Final Report
For Contract # AG-046W-P-05-0029

Soil Molecular Probe Field Sampling For Fungus *Albatrellus ellisii*

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Table of Contents

Introduction

Summary

Three sites inhabited by the rare fungus *Albatrellus ellisii* were monitored in three different seasons using a species-specific DNA marker. Occupancy was measured by taking soil samples from plots, extracting DNA from the soil samples, and testing these soil samples for the *A. ellisii* specific marker. Two of the sites showed no change in occupancy over the three sampling events, and one site showed an increase in site occupancy from spring to summer and from summer to fall, such that the fall occupancy level was significantly higher than the spring and summer levels. It is not clear if the change in occupancy at this site is seasonal, or if the fungus is changing size in response to some other environmental variable. The site that experienced the change in occupancy was located at a particularly low elevation for this species. Although a spatial analysis was not part of this study, inspection of the point data indicate that the physical distribution of this fungus changes over small spatial scales (1 m) over short time scales (months) at all of the sites. Interestingly, although the occupancy of the sites by *A. ellisii* was found to be stable over the course of the time of this study, new mushrooms were not produced during the study at any of the sites. Visual surveys for this species at these sites would have supported the hypothesis that the species is declining, while DNA testing for the species in the soil shows that this is not the case.

Background

A method that can detect the DNA of two fungal species, *Albatrellus ellisii* and *Albatrellus flettii*, was developed and tested in 2004 (see documents associated with BLM contract # HAP044122). Based on the success of this work, contract # AG-046W-P-05-0029, "Soil Molecular Probe Field Sampling for Fungus *Albatrellus ellisii*" was issued in 2006. The main purpose of the project funded by this contract is to determine if there is a seasonal variation in the extent of soil occupancy of individual colonies of *Albatrellus ellisii*, and if so, to determine the magnitude of the seasonal variation. Additional goals of this project are to develop and document an efficient method for collecting soil samples for DNA analysis, and to document the effectiveness of quality controls in sampling, sample handling, and lab work.

Materials and Methods

Study Areas

The contract called for the identification of three separate colonies of *Albatrellus ellisii* in "different climatic and vegetative habitats". The locations of known *A. ellisii* sites were reviewed by the contractor and COR, and three sites that fulfilled the requirements of the contract were selected. The site locations are shown in Figure 1, and Table I provides basic information on each of the sites. Further information on these sites can be found in the Supplementary Materials.

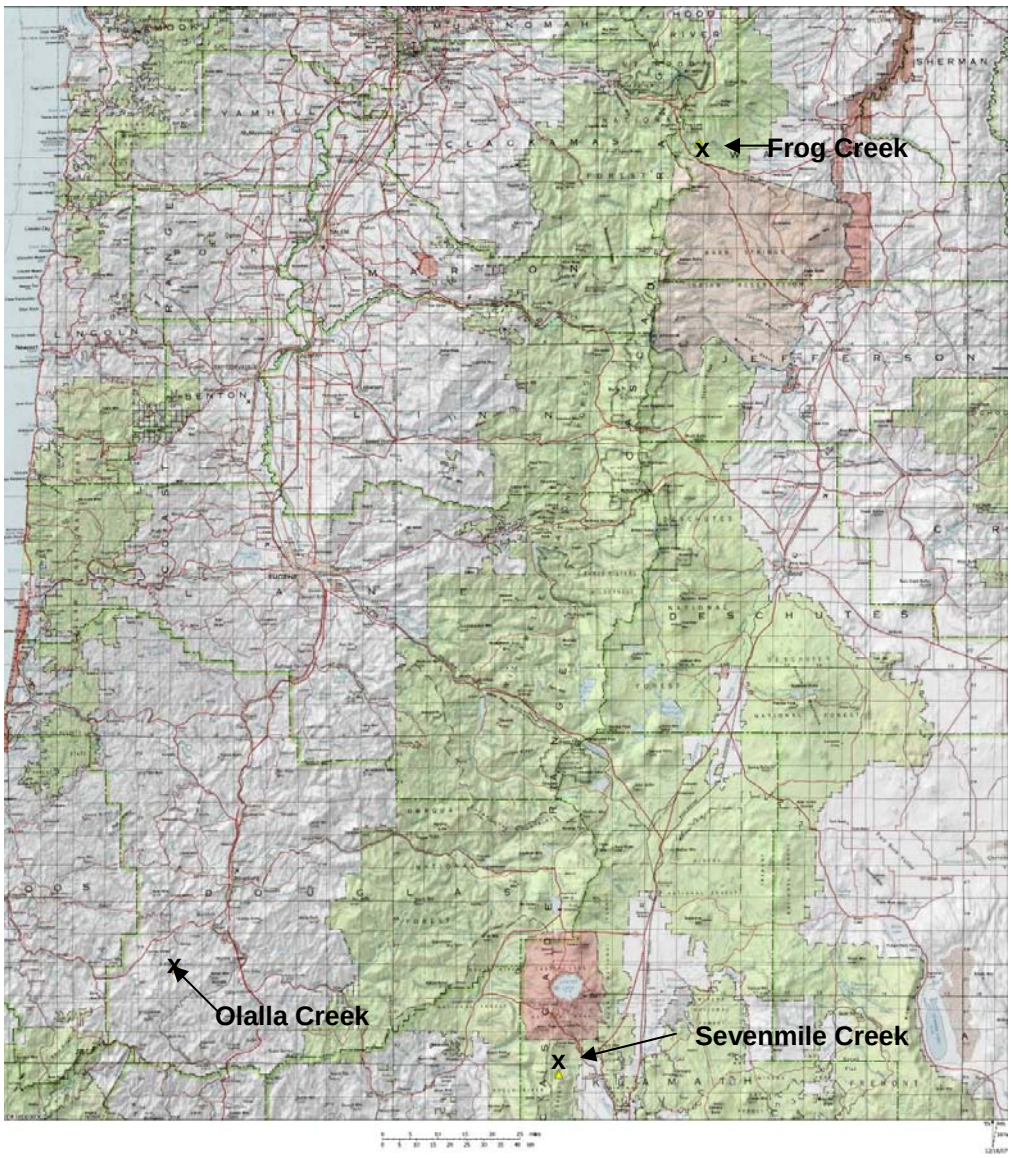


Figure 1. *Albatrellus ellisii* study site locations.

Table I Summary of Study Site Characteristics

Site Name	Administration	Latitude	Longitude	Elevation	Dominant Vegetation	Site Type
Frog Creek	Barlow R.D. Mt. Hood N.F.	5004465 N	608427 E	3400’	PSME, TSHE 100-200 yo	Flat, deep soil
Olalla Creek	S. Douglas R.A. Roseburg BLM	4759543 N	454391 E	1000’	PSME, ABCO 120-140 yo	Steep, deep soil with rocky patches
Sevenmile Creek	Klamath R.D. Winema N.F.	4728836 N	570413 E	5000’	ABCO, PIEN 100-200 yo	Flat, rocky, thin soil

Sampling

A form of spatially balanced sampling was done to avoid the clustering of randomly selected sample points in one area of the study site. In each of the study sites, 16 subplots, each measuring 2m by 10m were laid out. Seven sampling locations were randomly selected for the first subplot, and the same seven locations were sampled in all of the subplots. The pattern of subplot distribution at each study site varied from site to site, based on prior knowledge of the fungus distribution at the sites and surface features present at each site. Diagrams of the subplot arrangements are shown in Figures S1 through S3.

Samples were collected using a 2.5cm diameter plated steel soil probe. Samples were taken within 6 cm of each sample point of the previous visit. Some sample points were required at places where it was physically not possible to take a sample, for example where a large rock was located. These samples were taken as close to the required sample point as possible.

Soil samples were sealed in plastic bags and kept chilled while in the field. Samples were transported to the lab in a cooler at 2° C to 5°C. All samples were frozen within 72 hr of collection, and kept frozen until DNA extraction.

All spring samples were taken within two weeks of the site being snow free, the summer samples at the end of the summer dry period, and the fall samples after substantial fall rain. Because of the range of elevations of the study sites, the sampling date 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 the fall rains in 2006 and the sampling window was missed. The fall sampling for this site occurred in 2007. Details of sampling visit dates and some soil conditions can be found in Table II.

Lab Work

DNA Analysis

DNA was extracted from 1.0 to 1.3 g of soil from each sample using a standard Chelex extraction method. To minimize the effects of co-extracting interfering compounds in the soil, each DNA sample was cleaned using a DNA clean up kit (UltraClean GelSpin DNA Purification Kit, MoBio Inc.) Each cleaned DNA sample was run at least once in a PCR with *Albatrellus ellisii*-specific primers. PCR products were visualized on an agarose gel and photographed. Samples that showed a distinct 219 bp band were scored as positive (e.g. Figure 2). Results were entered into a spreadsheet.

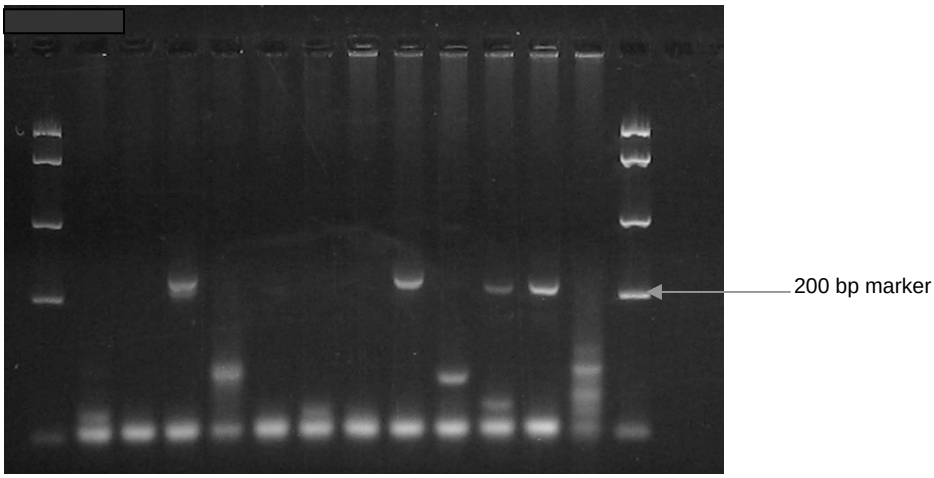


Figure 2. Typical gel of PCR products of soil DNA run with *A. ellisii*-specific primers. Lanes 1 and 14 are markers (2nd band from bottom is 200 bp), and lanes 4, 9, 11, and 12 are positive for *A. ellisii*. All soil DNA is from the Frog Creek site, except lane 8, which has no DNA (negative control).

Soil Properties

Soil water content was measured for each visit to each site. Water content was measured by heating a known weight of soil for 20 min at 200º C, and measuring the weight loss. For each visit, at least 4 samples from different parts of the site were measured.

The soil temperature for each site visit was measured by placing the sensor for a digital thermometer about 15 cm underground and recording the reading after it had equilibrated.

Statistical Analysis

All statistical analysis was done with SAS 9.1. The CATMOD (Categorical Data Modeling) procedure was used. This procedure models categorical data with weighted least squares analysis and can handle repeated measurements. All significance testing was done at the .05 level. Statistical analysis was provided by Carol Apple, Forest Service mathematical statistician.

Results

Soil water content is summarized in Table II. The results of the *A. ellisii* DNA test for all individual samples, sample times, and sites are found in the spreadsheet entitled "Albatrellus Data_071207". The data is summarized in Table III and Figure 3. The fraction positive for a site will also be referred to as the "site occupancy" in this report.

Table II Site sampling dates and soil conditions

Site	Sampling Date	Soil Water Content	Soil Temp. (ºC)
Frog Creek	5/22/06	25%	47.2
Olalla Creek	4/22/06	19%	43.4
Sevenmile Creek	7/01/06	25%	58.5
Frog Creek	9/07/06	12%	53.2
Olalla Creek	9/19/06	8%	53.0
Sevenmile Creek	9/25/06	15%	43.8
Frog Creek	10/12/07	22%	43.2
Olalla Creek	12/09/06	19%	43.0
Sevenmile Creek	11/12/06	25%	38.0

Table III Summary of Results for Each Study Site

Site	Season	Fraction Positive
Frog Cr.	spring	.31
Frog Cr.	summer	.26
Frog Cr.	fall	.25
Olalla Cr.	spring	.51
Olalla Cr.	summer	.63
Olalla Cr.	fall	.75
Sevenmile Cr.	spring	.28
Sevenmile Cr.	summer	.22
Sevenmile Cr.	fall	.33

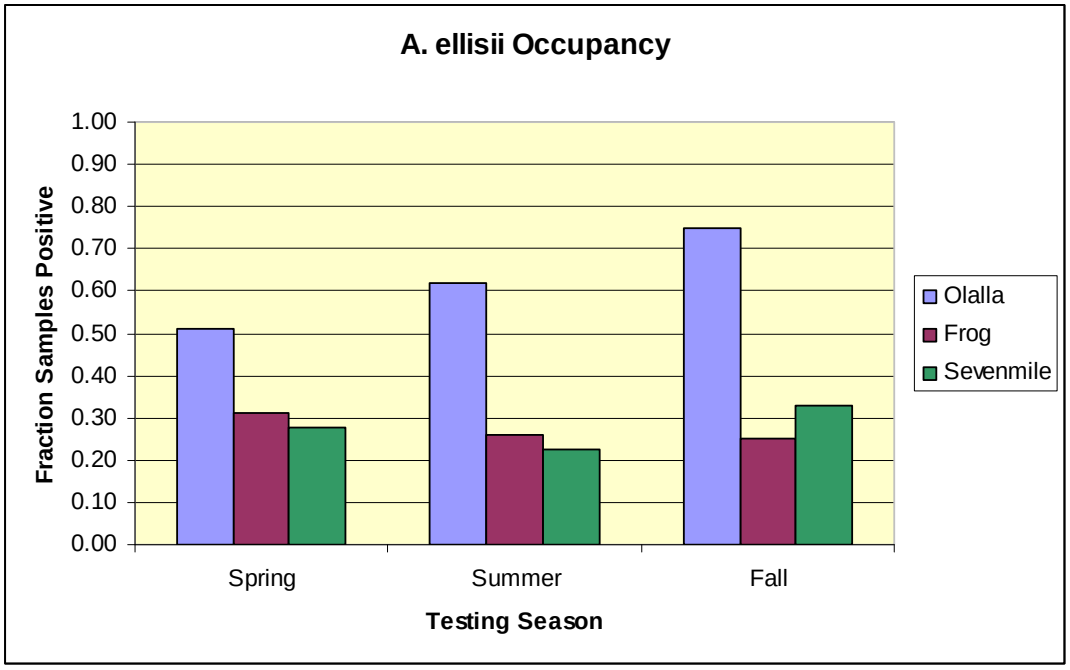


Figure 3. Positive DNA tests at each site for each season.

Statistical Analysis

The results were tested using a weighted least squares categorical model to determine if there are differences in the proportion of positive detections of *Albatrellus ellisii* among seasons, locations, and an interaction of location and season. The model includes all the locations and seasons. The results of this test (Table IV) show that the interaction between location and season is significant. With this interaction being significant, no conclusion can be made about the main effects of location and season.

Table IV

Analysis of Variance

Source	DF	Chi-Square	Pr > ChiSq
Intercept	1	659.73	<.0001
location	2	111.03	<.0001
season	2	6.81	0.0332
location and season interaction	4	13.70	0.0083
Residual		0	.

Since the interaction between location and season is significant, the model was modified to test for differences among seasons for each location. The results (Table V) show that Olalla Creek is the only location where season has a significant effect.

Table V

Analysis of Variance

Source	DF	Chi-Square	Pr > ChiSq
Intercept	1	659.73	<.0001
location	2	111.03	<.0001
seasons at Olalla	2	14.59	0.0007
seasons at Frog	2	1.41	0.4948
seasons at Sevenmile	2	3.12	0.2103
Residual		0	

Contrasts were used to look at the differences in proportion of positive detections among seasons for only Olalla Creek (Table VI). Significant test results indicate that the values being compared are significantly different. Contrasts were also used to check for linearity, a linear trend in the proportions across seasons (Table VI). A significant result means that there is a significant departure from linearity. A non-significant result means that there is no departure from linearity.

Table VI

Analysis of Contrasts

Contrast	DF	Chi-Square	Pr > ChiSq
spring vs summer	1	3.41	0.0646
spring vs fall	1	13.89	0.0002
summer vs fall	1	5.72	0.0167
linearity	1	0.01	0.9257

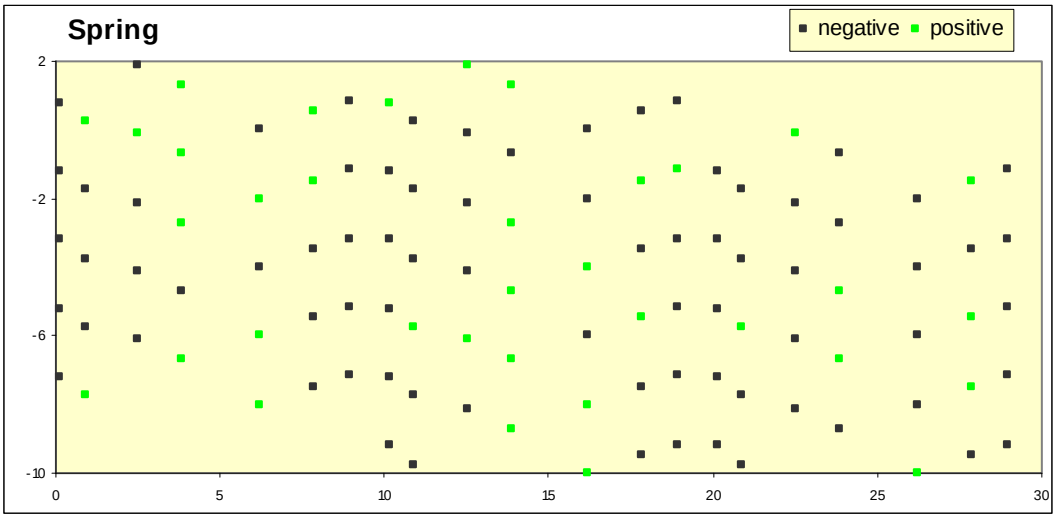
For Olalla Creek, the proportion of positive detections in the fall differs significantly from spring and summer. Spring and summer are marginally significantly different. The test for linearity shows that there is a linear trend across seasons for Olalla Creek.

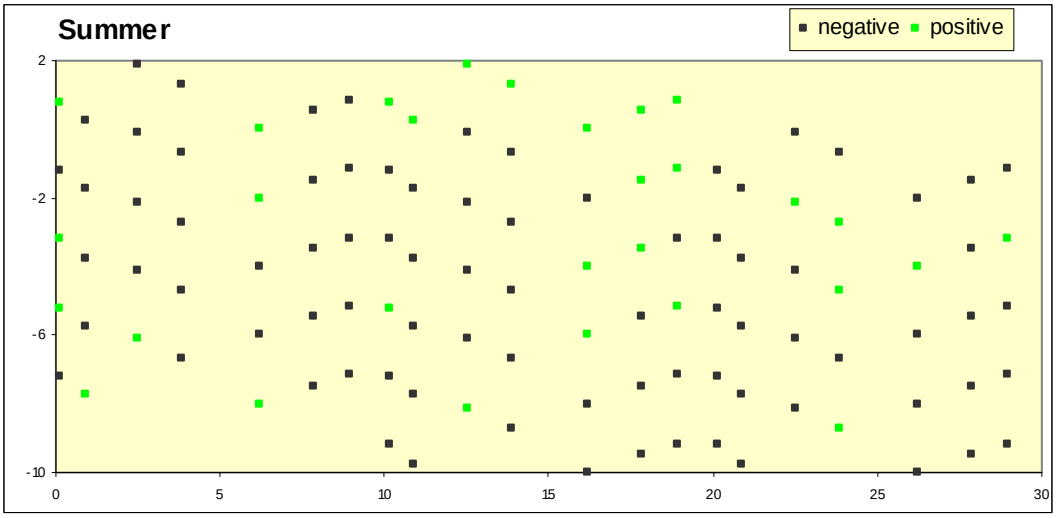
Result Maps

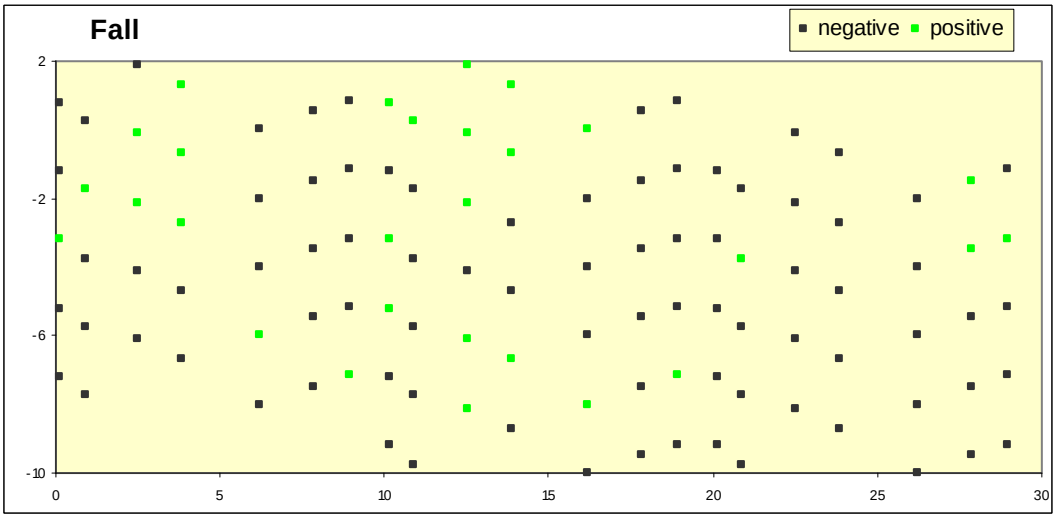
The location of *A. ellisii* at the three sites at the different sampling times is shown in Figures 4-12.

Figures 4-12 Maps of results for each sample point.

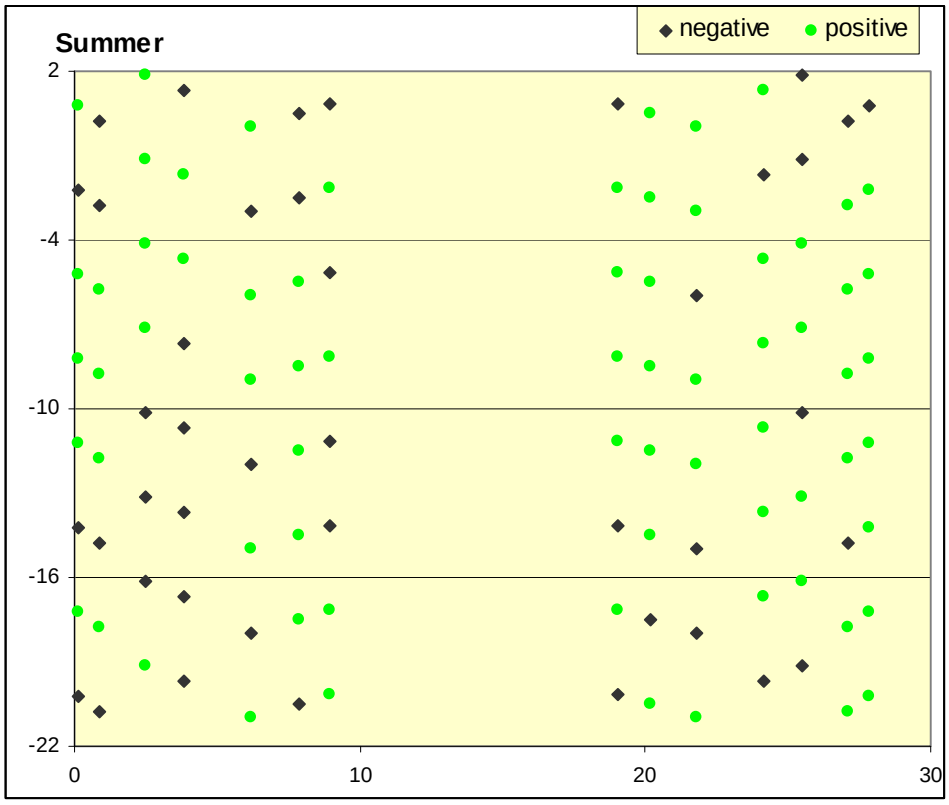
Frog Creek Results

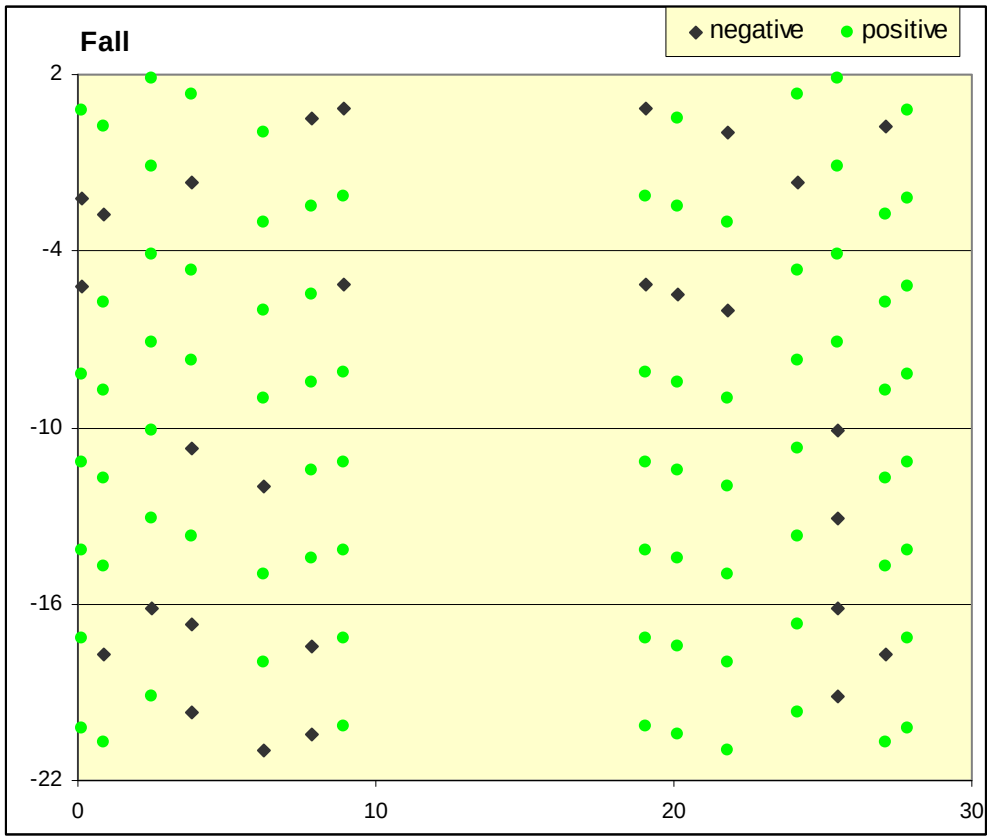




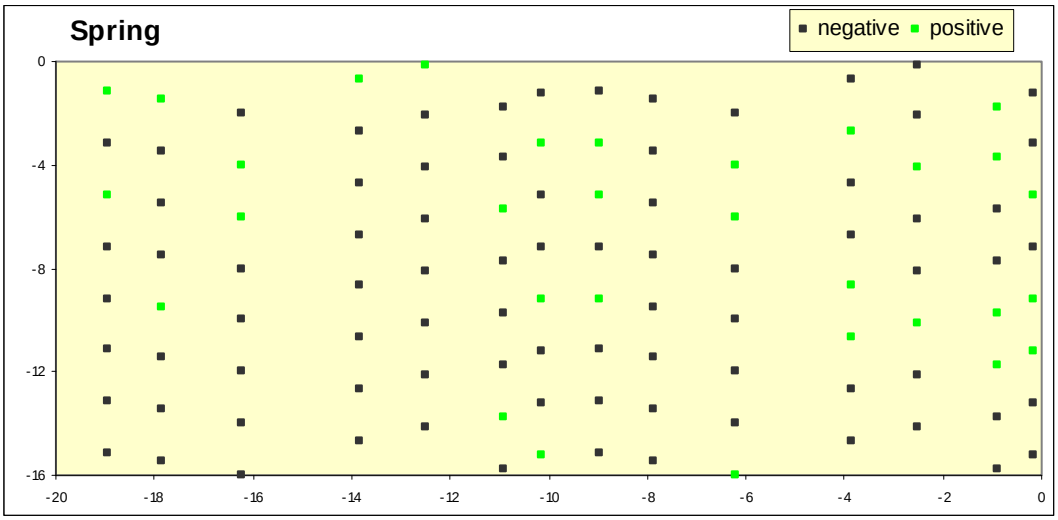


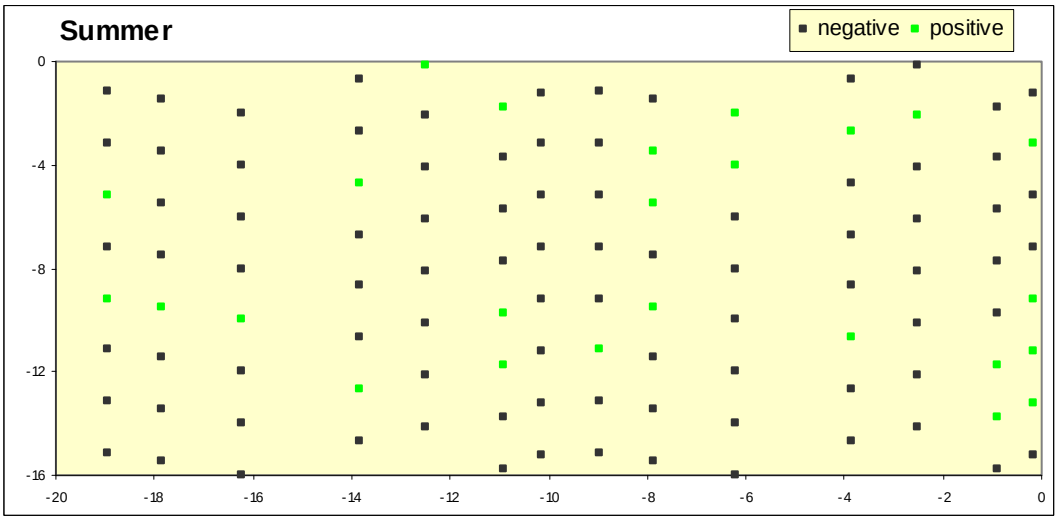
Olalla Creek Results

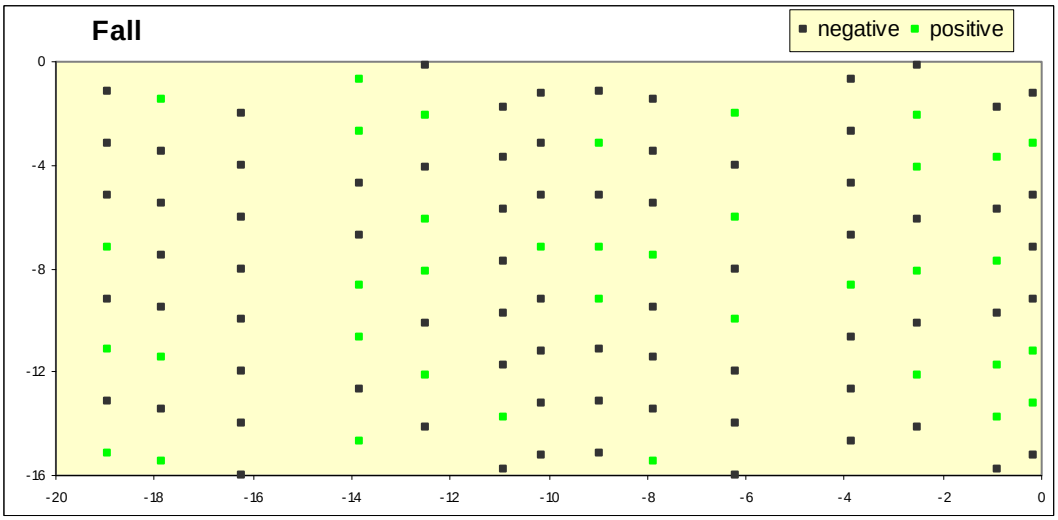



Fall
negative
positive
2
-4
-10
-16
-22
0
10
20
30

Sevenmile Creek Results







Discussion

In two of the three sites monitored in this study, the proportion of the sites occupied by the mycelium of *Albatrellus ellisii* did not change seasonally. The percent occupancy for these sites ranged from 22% to 33%. The third site, the Olalla Creek site at 1000' elevation in the Siskiyou Mtns., experienced an increase in the occupancy by *A. ellisii* in the fall such that the occupancy measured in the fall (75%) was significantly higher than both the spring (50%) and summer (63%) measurements.

The two sites that did not show significant change in occupation, the Frog Creek site at 3400' and the Sevenmile Creek site at 5000' are located at elevations more typical for *A. ellisii.* Of 21 *A. ellisii* locations with elevation data reported in an agency database for special status fungi, the Olalla Creek population is the only one reported from below 3000'. Eighteen of the other 20 populations were reported from above 4000'. It may be that the higher elevation populations of this fungus are more stable seasonally, or it may be that the Olalla Creek colony is experiencing environmental conditions that favor growth for reasons unrelated to seasonality. It would be interesting to test the spring occupancy of the Olalla Creek colony again. A hypothesis of seasonal change would be supported if occupancy was significantly below the fall 2006 level.

The most stable site, at Frog Creek, was sampled in the spring and summer of 2006 and the fall of 2007, so these results indicate that the population at this site was stable over the course of two growing seasons. Interestingly, although the occupancy of the sites by *A. ellisii* was found to be stable or increasing over the time of this study, new mushrooms were not produced during the study at any of the sites. The Sevenmile Creek population produced 2 mushrooms in 2005, and none in 2006. The Frog Creek population produced one mushroom in 2005, and none in 2006 or 2007. The Olalla Creek population was last known to produce a mushroom in 1997, and none was produced in 2005 or 2006. Visual surveys for this species at these sites would have supported the hypothesis that the species is declining, while DNA testing for the species in the soil shows that this is not the case.

A consideration of the spatial distribution of positive samples at each site over time indicates that there is significant change in the distribution of this fungus when measured at the meter scale over several month time intervals. For example, even though the total number of positive samples remained fairly constant at the Frog Creek site, the spatial distribution of the positive samples varied from one sampling time to another. This result is consistent with research on mycorrhizal fungi that indicates fungal structures in the soil are typically short-lived. Even on long-lived fungi, individual mycorrhizae only live for weeks or a few months before senescing. Moreover, research on the longevity of the extraradical mycelium indicates that individual hyphae are very short lived. One study on arbuscular mycorrhizal fungi found that

newly produced hyphae only live for 5 to 6 days (Staddon, et al., 2003), while more complex structures such as rhizomorphs appear to live for longer times.

The amount of change seen over time within each plot in this study is consistent with the idea of an individual fungus colonizing the soil as a dynamic, foraging organism (Rayner, 1991). Rather than thinking of the fungus as plant-like: occupying one location and slowly growing out from that spot in a quest for resources, it may be more useful to think of it as animal-like: exploring the underground environment with widely-ranging hyphae and directing its mass to areas where resources are found, then regressing and growing into different areas when that patch of resources is depleted.

In this study we assumed that target DNA detected in a sample was from living mycelium. It is possible that after a fragment of mycelium dies, the fungal DNA is released and persists in the soil at a detectable level. A thorough discussion of this issue is beyond the scope of this study; however a review of the literature indicates that it is an issue that requires further investigation.

Nuclease producing microbes are common in soil, and "on entry to soil both RNA and DNA are rapidly and extensively degraded" (Greaves and Wilson, 1970). Some clays may slow the action of the nucleases, but one study found significant degradation of DNA in montmorillonite-DNA complexes after 11 days (Greaves and Wilson, 1970). Even if some target DNA is protected in a clay complex within the soil, the DNA extraction procedure used must be capable of removing the DNA from the mineral complex.

Romanowski et al. (1992) monitored the degradation of a plasmid in 3 types of soil. They found that degradation began in the first hour in two of the soils. High molecular weight DNA was not detectable in these two soils after two days. High molecular weight DNA was not detectable in the third soil after five days.

Romanowski et al. (1993) found that when DNA was applied to soil, traces of it remained detectable for weeks, but at very low levels. After 60 days only 0.05% of a target gene was detectable in a clay soil, while 0.01% was detectable in a silty clay soil, and 0.2% was detectable in loamy sand. *Albatrellus ellisii* is not a mat forming fungus, and is not associated with high (visible) concentrations of mycelium in the soil. Since the starting amount of target DNA in the soil is low, it is unlikely that the target DNA would be detectable after a reduction of DNA concentration by more than a factor of 10, which occurred in 5 to 10 days in these soils.

Another aspect of this issue to bear in mind is that because the individual fungal hyphae have such a rapid turnover rate, fungi typically conserve resources by reabsorbing biochemicals from senescing hyphae, a process known as autophagy. The phosphorus and nitrogen in the DNA molecule are valuable resources that can be recycled from senescing hyphae into growing ones (Maheshwari, 2005). The importance of autophagy for *Albatrellus ellisii* is not known, but if

this process is present in *A. ellisii*, it may be that little DNA is released into the soil as parts of the fungus senesce.

In summary, fungi have mechanisms to recycle nucleic acids in senescing hyphae, preventing the release of DNA into the soil. If DNA is released into the soil after cell death, nucleases present in most soils act within days to reduce high molecular weight DNA to undetectable levels. At particular sites, biological or physical conditions may exist that allow extracellular DNA to exist for longer periods of time. It may be worthwhile to study fungal DNA degradation rates in study site soil to gain confidence that the DNA detected in a study is in fact from living tissue and not a remnant of past colonization of that soil sample.

References

Greaves, M. and M. Wilson. 1970. The degradation of nucleic acids and montmorillonite-nucleic acid complexes by soil microorganisms. Soil Biol. Biochem. 2: 257-268.

Maheshwari R. 2005. Nuclear behavior in fungal hyphae. FEMS Microbiology Letters 249 (1):7–14.

Rayner, A.D.M. 1991. The challenge of the individualistic mycelium. Mycologia 83 (1): 48-71.

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Romanowski, G. 1993. Use of polymerase chain reaction and electroporation of Escherichia coli to monitor the persistence of extracellular plasmid DNA introduced into natural soils. Appl. Envir. Microbiol. 59: 3438-3446.

Staddon, P., C. Ramsey, N. Ostle, P. Ineson, A. Fitter. 2003. Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of ^{14}C. Science, 300: 1138-1139.

Appendix I

Quality Controls

Soil Sample Collection

Testing was done to assess the effectiveness of the soil probe cleaning procedure. At each study site, pairs of test samples were taken according to the standard procedure. The first test sample (labeled X+) was taken at a spot that was thought to be positive for *A. ellisii*, either because of prior testing or because of proximity to a mushroom. The second test sample (labeled X-) was taken at a disturbed area near a roadside where the chance of colonization by *A. ellisii* was very low. The documented sampling and cleaning procedure was followed. The results of this testing are shown in Table QI.

Table QI Results of Cross-Contamination Testing

Site	Sample	Result
Frog Cr.	F1 X+	+
Frog Cr.	F1 X-	-
Olalla Cr.	O1 X1+	-
Olalla Cr.	O1 X1-	-
Olalla Cr.	O1 X2+	+
Olalla Cr.	O1 X2-	-
Sevenmile Cr.	S1 X+	+
Sevenmile Cr.	S1 X-	-
Frog Cr.	F2 X+	+
Frog Cr.	F2 X-	-
Olalla Cr.	O2 X+	+
Olalla Cr.	O2 X-	-
Sevenmile Cr.	S2 X+	+
Sevenmile Cr.	S2 X-	-

The results of this testing indicate that cross-contamination of samples is not occurring if documented sampling and cleaning procedures are used.

As a quality check, the temperature in the sample storage container was monitored from the time samples were added until the samples were placed in a freezer. For all sample collection trips, this temperature never went above 5° C.

Lab work

DNA Extraction

Known positive (100 ppm *A. ellisii* in soil) and negative soil samples were periodically extracted and analyzed to make sure that the extraction process was working properly and that contamination was not a problem. Each of the eight negative control tests gave a negative result, while each of the eight positive soil tests gave a positive result.

In addition, several soil samples were extracted twice, and the results of the two extractions compared (Table AI).

Table AI

Soil Sample	Extraction 1 Result	Extraction 2 Result
F1 6-4	+	+
F1 9-5	+	+
F1 10-1	-	-
F1 14-6	+	+
O1 6-5	-	-
O1 15-5	+	+
S1 3-6	-	-
S1 3-7	+	+
F2 2-3	+	+
F2 2-4	+	+
F2 5-6	-	-
F2 10-4	-	-
F2 14-7	-	-
O2 3-6	+	+
O2 11-3	+	+
O2 12-1	+	+
S2 2-2	+	-
S2 5-5	-	-
S2 5-6	+	+
S2 9-4	-	-
S2 10-1	-	-
S2 11-3	-	-
F3 4-5	-	+
F3 12-7	+	+
O3 1-1	+	+
O3 1-3	+	+
O3 9-7	+	+
O3 10-1	+	+
O3 10-2	-	-
S3 2-4	+	+
S3 2-6	+	+
S3 3-1	+	+
S3 3-3	+	-
S3 4-5	-	-

In three cases out of the 34 re-extractions (S2-2, S3 3-3, and F3 4-5), the first extraction gave a result different than the second result. This indicates that there may have been a segregation of target DNA among subsamples taken from the soil sample. Soil samples were 10 to 20g, and 1.0 to 1.3g of each sample is used for DNA extraction. Although each sample was carefully mixed before subsamples were taken, it is possible that in some cases subsamples were not representative of the sample from which they were taken. For example, it is possible that the target DNA concentration in a sample is just above the detection limit of the method, while some of the subsamples taken from this sample are slightly below the detection limit. This source of error could be reduced by mixing samples as thoroughly as possible before subsampling. However, the complete mixing of a heterogeneous material like soil can be time consuming. The use of a mechanical sample splitter may be beneficial in reducing this source of error.

PCR

Every batch of PCR reactions included a negative control, containing all of the PCR reagents but no DNA, and at least one positive control. If either of these controls failed, all samples in the batch were run again.

Appendix 2

Soil Sampling Procedure

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Purpose

The purpose of the activity described in this procedure is to collect soil samples from a site to be used in laboratory analysis of the DNA of organisms that are present in the soil. Some basic principles to keep in mind during this procedure are:

- The organisms and DNA in the soil are sensitive to temperature, moisture, and light conditions.

- DNA detection techniques are very sensitive, so extreme care must be taken to avoid cross-contamination of samples.

- Position data collection is a very important part of the sample collection process. Plot measurements, sample labeling, and field notes must be accurate.

Preliminary

This procedure assumes that plot corners have been installed at the study site as reference points, and that a sampling plan explaining how many samples are to be taken, and where they are to be taken has been written up. Before going out in the field, all field workers should understand the sampling plan. Any questions should be settled before going out to the field and each person should fully understand the goals of the project and the specific sampling plan. Each worker should understand his or her responsibilities. Safety and communication systems should be discussed and understood by all. Weather and possible hazards should be discussed. Any knowledge of the specific site should be shared.

The sampling plan includes a sample checklist, which is a list of samples to be taken and space for any notes on individual samples. For example, a sample checklist can take the following form:

Sample #	Transect #	Sample Location	Notes
1	1		
2	1		
3	1		
4	1		
5	1		
6	2		
7	2		
8	2		
9	2		
10	2		

The checklist above would be useful for a sampling plan that required, for example, 5 samples, 2 m apart, on each of two 10 m transects spaced 5 m apart.

The completed checklist might look something like this:

Sample #	Transect #	Sample Location	Notes
1	1	.4	
2	1	2.4 m	
3	1	4.4 m	
4	1	6.4 m	
5	1	8.1 m	Tree prevented sampling at 8.4 m
6	2	.4	
7	2	2.4 m	
8	2	4.4 m	
9	2	6.4 m	
10	2	8.4 m	

Deviations from the sampling pattern due to natural obstructions must be noted. The sampling plan will include instructions on how to deal with obstructions.

Equipment

For Sampling

>Two metal soil probe soil samplers, similar to the one shown in Figure 1. The probes must be capable of sampling to a depth of 12" (30 cm) and have a maximum internal diameter of 1" (2.5 cm).

Figure 1 Hoffer 1" diameter x 24" plated steel soil probe

>Soil sample bags. Bags must be at least 4 mil thick and have a "zip-lock" closure. Bags should be at least 10" long. Ideally, bags will be pre-numbered with an indelible marker.

>Wash solution. At least 1 gal. of 0.5 to 1.0 % Alconox detergent. Container should be at least 12" high.

>Rinse solution. At least 1 gal. of clean water (tap water). Container should be at least 12" high.

>Nylon brush, about 1" in diameter.

>Ice chest with ice or battery powered refrigerator large enough to hold filled sample bags.

>Notebook or PDA for data collection.

>Map indicating study site location.

>Written sampling plan

>2 copies of sample checklist

>Measuring tapes

>Small stakes or pin flags, pre-numbered with sample numbers.

For Mailing Samples to Lab

>Foam or gel refrigerant packs, e.g. Re-freez-r-brix (www.polar-tech.com).

>Insulated shipping container at least 1½ " thick (www.polar-tech.com).

Sampling Procedure

This procedure will describe the sampling procedure for a team of two people, the Sampler and the Assistant. Sampling can also be done effectively by one person. See the notes at the end of this section regarding one person sampling.

Typically a cleaning station will be set up just outside of the study area with the cleaning and rinse solutions and the cooler, and the Sampler will carry the empty sample bags, soil probe, and a notebook in a tool belt.

Using the map, the sampling plan, the established plot corners, and tape measures, set up the first transect or subplot. If this is a subsequent visit to a previously marked site, transects or subplots will not have to be set up.

Sampler Tasks

Move to next (first) sample point.
Remove numbered bag with the correct number and check the sampling table to make sure you are at the correct point.

Use the soil probe to pull a soil sample. Depending on the species of interest and location specific instructions will differ. Generally, the litter and duff layers are scraped away at the sample point and the soil probe is pushed into the soil using a twisting motion to a depth of about 10". Remove the probe with the soil sample in the tube.

Place the probe tube in the correctly numbered sample bag, and remove the soil from the tube to the bag through the wide slot in the tube. Wet soils may not drop out of the slot easily, and it may be necessary to knock the tube against a hard surface to get the soil out.

Hand the used soil probe to the assistant and take the cleaned soil tube. Mark the sample as collected on your sample list.

Repeat these steps until the plot has been sampled according to the sampling plan.

Assistant Tasks

The Sampler will hand you a soil probe that must be cleaned for the next sample. Take the probe to the cleaning station. The Alconox solution is very effective at removing soil from metal surfaces. Usually it is only necessary to swirl the tube in the wash solution for a few seconds, then swirl it in the rinse solution for 10 or 15 seconds and then dry it with a clean rag. After rinsing, inspect the tube, particularly the tip for any adhering dirt. If dirt is seen, either repeat the wash and rinse, or brush the soiled area with the nylon brush, then wash and rinse and dry.

Take the cleaned tube to the Sampler, and bring back the used tube for cleaning. Also bring back the last soil sample and place in the cooler. If the cooler is some distance from the sampling site, samples can be stored in a large plastic bag on the ground for up to 1 hour. At least every hour samples have to be put in a cooler between 2°C and 8°C. Do not freeze samples.

After placing the soil sample in the sample bag or cooler, the Attendant must check off that sample from the list of samples to be collected. Attendant and Sampler should periodically compare their sample checklists and resolve any discrepancies.

A gallon of wash solution and a gallon of rinse water are usually enough for 30 to 50 samples. When either solution gets extremely dirty, dispose of the used solutions and refill with fresh solutions. The rinse solution can be disposed of on the ground, at least 100 feet from any stream, but the wash solution must be emptied into a storage container for later disposal.

Continue collecting the filled sample bags and cleaning the two soil probes until all of the required samples are collected.

Note that if this is an initial sampling of a site, and subplots or transects have not been marked, it may be easier for one worker to set up the subplots or transects and mark sample locations with the pin flags or stakes, while the second worker follows behind taking samples at the marked locations and cleaning the soil probe between samples.

One person sampling

The above procedures can be done fairly easily by one person, although collection time will be somewhat greater. For sampling by one person, it is recommended that:

1) Lay out all pre-numbered sample bags at the corresponding sampling point before starting sampling.

2) Use three or four soil probes and collect three or four samples before bringing probes and soil samples to the cleaning station for cleaning and storage.

Sample Storage and Shipping

As soon as possible after sample collection, soil samples should be sent to the lab for analysis. If necessary, samples can be stored for up to 1 week before shipping to the lab. During storage they must be kept at a low temperature, but above freezing. Storage in an insulated container with ice or in a refrigerator is acceptable. The sample temperature should be kept between 0°C (32°F) and 8°C (46°F) at all times. Any deviation above 8°C or below 0°C should be noted.

Samples can be transported to the lab in an insulated container, or samples can be mailed using an insulating shipping container and a foam or gel refrigerant pack. Every 3 to 4 pounds of samples requires 1 pound of refrigerant. This is equivalent to a 1 pound brick of refrigerant for every 15 to 18 samples. Samples should be sent "next day" or 2nd day delivery. Shippers will not deliver on the weekend, so do not ship with 2nd day service after Wednesday, or "next day" service after Thursday.

Supplementary Materials

Albatrellus ellisii Study Sites

Frog Creek, Mt. Hood NF

This site is at about 3400' elevation in the Northern Cascade Mountains of Oregon, in the Eastern Cascades physiographic province. The site is on the Barlow Ranger District of the Mt. Hood N. F., in Clackamas County, with UTM coordinates 608427 (East), 5004465 (North). It is in a transition zone between the west and east sides of the Cascades, but seems more typical of a west-side site. The site is flat with deep soil. The forest consists of 100 to 200 year old *Pseudotsuga menziesii* and *Tsuga heterophylla*, with scattered *Taxus brevifolia*. A few small *Chrysolepis chrysophylla* were present on the site.

The contractor verified this site by the observation of a sporocarp in November 2004, and by the identification of the DNA of *A. ellisii* in the soil around the mushroom.

Olalla Creek, Roseburg BLM

The Roseburg BLM site is at about 1000' elevation in the Coast Range of Southern Oregon, in the Klamath Mountain physiographic province. The site is on the South Douglas Resource Area of the Roseburg District BLM, in Douglas County, with UTM coordinates 454391 (East), 4759543 (North). The site is on a 70% slope that faces east, with patches of deep soil intermixed with rocky patches. The site is near the base of a forested butte that rises about 2000' above the site. The forest is dominated by 120 to 140 year old *Pseudotsuga menziesii* and *Abies concolor* with younger *Abies grandis* and a few scattered *Taxus brevifolia*. The site also contains some large (> 10 m) specimens of *Chrysolepis chrysophylla*.

This site was identified by the observation of a sporocarp on a fungal survey in January of 1997. In December of 2005, the contractor took soil samples from a 20m by 20m plot in the approximate area of the 1997 sighting. The DNA of *A. ellisii* was detected in many of the soil samples, confirming its presence in the area.

Sevenmile Creek, Winema NF

The Winema NF site is near Sevenmile Creek at about 5000' elevation on the east side of the Southern Cascade Mountains of Oregon, in the Eastern Cascades physiographic province. The site is on the Klamath Ranger District of the Winema N. F., in Klamath County, with approximate UTM coordinates 570413 (East), 4728836 (North). The study site is on a flat

bench next to Sevenmile Creek. The soil on this site is thinner than the other two sites, with about ¼ of the site being very rocky. The forest is dominated by mature *Abies concolor*, *Picea engelmanii*, and *Pseudotsuga menziesii.* One large *Pinus ponderosa* was present on the site.

Sarah Malaby, a botanist on the Winema N.F., confirmed the presence of an *A. ellisii* sporocarp at this location in November 2005, and has flagged the site.

Site Diagrams

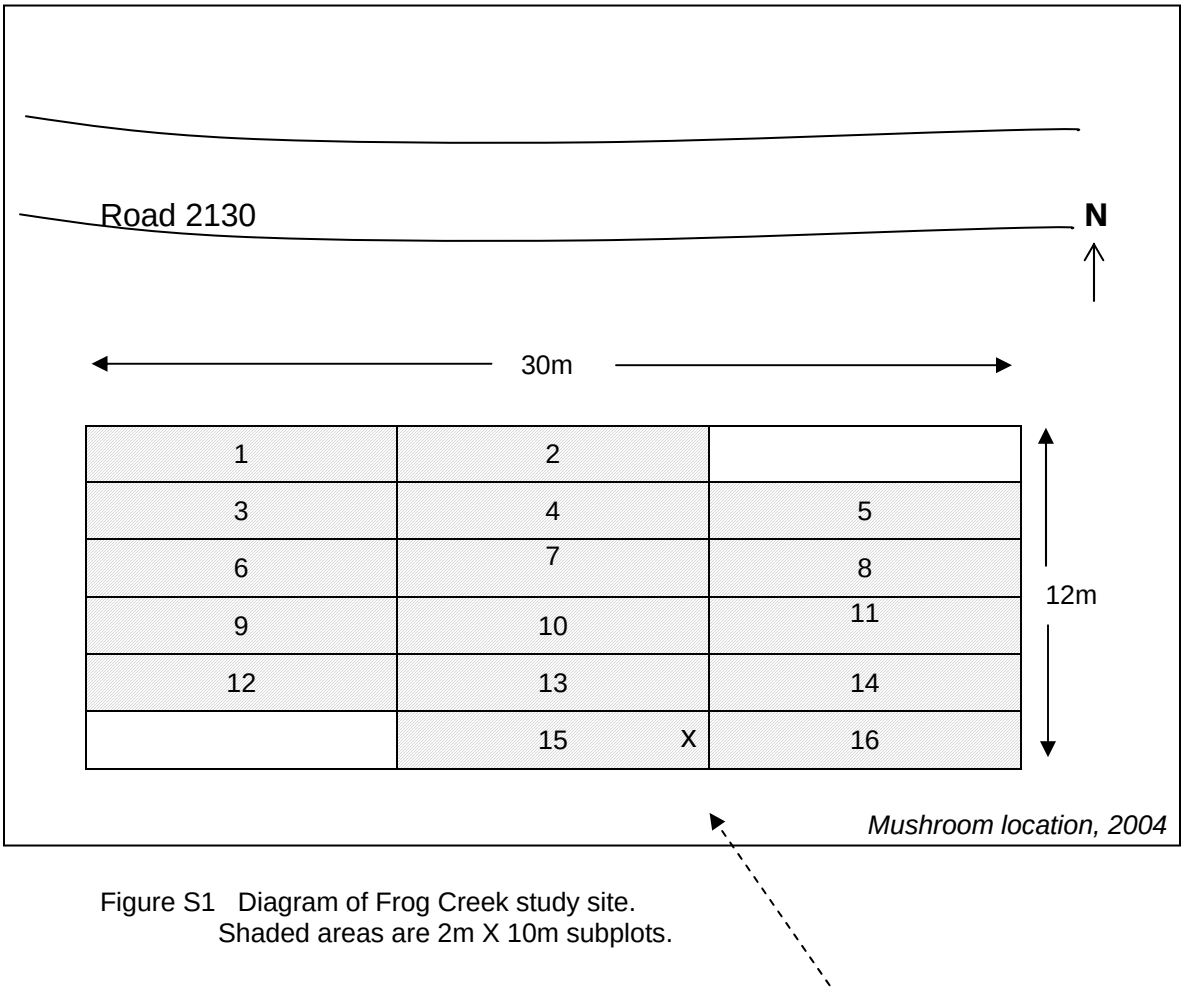

Figure S1 Diagram of Frog Creek study site.
Shaded areas are 2m X 10m subplots.

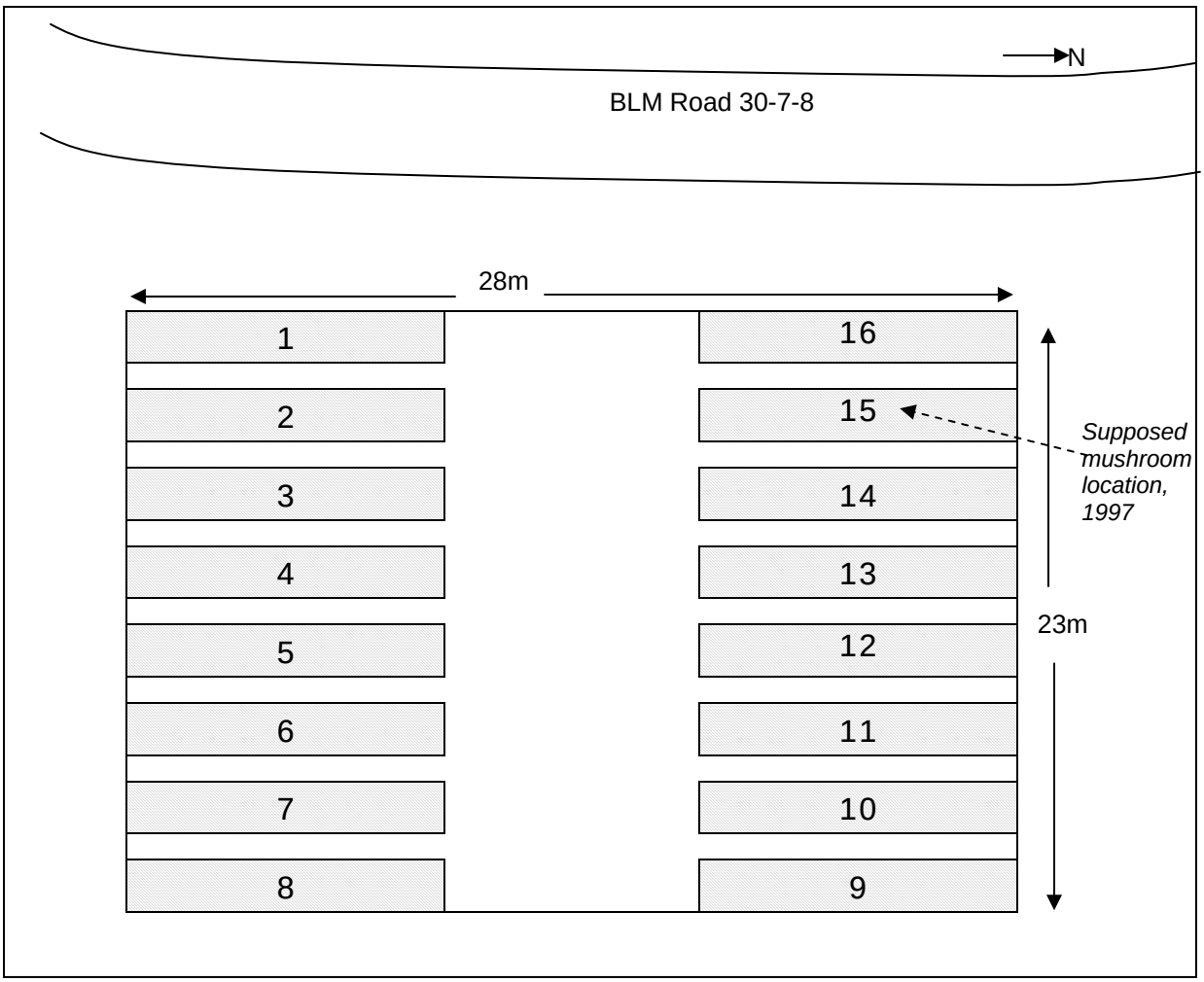

Figure S2 Diagram of the Olalla Creek study site. The 2m X 10m subplots are shaded.

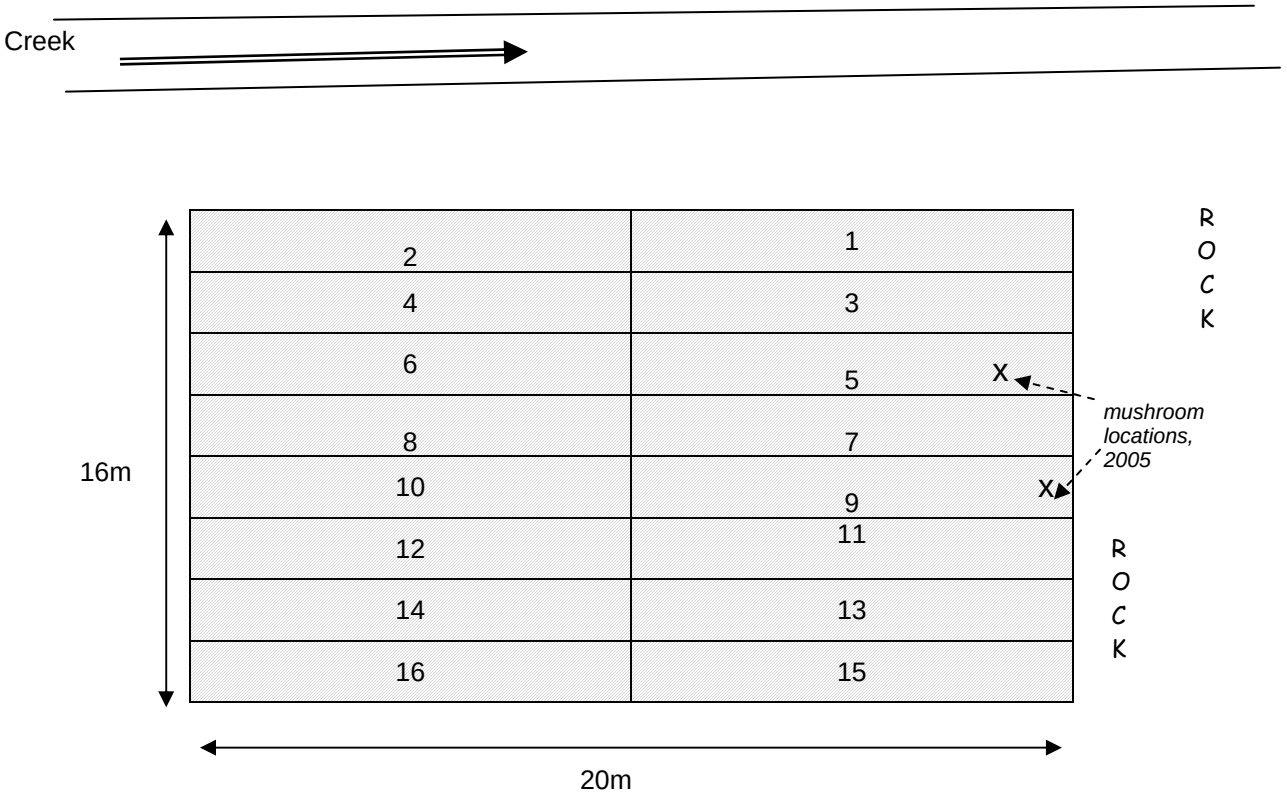

Figure S3 Diagram of the Sevenmile Creek site with 2m x 10m subplots.