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Field monitoring the seasonal variation in *Albatrellus ellisii* mycelium abundance with a species-specific genetic marker

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Abstract: The conservation of rare fungal sites occurring on actively managed forest lands requires efficient site monitoring methods. In this study species-specific primers for the putatively mycorrhizal *Albatrellus ellisii* (Russulales) were developed so that DNA extracted from soil samples at known sites of this fungus could be tested for the presence of *A. ellisii* mycelium with PCR. This method was used to measure seasonal changes in the levels of *A. ellisii* mycelium at three study sites while the utility of this monitoring method was assessed. We found that *A. ellisii* maintained a constant level of soil occupancy over three seasons, except at one site where mycelium abundance increased in the fall. No reduction in abundance was seen in the summer, although all three sites experienced significant summer drought. We found species-specific genetic marker detection to be an effective and practical method for monitoring the mycelial distribution of a target fungus in soil. The ability to obtain this data from rare fungal sites advances our capability to conserve these fungi, particularly within the managed forest landscape.

Key words: *Albatrellus ellisii*, fungal conservation, mycorrhizal, Pacific Northwest, soil DNA

INTRODUCTION

The protection of known sites of rare fungi is an important element of fungal conservation efforts, and successful site protection within actively managed forestland requires accurate and efficient site-monitoring methods. The need for effective site monitoring is evident in the USA Pacific Northwest, where large areas of federally managed forestland support a rich diversity of fungi (Molina et al. 2001) and where a mitigation measure in the Northwest Forest Plan (NFP), a 1994 federal ecosystem management program, mandated surveys for rare fungi and other organisms (USDA and USDI 1994). These surveys, conducted over 9 700 000 hectares of federal lands, have documented 14 384 sites of 219 rare and little known fungi over 12 y. Although 78% of the survey area is in reserves protected from commercial timber harvest (USDA and USDI 1994), for the 188 rarest species 57% of known sites are located outside reserved areas (Molina 2008). Moreover about 20% of exceptionally rare species (those with 10 or fewer known sites) were found only outside reserves (Molina 2008), indicating that areas open to commercial timber harvest are important habitat for rare fungi in this region.

Managers, concerned with the protection of an identified rare fungal site in an area where timber extraction is planned, have little information on which to base management decisions and face considerable difficulties in monitoring the results of their decisions. Monitoring with sporocarp surveys is problematic because even fungi that fruit annually are sufficiently unpredictable that repeated surveys over the fruiting season are required. Moreover many fungi do not reliably produce sporocarps every year, even though the underground mycelial mass of the fungus is present and active in the ecosystem (Gardes and Bruns 1996, Lodge et al. 2004). This irregular fruiting behavior requires site surveys over several years, with multiple site visits per year (O'Dell et al. 2004). In addition the location of the sporocarp gives only the approximate location of the fungus in the soil and gives no information about the extent of the mycelial mass that produced it or about spatial correlations between the fungus and landscape elements such as dead wood or particular tree species. Biologists relying on sporocarp surveys cannot make informed recommendations about buffer sizes, tree retention, or often even confirm the presence of a species at a particular location. These detection and monitoring difficulties can lead to management decisions that inadvertently harm species or needlessly prevent beneficial projects from going forward.

Studies indicate that the vegetative mycelial growth of a mycorrhizal fungus within the soil typically constitutes the vast majority of the biomass produced by these fungi (Colpaert et al. 1992, Wallander et al. 2001, Sims et al. 2007); so a detection method that targets this biomass should be a particularly effective monitoring tool for these fungi, providing unambig-

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uous presence/absence and location data. Mycelium detection methods based on species-specific PCR have been developed and field tested for *Hebeloma cylindrosporum* (Guidot et al. 2002), *Tuber melanosporum* (Suz et al. 2006), *Lactarius deliciosus* (Hortal et al. 2008) and *Tuber magnatum* (Zampieri et al. 2010). In this study we chose *Albatrellus ellisii*, one of 234 fungal species listed in the NFP as needing mitigation measures (USDA and USDI 1994), to develop and test a PCR-based method for detecting its presence in soil. Some named *Albatrellus* species are actually in the Polyporales (Binder and Hibbett 2002), however *A. ellisii* is a member of *Albatrellus* s.s. (Russulales), a mycorrhizal clade (Högberg et al. 1999, Larsson and Larsson 2003). *A. ellisii* is thought to be mycorrhizal with coniferous trees, but little is known about its ecological requirements (Kropp and Trappe 1982, Gilbertson and Ryvarden 1986, Ginns 1997).

In considering the practicalities of field monitoring concern was raised about seasonal variations in mycelium presence in the soil. In particular because the Pacific Northwest typically experiences summer drought forest soils become quite dry in summer and the coniferous mutualistic partners of *A. ellisii* typically experience significantly reduced photosynthesis due to stomatal closure (Waring and Franklin 1979). Although summer is the least problematic time to conduct field work in remote areas, it may also be a time when the soil-inhabiting mycelium of the target fungus is at a seasonal low. Little is known about the phenology of the mycelium of individual mycorrhizal taxa. Koide et al. (2007) monitored with molecular techniques the seasonal variation in the abundance of hyphae of individual mycorrhizal species in the soil over 13 mo in a red pine forest in Pennsylvania. They found a variety of seasonal growth patterns; one group of species had a constant relative frequency throughout the year, one group had a maximum in autumn and a third group had a maximum in winter or spring. Parrent and Vilgalys (2007) measured mycelial growth into sand-filled bags and identified species with molecular methods. They found that several taxa were more abundant in autumn than in summer in one year, but these trends were reversed the following year when a severe drought occurred. Hyphal response to sand might not have been reflective of responses in the native soil (Hendricks et al. 2006), particularly in the dry year. Other research has focused on the seasonal abundance of fungal species on mycorrhizal root tips (Courty et al. 2008, Smith et al. 2007, Buee et al. 2005), but we cannot assume that the phenology of the mycelium of a fungus is synchronous with that of its mycorrhizae (Koide et al. 2007).

Because the seasonal abundance patterns of the vegetative mycelium of mycorrhizal fungi are largely unknown and appear to be species specific we decided to investigate this issue for *A. ellisii*. In this study we developed species-specific PCR primers for *A. ellisii* and used them to detect the fungus in DNA extracted from soil samples taken at three *A. ellisii* sites in western Oregon, during three seasons. We hypothesized that we would find seasonal differences in the proportion of samples containing *A. ellisii*. At the same time we assessed the feasibility of using this method as a technique for fungal site monitoring and management.

MATERIALS AND METHODS

Specific primer development.—The development of a species-specific marker for *A. ellisii* for use in the Pacific Northwest required us to obtain DNA from representatives of *A. ellisii* and the eight other congeneric species that are found in the region. Fifty-four sporocarp collections of eight different *Albatrellus* species (*A. ellisii*, *A. flettii*, *A. ovinus*, *A. skamanius*, *A. caeruleoporus*, *A. subrubescens*, *A. dispansus* and *A. pescaprae*) were obtained from regional herbaria. In addition seven fresh collections were obtained from members of the Oregon Mycological Society. All sporocarps were collected from coniferous forests, and all were from Oregon and Washington, except one *A. caeruleoporus* collection from British Columbia, Canada. DNA was successfully obtained from 53 of the collections (TABLE I).

For DNA extraction about 30 mg sporocarp tissue was ground with a steel mandrel in a 1.7 mL microcentrifuge tube. A CTAB/borate buffer (0.2 M boric acid, 5 mM sodium tetraborate, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, 0.01 M ascorbic acid) was used in CTAB/chloroform extraction (Stewart and Via 1993). Any highly colored DNA solutions were cleaned with a glass milk DNA purification kit (Geneclean II, Q-Biogene Inc.). Genomic DNA was evaluated by gel electrophoresis with agarose gels and GelStar nucleic acid stain (Lonza Group Ltd.).

We used the successfully extracted *Albatrellus* DNA in a randomly amplified polymorphic DNA (RAPD) study to find a genetic marker specific to *A. ellisii*. First, a RAPD primer-screening study was done with DNA samples from five *Albatrellus* species run in PCR with each of six decamer primers in a RAPD analysis primer set (No. 27-9501-01, Pharmacia Biotech). Reactions were 25 μL and contained 1 U Taq polymerase and 1× Taq buffer (Sigma), 2.5 mM $MgCl_2$, 200 μM each dNTP and 2.0 μM primer. PCR conditions for all RAPD reactions were 94 C for 2 min, then 28 cycles of 94 C for 20 s, 35 C for 55 s, 72 C for 90 s, followed by final extension at 72 C for 4 min and cooling to 4 C. PCR products from the primer screening reactions were viewed on a 1.8% agarose gel containing GelStar nucleic acid stain.

Primer 06 produced a strong 790 base pair (bp) RAPD marker with *A. ellisii* that was not present in the PCR products from the other species tested in the primer

TABLE I. Sources of *Albatrellus* DNA used in this study

Albatrellus Species	Herbarium collection number[a,b]	Origin
A. ellisii	SMM 2009	OR, Klamath County
A. ellisii	SMM 61927	OR, Wasco County
A. ellisii	SMM 1951	OR, Klamath County
A. ellisii	SMM 4920	OR, Klamath County
A. ellisii	SMM 4927	OR, Klamath County
A. ellisii	SMM 1755	OR, Linn County
A. ellisii	SMM 2013	OR, Klamath County
A. ellisii	SMM 4932	OR, Klamath County
A. ellisii	SMM 4919	OR, Klamath County
A. ellisii	SMM 1972	OR, Klamath County
A. ellisii	SMM 6021	WA, Skamania County
A. ellisii	SMM ALEL-G1-102902	OR, Klamath County
A. ellisii	SMM ALEL64	WA, Chelan County
A. ellisii	WTU 10.4.00	WA, Chelan County
A. ellisii	OSC 67689	OR, Klamath County
A. ellisii	OSC 70308	OR, Klamath County
A. avellaneus	g0306 (fresh)	OR, Tillamook County
A. avellaneus	g0307 (fresh)	OR, Tillamook County
A. avellaneus	g 0308 (fresh)	OR, Tillamook County
A. avellaneus	g0309 (fresh)	OR, Tillamook County
A. avellaneus	g0310 (fresh)	OR, Tillamook County
A. avellaneus	g0311 (fresh)	OR, Tillamook County
A. caeruleoporus	WTU 10842	Canada, B.C.
A. caeruleoporus	WTU 10.14.94	WA, Snohomish County
A. dispansus	WTU 773	WA, Yakima County
A. flettii	SMM 3935	OR
A. flettii	SMM 1760	WA, Skamania County
A. flettii	SMM 3917	OR, Marion County
A. flettii	SMM 5276	OR, Douglas County
A. flettii	SMM 4901	OR, Klamath County
A. flettii	SMM 3934	OR
A. flettii	SMM 5277	OR, Douglas County
A. flettii	SMM 4899	OR, Klamath County
A. flettii	SMM 5252	OR, Douglas County
A. flettii	SMM 3514	OR, Lane County
A. flettii	SMM 5286	OR, Douglas County
A. flettii	SMM 6023	WA, Skamania County
A. flettii	SMM 6018	WA, Skamania County
A. flettii	SMM 6017	WA, Skamania County
A. flettii	WTU 12693	WA, Skagit County
A. ovinus	SMM 3919	OR, Coos County
A. ovinus	SMM 3922	OR, Lane County
A. ovinus	OSC 70322	OR, Klamath County
A. ovinus	OSC 74911	ID, Clearwater County
A. ovinus	WTU 11091	WA, Yakima County
A. ovinus	WTU 8497	OR, Tillamook County
A. ovinus	g0315 (fresh)	OR, Tillamook County
A. pescaprae	OSC 66233	OR, Linn
A. pescaprae	WTU 11840	WA, Skagit County
A. skamanius	OSC 70203	WA, Skamania County
A. skamanius	WTU 9750	WA, Skamania County
A. subrubescens	WTU 11397	WA, Chelan County
A. subrubescens	WTU 11406	WA, Chelan County

[a] SMM refers to collections (now at OSC) made under the survey and manage mitigation measure.
[b] Fresh collections were not archived.

screening. The specificity of this amplicon was confirmed with further testing of *Albatrellus* DNA samples with primer 06. One of the 790 bp *A. ellisii* amplicons was isolated from a gel of RAPD products and purified with Geneclean II (Qbiogene). The amplicon was cloned with the TOPO TA kit for sequencing (Invitrogen Corp., Carlsbad, California) according to the manufacturer's instructions. DNA was extracted from three separate transformed *Escherichia coli* cultures, and the plasmid insert from each of the three cultures was sequenced bidirectionally with T3 and T7 primers.

Amplicon sequences were found to be identical, and a forward and reverse primer targeting 219 bp of this sequence were designed with the assistance of the Netprimer (www. premierbiosoft.com) program. To avoid nontarget species interactions the primer sequences were checked for homology to sequences in the GenBank database with the primer BLAST algorithm.

Specific primer testing.—The putative specific primers were tested in PCR with all 53 successfully extracted *Albatrellus* DNA samples. Reaction chemistry and cycling parameters were the same as described earlier except 0.4 μM primers were used and the anneal temperature was 62 C. Reaction products were checked for the presence of the 219 bp amplicon in 1.9% agarose gels containing GelStar nucleic acid stain.

A dilution series of *A. ellisii* sporocarp tissue in soil was prepared by crushing fresh frozen sporocarp tissue with soil with a mortar and pestle. The initial dilution was 1000 μg fungus per gram soil, and subsequent dilutions were made with soil in 10-fold steps down to 1.0 μg fungus per gram soil. Three soil samples at each dilution were tested by extracting DNA and conducting PCR and gel electrophoresis as described below.

Study areas.—Data from herbarium collections indicate that *A. ellisii* is found in coniferous forests, usually above 1000 m. Habitat, when mentioned, typically was described as ''mature'' or ''old growth'' forest. In several cases collectors noted previous timber harvest at the collection site but always with a significant canopy of retained large trees. Three study areas, current or past *A. ellisii* sites, were chosen to represent the range of ecological conditions used as habitat by this fungus (FIG. 1). The Frog Creek site (10 608427E, 5004465 N), 1036 m, had 100–200 y old *Pseudotsuga menziesii* and *Tsuga heterophylla* as the dominant vegetation with an understory of *Taxus brevifolia*. This site was flat with deep soil. The Olalla Creek site (10 454391E, 4759543N), at (an unusually low) 305 m, was dominated by 120–140 y old *P. menziesii* and *Abies concolor*. The understory included *Acer circinatum* and *Taxus brevifolia*. This site was steep with deep soil; however a few rocky patches covered about 20% of the site. The Sevenmile Creek site (10 570413E, 4728836N), 1524 m, was dominated by 100–200 y old *A. concolor* and *Picea engelmannii* with a well developed herb layer but no tree or shrub understory. This site was flat with thin, rocky soil.

Soil sample collection.—A form of spatially balanced sampling was done to distribute the sample points in the

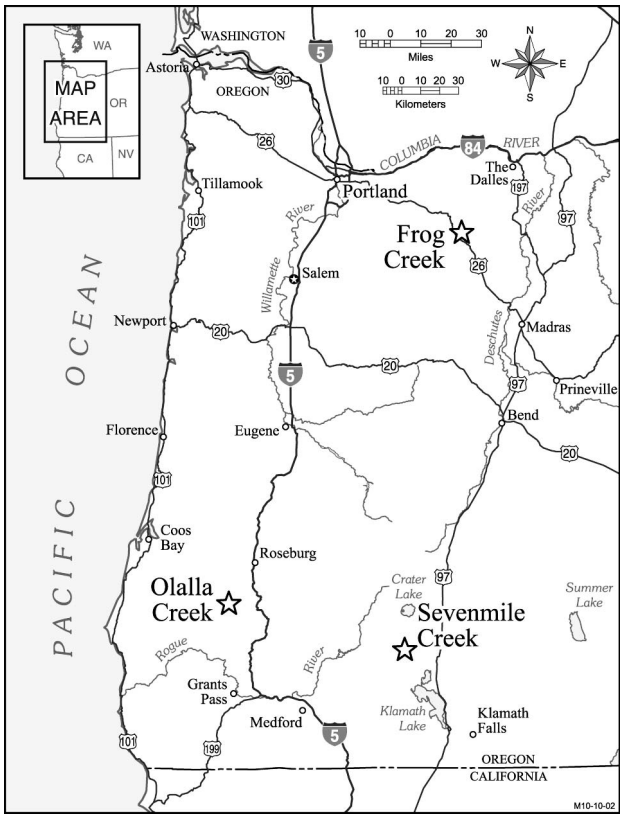

FIG. 1. *Albatrellus ellisii* study sites.

study sites. In each site 16 grid cells, each 2 × 10 m were laid out. Seven sampling locations were selected randomly within the first grid cell, and the same seven relative locations were sampled in all of the grid cells, giving a total of (seven samples per cell × 16 cells) 112 samples at each site for each sampling visit.

Samples were collected with a 2.5 cm diam chrome-plated steel soil probe. In sample collection the organic layer was scraped away then the soil probe was inserted perpendicular to the ground to remove a soil core 15–25 cm long. The organic layer (top 2–3 cm) was not sampled because preliminary testing in the vicinity of an *A. ellisii* sporocarp showed that *A. ellisii* was more reliably detected in the mineral layer than the organic layer (data not shown). Soil samples were sealed in plastic bags and kept chilled while in the field. The steel probe was thoroughly washed, rinsed and dried between samples to prevent carry-over of soil from one sample to the next. The cleaning procedure was tested by taking a sample near a previous sporocarp location, cleaning the probe, then taking a soil sample in a disturbed area near a road, where *A. ellisii* was not expected to be present. Nine pairs of these cleaning test samples were taken. These pairs of samples were tested for the presence of *A. ellisii* as described in *Soil DNA analysis* below.

The study design called for repeated measurements; so each sampling point was marked with a wooden stake on the first visit to each site. Samples on subsequent visits were

taken within 6 cm of the marked sampling points. Samples were transported to the lab in a cooler at 2–5 C. All samples were frozen within 72 h of collection and kept frozen until DNA extraction.

Spring samples were taken within 2 wk of snowmelt, the summer samples at the end of the dry period, and the fall samples after Oct 1 when at least 8 cm rain had fallen in the previous 2 wk. Because of the range of elevations of the study sites the sampling dates varied from site to site. All samples were taken in 2006, except for the fall sampling of the Frog Creek site. Snowfall began shortly after fall rains in 2006, and the sampling window was missed. The fall sampling for this site occurred in 2007.

Soil sample analysis.—Soil DNA analysis. After hand mixing the soil in a sample bag, 1.0–1.3 g soil was transferred to a 2.0 mL centrifuge tube, and DNA was extracted with a Chelex extraction buffer. To each tube containing a soil sample, 800 μL Chelex buffer (200 mM Tris pH 8.6, 8% Chelex 100 [Bio-Rad Laboratories], 1% Triton X-100) was added. Tubes were spun briefly, heated to 94–97 C for 5 min, spun 30 s, returned to 94–97 C for 10 min, then frozen. Before use samples were thawed then centrifuged 2 min before 100 μL supernatant was removed and cleaned with a DNA purification kit (UltraClean DNA Purification Kit, MoBio Inc.).

Cleaned soil DNA samples were tested in reactions containing 2 μL cleaned DNA solution, 1 U JumpStart Taq polymerase (Sigma). The recommended buffer with increased (2.5 mM) $MgCl_2$, 200 μM each dNTP, 0.4 μM Aell_f, 0.4 μM Aell_r, and 5.0 μg bovine serum albumin (Biotechnology Grade, Amresco). Total volume of each reaction was 25 μL. Reactions were subjected to 94 C for 90 s, followed by 40 cycles of 94 C for 20 s, 62 C for 50 s, and 72 C for 60 s, followed by 72 C for 2 min and cooling to 10 C. PCR products were viewed in agarose gels, and samples showing a distinct 219 bp band were scored as positive. In the first set of samples two positive bands were randomly selected, excised from the gel, cleaned and sequenced to confirm that the band being scored as positive was the expected *A. ellisii* sequence. Negative and positive controls were used for both DNA extraction and PCR.

Soil water content. After each soil sampling and after removing the subsample required for DNA analysis four of the soil samples were selected for water content measurement by randomly choosing one sample from each quadrant of the study site. On two occasions five samples were selected. Water content was measured by heating the soil 20 min at 200 C and measuring the weight loss.

Statistical analysis. All statistical analysis was done with SAS 9.1. The CATMOD (categorical data modeling) procedure was used to analyze the soil probe results. All significance testing was done at the 0.05 level.

RESULTS

Primer development and testing.—Usable DNA was obtained from all seven DNA extractions from fresh material and 46 of 53 extractions from herbarium collections (TABLE I). DNA was obtained from all nine *Albatrellus* species found in the Pacific Northwest.

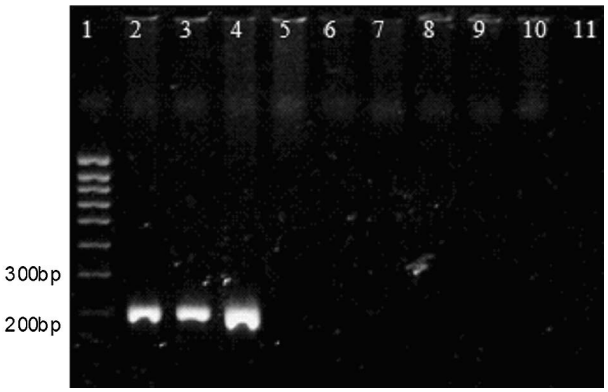

FIG. 2. Gel of products of PCR of Aell_f, Aell_r, and DNA from *Albatrellus* sporocarps. DNA sources were from left to right: molecular weight marker, *A. ellisii* (SMM 4927), *A. ellisii* (OSC 67689), *A. ellisii* (SMM 1972), *A. pescaprae*, *A. flettii*, *A. ovinus*, *A. avellaneus*, *A. skamanius*, *A. subrubescens*, PCR blank (no DNA).

All six sequences generated from the 790 bp *A. ellisii*-specific RAPD marker were compared visually, and only minor discrepancies were found. A single consensus sequence was generated from the alignment, and this sequence was submitted to GenBank (accession number EU069821). The candidate primers designed from this marker, Aell_f (5′TAGTCATGTCGGTATGGTCAAGGCTG) and Aell_r (5′CGAGCCTTTGTTTCTGACTGGGAC), generated a 219 bp amplicon in PCR with *A. ellisii* DNA.

A primer BLAST query of the GenBank sequence database yielded no potential amplicon-generating homologies among Aell_f and Aell_r and sequences in this database. When Aell_f and Aell_r were tested on *Albatrellus* DNA samples all *A. ellisii* samples (16 collections) tested positive while all other samples (37 collections from eight other *Albatrellus* species) tested negative. We illustrated a typical gel of PCR products from reactions containing *Albatrellus* DNA and Aell_f and Aell_r (FIG. 2).

All spiked soil samples at 1000 μg fungus per gram soil, 100 μg fungus per gram soil, and 10 μg fungus per gram soil were positive for the *A. ellisii* marker. The marker was not detected in the 1 μg fungus per gram soil samples, indicating that the protocol described here has a detection limit of 10 μg fungus per gram soil. The sequences of the two amplicons excised from a gel of PCR products derived from soil samples both were identical to the expected amplicon sequence.

Soil probe cleaning test.—Nine pairs of samples were taken in which the first sample of the pair was taken from the study site and the second sample in the pair

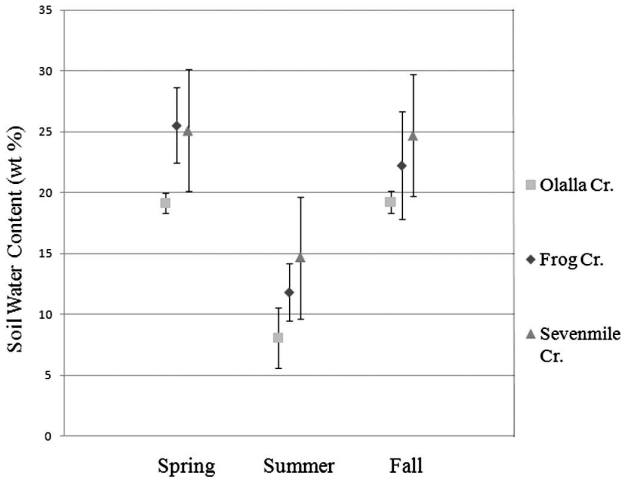

FIG. 3. Seasonal variation in soil moisture at the three study sites in 2006. Error bars represent one standard deviation.

was taken from disturbed soil where *A. ellisii* was not expected to be present. Of these nine pairs six of the first samples were positive while none of the second samples were positive, indicating that the procedure used to clean the soil probe between samples was effective at preventing DNA carryover between samples.

A. ellisii *site testing.—Soil water content.* Soil water content showed a similar seasonal trend at the three sites (FIG. 3), with summer values much lower than spring and fall. A general linear model (GLM) was run to determine whether season has a significant effect on soil water content. Season was found to have a significant effect at each site, so an analysis was done to test the differences between seasons. For each site summer was found to be significantly different from spring and fall. Low values of soil water content in summer are the result of the regional climate pattern of the Pacific Northwest, which includes a warm, dry summer. In the 10 wk before summer sampling visits, the Olalla Creek site received 0.3 cm rainfall, the Frog Creek site 1 cm and the Sevenmile Creek site 2 cm (data from the nearest National Weather Service station).

A. ellisii *occupancy.* Frog Creek and Sevenmile Creek sites showed little seasonal variation in site occupancy, while the Olalla Creek site had an increase in occupancy from spring to summer and again from summer to fall (TABLE II). The statistical model used to analyze the data was a weighted least squares categorical data model (CATMOD), capable of handling repeated measurements. The sample data has repeated measurements (seasons) from three study areas (locations) with categorical data (presence/absence) making it an appropriate model. The analysis

TABLE II. Fraction of 112 soil samples testing positive for *A. ellisii* each sampling visit

Site	Spring	Summer	Fall
Frog Creek	0.31	0.26	0.25
Olalla Creek	0.51[a]	0.63[a]	0.75[b]
Sevenmile Creek	0.28	0.22	0.33

[a,b] Different superscripts indicate significantly different values (at each location).

was done to determine whether there are differences in the proportion of positive detections of *A. ellisii* among seasons, locations, and an interaction of location and season. The model includes all the locations and seasons. The results of this test show that the interaction between location and season is significant ($P = 0.0083$); therefore no conclusion can be made about the main effects of location and season, and the model was modified to test for differences among seasons for each location. Olalla Creek was the only location where season had a significant effect ($P = 0.0007$). Contrasts were used to look at the differences in proportion of positive detections among seasons for only Olalla Creek. This analysis indicated that the proportion of positive detections in the fall differs significantly from spring ($P = 0.0002$) and summer ($P = 0.0167$). Spring and summer are not significantly different ($P = 0.0646$) at the .05 level.

This statistical analysis applies only to the study sites of Olalla Creek, Frog Creek and Sevenmile Creek, and no inferences outside these study areas can be made. Because grid cells were used only to spatially distribute the sample points and they are of no intrinsic interest they were not used in the analysis. As a consequence the variance of the points within the grid cells and the variance among the grid cells cannot be differentiated.

DISCUSSION

This study demonstrated the effectiveness of using a genetic marker to monitor the mycelial mass of a mycorrhizal fungus in its belowground habitat. Our detection limit of 10 μg of *A. ellisii* tissue per gram soil is comparable to that found in similar studies. With specific primers and DNA extracted from soil detection limits of 11.4 μg and 2 μg hyphae per gram soil were found respectively for *Tuber melanosporum* (Suz et al. 2006) and *Lactarius deliciosus* (Parladé et al. 2007). The method used in this study appears to be a practical field monitoring technique for fungi. Soil sample collection was straightforward; after the plots were laid out 100 samples could be collected by one person in about 6 h. The most difficult lab work was done in the genetic marker development phase, so

that only the most basic molecular procedures were needed to process soil samples to obtain results.

One issue affecting the interpretation of the results was the detection of spores that might be present in the soil samples. Assuming a mass of 2 ng ($2000\ \mu^3 \times 10^{-12}\ g/\mu^3$) per spore, our measured detection limit of 10 μg fungal tissue per gram soil corresponds to a detection limit of 5000 spores per gram soil or roughly 10^6 spores per soil core. Although *A. ellisii* sporocarps were not present at any of the sites, it is possible that spores produced in previous years remained in the soil in a viable state. Studies have shown that spores of *Rhizopogon* species can remain viable for many years within the soil spore bank (Bruns et al. 2009). Spore durability in *Rhizopogon* might have developed in association with its spore dispersal mechanism, which relies on small mammal mycophagy, and other fungi might have less resistant spores. For example Miller et al. (1994) measured soil spore levels in the spring at locations where epigeous mycorrhizal sporocarps (*Suillus*, *Lactarius*, *Russula*, *Tricholoma*) had fruited the previous fall. They found no spores at 3–6 cm deep in the mineral soil and greatly reduced or no spores in the first 3 cm. At the same time they found high numbers of spores at locations where two *Rhizopogon* species had fruited. In the current study we made no distinction between viable spores and mycelium, and for conservation purposes both are desirable and worthy of protection.

We also assumed that any *A. ellisii* DNA released into the soil after cell death would be quickly degraded. Romanowski et al. (1992, 1993) found that nucleases present in most soils act within days to reduce high molecular weight DNA to undetectable. However at particular sites biological or physical conditions may exist that let extracellular DNA exist for longer periods (Demaneche et al. 2001). Studies have shown both the quick destruction of extracellular DNA in soil and its prolonged persistence, depending on soil pH, cation valence and concentration, and type of mineral present (reviewed in England et al. 1998). In the future it would be worthwhile to experimentally evaluate the study site soil's ability to break down fungal DNA. In our study samples that were positive in one season often were followed by a negative result in the corresponding sample from the next season (data not shown), indicating that the soils had some capability to break down DNA released by senescing hyphae.

Our study did not find a reduction in *A. ellisii* mycelium in soil during the seasonal dry period. Because this study was not replicated over years the results must be taken as preliminary, but clearly *A. ellisii* is capable of maintaining its presence through periods of soil drying. Because of the fall increase in mycelium abundance at Olalla Creek and no seasonal change at the other two sites we found a significant interaction between season and location for mycelial occupancy. The only other study we are aware of in which soil mycelium of individual species of ectomycorrhizal fungi were monitored seasonally (Koide et al. 2007) was not replicated spatially, so it remains a question for further study whether the mycelial phenology of mycorrhizal fungi typically varies by site or is generally independent of ecosystem variables.

The genetic marker provided a more accurate assessment of the presence of *A. ellisii* than visual monitoring for sporocarps at the study sites. At the three sites a total of three mushrooms were produced in 2005 and none were produced in 2006. Visual surveys for this species in 2006 (confirmed in 2007 at Frog Creek) support the hypothesis that the species is declining or absent at these sites, while genetic probing of the soil shows that this is not the case. *A. ellisii* might be representative of a group of fungi that are long-lived but fruit infrequently, investing more in vegetative growth, competition and physiological adaptability (Gardes and Bruns 1996, Horton and Bruns 2001). This developmental pattern makes these fungi extremely difficult to study with sporocarp observation but particularly straightforward to monitor with a genetic marker. In the context of an actively managed forest with historically known sites of rare fungi, genetic marker probing can give biologists timely access to hard data that lets them make (and defend) rational management decisions such as buffer establishment, despite the sporadic nature of mushroom production.

Results from the Olalla Creek site demonstrate the use of this method in targeted surveys for rare fungi. There was a record of *A. ellisii* in this area in 1997 with imprecise location data. The next time a visual survey was done, in 2005, no sporocarps were found. Later in 2005 before this study 20 soil samples were taken in the general area of the 1997 sighting and the *A. ellisii* marker was found in the DNA extracted from several of these samples. In a few days the genetic test was able to determine the location of the colony referred to in the historical record, while 2 y of visual surveys could not even confirm its presence. Other molecular methods exist for monitoring the mycelium of individual species, such as terminal restriction fragment length polymorphism (Koide 2007) and sequencing of cloned amplicons (Landeweert et al. 2003, Parrent and Vilgalys 2007), and these methods give information on the broad fungal community. However the use of a species-specific PCR marker is much simpler and less expensive than other methods and so allows for more extensive sampling for a given

amount of financial support when interest is focused on one or a few species.

The use of species-specific primers to detect the DNA of a target fungus in DNA extracted from soil samples was found to be a practical method for the field monitoring of a fungus at known sites, with some cautions. Site-specific work should be done to determine the persistence time of fungal DNA released into the soil after cell death, and the possible effect of previously released spores of the target species should be recognized. In addition to monitoring this relatively simple molecular method should be useful in performing targeted surveys for rare fungi and in assessing the effects of experimental treatments in controlled studies.

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