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Molecular monitoring of protected fungi: mycelium persistence in soil after timber harvest☆

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ABSTRACT

The largely subterranean nature of the mycorrhizal fungal life form impedes efforts to monitor protected fungi and evaluate the effectiveness of mitigations in timber harvest areas. In this study we developed and tested a multiplex PCR system capable of detecting 14 listed *Phaeocollybia* species in soil. We then investigated the persistence of *Phaeocollybia* species at three sites in northwestern Oregon where *Phaeocollybia attenuata* and other *Phaeocollybia* sporocarps had been observed prior to timber harvest. The three sites had three different levels of protection for the fungi. One site was in a buffer zone next to a thinned area, one was within a thinned area, and one was in a clearcut area. The method was effective at detecting the target species in soil. We detected *Phaeocollybia* species in the soil at all sites, even in the clearcut after 12 yr, during which no sporocarps were observed.

Introduction

Despite the importance of conserving known sites of rare fungi within the managed landscape, it is difficult to determine the success of different conservation approaches when monitoring depends on sporocarp surveys. The appearance of sporocarps after site disturbance confirms colony persistence, but the lack of documented sporocarp production does not necessarily indicate an absence of fungi in the soil (Nara et al., 2003; Van der Linde et al., 2012). Many fungal species do not fruit regularly, and intermittent site monitoring may miss fruiting events (O'Dell et al., 2004). Mycorrhizal fungi are

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dependent on currently assimilated photosynthate for sporocarp production (Högberg et al., 2001) and the loss of phytobionts due to logging may inhibit fruiting, while not necessarily affecting the viability of the mycelium in the soil. Or the fungus may be temporarily eliminated from the disturbed area, then recruited back, either from spores or nearby underground inoculum sources, with a time lag in sporocarp production as mycelial or mycorrhizal establishment occurs. In all of these cases the species has persisted at the site, though this persistence goes unrecognized until the indeterminate time when a sporocarp is produced and observed.

One method for directly monitoring the fungal mycelium in soil is to test DNA extracted from soil samples for species-specific genetic markers (Anderson and Cairney, 2007; Van der Linde et al., 2008). This type of testing permits the assessment of the status of specific fungi at a variety of time points, chosen independently of poorly understood fungus biological cycles. For example, Guidot et al., (2002) developed a species-specific primer pair targeting the intergenic spacer region of *Hebeloma cylindrosporum* and then used this marker to track changes in mycelium locations over time at one site. Gordon and Apple (2011) used the sequence of a unique RAPDS generated amplicon to design specific primers for *Albatrellus ellisii* and then used these primers to monitor the mycelial occupancy of this fungus at three sites over three seasons. Zampieri et al., (2010) used β-tubulin-targeted specific primers to track the extent of the mycelium of *Tuber magnatum* in a truffle ground in two seasons. These studies indicate that species-specific PCR primers can be used as the basis of a monitoring protocol at conservation sites.

The conservation of rare and little known fungi over 9.7 million ha of forest land in the Pacific Northwest of the United States is guided by the Northwest Forest Plan (NWFP), a federal ecosystem management plan (Molina, 2008). In NWFP areas open to management activities, mitigation practices for known sites of rare fungi are implemented by the U.S. Forest Service Region 6 (USFS) and the Oregon/Washington Bureau of Land Management (BLM). These practices typically involve the establishment of a buffer around known sporocarp locations within which no habitat-disturbing activity is allowed. This provides a measure of protection for the fungal colony, but the subterranean fungal mycelium can extend well beyond sporocarp locations (Van der Linde et al., 2009; Zampieri et al., 2010; Gordon and Apple, 2011) and the physical effects of tree removal can reach hundreds of meters into the buffer (Chen et al., 1995). These concerns support arguments for large buffers, but requiring unnecessarily large buffers around multiple sporocarps in a planned project area can make it unfeasible to carry out any management activities at all. This could have long term negative impacts when management activities are intended to reduce the susceptibility of the stand to catastrophic fire, improve stand health, or accelerate the development of late successional characteristics. To better understand the responses of fungi to mitigation measures, our objectives in this study were to develop specific primers for a group of listed species, and to use these primers to determine persistence of these species at sites where management activities had taken place in the past, by testing DNA extracted from soil.

Representatives of the genus *Phaeocollybia* (Cortinariaceae) are distributed globally, but *Phaeocollybia* is particularly diverse in the coniferous forests of the Pacific Northwest of the United States, with 25 named species. These species appear to be largely or completely endemic to the region (Norvell and Exeter, 2008). Fifteen of these species are listed as sensitive or special status in Oregon and Washington. Norvell and Exeter (2008) provide strong evidence that the entire genus is mycorrhizal. Several *Phaeocollybia* species often fruit at the same site, indicating that the mycelium of more than one species could be found in one soil sample.

In a multiplex PCR system multiple primer pairs, each targeting a different species, are combined in each reaction, with amplicon size identifying the species present (Hamelin et al., 1996; Guglielmo et al., 2007). A multiplex system greatly reduces the testing burden when, as is the case for *Phaeocollybia*, several species could be found in one DNA sample. In this study we developed and tested a multiplex PCR system capable of detecting all 15 *Phaeocollybia* species falling under the protections of the NWFP. We then evaluated the method by testing DNA extracted from *Phaeocollybia* sporocarps and soil near sporocarps. Finally, in a case study we used the multiplex detection system to determine the persistence of *Phaeocollybia* mycelium at three sites in northwestern Oregon where *Phaeocollybia attenuata* and other *Phaeocollybias* had been observed prior to forest management activities. One site was in a buffer zone next to a thinned area, one was within a thinned area, and one was in a clearcut area. We hypothesized that the two less-disturbed sites would still support *Phaeocollybia*, while persistence in the clearcut area was considered unlikely.

Materials and methods

Marker development

Surveys over the last 15 yr (undertaken under provisions of the NWFP) have led to the compilation of several hundred *Phaeocollybia* collections for taxonomic and genetic analysis. The ITS sequences of the nuclear ribosomal DNA region of most of these collections have been deposited in GenBank, providing a valuable resource for phylogenetic study and specific primer design. The recent availability of this sequence data has also stimulated a refinement of the taxonomic work on this genus (L.L. Norvell, personal communication) that has not yet been formalized. Based on current (but incomplete) understandings of the genus, we recognize 21 *Phaeocollybia* species endemic to the Pacific Northwest (Table 1) rather than the 25 species recognized by Norvell and Exeter (2008). With respect to listed species, we combined *P. gregaria* with *P. olivaceae* in the *P. olivaceae* complex, since we found that the phylogenetic clustering of ITS sequences of these closely related species was inconsistent with current morphological definitions. This left 14 listed species requiring specific primers.

The ITS sequences for each of the 21 species were aligned. Within species similarity in ITS sequences was generally greater than 98 %, but some species contained outliers. In this study, sequences that had a pairwise identity of less than 95 % to others of the same named species were eliminated from analysis. This left a total of 239 ITS sequences, 181 from the 14

Table 1 – Number of ITS sequences used for each of the 21 species and their % identity

Species	Number of ITS sequences	% Pairwise identity	Primer group[a]
P. attenuata	16	99.4	B
P. californica	16	99.5	B
P. dissiliens	8	99.8	A
P. fallax	16	97.6	A
P. olivaceae complex	28	95.6	B
P. kauffmanii	12	99.0	B
P. lilacifolia	14	99.3	A
P. oregonensis	10	99.6	B
P. piceae	10	99.9	B
P. pseudofestiva	5	97.7	B
P. radicata	9	99.5	A
P. scatesiae	11	99.0	A
P. sipei	8	99.2	A
P. spadicea	18	99.5	A
P. ammiratii	13	99.7	nt
P. benzokauffmanii	9	99.9	nt
P. luteosquamulosa	10	99.6	nt
P. ochraceocana	5	99.5	nt
P. pleurocystidiata	9	99.9	nt
P. redheadii	7	97.2	nt
P. phaeogaleroides	5	99.8	nt

[a] nt indicates a non-target species.

listed *Phaeocollybia* species and 58 from the seven non-listed *Phaeocollybias* found in the Pacific Northwest.

To design specific primers for the 14 target species, the 21 species were divided into four groups, each group consisting of five or six closely related species. Sequences in each group were aligned and, by inspection, primers were designed for each listed species that targeted variable ITS1 regions in the group. These primers were then compared to the larger group of *Phaeocollybia* species to check their potential to prime other non-target *Phaeocollybias*. All sequence comparisons were done using the latest version of Geneious software (Biomatters Ltd.). Primer disagreements with non-target species were designed into the 3′ end of the primer to maximize the effects of the disagreements in PCR, and melting temperatures were targeted to be 63 °C ± 2 °C.

It became apparent that although the design of 14 specific ITS primers was possible, it would not be possible to design a uniquely-sized ITS-based marker for each of the 14 target species. For this reason, based on marker sizes generated by the candidate specific primers, the species were divided into two groups. Group A consisted of *P. sipei*, *P. dissiliens*, *P. radicata*, *P. spadicea*, *P. scatesiae*, *P. fallax*, and *P. lilacifolia*; group B consists of *P. pseudofestiva*, *P. piceae*, *P. californica*, *P. oregonensis*, *P. olivaceae*, *P. attenuata*, and *P. kauffmanii*. This division required the use of two PCRs for each sample tested.

Primers were tested for interactions with other primers in their group using the program OligoAnalyzer 3.1 (www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Potential primers were also compared to sequences in the GenBank database to check for interactions with other fungal species. To reduce the number of primers in each multiplex, and thus reduce primer-to-primer interactions, we combined seven species-specific forward primers with one reverse primer common to all species in the group. The reverse primer targeted the conserved 5.8S region. To keep the cost of testing as low as possible, we used standard agarose gel electrophoresis of PCR products to determine marker sizes. Because of closely sized amplicons, three markers were ambiguous, each possibly indicating two species. Primers were designed to distinguish these species pairs in a third PCR.

From previous work we had access to DNA from all PNW *Phaeocollybia* species. Using this DNA, putative specific primers were tested individually to confirm that they were effective with their target species but did not prime other *Phaeocollybias*. Primers that did not perform well were redesigned. Finally, the multiplex primer mixes were assembled and these were tested against the full range of *Phaeocollybia* DNA available. PCR was performed in 25 μl reactions with 1 μl sporocarp DNA, 0.4 μM each primer, 0.2 mM dNTP mixture, 2.0 mg ml^{-1} bovine serum albumin, and 0.6 U One*Taq* Hot Start DNA polymerase (New England Biolabs) in 1X One*Taq* standard buffer with 2.1 mM $MgCl_2$. Optimum PCR conditions were: 94 °C for 30 s, followed by 35 cycles of 94 °C for 15 s, 63 °C for 30 s and 68 °C for 50 s, followed by a final extension at 68 °C for 2 min. Products were visualized in a 2.6 % agarose gel with GelStar nucleic acid stain (Lonza Group Ltd.).

Later, in running the samples from the preliminary test plots, a couple of issues with primer design arose. First, in both primer sets A and B, several of the specific markers are in the 140 to 160 base pair (bp) range. Although it was possible to make size calls in this range after agarose gel electrophoresis, it was felt that confirmation of these calls was needed. Second, unlike when using sporocarp DNA, when soil DNA was used as the PCR template source, occasional stray amplicons appeared on the gel, with sizes not matching any *Phaeocollybia* marker, indicating the possibility of non-specific amplification. We have demonstrated that the primer sets do not prime closely related non-target *Phaeocollybia* species, but the possibility of generation of amplicons by chance from the multigenomic DNA extracted from soil, or chimera formation during PCR could not be eliminated (Shuldiner et al., 1989; Kanagawa, 2003). To address both of these issues a confirmation PCR was done for each positive sample. In the confirmation PCR, the single species-specific forward primer indicated by the marker in question was paired with a reverse primer targeting a variable part of the ITS2 region rather than the highly conserved 5.8S region. Confirming primers for this purpose were designed and tested for the particular species detected in this study.

Results were further validated by sequencing a subset of the positive amplicons obtained from soil samples from the persistence testing plots. For each persistence testing plot we randomly selected for sequencing one to several amplicons for each species detected at that site (33 amplicons total). Amplicons were sequenced in both directions using the same primers used in the initial PCR. The BLAST algorithm was used to compare amplicon sequences to sequences in GenBank.

Study sites – preliminary testing

Three locations where *Phaeocollybia* had been documented fruiting in the past were used for preliminary soil testing in November 2010. Two locations were on Paradise Ridge in the

Oregon coast range (Coos Bay District BLM). Transect 1 ran through a marked spot where *P. dissiliens* had been collected in 2009 and a currently fruiting group of *P. spadicea*. Transect 2 ran through a flagged *P. sipei* site from 2009 and a currently fruiting patch of *P. radicata*. Fifty samples were taken over 22.5 m on Transect 1, and fifty samples were taken over 18.0 m on Transect 2. The third location was on Mary's Peak, in the Oregon coast range (Salem District BLM), in an area where *Phaeocollybias* have been reliably collected over several years. Transect 3 was oriented to run through a fruiting patch of *P. attenuata*, and within 1 m of a group of *P. spadicea* mushrooms. Ninety-eight samples were taken along the 61.0 m length of Transect 3.

Study sites – persistence testing

For this case study three sites were identified where management activities had been completed at documented *P. attenuata* locations: a site within a buffer zone next to a thinned area, a site within a thinned area that was not protected, and a site within a small clearcut that was not protected (Fig 1). Since this is a case study and the results from the three plots cannot be directly compared, different plot configurations and sampling patterns were used at the three sites to assess the practicality and effectiveness of the different field methodologies.

The buffered site was on Moose Mountain in the western Cascades Range, north of the Santiam River, about 24 km east of Sweet Home, Oregon (10T 545398 4917093) at an elevation of 419 m. On Oct. 14, 2011 two approximately perpendicular transects were sampled at this site. The transects intersected at a flagged spot where *P. attenuata* sporocarps had been collected in 1999. A 15 m radius buffer had been set up around this point before a 50 % canopy thinning project was completed just downslope from the site in 2005. *Pseudotsuga fallax* had also been found at this site. Transect A was 36 m long and 80 equally spaced samples were taken from this transect. Transect B was 30.5 m long and 20 equally spaced samples were taken from this transect. No *Phaeocollybia* sporocarps were seen on the day of sample collection. Prior to thinning there had been no management activity in the area; the mature *Pseudotsuga menziesii* forest had naturally regenerated after a fire in 1859.

The thinned site, a mixed *P. menziesii/Tsuga heterophylla* forest, was on Green Peak in the Oregon Coast Range, about 29 km SW of Corvallis, Oregon (10T 464001 4912801) at an elevation of 558 m. The site was in a documented *P. attenuata* area that was thinned in 1999 to 300 trees ha^{-1}, and again in 2010 to a final density of 120 trees ha^{-1}. Soil samples were collected on Nov. 9, 2011 from two parallel transects. The transects were 20 m long and spaced 2 m apart. Fifty equally spaced samples were taken from each transect. *Pseudotsuga attenuata* was fruiting at the time of sampling, and various other non-*Phaeocollybia* sporocarps were present on the study site.

The clearcut harvest site was also on Green Peak, about 600 m WSW of the thinned site (10T 463420 4912592) at an elevation of 605 m. This 1 ha site had been clearcut in 1999 and re-planted with *P. menziesii*, *T. heterophylla*, and *Thuja plicata* in 2000. These and other young trees and shrubs were

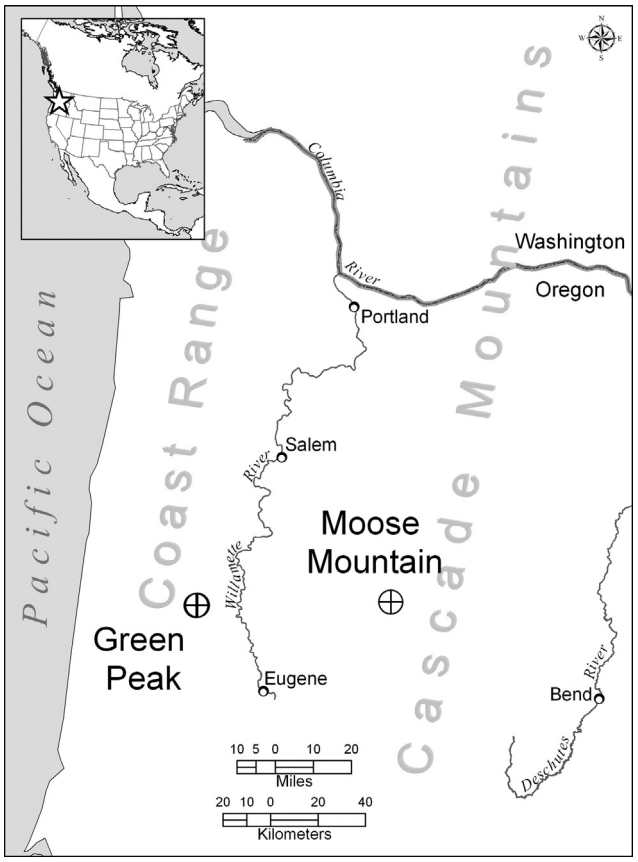

Fig 1 – Persistence testing study site locations.

growing throughout the site at the time of sample collection. Prior to harvest, *P. attenuata*, *P. spadicea*, *P. dissiliens*, *P. fallax*, and *P. sipei* had been found on a visual monitoring transect within the clearcut boundaries. None of these species were found in 6 yr of post-harvest monitoring, although *P. phaeogaleroides* (not a listed species) was seen. On Oct. 24, 2011, 12 yr after harvest, a plot was established, centered on the monitoring transect, with four parallel 10 m transects each 1 m apart on the north end where *P. attenuata* sporocarps had been seen before timber cutting at the site. The other *Phaeocollybia* species had been seen near or within the study plot, but the exact locations of these other sporocarps were not known. Twenty five equally spaced samples were taken from each transect. No mushrooms were seen on the day of sampling.

Field protocol

For both the preliminary testing and persistence testing an anchored tape measure was used to mark each transect. A 2.5 cm diameter stainless steel soil probe was used to obtain soil samples. At each sampling location, two adjacent probe samples (approximately 2 cm apart) were taken to increase the soil volume tested. The maximum depth of soil that could be taken by the probe was about 35 cm, but at times the probe hit obstructions in the soil and the full depth could not be reached. The top organic layer of each core sample was

Table 2 – *Phaeocollybia* species-specific primers and marker sizes

	Primer name	Species identified	Primer sequence	Paired with	Marker length (bp)
Primer set A	radf	*P. radicata*	CACACATAGTTTCCAAGGCTA	sr4	131
	disf	*P. dissiliens*	AAAGTTTGTAGCTTCACTTTTCAT	sr4	143
	sipf	*P. sipei*	CTGGAAAGTTATATTCCTTTACTTTTCTTT	sr4	153
	scaf	*P. scatesiae*	GACAAGAAAGAAAAAGGGATTTATTC	sr4	160
	spaf	*P. spadicea*	TTTGTAGACAGGAAAGACAAGG	sr4	192
	falif	*P. fallax/lilacifolia*	GCTGGCCTTTTGGCAAG	sr4	249
Primer set B	picf	*P. piceae*	TTCCTTAAATATTCCTTGTCTATTTTTTATATAC	sr4	134
	calf	*P. californica*	GAACTCATTTATTCCTCGAGTATTTC	sr4	147
	psef	*P. pseudofestiva*	GAAAAAGGGATTTATTCCTTGTG	sr4	151
	olgrf	*P. olivacea* complex	GCCATTTGGCCCCG	sr4	158
	kauf	*P. kauffmanii*	CCTTTTTGGCCACGTTTATT	sr4	159
	oref	*P. oregonensis*	GATTTATGTGGTGCCGTAATG	sr4	169
	attf	*P. attenuata*	TTGCAGCTGGCCCTTTT	sr4	262
Discriminating primers	lilf2	*P. lilacifolia*	AGACCAGAAAGAAAAAAGGGAT	r2	442
	falf2	*P. fallax*	GGCCCTCATGTAATCTCTAAA	r2	402
	kauf2	*P. kauffmanii*	TTGGTAGTAATATCAATGGCCTT	r2	117
	olgf2	*P. olivacea* complex	AGTGTCATTAAATTCTCAAAAGCTTT	r2	137
	calf2	*P. californica*	GCAGCTGGCCTTTCTTG	r2	477
	psef2	*P. pseudofestiva*	CAAGAAAGAAAAAGGGATTTATTCC	r2	397
Reverse primer	sr4	All	TTCATCGATGCGAGAGCC		
Confirming primers	r2	*P. spadicea*	GATAATTATCACACCAATAGACAAAGTC	spaf	434
		P. fallax		falf2	402
	r4	*P. attenuata*	GAACCTTTGGTAGTATTATCAATGGC	attf	415
	r3	*P. dissiliens*	GATAATTATCACACCAAATAGACCAAGTC	disf	400
		P. sipei		sipf	397
	radr	*P. radicata*	AGCCGACTCTAGTGAAAGAGCCTG	radf	325

removed to reduce the chance of collecting spores, and the soil from both samples was combined in a numbered heavy-duty reclosable plastic bag. After each pair of samples, the probe was cleaned in a detergent solution, rinsed in water, and dried. Samples were stored on ice during transport to the lab.

Lab protocol- soil

Soil samples were kept chilled (1–5 °C) until they were processed. After thoroughly hand mixing the soil in the plastic sample bag, from 1.0 to 1.2 g of soil was transferred to a 2.0 ml centrifuge tube, and DNA was extracted using a Chelex extraction buffer as follows. To each soil tube, 700 μl of Chelex buffer (200 mM Tris pH = 8.6, 5.5 % Chelex 100 (Bio-Rad Laboratories), 1 % Triton X-100) was added. Tubes were vortexed briefly, heated to 94–97 °C for 12 min, vortexed for 30 s, then frozen. Crude DNA solutions were cleaned using glass fiber filters in a method similar to that described in Ivanova et al. (2006). Extraction solutions were thawed and centrifuged for 2 min, then 120 μl of supernatant was removed from each tube, mixed with 400 μl of a binding solution (25 % ethanol with 4.5 M Guanidine hydrochloride, 15 mM EDTA, 7.5 mM Tris–HCl pH 6.4, 1 % Triton X-100) and drawn by vacuum through glass fiber filters in a 96-well plate (Pall Corp., PN 8032). The silica-bound DNA was washed twice (60 % ethanol with 50 mM NaCl, 10 mM Tris–HCl pH 7.4, 0.5 mM EDTA), then eluted with 70 μl of water. Every extraction batch included at least one positive control and every 16th sample extracted was a blank. Each DNA sample was tested with both group A and group B primers in two PCRs, and positive samples were tested further as previously described. The PCR protocol was the same as that used in marker development. Products were visualized in a 2.6 % agarose gel with GelStar nucleic acid stain (Lonza Group Ltd.).

Results

Primer design and testing

Our analysis of 239 ITS sequences from 21 *Phaeocollybia* species found enough inter-species sequence difference in the ITS1 region to design specific primers for each of the 14 target species in two multiplex sets of seven specific primers each. Primers that allowed us to discriminate between pairs of species with closely sized markers were also designed, as were confirming reverse primers with homologies to the ITS2 region (Table 2). Confirming primers were only designed for the six species detected in our field studies. Fig 2 shows a gel of the reaction products of primer set A with DNA from various *Phaeocollybia* species.

Preliminary soil testing

In the preliminary soil testing on transects running through patches of fruiting *Phaeocollybias*, all four fruiting species were detected (Table 3), indicating that the method can detect targeted fungi in soil samples. On Transect 2, *P. sipei*, which had been observed the previous year, was not detected, while on Transect 1 the marker for *P. dissiliens*, which fruited the previous year, was detected, indicating a continuing presence at

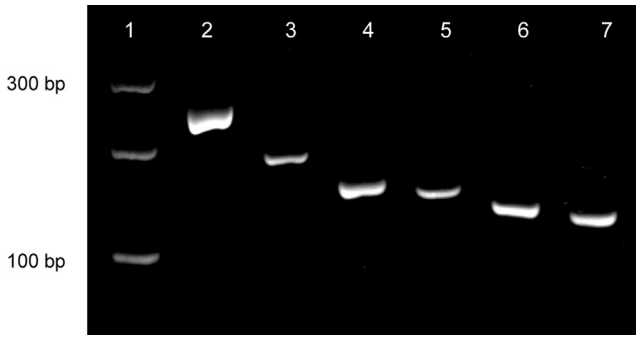

Fig 2 – A gel of PCR products from various combinations of *Phaeocollybia* DNA and primer set A, with 100 bp marker in lane 1. Lane 2, *P. lilacifolia*; lane 3, *P. spadicea*; lane 4, *P. scatesiae*; lane 5, *P. sipei*; lane 6, *P. dissiliens*; lane 7, *P. radicata*.

the site. Two species were detected on Transect 2 that had not been reported there, and one species was detected on Transect 3 that had not been reported there. All 198 soil samples from these transects were tested for all 14 *Phaeocollybia* species and ten species were not detected in any of the samples. Overall, 97 % of all tests were negative. Fifteen of the soil samples had two *Phaeocollybia* species present. Detections of DNA in soil were concentrated near sporocarps, but soil detections of *P. spadicea* were found as far away as 15 m from a sporocarp on Transect 1 and 25 m from a sporocarp on Transect 3.

Phaeocollybia *persistence soil testing*

Table 4 summarizes the detection results obtained using the *Phaeocollybia* species-specific primer sets. *Phaeocollybia attenuata* was fairly extensive in the soil at all of the sites, and was found in 60 % of the soil samples at the clearcut site. Two species (*P. spadicea* on the buffered site and *P. radicata* on the thinned site) that had not been seen at these sites were detected in soil samples. Three species that had been seen in or near the clearcut plot 12 yr before sampling were not detected in the soil samples. Overall, 95 % of all species tests were negative and nine of the target species were not seen in any of the 300 soil samples tested.

As noted earlier, all positive detections reported here produced an amplicon of the correct size in two separate PCRs with two different specific primer sets. The amplicons sequenced for further validation of the PCR results are shown in Table 5. All amplicon sequences were close matches with ITS sequences from the expected *Phaeocollybia* species.

Table 3 – Results of preliminary soil testing for *Phaeocollybia* DNA

Transect # (length)	Visual detection	DNA detected on transect	Fraction of samples with this species
1 (22.5 m)	*P. spadicea*	*P. spadicea*	0.24
	P. dissiliens	*P. dissiliens*	0.22
2 (18 m)	*P. radicata*	*P. radicata*	0.22
	P. sipei[a]	*P. spadicea*	0.22
		P. dissiliens	0.16
3 (61 m)	*P. attenuata*	*P. attenuata*	0.13
	P. spadicea	*P. spadicea*	0.12
		P. dissiliens	0.03

[a] Observed fruiting the previous season, not observed during sampling.

Discussion

Mycelium persistence

Many studies have shown the drastic reduction or elimination of mycorrhizal fruiting body production after heavy thinning or clearcutting (Luoma et al., 2004; Norvell and Exeter, 2004; Durall et al., 2006), and this was observed for *Phaeocollybia* species in the clearcut we studied. However, in this study we found, as others have (Gardes and Bruns, 1996; Dahlberg et al., 1997; Durall et al., 1999), that sporocarp surveys do not provide an accurate view of the underground fungal community. Twelve years after clearcutting, we could detect *P. attenuata* in 60 % of the soil samples taken from the area where sporocarps were previously produced, indicating species persistence through a significant mycelial or mycorrhizal presence. In studying primary succession in vegetation patches in a volcanic area, Nara et al. (2003) found that sporocarp production was not dependent on the density of mycorrhizal root tips in the patches, but on host size. It may be that phytobionts in the clearcut we studied have yet to reach the level of primary production necessary to support the fruiting of the *P. attenuata* and *P. spadicea* present in the soil. Although the *P. attenuata* appeared to be strictly vegetative at this point, fruiting potential persists, and this finding allows managers to make better informed conservation decisions. We now know that this site warrants continued monitoring for sporocarp production, and that the underground *P. attenuata* population should be considered when planning management activities in the area.

Table 4 – Results of soil testing for *Phaeocollybia* DNA at managed sites

Site	Visual detection (pre-disturbance)	DNA detection (post-disturbance)	Fraction of samples with this species
Buffered	*P. attenuata*	*P. attenuata*	0.43
	P. fallax	*P. fallax*	0.09
		P. spadicea	0.04
Thinned	*P. attenuata*[a]	*P. attenuata*	0.33
	P. spadicea	*P. spadicea*	0.43
	P. sipei	*P. sipei*	0.04
		P. radicata	0.03
Clearcut	*P. attenuata*	*P. attenuata*	0.60
	P. spadicea	*P. spadicea*	0.09
	P. dissiliens		
	P. fallax		
	P. sipei		

[a] *P. attenuata* sporocarps present during soil sampling.

Table 5 – Amplicons sequenced

Site	Specific amplicon	Number sequenced
Buffered	*P. attenuata*	2
	P. fallax	5
	P. spadicea	1
Thinned	*P. attenuata*	3
	P. spadicea	4
	P. radicata	1
	P. sipei	1
Clearcut	*P. attenuata*	12
	P. spadicea	4

The persistence of the *P. attenuata* mycelium in the clearcut may have been aided by particular characteristics of the clearcut that we studied. The cut was small (1 ha), our study plot was near an edge of the clearcut (less than 20 m), the harvest was done in the winter and replanted the next growing season, and no burning or clearing was done after timber removal so that understory plants remained largely undisturbed. In a review, Jones et al. (2003) cite at least ten studies that indicate ectomycorrhizal fungi colonize clearcuts most effectively by extension from an existing root system. In our case, the fact that likely refuges for *Phaeocollybia* mycorrhizas and mycelium remained undisturbed in the roots of shrubs and small trees within the clearcut and mature trees on the clearcut edge while new potential host trees were planted almost immediately, could have contributed to the persistence of the existing *P. attenuata* in the soil. Soil inoculum sources independent of living plants, such as mycorrhizas on dying roots and their associated extraradical mycelium, can also play an important role in reestablishing mycorrhizal fungi on newly planted trees (Teste et al., 2009). Since the clearcut site was not mechanically cleared or burned prior to replanting, these sources of *Phaeocollybia* inoculum probably remained intact.

The adoption of a saprotrophic mode of nutrition while awaiting the establishment of mature mycorrhizal partners should also be considered a possible means of fungal persistence. There is evidence that a wide range of mycorrhizal fungi can produce degradative enzymes (Bodeker et al., 2009) giving them a facultative saprotrophic capacity (Read and Perez-Moreno, 2003; Talbot et al., 2008; Koide and Malcolm, 2009) and allowing them to compete with saprotrophic fungi for carbon resources in woody debris (Kubartova et al., 2012). Given a saprotrophic ability, existing *Phaeocollybia* mycorrhizas could exploit the large carbon source found in the dying roots of harvested trees and the organic debris generated by logging activities.

Non-target sources of DNA

We assumed that DNA detected in soil samples was from living mycelium or mycorrhizas. Another possibility is that we detected DNA from spores of the target species. We attempted to minimize spores in our samples by discarding the upper organic layer of each sample. If spores were a significant contributor to our results, we would expect to see a positive correlation between sporocarp presence and positive soil samples. However, in the thinned persistence testing plot, where *P. attenuata* was fruiting during sample collection and *P. spadicea* was not, we detected *P. attenuata* less frequently than *P. spadicea*. Also, the other two persistence plots, where *P. attenuata* was not fruiting, had higher proportions of *P. attenuata* soil samples than the thinned site. Although individual spores are capable of traveling long distances, one recent study found that more than 95 % of spores generally fall within 1 m of the sporocarp that generated them (Galante et al., 2011). The likelihood of enough spores traveling from outside the clearcut in high enough numbers to cause 60 positive samples over a 40 m^2 plot seems remote, especially in light of the results from plots and transects containing fruiting *P. attenuata*. While multi-year spore viability has been observed for hypogeous mushrooms dependent on mycophagy for spore distribution (Bruns et al., 2009), long term spore viability does not appear to be typical for epigeous mycorrhizal fungi (Miller et al., 1994; Ishida et al., 2008; Nara, 2009).

Another possibility is that we detected decayed fungal tissue released into the soil and maintained there as a free molecule (Demanèche et al., 2001). In our preliminary soil testing, none of the soil samples collected from Transect 2, which ran through an area that had hosted a patch of *P. sipei* the previous year, showed any sign of *P. sipei* DNA (Table 3), indicating that DNA from both spores and dead mycelium had degraded to undetectable levels within 1 yr. In their investigations of mycelial extent of a fungus that reproduced from spores on a yearly basis, Guidot et al. (2003) also found the complete disappearance of target DNA from areas in 1 yr. RNA, a reactive molecule, indicates the presence of actively metabolizing organisms in the soil and, unlike DNA, is expected to degrade rapidly outside of living cells under all conditions (Prosser, 2002). Studies have shown discrepancies between communities indicated by RNA and those indicated by DNA, suggesting that DNA from inactive organisms can be detected with DNA probes (Anderson and Cairney, 2007; Bastias et al., 2007). As a check, we did return to the clearcut site in November, 2012 and took soil samples from the area where *P. attenuata* was detected for RNA analysis. cDNA generated from RNA extracted from several of these samples tested positive for *P. attenuata* RNA (data not shown) confirming that the fungus is physiologically active at this site.

Conclusion

The *Phaeocollybia*-specific multiplex PCR system tested in this study provides a subterranean view that is essential for the effective management of these fungi at conservation sites. Based on the success of this project, the BLM is using the molecular *Phaeocollybia* detection system to monitor an active management scenario in southwestern Oregon in 2013, both pre- and post-disturbance. Similar specific primer systems could also be developed for other fungal species or species groups, provided that enough sequence data is available to insure primer specificity.

In this study we obtained an unexpected result: the persistence of two species of *Phaeocollybia* in a clearcut area 12 yr

after timber harvest, without evidence of sporocarp production. This persistence may have been a result of continual occupation by the organisms documented 12 yr ago, or the dying back and recolonization of the area by mycelium, either through spore germination or regeneration from underground refuges. We cannot generalize from this observation, and it was likely the result of a virtuous combination of species- and site-specific characteristics, but it does indicate that some infrequently encountered fungal species have a robust survival capacity once established in the environment, and that prolonged periods without sporocarp production may be a normal part of the life trajectory of some fungi as they respond to local environmental changes. This last conjecture of course implies that these species are more abundant in the environment than generally recognized.

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