Pilot Project to Detect *Phaeocollybia* in Soil:

A Field Study

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Introduction

There are approximately 25 species of the fungus *Phaeocollybia* in the Pacific Northwest of North America (Norvell and Exeter 2008). Species-specific molecular markers were developed for 15 species of *Phaeocollybia* in preparation for conducting this pilot study. In this study soil samples were collected from two areas known to be colonized by *Phaeocollybia*. DNA was extracted from these samples and the recently developed species-specific PCR primers were used to test for the fifteen *Phaeocollybia species*, using PCR. This was a preliminary study to test the method and to gain some knowledge about the underground distribution of these fungi so that a more defined study can be done in the future.

Materials and Methods

Field Protocol

An anchored tape measure was used to mark a transect. Transect locations were chosen in the field to run through currently fruiting Phaeocollybia patches and marked locations of former sporocarp sites. Sampling points on the transect were also chosen in the field and varied from 5 feet apart where no sporocarps presence was known to 6" apart through known *Phaeocollybia* sporocarps patches. A 1" diameter stainless steel soil probe was used to obtain soil samples, while the location of each sample on the transect was written down. At each sampling location, two probe samples were taken 1 to 3" apart. The maximum depth of soil that could be taken by the probe was 13 to 15". However, frequently the probe hit obstructions in the soil and the full depth could not be reached. In these cases, the maximum depth reached by the deeper of the two samples was recorded.

For each sampling point, the soil from both samples was combined in a numbered heavy-duty recloseable plastic bag. After each pair of samples, the probe was cleaned in a detergent solution, rinsed in water, and dried with a rag. Samples were stored on ice during transport to the lab.

Study Areas

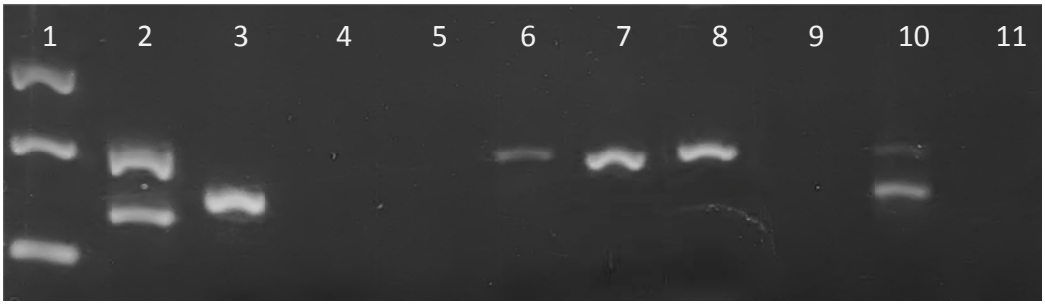
The first study area was Paradise Ridge in the Coos Bay District Bureau of Land Management (BLM). On Nov. 1, 2010, two transects were set up in this area. Transect 1 ran through a marked spot where *P. dissiliens* had been collected in 2009 and a currently fruiting group of *P. spadicea*. Transect 2 started at the edge of a road where a *P. sipei* site from 2009 had been flagged and ran downhill through a currently fruiting patch of *P. radicata*. Fifty samples were taken over 74' on Transect 1, and fifty samples were taken over 59' on Transect 2. The second study area was on Mary's Peak, in the Parker-Bear management unit of the Salem District BLM, where Phaeocollybias have been reliably collected over several years. On Nov. 9, 2010 Transect 3 was set up at Parker-Bear; it was oriented to run through a fruiting patch of *P. attenuata*, and within 3 ft of a group of *P. spadicea* mushrooms. Ninety-eight samples were taken along the 200' length of Transect 3.

Lab Protocol

Samples were kept chilled (2- 5 C) until they were processed. After thoroughly hand mixing the soil in a sample bag, 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 tube containing a soil sample, 800 µl of Chelex buffer (200 mM Tris pH= 8.6, 8% Chelex 100 [Bio-Rad Laboratories], 1% Triton X-100) was added. Tubes were vortexed briefly, heated to 94- 97 C for 5 min, vortexed for 30 s, then returned to 94- 97 C for 10 min, then frozen. Before use, samples were thawed, then centrifuged for 2 min, before 100 µl of supernatant was removed from each sample and cleaned using a DNA purification kit (UltraClean DNA Purification Kit, MoBio Inc.). PCR was run with typical reaction conditions. Preliminary tests showed that results improved when the concentration of DNA in the reaction was reduced.

Running a PCR with more than one primer set and targeting more than one sequence in the same reaction is referred to as multiplex PCR. In this work we were able to test for the presence of fifteen different species of *Phaeocollybia* in two multiplex PCRs, using specific primers designed and tested in previous work. Reaction products were run on an agarose gel, and gels were examined for specific bands corresponding to individual species. Figure 1 is an example of a gel of PCR products (using primer set 1) from ten samples.

Figure 1. Gel of PCR products of soil DNA and primer set 1.



Lane 1 is a DNA standard "ladder"; from bottom to top, bands are at 100 bp, 200 bp, and 300 bp. Lanes 2, 6, 7, 8, and 10 have the 190 bp marker indicating *P. spadicea*; lane 2 also has the 130 bp marker indicating *P. radicata*; lanes 3 and 10 have the 140 bp marker indicating *P. dissiliens*.

Results

Results are given in Appendix I. Each transect had at least two species of *Phaeocollybia* (Table I). *P. spadicea*, *P. dissiliens*, and *P. radicata* were seen in Paradise Ridge soil samples, while *P. spadicea*, *P. dissiliens*, and *P. attenuata* were seen in Parker-Bear soil samples. Transect 1 was routed through a 2009 *P. dissiliens* patch and a current *P. spadicea* patch. The soil samples indicated the presence of both of these species, although no *P. dissiliens* fruiting bodies were seen at the time of field sampling. Transect 2 ran through a flagged *P. sipei* patch of 2009 and a *P. radicata* patch that was fruiting. *P. sipei* was not found in any sample, although *P. spadicea* and *P. dissiliens* were found in addition to *P. radicata*.

Transect 3 was routed through patches of fruiting *P. attenuata* and near a patch of *P. spadicea*. *P. dissiliens* was found in addition to *P. spadicea* and *P. attenuata* in Transect 3 soil.

Out of 198 samples from the three transects, 66 were found to be positive. Fifteen of the 66 had two species present and interestingly, 14 of the two species samples had *P. spadicea* as one of the species. Transect 2 (Paradise Ridge) had the highest proportion of positive samples: 23/50 or 46%. This included 7 samples with two species, for a total of 30 individual detections in 50 samples.

Discussion

The purpose of this study is to test the methodology and point out any areas of concern for future work. In general the field work went well. However, soils at both sites had a high clay content, and this led to some difficulty in removing the soil core from the soil probes. Conditions at Parker-Bear were very wet which led to soil sticking to the soil probe, which in turn led to the need to refresh the wash and rinse water frequently. In this study, about 4 gal of water were used for cleaning for every 100 samples. This would be sufficient for dry conditions or loamy soils, but otherwise 6 to 8 gal of water is recommended.

Phaeocollybia species are putatively mycorrhizal, and so can be expected to maintain some level of activity throughout the year, including in the dry season. A study with *Albatrellus ellisii*, another putatively mycorrhizal fungus, found that this fungus maintained a fairly constant level of soil occupancy over three seasons, including during the summer drought season. Experience with other clay soils has shown them to be much less problematic to work with in dry conditions. Although this study showed that soil collection under wet conditions can be successfully accomplished, scheduling sampling in the dry season should be considered.

As previously mentioned, the standard PCR protocol was modified to reduce the amount of DNA extract in each reaction. Using the same protocol, another study in which soil from the Parker-Bear site was spiked with *P. radicata* and *P. spadicea* pseudorhiza tissue then serially diluted showed that the detection limit for the two species was 40 ppm, which is in the realm of what was expected.

Acknowledgements

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References

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Appendix I

Testing results data table

Column	Notes
C	Maximum depth reached for the 2 soil samples at each point
E	"+" indicates positive PCR result, "++" indicates 2 markers seen Primer set 1 covers the following species: *sipei, dissiliens, radicata, spadicea, scatesiae, fallax, and lilacifolia*
F	Any positive in column E is identified here
G	Primer set 2 covers the following species: *pseudofestiva, piceae, californica, oregonensis, gregaria, attenuata, "fallax B", kaufmannii*
H	Any positive in column G is identified here
J- M	Results for each individual species found in the study

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m1	0	13+	Next to 12" dbh hemlock on old roadbed	+	spa	-		1	0	0	0
m2	3	12		-		-		0	0	0	0
m3	6	13+		+	spa	-		1	0	0	0
m4	9	13+		-		-		0	0	0	0
m5	12	13+		-		-		0	0	0	0
m6	15	13+		-		-		0	0	0	0
m7	18	13+		-		-		0	0	0	0
m8	21	13+		-		-		0	0	0	0
m9	24	13+		-		-		0	0	0	0
m10	27	13+		+	spa	-		1	0	0	0
m11	28	13+	2009 *P. dissiliens* area	++	spa/ disA	-		1	1	0	0
m12	29	13+	2010 *P. dissiliens* area	+	disA	-		0	1	0	0
m13	30	13+	2011 *P. dissiliens* area	+	spa	-		1	0	0	0
m14	31	13+	2012 *P. dissiliens* area	+	spa	-		1	0	0	0
m15	32	13+	2013 *P. dissiliens* area	++	spa/ disA	-		1	1	0	0
m16	33	13+	2014 *P. dissiliens* area	+	spa	-		1	0	0	0
m17	34	13+		-		-		0	0	0	0
m18	35	13+		++	spa/ disA	-		1	1	0	0
m19	36	13+		+	disA	-		0	1	0	0
m20	37	13+		-		-		0	0	0	0
m21	38	13+		-		-		0	0	0	0
m22	39	13+		++	spa/ disA	-		1	1	0	0
m23	40	13+		-		-		0	0	0	0
m24	41	13+		+	disA	-		0	1	0	0
m25	42	13+		-		-		0	0	0	0
m26	43	13+		+	disA	-		0	1	0	0

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m27	44	6		+	disA	-		0	1	0	0
m28	45	7		+	disA	-		0	1	0	0
m29	46	13+		+	disA	-		0	1	0	0
m30	47	10		-		-		0	0	0	0
m31	48	13+		-		-		0	0	0	0
m32	49	13+		-		-		0	0	0	0
m33	50	7	*P. spadicea* at 49.9'	+	spa	-		1	0	0	0
m34	51	10	*P. spadicea* at 50.7'	-		-		0	0	0	0
m35	52	7		-		-		0	0	0	0
m36	53	13+	*P. spadicea* at 52.6'	-		-		0	0	0	0
m37	54	12		-		-		0	0	0	0
m38	55	13+		-		-		0	0	0	0
m39	56	4		-		-		0	0	0	0
m40	57	13+		-		-		0	0	0	0
m41	58	13+		-		-		0	0	0	0
m42	59	7		-		-		0	0	0	0
m43	60	13+		-		-		0	0	0	0
m44	62	13+		-		-		0	0	0	0
m45	64	13+		-		-		0	0	0	0
m46	66	7		-		-		0	0	0	0
m47	68	7		+	spa	-		1	0	0	0
m48	71	9	Tree at 70', sample taken at 71'	-		-		0	0	0	0
m49	72	13+		-		-		0	0	0	0
m50	74	13+	**End Transect 1**	-		-		0	0	0	0
m51	-5	13+	**Start Transect 2**	+	disA	-		0	1	0	0
m52	-4	13+		-		-		0	0	0	0

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m53	0	13+		+	disA	-		0	1	0	0
m54	1	6		+	disA	-		0	1	0	0
m58	2	6		++	spa/ disA	-		1	1	0	0
m55	3	6		+	disA	-		0	1	0	0
m56	4	13+	5 cm duff	+	disA	-		0	1	0	0
m57	5	13+		+	disA	-		0	1	0	0
m59	6	10		++	spa/ disA	-		1	1	0	0
m60	7	7		+	spa	-		1	0	0	0
m61	8	10		+	spa	-		1	0	0	0
m62	9	12	4 cm duff	-		-		0	0	0	0
m63	10	13+	2.5cm duff	-		-		0	0	0	0
m64	11	7		-		-		0	0	0	0
m65	12	13+		-		-		0	0	0	0
m66	13	13+		-		-		0	0	0	0
m67	14	13+		+	rad	-		0	0	1	0
m68	15	13+	2 cm duff	-		-		0	0	0	0
m69	16	13+	2 cm duff	-		-		0	0	0	0
m70	17	13+	air pocket	-		-		0	0	0	0
m71	18	13+		-		-		0	0	0	0
m72	19	13+	air pocket	-		-		0	0	0	0
m73	20	13+	class 5 log, went into it for sample	-		-		0	0	0	0
m74	21	13+	air pocket	-		-		0	0	0	0
m75	22	13+		-		-		0	0	0	0
m76	23	13+	air pocket 2.5cm duff layer	-		-		0	0	0	0
m77	24	13+		+	spa	-		1	0	0	0
m78	25	13+		-		-		0	0	0	0

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m79	26	13+		-		-		0	0	0	0
m80	27	13+		+	rad	-		0	0	1	0
m81	28	13+		-		-		0	0	0	0
m82	29	13+	P. radicata at 29' about 2' west of transect	++	spa/rad	-		1	0	1	0
m83	30	13+		++	spa/rad	-		1	0	1	0
m84	31	13+		++	spa/rad	-		1	0	1	0
m85	32	13+		+	rad	-		0	0	1	0
m86	33	13+		+	rad	-		0	0	1	0
m87	34	13+		+	spa	-		1	0	0	0
m88	35	13+	P. radicata at 34.5- 35' 3 cm duff layer	++	spa/rad	-		1	0	1	0
m89	36	13+		++	spa/rad	-		1	0	1	0
m90	37	13+		-		-		0	0	0	0
m91	38	13+		-		-		0	0	0	0
m92	39	13+		-		-		0	0	0	0
m93	40	13+		-		-		0	0	0	0
m94	42	7		-		-		0	0	0	0
m95	44	13+		+	rad	-		0	0	1	0
m96	46	9		-		-		0	0	0	0
m97	48	13+		-		-		0	0	0	0
m98	50	13+		-		-		0	0	0	0
m99	52	10	hit tree root	-		-		0	0	0	0
m100	54	13+	**End Transect 2**	+	rad	-		0	0	1	0
m101	0	7	rocky, airholes at top, sample from depth	-		-		0	0	0	0
m102	5	10		-		-		0	0	0	0
m103	10	9		-		-		0	0	0	0
m104	15	12		-		-		0	0	0	0

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m105	20	10	air	-		-		0	0	0	0
m106	25	4	air, taken 16" downhill from transect (stump)	-		-		0	0	0	0
m107	30	5	air and organics	-		-		0	0	0	0
m108	35	4	air, taken 16" downhill from transect (log)	-		-		0	0	0	0
m109	40	5		-		-		0	0	0	0
m110	45	12		-		-		0	0	0	0
m111	50	5		-		-		0	0	0	0
m112	52	7		-		-		0	0	0	0
m113	54	9		-		-		0	0	0	0
m114	56	10		-		-		0	0	0	0
m115	58	9		-		-		0	0	0	0
m116	60	5		-		-		0	0	0	0
m117	62	10		-		-		0	0	0	0
m118	64	10		-		-		0	0	0	0
m119	66	7	gravel	-		-		0	0	0	0
m120	68	5	gravel	-		-		0	0	0	0
m121	70	7	gravel	-		-		0	0	0	0
m122	72	7	gravel	-		-		0	0	0	0
m123	74	4		-		-		0	0	0	0
m124	76	11		-		-		0	0	0	0
m125	78	10		-		-		0	0	0	0
m126	80	6		-		-		0	0	0	0
m127	82	8		-		-		0	0	0	0
m128	84	10		-		-		0	0	0	0
m129	86	6		-		-		0	0	0	0
m130	88	4		-		-		0	0	0	0

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m131	90	10		-		-		0	0	0	0
m132	91	10		-		-		0	0	0	0
m133	92	12		-		-		0	0	0	0
m134	93	9		-		-		0	0	0	0
m135	94	10		-		-		0	0	0	0
m136	95	3		+	spa	-		1	0	0	0
m137	96	10		-		-		0	0	0	0
m138	97	4		-		-		0	0	0	0
m139	98	10	P. spadicea 3' from transect	-		-		0	0	0	0
m140	99	8		-		-		0	0	0	0
m141	100	5		-		-		0	0	0	0
m142	101	7	gravel	-		-		0	0	0	0
m143	102	8		-		-		0	0	0	0
m144	103	9	P. attenuata here	-		-		0	0	0	0
m145	104	5	P. attenuata here (root)	-		-		0	0	0	0
m146	105	5	P. attenuata here	-		-		0	0	0	0
m147	106	4		-		-		0	0	0	0
m148	107	7		-		-		0	0	0	0
m149	108	4		-		-		0	0	0	0
m150	109	7		-		-		0	0	0	0
m151	110	9		-		-		0	0	0	0
m152	111	10		-		-		0	0	0	0
m153	112	12		-		-		0	0	0	0
m154	113	8		-		-		0	0	0	0
m155	114	11		-		-		0	0	0	0
m156	116	11		-		-		0	0	0	0

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m157	118	8		-		-		0	0	0	0
m158	120	14		-		-		0	0	0	0
m159	122	11		+	spa	-		1	0	0	0
m160	124	10		+	spa	-		1	0	0	0
m161	126	7		-		-		0	0	0	0
m162	128	12		-		-		0	0	0	0
m163	130	10		+	spa	-		1	0	0	0
m164	132	5		+	spa	-		1	0	0	0
m165	134	6		+	spa	-		1	0	0	0
m166	136	6		+	spa	-		1	0	0	0
m167	138	9		-		-		0	0	0	0
m168	140	8	buried log	++	spa/ disA	-		1	1	0	0
m169	142	8		+	spa	+	attA	1	0	0	1
m170	144	11		-		-		0	0	0	0
m171	146	9		-		-		0	0	0	0
m172	148	7		-		-		0	0	0	0
m173	150	6		-		-		0	0	0	0
m174	152	7		-		+	attA	0	0	0	1
m175	154	3	P. attenuata here	-		+	attA	0	0	0	1
m176	156	6	P. attenuata here	-		+	attA	0	0	0	1
m177	158	6	P. attenuata here	-		+	attA	0	0	0	1
m178	160	11	P. attenuata here	-		+	attA	0	0	0	1
m179	162	12		-		+	attA	0	0	0	1
m180	164	13	P. attenuata here	-		-		0	0	0	0
m181	166	11	P. attenuata 3' from transect	-		+	attA	0	0	0	1
m182	168	10		-		+	attA	0	0	0	1

Sample #	Distance along tsect (ft)	max depth (in)	Collection Note	Primer Set 1 Final Result	Species	Primer Set 2 Final Result	Species	spa	disA	rad	attA
m183	170	10		+	spa	+	attA	1	0	0	1
m184	172	5		-		-		0	0	0	0
m185	174	10		-		-		0	0	0	0
m186	176	10		+	spa	-		1	0	0	0
m187	178	8		-		-		0	0	0	0
m188	180	10	P. attenuata 5' from transect	+	spa	-		1	0	0	0
m189	182	10		-		-		0	0	0	0
m190	184	10	P. attenuata here	+	disA	-		0	1	0	0
m191	186	3		-		-		0	0	0	0
m192	188	6		-		+	attA	0	0	0	1
m193	190	3		-		-		0	0	0	0
m194	192	8		-		-		0	0	0	0
m195	194	14		-		-		0	0	0	0
m196	196	12		-		+	attA	0	0	0	1
m197	198	7		+	disA	+	attA	0	1	0	1
m198	200	12	Last sample **End Transect 3**	-		-		0	0	0	0