Title: Final Report: Truffles of the Cascade-Siskiyou National Monument (FRF 2022-2023 cycle)

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Introduction

Truffles are hypogeous (underground-fruiting) fungi that form ectomycorrhizal relationships with trees. Because they complete their life cycle underground, truffles are often overlooked in traditional fungal surveys. However, they can contribute significant biodiversity to a regional flora, the Pacific Northwest being home to more known truffle species than all of Europe (Trappe et al. 2009 USFS Report PNW-GTR-772). Truffles are one of the most sought-after fungi and hold a near-mythical status among foragers and mycophiles due to their perceived scarcity and hidden growth habit. The most famous truffles of the Pacific Northwest are the Oregon black truffle (*Leucangium carthusianum*) and Oregon white truffles (*Tuber oregonense, T. gibbosum*) whose strong aroma makes an excellent addition to many dishes. However, there is a broad and understudied biodiversity of truffles outside of the culinarily valuable ones.

The flora of the Cascade-Siskiyou National Monument (CSNM) is an intersection of the classic Pacific Northwest flora and the northern California flora, and the region encompasses a fungal biodiversity sweet spot where many southern species are at their northernmost range, and many northern species are at their southernmost range. Truffles are ectomycorrhizal fungi that associate with specific plants, and the biodiversity of these florae combined makes this region ideal for truffle exploration. High summer temperatures and drought stress are posited to be one of the primary motivations for fungi to evolve hypogeous forms, a form which also offers protection against sudden freezes and snowfall (Trappe et al., 2009, USFS Report PNW-GTR-772). These conditions are characteristic of much of the CSNM across its variable ecoregions.

Fungi represent a tremendous spectrum of diversity on earth, and yet we struggle to quantify how many species there are and how many are at risk of extirpation or extinction (Tedersoo et al., 2021, *Fungal Divers*). We must explore the ecology and habitat requirements to better protect sensitive species. The first step is to locate and identify these overlooked fungi (Oliver, Molina, and Smith, 2009, *USFS PNW Science Findings*). Most fungal inventories are based on fungi that produce classic “mushrooms,” or aboveground fruiting bodies. By surveying the often ignored underground fungi, the results of this research promotes an understanding of the hidden diversity within the CSNM. Truffles are charismatic fungi that easily capture the imagination of scientists and the general public alike.

Out of the 350 known truffle species in the Pacific Northwest, 70 species are classified as rare or sensitive (Oliver, Molina, and Smith, 2009, *USFS PNW Science Findings*). Because truffle ecology is under-researched, "potentially all taxa [listed] are at risk from management and recreational activities that remove the mycorrhizal host or disturb the soil" (Castellano and O'Dell, 1997, *BLM Report*). Truffles are critical to ecosystem health not just as an ectomycorrhizal partner of trees, but also as valuable forage for small mammals such as the red-backed vole and can make up over three quarters of the mammals' diets (Mills, 1995, *Conserv Bio*). In analyzing the diets of small mammal mycophagists, truffle diversity seems to play an important nutritional role as their stomach contents generally contain the spores of multiple species at all times (Trappe et al., 2009, USFS Report PNW-GTR-772).

Truffles attract rodents and invertebrates by producing aromatic compounds that are attractive to their target audience, and are strong enough that these wildlife mycophagists find truffles by scent alone. Traditional survey methods involve scouting the forest floor for fresh truffle digs and peeling away the surface organic layer with a rake to locate any fruiting bodies that were left behind. This method is convenient because it allows any person with a rake to find truffles, but is biased towards larger, light colored truffles that are easily spotted with the naked eye, and takes a fair amount of trial and error. Dogs have been used reliably for many decades to find culinary truffles, and can also be trained to find truffles that produce any odor. With proper training, truffle dogs could represent the forefront of truffle diversity research.

Our study investigates the use of a trained truffle dog as an alternative method to raking. Dogs offer an advantage over raking because they target these fungi using the same method that wildlife does, and can thoroughly cover a large area in a short period of time. While it is not known how many unique truffle aromas a truffle dog will recognize and indicate, the dog we used in this study has found truffles from 26 different genera and continues to find more species with novel aromas. These different truffle species have highly variable aromas ranging from pleasant to foul, and some are so faint they are barely detectable to a human nose. Compared to raking, a dog would miss any hypogeous sporocarp that is either immature or does not produce volatile aroma. However, they remove any bias towards size or color and can pinpoint minute truffles and those that are visually indistinguishable from rocks in the soil.

The goal of this study was to develop a greater understanding of truffle diversity and abundance in the CSNM using a trained truffle dog, and our results are summarized in the following sections. We made three visits to the Monument, with 1-2 day surveys once in late October (fall), once in early February (winter), and once in mid-May (spring). While our surveys were limited by seasonal conditions, we were highly successful in finding a variety of truffles and made many valuable collections that will be an asset to ecological research and continued conservation of Monument lands.

Methods

Sites

The Cascade-Siskiyou National Monument straddles the Oregon-California border and contains an unusually high diversity of habitats (Friends of CSNM Monument Guide). Our study focused on mixed conifer and true fir forests located centrally and in the northeast corner of the CSNM, and oak woodlands on the western and southern sides of the Monument (Figure 1). Elevation ranged from 850 m to 1616 m (Table 1). True fir sites were dominated by ponderosa pine (*Pinus ponderosa*), white fir (*Abies concolor*), sugar pine (*Pinus lambertiana*), and Douglas-fir (*Pseudotsuga menziesii*), and one site near Surveyor Mountain which included Shasta red fir (*Abies magnifica* var. *shastensis*). Oak woodland sites were dominated by Oregon white oak (*Quercus garryana*) with occasional scattered California black oak (*Quercus kellogii*) and ponderosa pine. The mixed oak-conifer site had patches dominated by Douglas-fir, ponderosa pine, Oregon white oak, and California black oak, with some mature madrone trees (*Arbutus menziesii*).

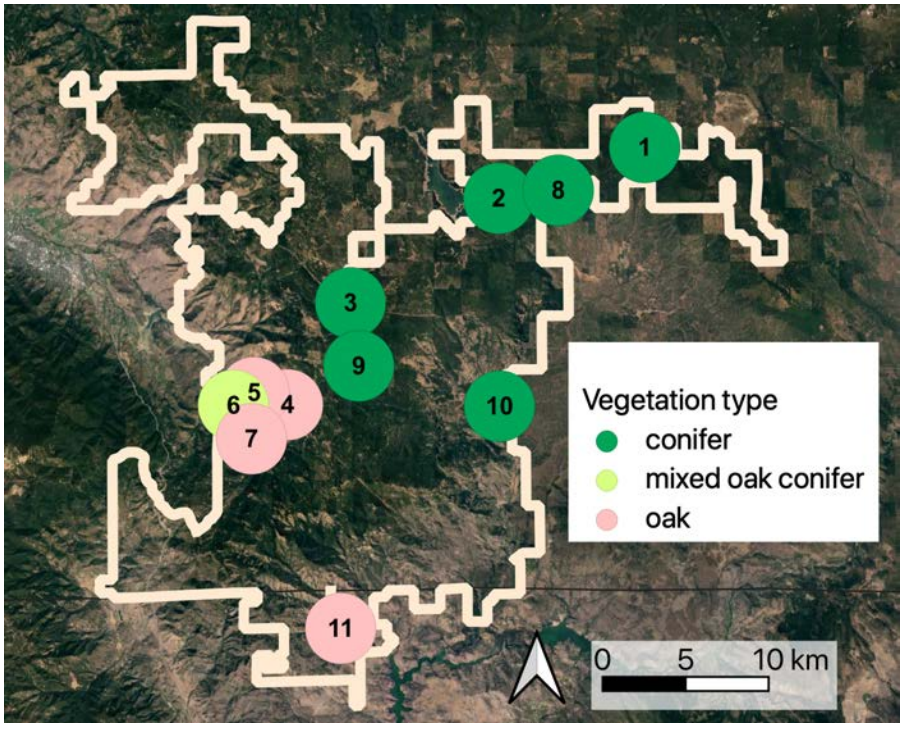

Figure 1: Map of rough locations of all sites in relation to the Cascade-Siskiyou National Monument. Satellite imagery from Google. Site numbers correspond to Table 1.

Table 1: Site survey dates and characteristics.

Site	Habitat	Survey date	Elevation (m)	Mean annual precipitation (mm)	Mean temperature (ºC)
1	Mixed conifer	Oct 31, 2022	1616	1149.92	6.5
2	Mixed conifer	Oct 31, 2022	1616	1149.92	6.5
3	Mixed conifer	Nov 1, 2022; May 13, 2023	1540	844.11	7.8
4	Oak	Feb 4, 2023	858	598.15	10.5
5	Oak	Feb 4; May 13, 2023	934	590.6	10.5
6	Mixed oak/conifer	Oct. 31, 2022; Feb 4, 2023	998	613.6	10.3
7	Oak	Feb 4, 2023	1141	666.82	9.1
8	Mixed conifer/true fir	May 13, 2023	1550	744.45	7.5
9	Mixed conifer, Doug-fir plantation	May 13, 2023	1460	740.44	8.8
10	Mixed conifer, pine plantation	May 13, 2023	1100	782.19	8.4
11	Oak	May 14, 2023	850	538.98	10.8

Sites were chosen by consulting truffle experts in the area who had experience surveying for fungi in Monument lands. When possible, sites visited earlier in the season were revisited during later surveys, but this was often not feasible or practical. As an exploratory study, the main priority during each visit was to target the areas most likely to contribute to our list of species and add the greatest diversity. Our fall foray was timed just after the first fall rains in late October, and was cut short by an early arrival of snow. These sites were inaccessible when we visited in early February, and during that visit we focused on surveying the lower elevation oak woodland areas. The snowpack and cold weather extended through the beginning of May, and during our spring visit in mid-May we surveyed conifer sites that were at the edge of the melting snowpack as well as some lower elevation oak woodland sites where the trees had recently put on new spring leaves.

Truffle hunting

All truffles were located by trained truffle diversity dog "Rye", a golden retriever with two years experience in diversity truffling (Figure 2). While most truffle dogs focus on culinary truffles (*Leucangium carthusianum*, *Tuber oregonense*, and *T. gibbosum*), Rye will indicate any type of hypogeous sporocarp that produces mature aroma. At each site, he was given the command to search and allowed to roam free to follow his nose. Mature truffles were indicated by digging until the truffle was excavated, then located from the surroundings. We collected each truffle that was presumed to be a unique species and documented its fresh characteristics with photos and field notes. Any duplicate specimens were noted and placed back in the soil where they were found. We continued each search until the dog expressed no further interest (a general indication that he has found a majority of the truffles). We carried a Garmin eTrex hand-held GPS unit that tracked our path while truffling and marked each truffle found.

During our fall survey, we tested Rye's thoroughness at each site by performing a separate truffle search with a hand rake. However, we discontinued raking as inefficient in subsequent surveys because we only found a single immature sporocarp of a species found many times by the dog. Truffles were dried at the end of each day in a dehydrator at 35ºC. Each collected truffle has an associated iNaturalist observation (with obscured coordinates) with color images of fresh characteristics. Truffle pictures and descriptions can be found in the attached visual guide.

Figure 2: Rye the truffle diversity dog finding truffles in the CSNM. Rye is trained to locate truffles in exchange for a ball.

DNA analyses

Sporocarp DNA extraction was performed using the Extract 'n Amp method (Truffle Smith lab protocol) by placing a fragment of fresh or dry tissue in 50 µL of the Extraction Solution, incubating at 95ºC for 10 minutes, then diluting 1:1 with 3% Bovine Serum Albumin (BSA). The majority of the sporocarp collections were prepared for PacBio SMRT sequencing with rDNA target regions including the Internal Transcribed Spacer (ITS) region into the Large Sub-unit (LSU), using primers ITS1catta and LR5 (TW14) with attached PacBio barcodes. PCR amplification was performed in 25 µL reactions containing 12.5 µL GoTaq® Green Master Mix, 0.4 µL 2% BSA, 1 µL unique primer/barcode pairs, 0.8 µL DNA template, and 10.3 µL nuclease-free water. Reaction conditions were set to an initial 2 min denaturation at 95ºC, followed by 30 cycles of 30 s at 95ºC, 30 s at 51ºC, and 90 s at 72ºC, then a final 7 min at 72ºC. Gel electrophoresis was performed with 5 µL of the PCR product, and reactions were quantified with a SpectraMax® M Series Microplate Reader and pooled for 3 ng DNA per sample. Pools were purified using Omega Mag-Bind® TotalPure NGS Beads at 0.8X to remove small DNA fragments, and were sent to the UO Genomics and Cell Characterization Core facility for final preparation and sequencing.

An additional round of PCR was performed to prepare the truffles from our final spring survey for Sanger sequencing, as we did not have the time to include them on a second PacBio sequencing run. The target region for these collections was only the ITS region, using primers ITS1F and ITS4. Similar PCR methods were used for these samples, and 15 µL of the unpurified products were sent to MC Lab (San Francisco, CA) for purification and sequencing with ITS1 and ITS4. Editing and assembly of contigs was done in Geneious, and final sequences were compared with similar sequences in the GenBank nucleotide database using Megablast. Along with morphological characteristics, identifications were based on percent similarity to vouchers in the GenBank database, with >99% being considered a species match and <99% as inconclusive or potentially undescribed.

Findings

Truffle diversity

We found truffles at all of the sites we visited representing both the remarkable abundance of truffles in the region and the efficiency of the truffle dog method. Rye found 144 individual truffles at a total of 11 sites. We collected 46 of those for DNA analysis, with collections including 13 different genera (6 from Ascomycota, 7 from Basidiomycota). We believe that this represents around 24 species, at least two of which are undescribed (Table 2). We are still waiting for DNA analyses to place most of the species identifications. The initial PacBio submission with most of our collections was submitted mid March

2023 and the raw data was received late May, and is currently being processed and demultiplexed. We anticipate being able to fill out our collection data with species identification very shortly.

Table 2: A list of all the truffle species that were found during our truffle dog surveys in the CSNM. Species names are based on sequence data and/or morphology; the majority of collections have not been identified to species level in anticipation of sequence data for genetic analysis. **Listed incorrectly as Trappea sp. on GenBank.*

Species	**Habitat**	**Season**	**Closest GenBank species match**	**Percent match**	**Sequencing method**
Balsamia latispora	Conifer	Spring	*Balsamia latispora* (MK991863.1)	99.39%	Sanger
Balsamia sp.	Oak; mixed oak/conifer	Winter	-	-	PacBio
Choiromyces alveolatus	Conifer	Fall	-	-	PacBio
Elaphomyces cf. *muricatus*	Oak; mixed oak/conifer	Fall, Winter	-	-	PacBio
Elaphomyces sp. (section *Ceratogaster*)	Oak	Spring	*Elaphomyces aculeatus* (KR029777.1)	93.94%	Sanger
Gautieria cf. *monticola*	Conifer	Fall	-	-	PacBio
Genea arenaria	Oak; mixed oak/conifer	Winter, Spring	-	-	PacBio
Genea harknessii	Oak; mixed oak/conifer	Fall, Winter, Spring	*Genea harknessii* (FJ235150.1)	99.23%	Sanger
Hymenogaster raphanodorus	Oak	Spring	*Hymenogaster raphanodorus* (NR_119531.1)	99.02%	Sanger
Hysterangium sp.	Conifer	Fall	-	-	PacBio
Hysterangium sp.	Oak	Spring	*Hysterangium sp.** (ON256876.1)	97.80%	Sanger
Lactarius gardneri	Oak	Winter	-	-	PacBio
Leucangium carthusianum	Conifer	Spring	*Leucangium carthusianum* (MZ970467.1)	99.72%	Sanger
Leucogaster cf. *citrinus*	Conifer	Fall	-	-	PacBio
Leucogaster sp.	Conifer	Fall	-	-	PacBio
Rhizopogon sp.	Conifer	Fall	-	-	PacBio
Rhizopogon sp.	Oak	Winter	-	-	PacBio
Russula sp.	Conifer	Fall	-	-	PacBio
Tuber sp. (1)	Conifer	Fall	-	-	PacBio
Tuber sp. (2)	Conifer	Fall	-	-	PacBio
Tuber sp. (3)	Conifer	Fall	-	-	PacBio
Tuber sp. (4)	Conifer	Fall	-	-	PacBio
Tuber sp. (5)	Conifer	Fall	-	-	PacBio
Tuber sp. (rufum clade)	Oak	Winter	-	-	PacBio

Based on identifications primarily at the genus level, we found that oak and conifer habitats held a similar diversity, but individual sites had large variation. For example, the first fir site we surveyed had high diversity in the fall (7 genera), while another site surveyed a day later had lower diversity (3 genera). The highest diversity between oak sites was 4 genera, and the lowest was 2 genera.

Rare, unusual, and undescribed species

One of our aims with this project was to shed light on some of the lesser known truffle species that may be found in the unique habitat of the CSNM, and bring greater recognition to this region as a biodiversity hotspot. A few of our particularly rare or interesting finds are described below, and we expect to fill this section out when we receive sequence data for our other collections.

An oak woodland at the southern region of the Monument (site 11) provided three valuable collections: an undescribed *Elaphomyces* sp. and a likely undescribed *Hysterangium* sp. We also found the recently described *Hymenogaster raphanodorus* that only has one other accessioned collection, the type specimen. Sequence data seems to indicate that the *Elaphomyces* sp. may be a member of the section *Ceratogaster,* which includes some of the rarest known *Elaphomyces* species. These species are typically characterized by a black cortex, and the specimen we found under *Quercus garryana* had a black, warty exterior. The closest species match is only ~94% for the European species *E. aculeatus*, a species listed as Critically Endangered by the IUCN Red List. There is one other black *Elaphomyces* species known from this region, an exceptionally rare species that has historically been called the European name *E. anthracinus*. The new *Elaphomyces* sp. we found does not match well with the description of this other species, but there is no sequence data yet to compare their genetic similarity.

The *Hysterangium* sp. that we found under *Q. garryana* was fruiting productively in one small patch, but absent across most of the territory that the dog searched. Old specimens had turned to liquid in the soil and smelled strongly unpleasant, while younger specimens were firm with a less offensive odor. Sequence data places this collection closest (97.8%) to a single voucher specimen in GenBank that is incorrectly listed as a *Trappea* sp., and is actually a *Hysterangium* sp. (B. Roy, pers. comm.). There are no described species matches in the 90% range, suggesting that this may be an undescribed species.

The sequence data of our *Hymenogaster* collection from the white oak woodland has a 99% match to the holotype specimen of *Hymenogaster raphanodorus*, a species that was described in 2005 from California and so far still seems to be only known from the holotype collection (Smith et al., 2005, *Mycotaxon*). The specimen that we found was growing under a *Ceanothus* shrub in a dry, exposed area near a young Oregon white oak. It had a strong, unique odor of curry, with some undertones of crushed

weedy plants. This collection will help create a better understanding of the distribution and phylogeny of this species.

Although we are still waiting for sequence data to determine most of the species from the conifer sites we visited, we are confident in our identification of the truffle *Choiromyces alveolatus,* which we found multiple times across two different conifer sites in the fall. *C. alveolatus* has historically been considered rare to uncommon, making this a notable find for the Monument. Our personal experience finding this truffle indicates that it is less rare than has been assumed in the past, but this genus is of substantial interest in North America as it is the only other member of the family *Tuberaceae* (the genus *Tuber* being the most prominent member) found in this region (Bonito et al., 2013, *PLoS One*). While *Tuber* is an incredibly diverse genus across the northern hemisphere, *C. alveolatus* is one of only three likely species of *Choiromyces* found in North America (Wang et al., 2022, *Diversity*).

While the esteemed Oregon black truffle is considered one of the most common truffle species in Oregon, finding it as far south as the CSNM and in high elevation forest is one of the more unexpected places for this truffle to be found. We found it only once in the spring, during the edge of the May snowmelt. It was buried exceptionally deep in the soil, over a foot deep, illustrating the protection that a hypogeous habit can provide from the harsh winters of living at 1500 m. Given the reasonably large size of the specimen and life history of this species, it likely grew over a longer period of time and ripened when the soil thawed. There should not be any concern of truffle hunters attempting commercial success with this species in the Monument, as it appears to be quite sparsely distributed and unlikely to be found with a rake.

We are certain that many more of our collections are considered rare or unusual, and look forward to filling out this section when we are able to complete the rest of the genetic analyses.

Truffle seasonality

Truffle fruiting was limited by a long dry season and sudden onset of a snowpack, providing us with a short window to survey in late October during the first year of study. During this fall survey, we focused on the higher elevation mixed conifer and true fir forests in the central area of the Monument and in the northeast corner. Truffle productivity in the fall was high, both in general abundance and diversity. This area was under snowpack until mid-May during our final foray, with the most productive fir site unreachable, but we surveyed a similar habitat a few miles down the same road. All of the mixed conifer sites visited in the spring had markedly fewer truffles and truffle diversity compared to the fall, and there were no shared species across the two seasons. The lack of spring truffles may be due to the very recent snowmelt, and a later survey could reveal more productivity.

During the fall survey, the fir sites were dominated by a truffle in the genus *Russula,* followed by a number of different *Tuber* spp., and several finds of *Choiromyces alveolatus, Gautieria* sp.*,* and *Leucogaster* cf. *citrinus*. Additionally, *Leucogaster* sp.*, Hysterangium* sp. and *Rhizopogon* sp. were found, but were more rare. Conversely, only two truffle species were found in the conifer sites in the spring. *Balsamia latispora* was the most common while still only found a handful of times across all sites surveyed, and we found one single *Leucangium carthusianum* (Oregon black truffle) (Figure 3a). Across the fall and spring surveys, we collected truffles from a total of six separate conifer sites.

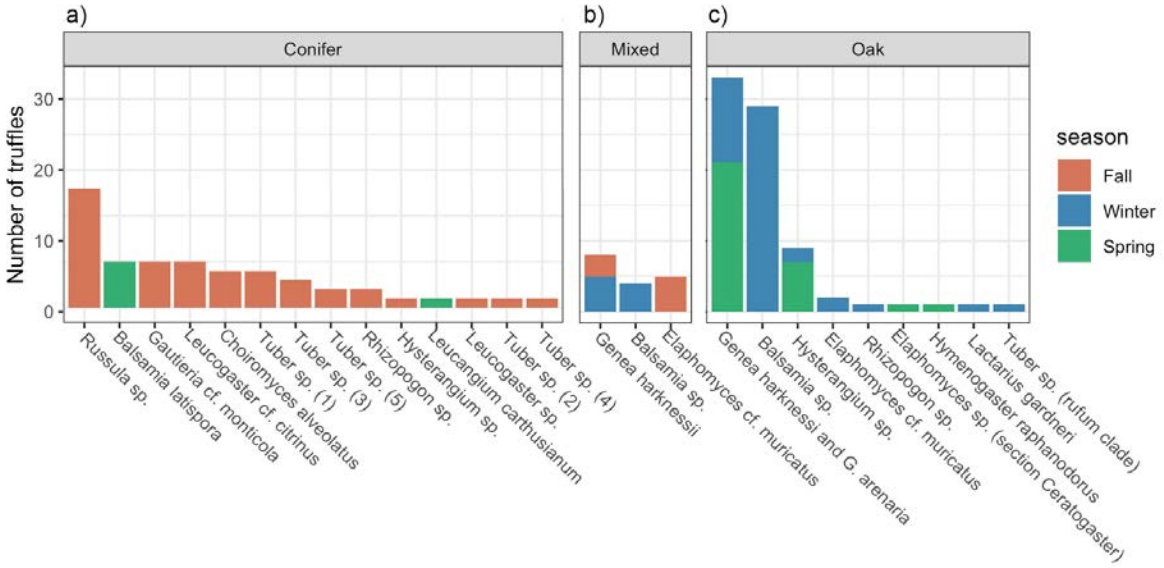

Figure 3. Number of truffles found by site and across seasons. a.) Conifer sites surveyed across two seasons, showing higher abundance and diversity in the fall. **b.)** Three species were found in the conifer/oak forest site we surveyed. **c.)** Winter and spring surveys at oak woodland sites showed high productivity of *Balsamia* and *Genea.* We could not consistently distinguish *G. harknessii* and *G. arenaria* in the field and chose to group them together.

We visited one low elevation mixed oak and conifer site in both fall and winter, where we found *Genea harknessii* fruiting during both seasons, *Elaphomyces* cf. *muricatus*. fruiting only in the fall and *Balsamia* sp. fruiting only in the winter (Figure 3b)

Our winter survey focused on lower elevation oak woodland sites (pre-budburst), where we found high truffle diversity but several of these species were found only once or twice (*Tuber* sp., *Rhizopogon* sp., *Lactarius gardneri*, *Elaphomyces* cf. *muricatus*, *Hysterangium* sp.) suggesting it was not peak season for many of them. However, one or two species in the genus *Balsamia* were fruiting in large numbers at nearly every site we visited, closely followed by many *Genea arenaria* and a few *Genea harknessii*. Spring surveys (post leaf-out) in the oak woodlands did not reveal any *Balsamia* truffles in the oak sites,

but the areas we visited were all dominated by *Genea* truffles with discrete patches of *Hysterangium* sp. Single collections were made of *Elaphomyces* sp. (undescribed, section *Ceratogaster*) and *Hymenogaster raphanodorus* (Figure 3c).

The fruiting pattern of the *Balsamia* sp. found in the oak sites was particularly notable, as during the winter survey these truffles were present in exceptional numbers and consistently found very close to or directly under *Ceanothus cuneatus* shrubs (Figure 4). The genus *Balsamia* is ectomycorrhizal (Southworth, D et al., 2018, *Fungal Systematics and Evolution*) and our assumption is that these truffles were ectomycorrhizal associates with the oak trees at these sites, but the location of the fruiting bodies had little to do with the proximity of the oak host and was more closely tied to *C. cuneatus*. This shrub is not known to form ectomycorrhizal partnerships, and we speculate that there could be a connection related to its nitrogen-fixing capabilities. The fruiting of *Balsamia* in these conditions seems to have a specific seasonality, as we did not find any *Balsamia* truffles during a revisit in the spring when the shrubs were flowering. Chemical and isotopic analyses of the soil as well as plant and truffle tissue could provide further insight, along with an examination of root associates of both the *C. cuneatus* shrubs and oak trees.

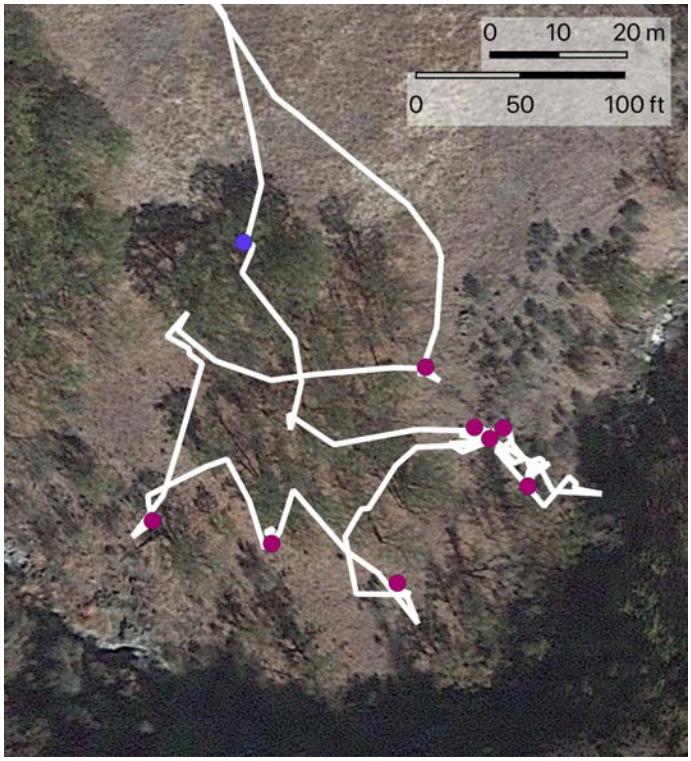

Figure 4: *Balsamia* were limited to *Ceanothus* shrubs around an oak (*Quercus*) stand at site 4. White line indicates truffle dog track. Magenta dots indicate where *Balsamia* were found. Purple dot indicates where a *Genea* sp. was found. Satellite imagery from Google.

Dog-led truffling for biodiversity

Rye successfully found truffles at each site that we visited. His success rate was much greater than our raking success, which only found a single sporocarp at a single site. Looking at the GPS tracks for each site, it was clear that Rye was targeting truffles, rather than wandering aimlessly (Figure 5). This reflects our broader experience with truffle dogs. Truffles that Rye located could be up to 30 cm deep underground, but most were found in the upper regions of the mineral soil or in the interface between the organic layer and mineral soil. While some of the truffles he found were large and contrasted with the soil, many others were small and unrecognizable against a soil-colored background and would have been difficult if not impossible to spot if uncovered with a rake.

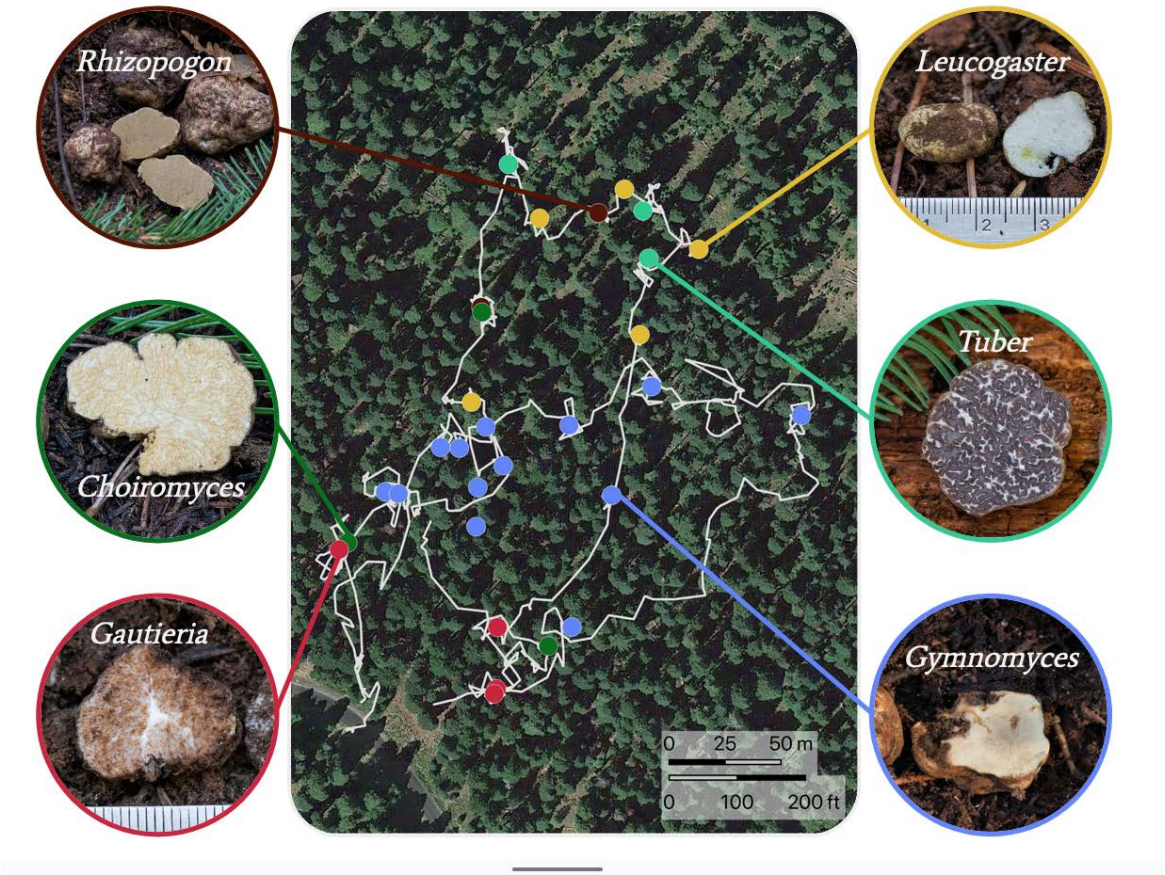

Figure 5: Map from a fall survey of site 1 showing Rye's path between truffles and the distribution of different truffle species. Satellite imagery from Google.

Importantly, Rye found several uncommon truffles and an established culinary truffle outside of its primary range. Our truffle collections and occurrence data offer important insight into the distribution and fruiting patterns of these cryptic and vulnerable organisms during a time of rapid climate change. Traditional truffle survey methods have yielded the wealth of knowledge we have on truffles today, but

these methods take a keen eye and patience and it is worth recognizing that a large majority of the work on truffles in the Northwest has been performed by only a handful of field experts. Surveying with a trained dog allowed us as relative newcomers to this field to thoroughly cover each site we visited with a minimal amount of time and effort, reduced unnecessary ground disturbance, and produced only mature specimens allowing for a better description of truffle characteristics and spore examination.

Acknowledgements

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The biggest thanks goes to the best ever truffle diversity dog, without whose talent this project would never have been possible. Good dog, Rye.