vinicolor* (Kretzer *et al*., 2000). Here, we report the development of 11 additional markers for R vesiculosus and the use of selected markers to investigate the belowground distribution of genets in both *R. vinicolor* and *R. vesiculosus*. Preliminary data from populations sampled at Mary's peak in the Oregon Coast Range also suggest that both species are largely in Hardy-Weinberg and linkage equilibrium.

Materials and Methods

Development of microsatellite markers

A culture of *R. vesiculosus* strain T20874 was kindly provided by Dr Ari Jumpponen (Department of Biology, Kansas State University, Manhattan, KS, USA), and was grown on liquid MMN medium (Marx, 1969) for approx. 3 wk. Mycelium was harvested, freeze dried, and ground in liquid nitrogen. High molecular weight genomic DNA was extracted as previously described (Kretzer *et al*., 2000). Approximately 3.5 µg of DNA was digested with M*sp*I and fragments in the 600-1200 by size range were isolated by gel purification. Fragments were subsequently ligated to short, double-stranded DNA linkers composed of two complementary oligonucleotides with the sequences 5'-pCGCCAAGCTTCCCGGGTACCGC and GCGGTACCCGGGAAGCTTGG. The linker-ligated, size-selected MspI fragments were PCR amplified in a reaction containing 50 mM KCl; 10 mM Tris/HCl (pH 9), 0.1 % Triton X-100, 2.5 mM $MgCl_2$, 200 µM of each of the four dNTPs, 0.5 µM of the GCGGTACCCGGGAAGCTTGG-oligo as primer, 25-50 U ml^{-1} Taq polymerase, and empirical amounts of template DNA. Cycling conditions were 25 cycles of 45 s at 94°C, 30 s at 65°C, and 120 s plus 1 s per cycle at 72°C, followed by a final extension of 10 min at 72°C. The PCR products were subsequently enriched for $(GTG)_n$ repeats using the modified procedures of Kijas *et al.,* (1994) and Hamilton & Fleischer (1999). Briefly, the PCR products were denatured at 94°C and hybridized to a 5'-biotinylated $(CAC)_{10}$ probe in 6× standard saline citrate (SSC) +0.1.% sodium dodecyl sulphate (SDS) at 70°C. Probes were subsequently bound to streptavidin-coated magnetic beads (Promega, Madison, WI, USA), and the beads were washed three times in 1× SSC buffer at room temperature and three times at 55°C. Enriched fragments were eluted in 10 mM Tris/HCl (pH 8.5) and were amplified by 40 cycles of PCR under the conditions described above. Products of the second PCR reaction were digested with M*sp*I at a restriction

Table 1 Microsatellite markers used in this study; *Rv* loci were previously cloned and sequenced from *Rhizopogon vinicolor* T20787 (Kretzer *et al*., 2000), while *Rve* loci were cloned and sequenced from *Rhizopogon vesiculosus* T20874 in this study

			R. vesiculosus			*R. vinicolor*		
Locus	Repeat	Primer sequence[1]	Alleles	*D*	F_{IS}	Alleles	*D*	F_{IS}
Rv02	$(CAC)_7$	'F' GTGAAACTGTTCAACGCACG GTAACTCGTGCTCTTCATCG	3	**0.49**	**-0.03**	8	0.76	+0.24**
Rv15	$(CAC)_{11}$	'F' TCCACCTATCCACCTATTCG AACGTAGACCCAACATAAGAC	5	0.71	+0.23**	3	**0.29**	**-0.13**
Rv46	$(CCG)_5(CCA)_6$	CATCTGGTCTAGTGCTGACTGC 'H'CTCGGCAGCAGAAACCTCAGG	nd	nd	nd	5	**0.69**	**-0.01**
Rv53	$(CGA)_6N_{24}(CGA)_4$	'F' GTTGCGTGGGAAGTCATTC GGTACAGGGAATGCTTCG	nd	nd	nd	5	**0.67**	**+0.02**
Rve1.21	$(ACC)_8$	'E' TGGATGATACTCGGGTACAG AGTTCCATCGCCTGAAGTAC	4	**0.66**	**+0.16**	1	0.00	nd
Rve1.34	$(CAC)_6N_{51}(CAC)_4$	'H' CTTGATTTACAACATGCAGCG CGAGACCAAGGTGATTTCT	2	**0.16**	**-0.09**	2	**0.37**	**+0.15**
Rve1.41	$(CAC)_2N_3(CAC)_2N_3(CAC)_2$	'F' CCAGTGTATCGCGTTTTCAG CGCATTGAACCTGGGTAAG	1	0.00	nd	3	0.29	+0.02
Rve2.10	$(CAC)_7(CGC)_2$	'H' CCGAAGGTACTCAACTGTTG GAACGCTCTACGCTCATG	3	**0.49**	**-0.08**	1	0.00	nd
Rve2.14	$(CAC)_5CTC(CAC)_2$	'F' CGAGTAATACTGAGCCGC CTTCGTGGTACTATGAGTTAC	2	**0.47**	**-0.12**	1	0.00	nd
Rve2.44	$(SAC)_4N_{27}(CAC)_2N_{12}(CAC)_6$	'E' GCCCATGTTGCCACGTG CCTACATGGTTGGAAAGCG	3	**0.57**	**-0.02**	1	0.00	nd
Rve2.67	$(CAC)_2(CACCCAWTCCAC)_3$	'F' TGTTGAACGTTAGTAATCCCG CAGCGGAATGAGAGGC	3	0.12	+0.79**	1	0.00	nd
Rve2.71	$(CAC)_5N_{26}(CAC)_3$	'H' CAGAAGTTCCTTGCTGGTC CCTGAATCGGTGACATCG	3	0.07	-0.02	1	0.00	nd
Rve2.74	$(CCA)_4ACA(CCA)_4$	'H' CTAAATATCCGCCAGTCACG CATTTTCGAGCGATTCTCCC	2	**0.14**	**-0.07**	2	0.03	nd
Rve2.77	$(CGC)_3(CAC)_6$	'E' GCCATCGAGCTAACAGAG GAGAACAATACCCATGCCG	3	0.66	-0.16*	3	**0.42**	**-0.03**
Rve3.21	$(ACC)_8$	'E' AGCGTGCTACTCTCGAAC CCTTCGAGTCGTCAAATGG	6	0.68	-0.13*	3	**0.43**	**-0.01**

Numbers of alleles, gene diversities (*D*), and F_{IS} values are those observed in the larger MP1 population (n = 39 for *R. vesiculosus* and n = 34 for *R. vinicolor*). Asterisks indicate significance of homozygote excess if F_{IS} value is positive and significance of heterozygote excess if F_{IS} value is negative (*P < 0.10; * *P < 0.05); nd = not determined. Numbers printed in bold refer to loci that were subsequently used to delineate genets in either *R. vesiculosus* or *R. vinicolor*. 'H', HEX dye, 'F, fluorescein, 'E', TET dye.

site located within the linker sequence and subsequently cloned into the A*cc*I site of pUC19 using competent *Escherichia coli* DH5α cells from Invitrogen Life Technologies (Carlsbad, CA, USA). Screening of transformants was done as described previously using blue/white selection as well as colony hybridization with a $(CAC)_{13}$ probe and the AlkPhos^{DIRECT} labeling and detection kit from.Amersham Biosciences (Piscataway, NJ, USA). (Kretzer *et al*., 2000). Sequencing of inserts from positive clones has also previously been described (Kretzer *et al*., 2000). Locus-specific primers were obtained from Qiagen (Alameda, CA, USA), and one primer per locus was synthesized with a fluorescent label as indicated in Table 1.

Study sites and sampling

Tuberculate ectomycorrhizas analysed in this study were collected in the spring of 1998 from two plots, one located at Mary's Peak (44°31.86' N and 123°32.86' W) in the Oregon Coast Range and the other at Mill Creek (44°12.15' N and 122°13.95' W) in the Oregon Cascade Mountains, USA. Plots were within 40- to 80-yr-old and 40- to 50-yr-old second-growth forests, respectively, that were dominated by Douglas fir (*Pseudotsuga menziesii*), western hemlock (T*suga heterophylla*) and western red cedar (*Thuja plicata*). Both stands were selected for their similarity in age and vegetation, and because exploratory sampling efforts had demonstrated the abundance of tuberculate ectomycorrhizas. Collections made during the exploratory sampling were subsequently included in this study and account for samples shown in Fig. 1 that are either not located on regularly spaced grid points or located outside the core plots. Core plots were 10 x 10 m in size and overlaid with an 11 x 11 point grid (1-m intervals between points). Soil cores (5 cm wide and approx. 30 cm deep) were taken at each grid point (121 cores per plot), and soils were sifted through a W .S. Tyler Company 2 mm sieve. Tubercu-

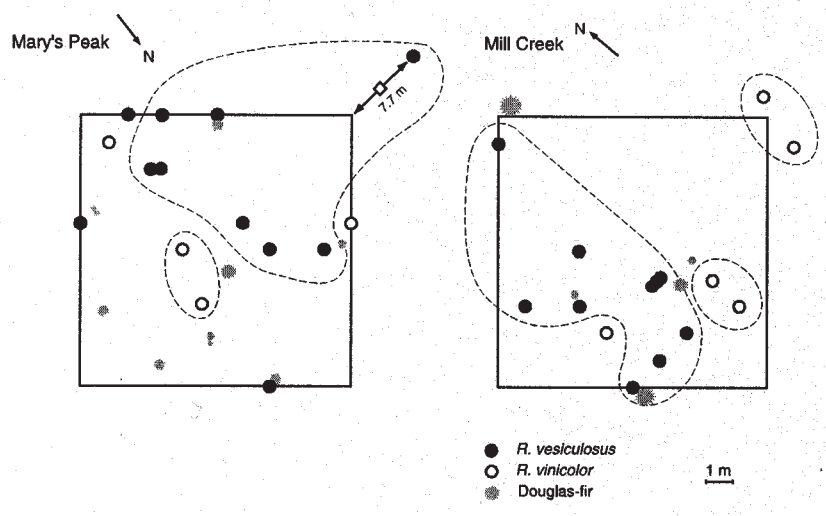

Fig. 1 Map of tuberculate ectomycorrhizal samples collected within the Mary's Peak and Mill Creek (OR, USA) core plots. Samples representing the same multilocus genotype (genet) are encircled with dashed lines; others represent unique multilocus genotypes. Since both *Rhizopogon vesiculosus* (closed circles) and *Rhizopogon vinicolor* (open circles) *sensu* Kretzer *et al.* (2003) are host-specific to Douglas fir (*Pseudotsuga menziesii*; gray stars), only Douglas-fir trees present within the plots and on the perimeters are shown. In addition, two small hemlock trees (*Tsuga heterophylla*) and two small red cedar trees (*Thuja plicata*) were present within the Mary's Peak core plot. Douglas fir trees are approximately drawn to scale, but ectomycorrhizal samples are not.

late ectomycorrhizas were freeze-dried and stored at -20°C before molecular analysis.

A larger, approx. 50 x 100 m plot that included the core plot was sampled in 1999 and 2000 at the Mary's Peak site for the purpose of increasing the number of *R. vinicolor* and *R. vesiculosus* genets sampled. Larger sample numbers were required in order to determine gene diversity of the microsatellite markers developed and to test for deviations from Hardy-Weinberg expectations as well as linkage disequilibrium (see below). Other than the 10 x 10 m core plot, the larger plot at Mary's Peak was not sampled by soil coring but by random raking of the top organic soil layers. The latter proved to be a much faster and more efficient sampling technique for tuberculate ectomycorrhizas; in addition to ectomycorrhizas, it, routinely also turned up a few sporocarp collections. The same multilocus genotyping technique outlined in the Results section was used to genotype the samples from the larger Mary's Peak plot. Some multilocus genotypes proved to be represented by single samples, while others were represented by multiple samples including both ectomycorrhizas and sporocarps. Only one representative per multilocus genotype was used to calculate population genetic parameters and to test for disequilibria. The total number of multilocus genotypes (genets) sampled at the larger Mary's Peak site was n = 39 for *R. vesiculosus* and n = 34 for *R. vinicolor.*

Molecular analyses

Extraction of DNA from tuberculate ectomycorrhizas, PCR amplification of microsatellite loci and scoring of alleles was performed as described by Kretzer *et al.*, (2000, 2003). Sizing of bands was done on an ABI 377 automated sequencer using the 'GS500 Tamra' internal size standard and GeneScan software (Applied Biosystems, Foster City, CA, USA).

Restriction fragment length polymorphisms of the internal transcribed spacer region (ITS-RFLPs) were used for species level identification of the fungal symbionts and were detected as described in Kretzer *et al* (2003). Briefly, ITS-RFLPs were produced with the fungal-specific PCR primer pair ITS1f and ITS4 (Gardes & Bruns, 1993) and the restriction enzyme AluI, which had previously been shown to allow for easy differentiation between *R. vinicolor* and *R. vesiculosus* (Kretzer *et al.*, 2003). Restriction fragments were separated on agarose gels (1% SeaKem GTG and 2% NuSieve GTG agarose from Cambrex Bio Science, Rockland, ME, USA) and stained with ethidium bromide.

Population genetics

Gene diversity D at locus j was calculated $D_j = \sum p^2_i$, (p_i = frequency of allele i at locus j), which is the same as expected heterozygosity under Hardy-Weinberg equilibrium. Nei (1973) introduced the term 'gene diversity' as a measure of genic variation, because 'heterozygosity' is not appropriate in nonrandom mating populations (or in populations where a priori knowledge of random mating does not exist). F_{IS} was calculated according to Weir & Cockerham (1984). Heterozygote excess and deficiency were tested for independently using the method of Rousset & Raymond (1995). Fisher's exact test was used to analyse genotypic linkage disequilibrium between loci. All statistics were calculated in GENEPOP (Raymond & Rousset, 1995) using the web version 3.1c at http://wbiomed.curtin.edu.au/genepop/index.html.

Results

Development of microsatellite markers

A total of 230 recombinant *E. coli* clones from the enriched library were screened by colony hybridization. After sequencing of the inserts from positive clones, 14 different loci were identified that contained either perfect or imperfect

microsatellite repeats. Of those 14 loci, two were discarded because primer design proved difficult due to short flanking sequences, and another one was discarded because more than two PCR bands were occasionally obtained from individual samples. Complete sequences of the remaining 11 loci have been submitted to GenBank under the accession numbers AY117115 to AY117125. The PCR primers were developed based on nucleotide sequences flanking the repeats in *R. vesiculosus* strain T20874, but all primer pairs developed in this study also produced specific PCR products from collections of *R. vinicolor.* Gene diversities ranged from 0.00 to 0.68 in *R. vesiculosus,* and from 0.00 to 0.43 in *R. vinicolor* (Table 1).

At a number of loci, short alleles were preferentially amplified, as indicated by the fact that long alleles generally appeared as weaker bands. This effect was particularly pronounced at loci *Rve1.21, Rve2.77* and *Rve3.21* in *R. vesiculosus* and at the previously published locus Rv02 in *R. vinicolor.* A similar phenomenon has been described as 'short allele dominance' and can lead to false homozygotes and thus an artificial heterozygote deficiency if long alleles go undetected (Warder *et al*., 1998). We have therefore tested all loci published here as well as four previously published loci (Kretzer *et al*., 2000) for deviations from Hardy-Weinberg expectations according to Rousset & Raymond (1995). Of the four loci expected to be affected by false homozygotes, only *Rv02* was found to exhibit a significant excess of homozygotes in *R. vinicolor* at the $P<$ 0.05 level. Excess of homozygotes was also observed at loci *Rv15* and *Rve2.67* in *R. vesiculosus*. Because there was no general trend towards heterozygote deficiency across loci, it seems that the excess in homozygotes observed at locus *Rv02* in *R. vinicolor* and at loci *Rv15* and *Rve2.67* in *R. vesiculosus* is most likely due to short allele dominance or null alleles.These loci are therefore likely to be unreliable for any type of population genetic analysis. By contrast to the heterozygote deficiency described above, a mild heterozygote excess was detected at loci *Rve2.77* and *Rve3.21* in *R. vesiculosus* ($0.05 < P < 0.10$). As mentioned above, we regularly noticed less efficient PCR amplification of long alleles at these loci and thus scored minor and sometimes weak bands as long alleles. This strategy might. occasionally result in contaminants being scored as alleles and homozygote samples being scored as false heterozygotes. Loci with significant deviations from Hardy-Weinberg expectations in either direction were not used to investigate genet size and distribution. See Table 1 for a summary of F_{IS} values and significant deviations.

Loci were also tested for linkage disequilibrium. Of 66 pairwise comparisons possible in *R. vesiculosus*, five were significant at the $P < 0.05$ level (see Table 2). However, when loci *Rv15* (heterozygote deficiency) and *Rve2.71* (low gene diversity) were ignored, only loci *Rve1.34* and *Rve2.14* appeared linked in the Mary's Peak population of *R. vesiculosus* ($P =$ 0.012). Linkage, however, may not be tight, because *Rve1.34* and *Rve2.14* were not consistently linked in other populations of *R. vesiculosus* (data not shown),. neither were they linked in *R. vinicolor.* Inconsistent results with respect to linkage disequilibrium are likely due to the comparably small sample sizes. None of the loci tested appeared linked at the $P < 0.05$ level in *R. vinicolor* (see Table 2).

Genet study

We have subsequently used selected microsatellite markers to differentiate genets of both *R. vesiculosus* and *R. vinicolor* from tuberculate ectomycorrhizas. Fresh tuberculate ectomycorrhizas were found in 15 soil cores at Mary's Peak and in 15 soil cores at Mill Creek (Fig. 1). Tuberculate ecto-

Table 2 Test for linkage disequilibrium between loci; *P*-values in the upper right 'triangle' are for *Rhizopogon vesiculosus*, and *P*-values in the lower left triangle are for *Rhizopogon vinicolor*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *Rv02*	-	0.668	nd	nd	0.826	0.397	nd	0.847	0.693	0.552	0.273	**0.067***	0.172	0.610	0.568
2 *Rv15*	0.868	-	nd	nd	0.506	0.375	nd	**0.032****	0.962	0.761	**0.060***	0.346	0.384	0.457	0.504
3 *Rv46*	0.821	0.450	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4 *Rv53*	0.929	0.838	0.695	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5 *Rve1.21*	nd	nd	nd	nd	-	0.633	nd	0.284	0.881	0.507	**0.057***	0.127	0.205	0.950	0.499
6 *Rve1.34*	0.114	0.686	0.681	0.108	nd	-	nd	1.000	**0.012****	0.851	0.473	1.000	1.000	0.280	0.744
7 *Rve1.41*	0.368	**0.081***	0.933	0.828	nd	0.804	-	nd	nd	nd	nd	nd	nd	nd	nd
8 *Rve2.10*	nd	nd	nd	nd	nd	nd	nd	-	0.683	0.395	0.149	**0.034****	1.000	0.603	0.899
9 *Rve2.14*	nd	nd	nd	nd	nd	nd	nd	nd	-	**0.070***	0.788	1.000	0.441	0.294	0.404
10 *Rve2.44*	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	0.365	**0.021** **	0.104	0.839	0.370
11 *Rve2.67*	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	**0.034****	1.000	0.759	0.407
12 *Rve2.71*	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	0.406	0.745	0.732
13 *Rve2.74*	0.500	1.000	0.674	0.672	nd	1.000	1.000	nd	nd	nd	nd	nd	-	0.303	0.479
14 *Rve2.77*	0.301	0.407	0.470	**0.079***	nd	0.856	0.332	nd	nd	nd	nd	nd	0.236	-	0.994
15 *Rve3.21*	0.981	0.342	0.702	0.831	nd	0.793	0.352	nd	nd	nd	nd	nd	0.499	0.974	-

Asterisks indicate level of significance with *$P < 0.10$ and * *$P < 0.05$; nd = not determined (linkage disequilibrium could not be tested, because data for at least one locus were missing, or because at least one locus was monomorphic).

mycorrhizas formed by either *R. vesiculosus* or *R. vinicolor* are morphologically slightly different (Kretzer *et al*., 2003), but species level identification was confirmed using ITS-RFLPs. Briefly, when using ITS primers 1f and 4, and the restriction enzyme A*lu*I *R. vesiculosus* is characterized by a single band of size 743 bp, while *R. vinicolor* is characterized by either three bands of sizes 419 bp 224 by and 97 bp or two bands of sizes 516 by and 224 by (Kretzer *et al*., 2003). No soil core was found to contain tuberculate ectomvcorrhizas of both *R. vesiculosus* and *R. vinicolor.* When multiple tuberculate ectomycorrhizas were found in a single core, they were often connected by rhizomorphs and preliminary analysis indicated that they belonged to the same genet (data not shown). Hence only one tuberculate ectomycorrhiza from each soil core was subsequently analysed. Tuberculate ectomycorrhizas of R vesiculosus were genotyped at microsatellite loci *Rv02 Rvel.21, Rve1.34, Rve2.l0, Rve2.14, Rve2.44*, and *Rve2.74*, and tuberculate ectomycorrhizas of R vinicolor-were genotyped at loci *Rv15, Rv46 Rv53, Rvel.34, Rve2.77,* and *Rve3.21.* Samples sharing the same multilocus genotype (= same alleles at all loci analysed) are encircled in Fig. 1. For *R. vesiculosus* and *R. vinicolor*, four and three different multilocus genotypes were identified in the Mary's Peak core plot, respectively, and one and three, respectively, at Mill Creek. The greatest distance observed between tuberculate ectomycorrhizas collected in the Mary's Peak and Mill Creek core plots that represent the same multilocus genotype was approx. 13.5 m for *R. vesiculosus*, and approx. 2 m for *R. vinicolor.*

Multilocus genotypes are assumed to represent individual genets of *R. vesiculosus* and R. viuicolor. In order to assess the power of the multilocus genotypes detected in this study to resolve genets of *R. vesiculosus* and *R. vinicolor*, we have used allele frequencies observed in the larger Mary's Peak population to calculate expected frequencies of multilocus genotypes under Hardy-Weinberg expectations and linkage equilibrium (multilocus genotype frequency $f = \prod 2\, p_i q_i \times \prod r^2_j$, where p_i and q_i are the frequencies of two alleles observed at heterozygous locus i, and $r_{j.}$ is the frequency of the allele observed at homozygous locus j). Expected frequencies for multilocus genotypes observed in the Mary's Peak and Mill Creek core plots ranged from 1.4×10^{-5} to 4.2×10^{-3} in *R. vesiculosus* (seven loci) and from 4.2×10^{-6} to 4.1×10^{-3} in *R. vinicolor* (six loci). Thus, the probability that two samples may have the same multilocus genotype by chance rather than by clonal propagation is very low (≤ 0.004).

A second approach was taken to assess the power of the microsatellite markers used in this study to resolve genets of *R. vesiculosus* and *R. vinicolor* that is independent of assumptions of Hardy-Weinberg equilibrium. Figure 2 shows a plot of the average number of genets resolved over the number of loci analysed. In both *R. vesiculosus* and *R. vinicolor*, the maximum number of genets could be resolved using any five of the microsatellite markers. Use of any additional markers (seven total in *R. vesiculosus* and six total in *R. vinicolor*) did not increase the number of genets observed.

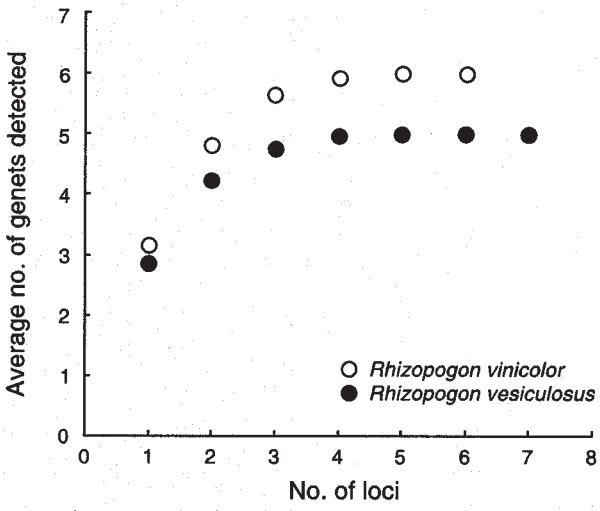

Fig. 2 The average number of multilocus genotypes (genets) detected within the two 10 × 10 m core plots at Mary's Peak and Mill Creek (OR, USA) was plotted over the number of loci analysed. Closed circles, *Rhizopogon vesiculosus*; open circles, *Rhizopogon vinicolor sensu* Kretzer *et al*. (2003).

Discussion

Our choice to enrich and screen for $(CAC)_n/(GTG)_n$ repeats was based on preliminary experiments that used primers with various microsatellite motifs as ISSR primers to estimate which repeat types were common (and polymorphic) in *R. vesiculosus* and *R. vinicolor* (data not shown). The ISSR markers indicated that $(CAC)_n/(GTG)_n$ repeats were fairly common and polymorphic in our target species. This result is consistent with many other reports using $(GTG)_n$ primers to produce polymorphic ISSR markers in a diverse array of fungi (Longato & Bonfante, 1997; Anderson *et al*., 1998; Liu *et al*., 1998; Gherbi *et al*., 1999; Sawyer *et al*., 1999; Zhou *et al*., 1999).

The microsatellite markers developed here as well as four previously published loci (Kretzer *et al*., 2000) were tested for deviation from Hardy-Weinberg equilibrium and linkage dis equilibrium. The majority of markers did not appear to be linked, nor did they deviate significantly from Hardy Weinberg expectations. As a result, we consider the significant heterozygote deficiency observed at loci *Rv15* and *Rve2.67* in *R. vesiculosus* and at locus *Rv02* in *R. vinicolor* as likely artefacts of null alleles or short allele dominance; these loci are not being used further in our population genetic work. When loci *Rv15* and *Rve2.67* were omitted from the *R. vesiculosus* data set and locus *Rv02* was omitted from the *R. vinicolor* data set, there was no global excess of either homozygotes or heterozygotes in either species.

Overall, the data indicate that the larger Marys Peak populations of both *R. vesiculosus* and *R. vinicolor* are largely in Hardy-Weinberg equilibrium. As stated in the introduction, both taxa form hypogeous sporocarps, and their spores are dis-

persed primarily by small mammal mycophagy. However, it is generally thought (although unproven) that only a fraction of all sporocarps are actually consumed by mammals, leaving many to decompose in the soil. Under the latter scenario, high rates of selfing might be expected (because basidiospores germinate to form monokaryons before they mate, selfing is often alternatively referred to as mating of sibling monokaryons). Although the mating system has never been investigated in *Rhizopogon*, it was shown to be bipolar in several species of *Suillus*, which are closely related to *Rhizopogon* (Fries & Neumann, 1990; Fries & 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. However, heterozygote deficiency, as expected under high rates of selfing, was not observed in either species. This finding contrasts with a study on *Hebeloma cylindrosporum* where two large genets were observed to occasionally produce inbred progeny apparently by selfing (Gryta *et al*., 2000); however, while the two parent genets persisted and spread over four years of study, inbred progeny genets were always small and apparently short-lived. It is possible that selfing occurs in both systems without affecting population genetic parameters, because well-established parental genets quickly outcompete inbred progeny. Smith *et al*., (1992) point out that sibling genets created by selfing would be genetically very similar and hard to differentiate with genetic markers. However, as noted above, frequent selfing should lead to a heterozygote deficiency that was not detected in this study.

Genets of ectomycorrhizal fungi can range in size from less than 1 m^2 (Gherbi *et al*., 1999) to, 300 m^2 or more (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). By contrast, *Laccaria amethystina* forms small and potentially short-lived genets in a 150-yr-old forest (Gherbi *et al*., 1999). It is generally assumed that ectomycorrhizal fungi such as *S. bovinus* and *variegatus* and *L. amethystina* represent two extremes in a spectrum of life strategies that are dominated either by long-lived mycelia and abundant vegetative growth or by short-lived mycelia and frequent reinoculation from spores. However, frequency of sporocarp formation is not a reliable indicator for either strategy. For example, although, *S. pungens* was the most abundant sporocarp producer in a *Pinus muricata* stand in California (Garden & Bruns, 1996), it also formed a very large genet in the same stand (Bonello etat, 1998).

Various *Rhizopogon* species infect readily from spores and their spores are routinely used to infect nursery seedlings. The widespread natural occurrence of *Rhizopogon* spores has also been documented by soil bioassays (Molina *et al*, 1999). In this procedure, sterile seeds or nonmycorrhizal seedlings are planted into field-sampled soils and grown in the greenhouse as a 'bait' for ectomycorrhizal spores and other types of resistant inoculum (Taylor & Bruns, 1999). In the field, *Rhizopogon* ectomycorrhizas are common in forests of all age, but are often particularly dominant on recently disturbed sites (Baar *et al*.,, 1999; Molina *et al*, 1999). Based on these aspects of spore ecology, we expected *R. vesiculosus* and *R. vinicolor* to form abundant small genets and designed a dense, small scale sampling scheme to map genets in this study. Interestingly, our data from two 10 x 10 m plots revealed a somewhat unexpected result: In both the Mary's Peak and the Mill Creek plot, *R. vesiculosus* was found to form at least one fairly large genet with maximum distances between samples of 10.3 m and 13.4 m, respectively. By contrast, only much smaller genets were observed for *R. vinicolor*. with maximum distances between samples of approx. 2 m in both plots. Owing to the small plot size, however, genet size in both species could have easily been underestimated; a more extensive study is currently under way.

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